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ROLE OF HAIRY-RELATED (HER) GENES DURING VERTEBRATE RETINAL DEVELOPMENT AND REGENERATION

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ROLE OF HAIRY-RELATED (HER) GENES DURING VERTEBRATE RETINAL DEVELOPMENT AND REGENERATION

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By

Stephen G. Wilson
Lexington, Kentucky

Director: Ann C. Morris, Associate Professor of Biology
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2016

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ABSTRACT OF DISSERTATION

ROLE OF HAIRY-RELATED (HER) GENES DURING VERTEBRATE RETINAL DEVELOPMENT AND REGENERATION

Development and regeneration of the vertebrate eye are the result of complex interactions of regulatory networks and spatiotemporally controlled gene expression events. During embryonic retinal development, the coordination of cell signaling and transcriptional regulation allows for a relatively homogenous sheet of neuroepithelial cells to proliferate and differentiate into a multilayered, light sensitive retinal tissue. Following injury, the retinas of many cold-blooded vertebrates, such as the zebrafish, undergo a proliferative response that results not only in new retinal cells of the correct type in the correct location, but also functional integration of these cells and restoration of vision. In order for embryonic retinal neurogenesis to proceed correctly, systems must be in place that restrict subsets of progenitor cells from differentiation. Pools of actively proliferating retinal progenitor cells are maintained to fill the needs of developmental processes and normal growth of the retina. In addition, subsets of radial glia in the retina retain the ability to de-differentiate into proliferating progenitor cells to meet the demands of the regenerating retina. All of these processes rely on the tight coordination of extrinsic and intrinsic cues, as well as regulation of gene expression by transcription factors. Although a considerable amount of work has been conducted to identify key regulators of retinal development and regeneration, many gene regulatory networks which include both master signaling pathways as well as individual transcription factors remain poorly characterized.

Some of these factors implicated in retinal development and regeneration are members of the Hairy/Enhancer of Split (Hes) superfamily of genes, including the Hairy-related (Her) factors Her4 and Her9. Her transcription factors are basic-helix-loop-helix-orange (bHLH-O) transcription factors that bind to palindromic E- and N-box canonical sequences in the promoters of target genes. Her factors have been previously shown to play roles in a diverse array of developmental and neurogenic processes, including neural tube closure, floor plate development, somitogenesis, and development of various components of the central nervous system as well as the cranial sensory placodes. The roles of her4 and her9 in retinogenesis, however, remain undefined. To determine the possible roles of her4 and her9 factors in the retina, I characterized the expression patterns of these factors during developmental retinal neurogenesis and/or regeneration, examined loss of function phenotypes, and identified signaling pathways that modulate expression of these factors.

Chapter 1 of this dissertation provides an overview of vertebrate retina and retinal development, the known functions of her4 in other tissues, and the Notch-Delta signaling pathway. Chapter 2 provides evidence that her4 is a primary effector of the Notch pathway during retinal development, and examines the role of her4 expressing cells during regeneration of the mature zebrafish retina within the context of both chronic and acute photoreceptor damage paradigms. In addition, generation and validation of the transgenic her4:Kaede zebrafish which was used to identify the lineage of her4-expressing cells is described. Characterization of her9 during retinal development, identification of the retinoic acid signaling pathway as a regulator of her9 expression in the retina, and the role her9 plays during retinal vasculogenesis are discussed in Chapter 3. Chapter 4 discusses the generation of her9 knock-out zebrafish lines using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology and characterization of mutant phenotypes in mosaic her9 mutant F0 fish. In addition, in Chapter 4 I also discuss the screening processes used to identify and characterize genetic lesions in the her9 allele and establish various lines that stably transmit deleterious her9 alleles in the germline, and provide preliminary data of the her9 mutant phenotype. Finally, in Chapter 5 I discuss conclusions from the data generated from this dissertation, additional studies that would
expand upon this work, and the implications of these results on the broader understanding of retinal development and regeneration.

My dissertation incorporates reverse genetic analysis in zebrafish, biochemical analysis, transgenesis, and various molecular approaches to help better understand the roles of *her4* and *her9* during retinal neurogenesis. Moreover, these studies may also contribute to a better understanding of retinal development, and disease pathogenesis. It is my hope that this work could also ultimately contribute, even if in some small way, to the goal of enabling human patients who have suffered from vision loss a means by which a damaged retina could be regenerated and functional vision restored.

KEYWORDS: Retina, Development, Regeneration, Vasculogenesis, Notch-Delta, Retinoic Acid, *her4, her9*, Zebrafish, CRISPR/Cas9

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April 25, 2016
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“Things are only impossible until they are not” – Captain Jean-Luc Picard

I love the simplicity and implication of one of Captain Picard’s more famous quotes. Truly, things are only impossible until they are not, and I find this to be especially relevant to the field of biology. I find it absolutely amazing that in the span of roughly one hundred years, our understanding of the concept of a gene went from mathematical abstractions of heritable traits to now being able to precisely describe the molecular interactions of nucleic acids and proteins within context of larger networks that lead to fundamental biological principles. I think that one of the things that really drove me into this field is the idea that it is now possible to not only understand but to fix or even improve various aspects of our own biology. I strongly believe that what we now consider to be impossible will indeed be possible in the near future, and we will all live long and prosper.

First and foremost, I want to acknowledge my amazing wife, Hilary. I don’t think that any of this would have been remotely possible without her encouragement, love, and understanding. Hilary has been unbelievably supportive and understanding throughout this whole process. Not only has she had to put up with me in general, but she is also an amazing mother and has somehow managed to take excellent care of both of her Garretts. She has graciously endured the sometimes strange work hours, the late nights of study and writing, and the numerous absences from family or social obligations. Not only does Hilary have a very successful career, but she also takes care of nearly everything at home, including our rather rambunctious son, Asher, with perfection. I admire her, she is so intelligent and such a hard worker that she makes everything look easy. Hilary has kept me sane and happy. All of this would have been absolutely impossible without her. How she puts up with my nonsense is pure mystery, but I am happy that she does and I hope that she continues to do so for the rest of our lives.
Next, I would like to express my immense gratitude to my doctoral advisor, Dr. Ann Morris. Ann has taught me how to not only be a scientist, but a good scientist. Ann has taught me how to ask the right questions, how to design and carry out appropriate experiments, and to be consistently vigilant when interpreting results. I consider myself to be very lucky to have had her as my mentor, and have benefited greatly from my time in her lab. I admire her enthusiastic approach to science, how she manages the lab, and how easily approachable and helpful she has been. She encourages independent thought but is always there to help if any problems are encountered. In addition to professional development, Ann really cares about her student’s well-being, and has always been there for us whenever we have needed her. Any time I have been sick, injured, or hospitalized for any reason, Ann was there immediately. When Asher was born, Ann was one of the first people there to hold him and insist that if we needed anything at all, she was just a phone call away. My time in Ann’s lab, and my interactions with her have been essential to my growth and success, and I am grateful.

I would also like to thank the members of my committee for all of their help and suggestions. When initially forming my committee, I knew that it had to include Dr. Pete Mirabito. Dr. Mirabito was one of the first people I had interacted with at UK, and I am constantly impressed by his breadth and depth of knowledge as well as his emphasis on the big picture. Dr. Doug Harrison taught me the importance of not only knowing techniques and interpreting results, but to appreciate the how and the why. I was always eager to talk to Dr. Bruce O’Hara about my work because he would carefully look at the data and challenge my interpretations and enable me to find possible alternative explanations. Dr. Brett Spear’s critical analysis of my data and helpful suggestions have also helped tremendously in my research.

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CHAPTER 1: Vision, retinal development, and bHLH-O transcription factors

KEYWORDS: Vision, retinal development, Notch-Delta signaling, Her4

1.1 Introduction

A primary function of all living organisms is the ability to perceive and act upon environmental cues and information in an effort to collect resources, avoid predation, and in many cases, reproduce. One of the most heavily relied upon means by which vertebrates survey their world is through vision, which can be described as the process by which an organism collects, organizes, and interprets quanta of electromagnetic radiation emanating from the environment (Lamb et al., 2007). Vision, or the ability to “see”, is a critically important biological process in nearly every aspect of our lives. In addition, retaining the capacity to detect visual information from the environment has proven to be so evolutionarily advantageous, that analogous traits that allow for various forms of light-sensing have arisen independently multiple times in every biological domain (Nilsson, 1996).

Functional vision in vertebrates requires the coordinated efforts of multiple complex tissues, including both neural and non-neural tissues derived from different embryonic origins, as well as establishment and refinement of connections between the numerous cell types found in the retina and brain. There is a multitude of individual genetic, cellular, and tissue level events that must take place in near spatiotemporal precision during initial embryonic eye development for proper vision to occur. In addition, there are numerous eye pathologies that can occur after the visual system has already been established that can lead to various types of vision loss. Due to these factors, the National Eye Institute estimates that over 1 in 10 Americans currently suffer from inadequate vision or eye disease, at an estimated cost of over $68 billion per year. Because the United States, like other first world nations, is experiencing the results of an increasingly large aging population, visual pathologies such as Age-related Macular Degeneration (AMD), diabetic retinopathies, and slow onset Retinitis Pigmentosa (RP) are becoming much more common and are likely to exacerbate these numbers. Although in this modern era
it is certainly possible to live a long and fulfilling life that doesn't include functional vision, retaining and/or acquiring the ability to see can profoundly affect the quality of life of those afflicted with vision loss.

The retina is the photosensitive neural tissue that lines the inner part of the back of the eye and is an outgrowth of the vertebrate brain, making it the outermost portion of the central nervous system (CNS). Although the various retinal cell types as well as the overarching structural themes are very similar in cold- and warm-blooded vertebrates, warm-blooded mammals lack the ability to regenerate neurons of the CNS (Fischer and Bongini, 2010). Whereas both acute and chronic damage to the mammalian retina is generally permanent, the damaged zebrafish retina undergoes a robust regenerative response (Vihtelic and Hyde, 2000, Morris and Fadool, 2005, Goldman, 2014, Gorsuch and Hyde, 2014). Although there have been recent advances in cell transplantation therapies as well as gene therapies designed to stimulate some degree of regeneration in the mammalian retina, restoration of vision in human patients remains a distant goal. In order to properly implement a biological therapy that restores vision, one promising avenue of research is to uncover exactly what molecular and cellular mechanisms enable a cold-blooded vertebrate such as the zebrafish to have the remarkable ability to fully regenerate damaged retinal tissue.

This chapter will provide an overview of the visual system and retinal development, including relevant signaling pathways and transcription factors that are essential for proper retinal neurogenesis. Additionally, we will discuss the utility of the zebrafish as an appropriate model organism for these studies, and review the known functions of basic-helix-loop-helix-orange (bHLH-O) proteins, including the Hairy-related 4 (her4) transcription factor. Throughout this text, we will follow established nomenclature, designating human genes in all capital letters, rodent genes with the first letter capitalized, and lower case letters for all other vertebrates; italic letters indicate genes, whereas non-italicized names refer to proteins.
1.2 Vertebrate retinal development

1.2.1 Retinal cell types, functions, and connections

The vertebrate retina is a highly conserved and specialized extension of the central nervous system (CNS) that enables vision by allowing photons of light to be captured and transduced into electrical signals that output to and are interpreted by the brain (Figure 1.1). The neural retina is a complex multi-layered tissue that consists of three nuclear layers; the Outer Nuclear Layer (ONL), Inner Nuclear Layer (INL), and Ganglion Cell Layer (GCL). In addition, there are two synaptic layers that enable electrical and chemical signal transmittance of visual information between the cell layers to occur. The processes of cells in the ONL and INL synapse in the outer plexiform layer (OPL), and in the inner plexiform layer (IPL), the processes of INL neurons synapse with ganglion cells (Fadool and Dowling, 2008).

There are six major classes of retinal neurons, each containing multiple subtypes, and one intrinsic glial cell type (Stenkamp, 2007, Fadool and Dowling, 2008). The two general classes of photoreceptor cells (PRCs), the light receiving cells responsible for phototransduction which are found in the ONL, are the rods and cones. Rods are exquisitely sensitive to light, allow for the detection of as little as one quanta of photons, and are responsible for scotopic vision. Rod PRCs, however, are not able to discriminate spectral information. Cones allow for color vision in photopic conditions. They require significantly more light to induce a signal transduction cascade than rods, but enable high visual acuity and operate at significantly faster response times (Joselevitch and Kamermans, 2009). Multiple cone subtypes that are spectrally tuned to respond to different wavelengths of light have been identified. This wavelength sensitivity and specificity is the result of expression of several variants of the opsin visual pigment in the different cone PRC types. The different cone cell types each containing a different opsin enables an organism to differentiate between wavelengths of light and is the basis of color vision. The soma of rod and cone PRCs (basal side of the PRCs) reside in the ONL, but it is the outer segments of the
PRCs (apical side of the PRCs) that are responsible for initial photon detection. The photoreceptor outer segments are modified sensory cilia composed of numerous lipid bilayer disks or folds (in rods and cones respectively) that contain the photosensitive opsin transmembrane proteins. These long outer segments are embedded into and closely associate with the retinal pigmented epithelium (RPE), a part of the non-neural retina that is responsible for absorption of scattered light, phagocytosis and turnover of shed outer segment tips, ion buffering, transport, secretion, and recycling and storage of 11-cis retinal, the chromophore bound to opsins that undergoes a conformational change upon photon capture (Strauss, 1995, Navid et al., 2006). The PRC terminals project into the OPL, where they form synapses with the interneurons located in the apical part of the INL.

There are three types of interneurons in the retina that are located in the INL: horizontal, bipolar, and amacrine cells. These cells are responsible for organizing, refining, and transmitting signals that originated from the photoreceptor cells to the ganglion cells in the GCL. In addition, the nuclei of the only intrinsic retinal glial cell, the Müller glia, is found in the INL. The cell bodies of horizontal interconnecting neurons can be found on the apical side of the INL, and bipolar cell soma can be found throughout the INL. The horizontal cells synapse with the processes of multiple photoreceptors in the OPL as well as processes from bipolar cells. There are 3 horizontal cell subtypes, and 13 bipolar cell subtypes that have been identified in the primate retina (Kolb et al., 1992, Euler et al., 2014). At the basal side of the INL, the cell bodies of more than 25 subtypes of amacrine cells can be found (Wassle et al., 2009). The amacrine cells further refine output from the bipolar cells, and interconnect with both the bipolar cell processes and the ganglion cells in the IPL. The ganglion cell bodies are found in the GCL, and their axons bundle together to form the optic nerve which serves as the terminal output of visual signals from the retina to the brain (Sanes and Masland, 2015).
Figure 1.1: Schematic of the vertebrate retina. The vertebrate retina is composed of three nuclear layers and two plexiform layers: the Outer Nuclear Layer (ONL) which contains the cell bodies for rod and cone photoreceptors, the Inner Nuclear Layer (INL) which contains the cell bodies of horizontal, bipolar, and amacrine interconnecting neurons, and the Ganglion Cell Layer (GCL) which contains ganglion cells whose axons form the optic nerve. The Outer Plexiform Layer (OPL) contains synapses of photoreceptors, horizontal cells, and bipolar cells, and the Inner Plexiform Layer (IPL) contains synapses of amacrine, bipolar, and ganglion cells. The Müller glia cells are the only intrinsic retinal glial cell and expand the entire thickness of the neural retina. The Retinal pigment epithelium (RPE) envelops the photoreceptor outer segments, and the choroid contains blood vessels that nourish the retina. Adapted from webvision.com.

Müller glia are the intrinsic radial glial cells of the retina. Although their cell bodies are found in the INL, Müller glia processes extend throughout the full thickness of the retina, from the inner limiting
membrane in the basal retina to the PRC outer segments and RPE. The Müller glia act as support cells for the retinal neurons by phagocytosing cellular debris, regulating concentrations of neurotransmitters, ions, and signaling factors, have neuroprotective properties, and serve as a scaffold that provides structural support to all of the neurons throughout all of the retinal layers (Reichenbach et al., 1989, Newman and Reichenbach, 1996, Garcia and Vecino, 2003, Bringmann et al., 2006). In addition, the Müller cells in the mature retina of many cold-blooded vertebrates as well as embryonic avian retinas can serve as a source of retinal stem cells that contribute to regeneration of the damaged retina (Fischer and Reh, 2001, Fischer and Reh, 2003, Garcia and Vecino, 2003, Bernardos et al., 2007, Fischer and Bongini, 2010, Lenkowski and Raymond, 2014).

1.2.2 Development of the vertebrate retina

Initial embryonic development of the vertebrate retina has been intensely studied, in part because it can serve as an experimentally tractable proxy for understanding the development in of other parts of the CNS including the brain. Here, we focus primarily on development of the zebrafish retina, which serves as an excellent example of the general paradigm of retinal development in all vertebrates because the overall order of cell signaling, proliferation, cell cycle exit, and differentiation are highly conserved within the subphyla. The zebrafish retina has the same superstructure, lamination, morphology, cell types and function as the human eye (size and regenerative capacity notwithstanding).

1.2.2.1 Order of zebrafish retinal cell differentiation

Development of the zebrafish retina proceeds in an ordered and predictable spatiotemporal pattern. By 11 hours post fertilization (hpf), the primordial eye field of the zebrafish is identifiable as an evagination from the anterior neural keel. Development proceeds rapidly; at 24 hpf the presumptive retina exists as a single, pseudostratified neuroepithelial layer of proliferating cells (Malicki, 1999, Schmitt and Dowling, 1999, Stenkamp, 2007). Cell cycle exit and differentiation of the first born cell type occurs first in the ventral patch of the inner retina then proceeds towards the outer retina, and
neurogenesis within each respective nuclear layer occurs asynchronously. In the developing zebrafish retina, differentiation of retinal cell types in their respective layer is mostly complete before cells in the next nuclear layer start to differentiate (Figure 1.2A). In addition, whenever differentiation first begins in the ventral portion of the retina within a respective nuclear layer, neurogenesis occurs in a fan-shaped wave and proceeds through the ventro-nasal to the dorsal and the ventro-temporal regions of the retina (Figure 1.2B) (Stenkamp, 2007). Starting at 28 hpf, the first cells to differentiate are the ganglion cells. This is followed by differentiation of cells that make up the INL (amacrine and horizontal cells) from 36-48 hpf, with bipolar cell differentiation starting around 60 hpf (Schmitt and Dowling, 1999, Connaughton, 2011). Cone PRCs develop from 48-60 hpf. Rod PRCs follow a developmental program that is different from cones. Rods develop from a defined lineage of retinal progenitor cells, and their neurogenesis is protracted and delayed relative to cone photoreceptors (Raymond, 1985, Raymond and Rivlin, 1987, Morris and Fadool, 2005, Morris et al., 2008a). Müller cells are the last retinal cells to differentiate. Although they are the principle glial cell type in the retina, they differentiate from the same pools of progenitor cells that give rise to the retinal neurons (Turner and Cepko, 1987, Bernardos et al., 2005, Jadhav et al., 2009).

After initial embryonic retinal neurogenesis in the zebrafish, there remains a specialized domain within the retina that contains continuously proliferating stem cells and retinal progenitor cells to meet the demands of a continuously growing retina. Because the zebrafish displays indeterminate growth, the eye gets larger throughout the life of the animal. As a result, the retina must expand, and new neurons and glia must be incorporated into the growing retinal tissue to maintain visual acuity. The source of these new retinal cells is the ciliary marginal zone (CMZ), a specialized area at the far periphery of the neural retina where populations of retinal stem cells are maintained that give rise to both new retinal cells as well as RPE cells (Raymond et al., 2006, Stenkamp, 2007, Reinhardt et al., 2015).
Figure 1.2: The order of differentiation of retinal cell types in vertebrates is highly conserved. A) Differentiation of subsets of zebrafish RPCs to GCs begins around 24 hpf and is largely completed by 36 hpf, prior to the onset of AC and HC differentiation at 36 hpf. The other INL neuron type (BPCs) begin to differentiate around 60 hpf. In the ONL, cone PRCs start to differentiate at 48 hpf, and rod PRCs start to appear at 55 hpf. Müller glia, that last retinal cell type to develop, start to differentiate in the INL around 60 hpf. B) Saggital view of (outward facing zebrafish eye) diagram depicting sequential, fan-shaped waves of differentiation of RPCs into retinal neurons starting at the ventral patch. The cells of the GCL are generated from 24-36 hpf, INL neurons (besides BPCs) are generated from 36-48 hpf, and ONL neurons start to differentiate from 48-60 hpf.
1.2.2.2 Regulation of proliferation of retinal progenitor cells during development

The relatively homogeneous population of proliferating, pseudo-stratified neuroepithelial cells of the primordial eye that form the retina are derived from the inner layer of the optic cup. These progenitor cells are maintained in the cell cycle until the appropriate extrinsic and intrinsic cues push subsets of retinal progenitor cells (RPCs) within a respective nuclear layer towards cell cycle exit and differentiation. The RPCs balance proliferation and cell cycle exit with expression of fate determinant genes to ensure the correct number and proportion of each neuron type as well as Müller glia are generated (Jusuf et al., 2011, Ng Chi Kei et al., 2016). Proliferation of RPCs is highly regulated, and keeping RPCs in the cell cycle depends upon upregulation of proliferative signals from the Notch-Delta pathway which results in increased expression of downstream effectors that repress typically pro-neural and pro-differentiation factor expression (Dvoriantchikova et al., 2015, Taylor et al., 2015). In addition, expression of intrinsic cell cycle progression factors such as cyclinD1, repression of cell-autonomously acting factors such as p27kip1 and p57kip2, as well as extrinsic cues from factors such as transforming growth factor alpha (TGFα), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), Wnt/Frizzled and epidermal growth factor (EGF) are all required to keep a RPC in the cell cycle (Lillien and Cepko, 1992, Van Raay and Vetter, 2004, Hashimoto et al., 2006).

1.2.2.3 Cell cycle exit and retinal cell specification during development

Neurogenesis in the developing zebrafish retina occurs in several waves of differentiation of post mitotic progenitor cells. These neurogenic waves are highly conserved, and arise from many of the same mechanisms and expression of orthologous factors observed in the morphogenetic wave during Drosophila retinogenesis (Neumann and Nuesslein-Volhard, 2000, Kay et al., 2005). The neurogenic waves utilize hedgehog (hh) signaling via the vertebrate homolog sonic hedgehog (shh) with minor contributions from tiggy winkle hedgehog (twhh) as well as indian hedgehog (ihh). During the initial differentiation of RPCs, extrinsic cues stimulate expression of intrinsic factors that regulate retinal
neurogenesis. The key transcription regulators that are expressed as a result of extrinsic cues belong predominantly to three major classes: the homeobox-containing factors, members of the basic helix-loop-helix (bHLH) motif containing family of transcription factors, and nuclear hormone receptors (Vetter and Brown, 2001, Dyer, 2003, Stevens et al., 2011, Suzuki et al., 2013).

A transition zone that defines which cells will differentiate into the retina and RPE versus the optic stalk is created by expression of pax6 and pax2 respectively, and is generated and maintained by midline hh signals (Macdonald et al., 1995). In the early eye primordia, the homeodomain containing transcription factor pax6 is expressed in all retinal precursor cells, and this expression persists through the early stages of retinal neurogenesis when pax6 is downregulated in all retinal cell types except ganglion and amacrine cells (Hitchcock et al., 1996, Nornes et al., 1998). There are retinal homeobox (rax or rx) genes expressed during zebrafish eye development. Rx1 and rx2 are expressed in the anterior neuroepithelium and become restricted to retinal progenitor cells where expression persists until they are downregulated as neurogenesis proceeds and are then re-expressed in cone PRCs and subsets of INL neurons. Rx3 is also expressed in anterior neuroepithelial cells but only appears in a few INL cells later in development, and is the only rx gene shown to be required for eye development in zebrafish (Chuang et al., 1999, Loosli et al., 2003).

The bHLH genes that are expressed during retinogenesis are pro-neural; their expression in RPCs promotes the adoption of a neuronal fate. The first neurogenic wave is initiated by hh signaling in a small portion of precursor cells adjacent to the optic stalk and the pax6/pax2 expression boundary in the ventronasal quadrant of the retina (Stenkamp et al., 2000). Differentiation of ganglion cells is initiated by upregulating expression of the bHLH transcription factor ath5/atoh7 in conjunction with expression of the POU-domain homeobox transcription factor pou4f (DeCarvalho et al., 2004, Kay et al., 2005).
The second wave of $hh$ signaling initiates differentiation of amacrine, horizontal, and bipolar cells in the INL and arises independently of $hh$ signaling from the GCL and is not dependent upon expression of $ath5$ from the ganglion cells (Shkumatava et al., 2004). RPCs in the INL retain the plasticity to produce the three types of neurons found in the INL, and the type of neuron generated depends upon the relative levels of various transcription factors that are expressed in INL RPCs. Here, expression of $foxn4$ promotes RPC differentiation towards the amacrine and horizontal cell fate and is negatively regulated by $vsx2$ (Li et al., 2004, Vitorino et al., 2009). In addition, continued expression of $vsx2$ as well as expression of $foxn4$, $pax6$, and $neuroD$ in RPCs in the INL result in amacrine cell fate determination. To specify subtypes of amacrine cells, expression of the bHLH transcription factors $bhlhb5$ and $garhl2$ specify GABAergic or glycinergic amacrine cell fates respectively (Duquette et al., 2010, Jusuf et al., 2012). Expression of $prox1$ is required for horizontal cell specification in INL RPCs that express $vsx2$, $foxn4$, low levels of $pax6$, and not $neuroD$ (Dyer, 2003). Bipolar cell specification requires $vsx2$ and $ascl1a$ expression (Stenkamp, 2007).

The last retinal neurons formed are the PRCs in the ONL. During embryonic retinal development, both rod and cone PRCs are derived from a common progenitor cell pool, but in the mature retina, rod PRCs are derived from a separate lineage than cones (Morris and Fadool, 2005). The RPCs that give rise to PRCs express the required transcription factors $otx2$, $crx$, $neuroD$, $ascl1a$, and the retinoid receptors $rar$ and $rxr$ (Stenkamp, 2011, Suzuki et al., 2013, Wang et al., 2014). Expression of $otx2$ causes transactivation of $crx$, which in turn acts with $rorβ$ to induce expression of pro-rod genes such as $nrl$ and $nr2e3$ which leads to suppression of cone-specific genes biasing the RPC towards the rod PRC fate (Jia et al., 2009, Wang et al., 2014). $Crx$-expressing cells that do not receive the necessary signals to drive them towards a rod PRC fate and do not express pro-rod factors will express cone-specific factors and differentiate into the various cone subtypes instead. Specification of the four cone subtypes found in zebrafish relies upon expression levels of the thyroid hormone receptor $trβ2$ as well as various retinoid X
receptor (rrx) genes and various retinoic acid receptor related orphan receptor (ror) genes during terminal symmetric divisions of precursor cells (Suzuki et al., 2013). Patterning of the cone mosaic in the tangential plane of the ONL relies upon cell-cell and cell-surface interactions by Notch-Delta mediated signaling (Bernardos et al., 2005).

The only intrinsic glial cell in the retina, and the last type of retinal cell to differentiate are the radial Müller glia. Activated Notch-Delta signaling typically favors the maintenance of proliferating stem and progenitor cells as well as glial cell differentiation while precluding differentiation to neuronal cells. (Livesey and Cepko, 2001). During development, the RPCs that retain relatively higher levels of Notch activity differentiate into Müller glia. Transgenic zebrafish embryos that globally express constitutively active Notch signaling experience a repression of neuron differentiation and increased Müller glia populations in the retina (Scheer et al., 2001).

The tightly regulated and highly coordinated processes of proliferation, specification, migration, differentiation, and synaptogenesis are necessary for proper retinal development to occur. In addition, multiple series of cell-specific apoptotic cell death further refine each retinal layer, although the amount of apoptosis in the developing zebrafish retina is minimal when compared to mammals (Biehlmaier et al., 2001). After the initial embryonic development and establishment of the retina, there are also a variety of processes that regulate maturation, maintenance, and cell homeostasis in the neural retina as well as the RPE. Misregulation of or failure to complete any of these functions could lead to cell-type specific or global retinal cell degeneration and multiple types of retinal degenerative diseases that ultimately result in disruption or complete loss of visual function.

1.3 The Notch-Delta signaling pathway during retinal development

1.3.1 Notch-Delta signal transduction
The Notch-Delta signaling pathway is highly conserved juxtacrine signaling system present in most multicellular organisms that establishes embryo polarity, coordinates cell clocks during somitogenesis, and regulates lateral inhibition, proliferation, and gliogenesis (Furukawa et al., 2000, Scheer et al., 2001). In addition, components of the Notch pathway are upregulated in the retina following injury (Kassen et al., 2007, Ghai et al., 2010). The Notch signaling pathway is a master regulator during neurogenesis, because it influences expression of transcription factors at or near the very top of entire gene regulatory networks. Activation of Notch signaling influences expression of thousands of genes at various downstream regulatory levels, and modulates numerous other intracellular and extracellular signaling events.

During canonical Notch-Delta signaling, initially all cells within an equivalence group will express both the Delta ligand and the Notch receptor. However, once lateral inhibition starts to take place, a signaling cell will begin to express a larger proportion of a ligand of the DSL (Delta/Serrate/LAG-2) class of single-pass transmembrane proteins (the vertebrate orthologs include multiple Delta, Delta-like, and Jagged ligands) than its neighboring cells. Typically, the signaling cell will be the most differentiated cell in a pool of equipotent progenitors that will eventually give rise to a specific tissue type or large structure. Since all of the cells within an equivalence group are expressing both the ligand and the receptor, and because proximal cells in the group are continuously signaling and re-expressing components of the Notch pathway, the system is always in flux until the most differentiated cells start to express predominantly the Delta ligand. The signal-receiving cell expresses the single-pass transmembrane Notch receptor (of which there are multiple paralogs in vertebrates). The neighboring cells (which also still express limited numbers of the Delta ligands) now contain a relatively higher number of Notch receptors embedded in the plasma membrane, and as a result will receive a higher dose of Notch activation (Bray, 2006).
Both the Delta ligand and the Notch receptor contain a large extracellular domain, as well as a small transmembrane and intracellular domain. There are numerous epidermal growth factor like (EGF-like) repeats in the long extracellular domain of the protein. The EGF-like repeats (typically 36-42 on the Notch receptors, and 25-30 on the Delta ligands) consist of 40 amino acid residues per repeat, and their structure is defined by six conserved cysteine residues that form three conserved disulfide bonds per EGF-like repeat (Kopczynski et al., 1990, Nye and Kopan, 1995). Furthermore, the EGF-like repeats of the extracellular domain can be further modified by the addition of various sugar groups by fringe genes which influence the binding affinity of the Notch receptor to either Delta or Jagged ligands (Shao et al., 2002, Taylor et al., 2014).

When a signaling cell expressing a Delta-like ligand comes into direct physical contact with a signal-receiving cell expressing a Notch receptor, a series of proteolytic cleavage events liberates the Notch intracellular domain (NICD) from the cell membrane and the soluble NICD (which contains two flanking nuclear localization sequences) diffuses freely through the cytoplasm where it is trafficked into the nucleus (Lai, 2004). ADAM-family metalloproteases initiate cleavage at the S2 site at the extracellular side of the transmembrane domain, releasing the majority of the extracellular domain to the Delta ligand which then undergoes endocytosis and re-processing by the signaling cell. A second proteolytic cleavage event at the S3 site on the intracellular side of the transmembrane domain of Notch is mediated by γ-secretase, freeing the NICD from the membrane into the cytosol. In the nucleus, the NICD interacts with various members of the CBF1/Su(H)/Lag1 (CSL) family of proteins and the resulting NICD/CSL complex acts as a transcriptional activator by binding to Suppressor of Hairless (Su(H)) sites on the promoters of target genes (Lai, 2004, Bray, 2006, Nelson et al., 2007). Targets of Notch signaling include members of the hairy/enhancer of split (hes) and hairy-related (her) gene families (Fischer and Gessler, 2007).
Notch activity in the retina has been shown to be necessary for maintenance of pools of stem and progenitor cells and mediates lateral inhibition of neuron differentiation and synchronizes differentiation of RPCs (Nelson et al., 2007). Notch signaling plays a role in repressing the specification of earlier born cell types to maintain the necessary RPC pool size so that later born retinal cells can differentiate, and is one mechanism of selecting photoreceptor subtypes and Müller glia (Scheer et al., 2001, Bernardos et al., 2005, Dvoriantchikova et al., 2015).

1.3.2 Notch-Delta signaling during interkinetic nuclear migration in the developing retina and in retinal stem cell niches in the mature retina

Although it is a juxtacrine signaling pathway and therefore doesn’t rely upon secreted signals, the effects of Notch mediated signaling are surprisingly dosage dependent. The dosage of Notch signaling that an individual proliferating RPC or differentiating progenitor will receive depends upon migration of the nucleus within a defined apical-basal boundary in a process called interkinetic nuclear migration (Buchman and Tsai, 2008). Throughout the nervous system, nuclear migration is prominent during both proliferative and post-mitotic phases of development. During embryonic retinal development, proliferating progenitor cells have processes that extend the width of the neural retina. The cell body and nucleus are on the apical side of the neuroepithelium closest to the RPE upon entry into G1 phase. Progression of G1 phase proceeds as the cell nuclei move toward the basal side of the retinal neuroepithelium. They pass through G1 and enter S-phase near the end of this migration near the lens. Upon completion of the basal migration, the cell nuclei migrate back to the apical side during G2, where they enter M-phase at the apical boundary. Notch expression in these cells and subsequent Notch activity increase nearer the apical side of the neuroepithelium, and Delta ligands are expressed in a gradient with the opposite orientation (Baye and Link, 2008). Upon completion of mitosis, the daughter cells repeat this entire process. This nuclear migration of RPCs and differentiating precursors leads to regulation of retinal neurogenesis of the various retinal cell types by altering exposure to Notch.
signals through the apical-basal Notch gradient while at the same time individual cells are moving through different competence states in a time dependent manner (Clark et al., 2012, Aggarwal et al., 2016).

The circumferential germinal zone (CGZ), also referred to as the ciliary marginal zone (CMZ) is found at the margin where the neural retina and RPE meet the iris epithelium. It consists of stem cells and retinal progenitor cells and has long been considered a remnant of the embryonic retina due to the proliferative nature of the cells found here (Johns, 1977). Recent studies, however, have shown that although at the genetic level there are many of the same regulatory networks and transcription factors are expressed in both the embryonic retina and the CMZ, cells in the ciliary margin have many distinctive features from those of the RPCs in the developing retina (Wehman et al., 2005). In the teleost retina, it is the CMZ that provides new RPCs as the retina grows, and these RPCs have the capability to contribute to every retinal cell type during retinal growth except for rod PRCs. Notch-Delta signaling persists in the CMZ of the teleost retina throughout the life of the animal (Zupanc and Sirbulescu, 2011). In addition, it has been shown that functional Notch-Delta signaling is required for not only for normal retinal growth and neurogenesis, but also for stimulating proliferation in the CMZ following retinal damage (Uribe et al., 2012, Fischer et al., 2013). In the CMZ, many of the downstream effectors of the Notch-Delta signaling pathway are persistently expressed, and act to keep stem cells and RPCs in the cell cycle in the peripheral most areas of the retina (Raymond et al., 2006).

Another retinal stem cell niche found in the teleost retina that is heavily dependent upon the Notch-Delta signaling pathway is associated with the cell bodies of Müller glia in the INL (Raymond et al, 2006, Lenkowski and Raymond, 2014). In the uninjured zebrafish retina, rod precursor cells are derived from slowly proliferating progenitors in the INL associated with subsets of Müller glia. Notch-Delta signaling is required for the postmitotic retinal progenitor cells that differentiate into Müller glia during initial embryonic development to maintain a glial cell fate (Nelson et al., 2011). In the mature retina,
there are subsets of slowly proliferating Müller glia in which Notch-Delta signaling is still active, and these cells give rise to the rod PRC lineage (which will be discussed in detail in chapter 2). In addition, the Müller cells in the zebrafish retina are capable of de-differentiating, proliferating, and generating RPCs that will give rise to all retinal cell types following injury (Thummel et al., 2008).

1.4 Zebrafish as a model organism

The zebrafish, *Danio rerio*, is a small freshwater teleost fish native to the streams of the southeastern Himalayan region and belongs to the minnow family. In addition to being a popular fish for aquarists, the zebrafish has also been used extensively in scientific studies (Li et al., 2013). The zebrafish presents an excellent model system for studying a wide variety of biological processes, including development and regeneration of the vertebrate retina.

As a model organism, the zebrafish possesses many desirable characteristics. It has remarkably high fecundity for a vertebrate; a typical cross between a single mating pair can generate well over a hundred viable offspring. Due to the large number of embryos per clutch, experiments can be designed and performed with results that yield high statistical power. In addition, the zebrafish exhibits external fertilization and development, and the fertilized embryos remain optically transparent until onset of pigmentation (which can be blocked chemically or avoided altogether through use of albino mutant strains), thus permitting direct observation of the developing embryos without the requirement of sophisticated imaging techniques. Development of the embryos occurs rapidly, with the first cell division taking place as early as 0.5 hours post fertilization (hpf), and successive cell divisions taking place roughly every half hour. As quickly as 24 hpf the embryo is already immediately identifiable as a small vertebrate; it has a recognizable head, tail, eyes, recognizable somites, a beating heart, as well as a yolk sack that provides nourishment until the larval stage. By 72 hpf, embryonic development is largely completed, and most of the major organs and essential tissues have already been established and are
At this point, the embryos respond to touch stimuli and each individual embryo has already exited the protective chorion through lateral swiveling of the tail. In addition, visually evoked responses can be measured and quantified at 5 dpf through relatively high throughput methods such as visualization of the optokinetic response which can be easily observed through various low tech devices (Mueller and Neuhauss, 2010). By 5 days post fertilization, the zebrafish larvae is actively swimming, hunting/scavenging, and avoiding predation. The zebrafish larvae begin metamorphosis from 10-14 days post fertilization (dpf), at which point they start to refine fin epithelial tissues, start scale formation, and show early signs of sexual dimorphism and sexual maturation. With optimal growth conditions, the zebrafish can achieve sexual maturity as quickly as 6 weeks post fertilization (Hsiao and Tsai, 2003).

1.4.1 Zebrafish as a genetic and molecular vertebrate model

Although it is a relatively new model organism within the field of embryology and developmental biology when compared to classically studied animals (various species of *Drosophila*, *Gallus*, and *Mus* genera), the zebrafish quickly came to the forefront of study within the field due to the relative ease of genetic manipulation within this vertebrate model and the advent of modern molecular techniques.

The zebrafish has a fully sequenced and nearly fully annotated genome which is currently on the 10th iteration of the reference assembly (Howe et al., 2013). The zebrafish has well developed genetic resources, and many mutant and transgenic lines are available to the research community. The zebrafish has been a go-to model organism for forward genetic screens in vertebrates due to the large clutch size, fast generation time, and ease of mutagenesis through application of either N-ethyl-N-nitrosourea (ENU) or ethyl methansulfonate (EMS) alkylating agents to sperm. In addition, knockdown (via morpholino mediated RNA splicing or translation interference) as well as misexpression of selected genes via mRNA or plasmid injection are common methods of studying the effects of specific genes in zebrafish.
(Nasevicus and Ekker, 2000). Besides gene knockdown, genomic knockout techniques have also recently become available in the zebrafish, thus greatly expanding the types of reverse genetic approaches to that can be employed. Both transcription activator-like effector nuclease (TALEN) and clustered regularly-interspaced short palindromic repeats with Cas9 nuclease (CRISPR/Cas9) have been successfully used to generate targeted mutations and site-specific knock-in lines (Hisano et al., 2015, Varshney et al., 2015, Prykhozhij et al., 2016).

Another advantageous aspect of zebrafish as a vertebrate model organism that is permitted by their small size and high fecundity as well as being an aquatic species is their use in quantitative, high-throughput drug screens and drug discovery applications. A zebrafish embryo easily survives in a very small volume of fluid, enabling small quantities of potentially expensive compounds to be added to the media to determine efficacy and dosage requirements in a developing vertebrate with relatively little expense (Miscevic et al., 2012). Although it is comparatively more labor intensive and expensive to use zebrafish for chemical screens rather than cultured cells, it has been repeatedly been documented that traditional high-throughput drug screening methods such cell-based assays are often unreliable because of limited physiological relevance and do not yield nearly as much useful information as organismal level studies (Ryan et al., 2016). In addition, the use of zebrafish in drug screens permits direct observation of the effects of compounds within the context of the biological networks in which they act (Wist et al., 2009). Numerous biological processes have been studied and potential therapeutic compounds for diseases ranging from cancer, cardiac disease, depression, and even hair loss and have been evaluated using zebrafish (Miscevic et al., 2012, Zulkhernain et al., 2014, Stawicki et al., 2015, Fonseka et al., 2016).

1.5 Hairy-Related 4

1.5.1 Overview of basic-Helix-Loop-Helix-Orange (bHLH-O) transcription factors
*Hairy-related (her)* genes (also referred to as *hairy* and *enhancer of split related*) are the highly evolutionarily conserved zebrafish counterparts of the *Hairy* and *Enhancer-of-split* type genes in *Drosophila*, and of the *Hes/Hey* genes in mammals, all of which are members of the basic-Helix-Loop-Helix-Orange (bHLH-O) class of DNA binding proteins (Muller et al., 1996). Genes in the *bHLH-O* family are ancient eukaryotic transcription factors that can only be found in metazoans. They have undergone numerous duplication events throughout metazoan evolution which has resulted in not only a large number of these types of genes per species, but also extensive subfunctionalization and differential expression even within the same tissue (Davis and Turner, 2001, Zhou et al., 2012). There are four general types of *bHLH-O* proteins found throughout the animal kingdom: Stra13, Hey, E(*spl*), and Hairy (which include Hes and Her), all of which have very similar residue sequences and protein structure (Davis and Turner, 2001).

Members of the *bHLH-O* family, including the zebrafish *Hairy-related 4* (Her4), are transcriptional regulators known to play critical roles in many physiological processes including cellular differentiation, cell cycle arrest, apoptosis, self-renewal ability, and have also been extensively studied for their roles in metastasis, antagonizing drug-induced apoptosis, and crosstalk with carcinogenic signaling pathways (Liu et al., 2015). The *bHLH-O* genes act as downstream effectors of various signaling pathways, but are most commonly associated with being effectors Notch-Delta signaling and mediating cross-talk between Notch and other signaling pathways (Delidakis et al., 2014).

The first *bHLH-O* gene to be identified (*E(spl)*) was a spontaneous dominant mutation in irradiated *Drosophila* that enhanced the small eye phenotype caused by a recessive allele of *Notch* (Welshons, 1956). After numerous other *bHLH-O* genes were identified, it was recognized that the loss of function of these genes commonly resulted in hyperplasia of the embryonic nervous system at the expense of larval epidermis, caused by misspecification of most ectodermal cells as neural precursors (what is termed a “neurogenic” phenotype) (Knust et al., 1987, Knust and Campos-Ortega, 1989, Knust
et al., 1992). These observations lead to the early conclusions that bHLH-O genes primarily mediated lateral inhibition downstream of the Notch pathway, although it was soon discovered that they have a much more diverse role in regulation of gene function.

During development, bHLH-O genes have been shown to be critically important in regulating a variety of processes including the segmentation clock, somitogenesis, neural tube development, floor plate development, CNS development, primary neuron, hypochord development, and are transiently expressed in most developing tissues (Takke et al., 1999, Sun et al., 2007). In addition, they have been shown to play similar roles in the regeneration of CNS tissues (Zupanc and Sirbulescu, 2011).

1.5.2 Structure and function of the bHLH-O transcription factor, her4

Her4 is the zebrafish ortholog of the mammalian HES5 (Hortopan and Baraban, 2011), a basic-Helix-Loop-Helix transcriptional repressor directly regulated by the Notch-Delta signaling pathway in mouse (Kageyama and Ohtsuka, 1999). The DNA-binding basic domain (b) of her4 binds to palindromic E-box (CANNTG) and N-box (CACNAG) sequences on the promoters of target genes (Massari and Murre, 2000). Adjacent to the basic domain are two amphipathic α-helices separated by a loop (HLH) that serve as a dimerization domain as well as an interface for additional protein-protein interactions (Massari and Murre, 2000). Following the HLH region is the orange domain (O). It is composed of two additional α-helical structures that serve as a site for additional protein interactions and can act as a transcriptional repressor when fused to a DNA-binding domain (Dawson et al., 1995, Fischer and Gessler, 2007). The C-terminal end of Her4 contains the characteristic WRPW (Trp-Arg-Pro-Trp tetrapeptide) motif of bHLH-O transcription factors, and is required for strong transcriptional repression (Fisher et al., 1996). The WRPW motif recruits various transducin-like enhancer of split (tle) co-repressors (orthologs of Groucho in Drosophila) as well as histone deacetylases, and once bound to the WRPW tetrapeptide, greatly increase the transcriptional repressive properties of Her proteins (Fischer and Gessler, 2007).

1.5.3 Mechanisms of Her mediated gene regulation
The targets and mode of Her mediated regulation are varied. Expression of different bHLH-O genes in different combinations (and in different tissues) can result in a vastly different transcriptional profile through various mechanisms (Gazave et al., 2014). Numerous genes contain E-box (CANNTG) and N-box (CACNAG) sequences in their promoter regions as well as throughout the gene in which the bHLH domains of various Her dimerization partners bind. Active repression is dependent upon binding of the Her to DNA is dependent upon homo- or heterodimerization of Her transcription factors, but not dependent upon interaction with a different heterodimerization partner with the Orange domain of Her (Kageyama et al., 2007). Once bound to an E- or N-box, the Orange domain can repress transcription directly by blocking access of other transcription factors to an adjacent DNA target sequence. In addition, the Orange domain interacts with numerous other proteins to form heterodimers which in turn can both repress or activate transcription (Dawson et al., 1995). In addition, when Her transcription factors are bound to DNA, the WRPW motif will recruit the co-repressors or histone deacetylases which results in repression of a target gene or of multiple genes within a given loci (Fisher et al., 1996).

When not bound to the DNA, Her still has the capability to passively regulate gene expression. The bHLH and Orange domains of Her still readily interact with binding partners (usually pro-neural bHLH transcription factors), sequestering them from the DNA binding sites of their own target genes through steric hindrance. The WRPW motif, in addition to recruiting repressors, acts as a polyubiquitylation signal (Kang et al., 2005). When not bound to DNA, either free in solution or dimerized with a binding partner, Her proteins are rapidly polyubiquitylated and quickly undergo proteosomal lysis. The Her proteins themselves have very short half-lives and a high turnover rate (~20 minutes), but the heterodimerization partner may be quickly titrated out if it does not have an equally high turnover rate itself (Hirata et al., 2002, Coglievina et al., 2010).

1.5.4 The zebrafish hairy/enhancer of split gene superfamily
The zebrafish genome contains 26 members of the *hairy/enhancer of split* gene superfamily of bHLH-O transcriptional repressors. These include 3 of the *hairy/enhancer of split* (*hes*) class, 4 of the *hes-related with YRPW motif* (*hey*) class, and 19 of the *hairy-related* (*her*) class bHLH-O genes (Table 1.1). These three classes of *hairy*-type genes are structurally very similar to each other at the protein level when compared to each other, and many have redundant functions and expression patterns across the three groups (Figure 1.3). The peptide sequences in the major domains contain few polymorphisms between the three *hairy*-type classes. There are, however, numerous polymorphisms (both in residue and sequence length) in inter-domain regions of the proteins. At the nucleotide sequence level, there are numerous polymorphisms throughout the entire gene, although less so in conserved domains. Intron regions vary widely in number, sequence, and length between classes. In addition, the only common promoter elements to be found (and only among the Notch-responsive genes) are consensus Su(H) binding sites which are necessary for NICD/ CSL complex binding.

Various members in the three *hairy*-type classes have been shown to be regulated not only by Notch-Delta signaling, but also by Hh, VegF, Nodal, FGF, and retinoic acid signaling (Holzschuh et al., 2005, Latimer et al., 2005, Morrow et al., 2009, Radosevic et al., 2011) (Table 1.1). *Hairy-related* genes tend to be self-regulating due to having numerous E- and N-box sequences within their respective promoters that lead to feedback loops which result in cyclic gene expression. *Her* genes are also key interconnecting factors that allow for crosstalk between multiple signaling pathways (Hue et al., 2012). Another level of regulatory complexity is added when considering that these transcription factors are co-expressed or differentially expressed within the same tissues in various spatiotemporal patterns. This is due to heterodimers (with respect to bHLH-O proteins dimerizing via the bHLH domain) having different binding affinities for the E- and N-box sequences in target promoters than homodimers or other permutations of heterodimers involving the numerous Her transcription factors (Davis and Turner, 2001). It must also be noted that because the zebrafish contains such a large compliment of *her* genes
(in part due to a whole genome duplication in the teleost lineage), there is much functional redundancy. As with many other genes in the zebrafish, occasionally only very mild (or no observable) phenotypes can be observed when knocking down or knocking out single her genes that are expressed in the same spatiotemporal pattern as other her genes within the same developing tissue.

**Figure 1.3: The zebrafish bHLH-O proteins.** The zebrafish genome contains 3 classes of bHLH-O transcription factors; 3 Hairy (Hes), 4 Hairy/Enhancer of Split-Related with YRPW motif (Hey), and 19 Hairy/Enhancer of Split-Related (Her). The DNA-binding basic domain (b) of her4 binds to palindromic E-box (CANNTG) and N-box (CACNAG) DNA sequences. The HLH domain is composed of two amphipathic α-helices (H) separated by a loop (L) that serve as a dimerization domain as well as an interface for additional protein-protein interactions. The Orange domain (O) is composed of two additional α-helical structures that bind to DNA to inhibit binding of pro-neural bHLH genes and also serves as a site for additional protein interaction. The WRPW tetrapeptide motif (in Hes and Her proteins) and YXXW motif (in Hey proteins) associates with co-repressors and HDACs which lead to strong repression of target genes.

**1.6 Rationale, hypotheses, and specific aims**

Previous studies have shown that bHLH-O genes play diverse roles in many physiological processes including cellular differentiation, cell proliferation, cell cycle arrest, oscillation clock cycling, and crosstalk between signaling pathways. In addition, bHLH-O genes are transiently expressed in most tissues during embryonic development. Many members of the bHLH-O family have been shown to be
downstream effectors of the Notch-Delta signaling pathway and typically repress expression of pro-
neural genes and keep stem and progenitor cells in the cell cycle. There are, however, multiple bHLH-O
genes that are negatively regulated by Notch, or are downstream of other signaling pathways
altogether. To add to the complexity of understanding the gene regulatory networks that individual
bHLH-O genes are involved with, expression of a single bHLH-O transcription factor can be regulated by
different signaling pathways depending on what tissue it is expressed in. The hairy/enhancer of split-
related 4 and 9 (her4 and her9) bHLH-O transcription factors have been found to be upregulated in the
mature zebrafish retina following rod PRC degeneration, but the roles that these genes play in the retina
have remained largely unexplored. The main questions addressed in this dissertation are: What are the
gene regulatory networks that control expression of her4 and her9 in the retina? What are the roles of
her4 and her9 during retinal development? What are the roles of these genes during regeneration of the
zebrafish retina? Reverse genetic analysis, biochemical analysis, transgenesis, and various molecular
approaches have been used to address these questions in this study.

The aims of this dissertation were to determine the regulatory networks that control her4 and
her9 expression in the retina and to determine their functions during retinal neurogenesis. The initial
hypothesis was that her4 and her9 play a role in retinal neurogenesis, specifically by maintaining pools
of proliferating stem and progenitor cells downstream of the Notch-Delta signaling pathway. Presented
here is evidence that her4 is a primary effector of Notch-Delta signaling during retinal development, and
that her4-expressing cells in the INL of the mature retina give rise to the rod PRC lineage. Separately, we
have shown that her9 expression in the developing retina is regulated by retinoic acid signaling, and that
her9 plays a role in the development of the retinal vasculature. This work will be organized into the
following aims:
Specific Aims:

I. Determine if her4 is downstream of the Notch-Delta signaling pathway.
   a. Assess expression her4 following manipulation of Notch-Delta signaling.

II. Characterize the effects of her4 misexpression in the developing retina at the molecular and cellular level.
   a. Using morpholino mediated knockdown and mRNA overexpression, determine the function of her4 during embryonic retinal development.
      i. Comparison of her4 morphant with genetic and pharmacological based knockdown of the Notch pathway.
      ii. Comparison of her4 morphant and control retinas using immunohistochemistry for cell identity, morphology, and arrangement.

III. Determine the contribution of her4-expressing cells in the mature zebrafish retina.
   a. Characterize her4 expression in the adult retina.
   b. Generate a transgenic line of zebrafish that expresses a photoconvertible fluorescent reporter driven by the her4 promoter.
   c. Track the lineage of her4-expressing cells in the INL of a retina experiencing constitutive rod degeneration and regeneration.
   d. Track the lineage of her4-expressing cells in the CMZ.
   e. Characterize expression of her4 in the retina following acute light damage.

IV. Determine if her9 is regulated by retinoic acid signaling.
   a. Assess expression of her9 following manipulation of retinoic acid signaling.
   b. Determine if retinoic acid signaling plays a role in retinal vasculogenesis.

V. Characterize the effects of her9 misexpression in the developing retina and retinal vasculature network.
Using morpholino mediated knockdown and mRNA overexpression, determine the function of her9 during development of the retinal vasculature.

VI. Generate her9 knockout lines using CRISPR/Cas9 genome editing technology.
   a. Characterize genome lesions and find germline transmitted her9 mutant alleles.
   b. Characterize the her9 mutant phenotype.

Aims I, II, and III are included in Chapter 2.
Aims IV, and V are included in Chapter 3.
Aim VI is included in Chapter 4.

Chapter 1 Tables

Table 1.1: The zebrafish bHLH-O superfamily of transcription factors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Regulatory Pathway(s)</th>
<th>Human Ortholog</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hes2.1 (hairy/enhancer of split 2.1)</td>
<td>Notch</td>
<td>HES2</td>
<td>(Zhou et al., 2012)</td>
</tr>
<tr>
<td>hes2.2 (hairy/enhancer of split 2.2)</td>
<td>Notch</td>
<td>HES2</td>
<td>(Zhou et al., 2012)</td>
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<tr>
<td>hes6 (hairy/enhancer of split 6)</td>
<td>FGF</td>
<td>HES6</td>
<td>(Kawamura et al., 2005)</td>
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<tr>
<td>hey1 (hairy/enhancer of split-related with YRPW motif 1)</td>
<td>Notch</td>
<td>HEY1</td>
<td>(Fischer et al., 2004)</td>
</tr>
<tr>
<td>hey2 (hairy/enhancer of split-related with YRPW motif 2)</td>
<td>Hh, VegF</td>
<td>HEY2</td>
<td>(Rowlinson and Gering, 2010)</td>
</tr>
<tr>
<td>hey1 (hairy/enhancer of split-related with YRPW motif-like)</td>
<td>Notch</td>
<td>HEYL</td>
<td>(Zhou et al., 2015)</td>
</tr>
<tr>
<td>hey4 (hairy/enhancer of split-related with YRPW motif 4) (a.k.a. helt)</td>
<td>unknown</td>
<td>HELT</td>
<td>(Peukert et al., 2011)</td>
</tr>
<tr>
<td>her1 (hairy/enhancer of split-related 1)</td>
<td>Notch</td>
<td>HES7</td>
<td>(Takke and Campos-Ortega, 1999)</td>
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<tr>
<td>her2 (hairy/enhancer of split-related 2)</td>
<td>Notch</td>
<td>HES5</td>
<td>(Cheng et al., 2015)</td>
</tr>
<tr>
<td>her3 (hairy/enhancer of split-related 3)</td>
<td>Negatively regulated by Notch</td>
<td>HES3</td>
<td>(Hans et al., 2004)</td>
</tr>
<tr>
<td>her4.1 (hairy/enhancer of split-related 4.1)</td>
<td>Notch</td>
<td>HES5</td>
<td>(Takke et al., 1999)</td>
</tr>
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<td>her4.2 (hairy/enhancer of split-related 4.2)</td>
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<td>HES5</td>
<td>(Takke et al., 1999)</td>
</tr>
<tr>
<td>her4.3 (hairy/enhancer of split-related 4.3)</td>
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<td>HES5</td>
<td>(Takke et al., 1999)</td>
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<tr>
<td>her4.4 (hairy/enhancer of split-related 4.4)</td>
<td>Notch</td>
<td>HES5</td>
<td>(Takke et al., 1999)</td>
</tr>
<tr>
<td>her4.5 (hairy/enhancer of split-related 4.5)</td>
<td>Notch</td>
<td>HES5</td>
<td>(Takke et al., 1999)</td>
</tr>
<tr>
<td>her5 (hairy/enhancer of split-related 5)</td>
<td>FGF, negatively regulated by Notch</td>
<td>HES7</td>
<td>(Dyer et al., 2014)</td>
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<td>her6 (hairy/enhancer of split-related 6)</td>
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<td>HES1</td>
<td>(Pasini et al., 2004)</td>
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<td>her7 (hairy/enhancer of split-related 7)</td>
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<td>HES7</td>
<td>(Oates et al., 2005)</td>
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<tr>
<td>her8a (hairy/enhancer of split-related 8a)</td>
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<td>HES6</td>
<td>(Chung et al., 2011)</td>
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<td>her8.2 (hairy/enhancer of split-related 8.2)</td>
<td>Unknown</td>
<td>HES6</td>
<td>(Wang et al., 2009)</td>
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<td>her9 (hairy/enhancer of split-related 9)</td>
<td>Nodal, BMP, RA</td>
<td>HES1, HES4</td>
<td>(Bae et al., 2005, Latimer et al., 2005, Radosevic et al., 2011)</td>
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<td>her11 (hairy/enhancer of split-related 11)</td>
<td>Negatively regulated by Notch</td>
<td>HES7</td>
<td>(Sieger et al., 2004)</td>
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<td>her12 (hairy/enhancer of split-related 12)</td>
<td>Notch</td>
<td>HES5</td>
<td>(Shankaran et al., 2007)</td>
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<td>HES7</td>
<td>(Dias et al., 2012)</td>
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<td>HES5</td>
<td>(Shankaran et al., 2007)</td>
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<td>her15.2 (hairy/enhancer of split-related 15.2)</td>
<td>Notch</td>
<td>HES5</td>
<td>(Shankaran et al., 2007)</td>
</tr>
</tbody>
</table>
Chapter 2: Tracking the fate of her4 expressing cells in the regenerating retina using her4:Kaede zebrafish

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Key words: Her4; Hes5; Müller glia; Regeneration; Retina; Stem cells; Zebrafish


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N.B. For this dissertation, figure numbers, headings, and text were modified to match dissertation style. Characterization of her4 during embryonic retinal development (Sections 2.3.1-2.3.2, and Figures 2.1-2.6 and 2.S1-2.S2) were added. Supplemental Figure 2.S7 was added.

2.1 Abstract

The Basic-Helix-Loop-Helix-Orange (bHLH-O) transcription factor Hairy-related 4 (her4) is a downstream effector of Notch-Delta signaling that represses expression of typically pro-neural genes in proliferative domains of the central nervous system. Notch-Delta signaling in the retina has been shown to increase in response to injury and influences neuroprotective properties of Müller glia. In contrast to mammals, teleost fish are able to regenerate retinal neurons in response to injury. In zebrafish, her4 is upregulated in the regenerating neural retina in response to both acute and chronic photoreceptor damage, but the contribution of her4 expressing cells to neurogenesis following acute or chronic retinal damage has remained unexplored. Here we investigate the role of her4 in the regenerating retina in a background of chronic, rod-specific degeneration as well as following acute light damage. We
demonstrate that her4 is expressed in the persistently neurogenic ciliary marginal zone (CMZ), as well as in small subsets of slowly proliferating Müller glia in the inner nuclear layer (INL) of the central retina. We generated a transgenic line of zebrafish that expresses the photoconvertible Kaede reporter driven by a her4 promoter and validated that expression of the transgene faithfully recapitulates endogenous her4 expression. Lineage tracing analysis revealed that her4-expressing cells in the INL contribute to the rod lineage, and her4 expressing cells in the CMZ are capable of generating any retinal cell type except rod photoreceptors. Our results indicate that her4 is involved in a replenishing pathway that maintains populations of stem cells in the central retina, and that the magnitude of the her4-associated proliferative response mirrors the extent of retinal damage.

**Highlights:**

- We provide evidence that her4 is a primary effector of Notch-Delta signaling during embryonic retinal development.
- We characterize the expression of her4 in the regenerating zebrafish retina in response to chronic and acute photoreceptor degeneration.
- We describe a novel transgenic her4:Kaede reporter line for tracing the lineage of her4-expressing cells.
- We demonstrate that her4-expressing cells in the inner nuclear layer of the retina contribute to the rod photoreceptor lineage.
- We show that the magnitude of her4 expression during regeneration correlates with the extent of damage to the retina.
2.1 Introduction

The vertebrate retina is a highly conserved and specialized extension of the central nervous system that enables vision by transducing photons of light into an electrical signal. It is a complex, multi-layered tissue that consists of three nuclear layers: the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). In addition, there are two plexiform layers where synaptic transmission between retinal neurons occurs (Fadool and Dowling, 2008). There are six major classes of retinal neurons and one intrinsic glial cell type (Stenkamp, 2007, Fadool and Dowling, 2008). The two general classes of photoreceptor cells in the retina are the rods and cones. Rods are exquisitely sensitive to light, allow for detection of as little as one quanta of photons, and are responsible for scotopic vision. Cones allow for color vision in photopic conditions. Mutations in several photoreceptor specific genes cause chronic damage to the retina, ultimately leading to blinding retinopathic diseases such as retinitis pigmentosa (RP) (Hartong et al., 2006, Morris, 2011). In addition, photoreceptor cells are sensitive to both chronic and acute damage from natural ionizing radiation such as ultraviolet light or simply from exposure to intense white light (Abler et al., 1996).

Although the various retinal cell types as well as the overarching laminar organization are similar in cold- and warm-blooded vertebrates, mammals lack the ability to regenerate neurons of the CNS (Fischer and Bongini, 2010). Whereas both acute and chronic damage to the mammalian retina is generally permanent, the damaged zebrafish retina undergoes a robust regenerative response (Vihtelic and Hyde, 2000, Morris et al., 2005, Goldman, 2014, Gorsuch and Hyde, 2014). Following injury, Müller glia in the central regions of the zebrafish retina de-differentiate and proliferate to produce neural progenitor cells that migrate to the correct location, differentiate into the correct cell type, and functionally integrate into the existing tissue. In contrast, in response to injury or chronic degeneration, the mammalian retina undergoes gliosis, scarring, and various degrees of vision loss (Raymond and Hitchcock, 1997, Fischer and Reh, 2001, Fischer and Reh, 2003, Fischer and Bongini, 2010, Nelson and
In the undamaged, but continuously growing zebrafish retina, new retinal neurons arise from proliferating stem cells in the ciliary marginal zone (CMZ) in the peripheral retina (Raymond et al., 2006). Progenitors in the CMZ, however, are not thought to produce rod photoreceptors. Rod photoreceptors are generated from a discrete population of rod progenitor cells located in the ONL, which are themselves seeded from more slowly dividing stem cells associated with radial Müller glia in the INL (Johns and Fernald, 1981, Johns, 1982, Raymond and Rivlin, 1987, Otteson et al., 2001, Raymond et al., 2006, Morris et al., 2008a, Morris et al., 2008b).

It has been observed that the zebrafish retina is capable of matching its regenerative response to the amount of damage detected. For example, the XOPS:mCFP transgenic line of zebrafish exhibits selective degeneration of rod photoreceptor cells without any secondary effects on other retinal neurons (Morris et al., 2005). In contrast to acute damage models, in this background of chronic, rod-specific degeneration and regeneration, there is no reactive gliosis and the number of proliferating Müller glia cells in the INL appears to be unchanged. Rather, rod regeneration in this model is mediated solely by an increase in the number of proliferating rod progenitor cells in the ONL (Morris et al., 2005).

A microarray analysis of mRNA from adult wild-type and XOPS:mCFP retinas identified several transcription factors with increased expression in response to the chronic rod damage and regeneration. The present study focuses on one of those transcription factors: Her4, an effector of Notch-Delta signaling (Takke et al., 1999, Morris et al., 2011).

The Notch-Delta pathway is a highly conserved juxtacrine signaling system that regulates lateral inhibition, proliferation, and gliogenesis (Furukawa et al., 2000, Scheer et al., 2001), and components of the Notch pathway are upregulated in the retina following injury (Kassen et al., 2007, Ghai et al., 2010). When a signaling cell expressing a Delta-like ligand comes into physical contact with a signal-receiving cell expressing a Notch receptor, a series of proteolytic cleavage events liberates the Notch intracellular domain (NICD) from the cell membrane. The soluble NICD then diffuses through the cytoplasm and
binds to Suppressor of hairless (Su(H)) sites where it interacts with the CBF1/Su(H)/Lag1 (CSL) family of proteins and activates transcription of target genes (Campos-Ortega, 1995, Lai, 2004). Notch activity in the retina has been shown to be necessary for maintenance of pools of stem cells and progenitor cells and mediates lateral inhibition during neural differentiation (Nelson et al., 2007). During retinal development, Notch-Delta signaling functions to prevent the premature depletion of progenitor cells before all of the retinal neurons and glial cells have differentiated in their correct proportions (Bao and Cepko, 1997, Bernardos et al., 2005, Nelson et al., 2007). In the regenerating retina, the Müller glia are directed by various extrinsic signals, including Notch-Delta signaling, to dedifferentiate and re-enter the cell cycle, acquiring progenitor-like phenotypes (Gorsuch and Hyde, 2014, Lenkowski and Raymond, 2014).

_Hairy-related (her) genes_ are the highly evolutionarily conserved zebrafish counterparts of the _Hairy and Enhancer-of-split_ type genes in _Drosophila_, and of the _Hes/Hey_ genes in mammals (Muller et al., 1996). Her4 is an ortholog of mammalian _HES5_, and represents one of over 20 members of the _hairy/enhancer of split_ gene superfamily of transcription factors found in the zebrafish genome, not including duplicate variants of an individual gene (Davis and Turner, 2001). The _her4_ gene is comprised of five tandem duplicate repeats on linkage group 23 of the zebrafish genome. All variants of _her4_ have nearly identical transcripts with minor sequence polymorphisms in the 3’ untranslated region (UTR) and are translated into identical peptides. Her4 is a basic-helix-loop-helix-orange (bHLH-O) transcriptional repressor that is directly regulated by the Notch-Delta signaling pathway (Takke et al., 1999). _Her4_ is expressed throughout the developing nervous system and hypoblast where it has been shown to be necessary for primary neuron and hypochord development, as well as maintaining cyclic gene expression during somitogenesis (Takke et al., 1999, Pasini et al., 2004). In the developing CNS, Her4 is required for establishing peripheral outgrowth of subsets of sensory neurons in the trigeminal ganglia as well as regulating the number of _neurog1_ and _deltaB_-positive otic neurons during inner ear development.
development (So et al., 2009, Radosevic et al., 2014). In the adult zebrafish CNS, subsets of Her4 and GFAP-GFP positive glia in the optic tectum act as proliferating neural precursors (Jung et al., 2012). In addition, her4-expressing radial glia in the adult zebrafish CNS have been shown to proliferate and generate neuroblasts in response to brain lesions resulting in efficient regeneration of neurons without glial scarring (Kroehne et al., 2011, Skaggs et al., 2014). In previous studies of adult retinal regeneration, her4 has primarily been used as a marker for active Notch-Delta signaling in response to acute damage (Conner et al., 2014). However, her4 has not been studied in the context of chronic damage, and the fate of her4-expressing cells during retinal regeneration has not been determined.

In this study, we investigated the role of her4 during regeneration of photoreceptor cells in a chronic, rod-specific degeneration background and in an acute light damage model. We generated a transgenic zebrafish line that expresses the photoconvertible protein Kaede in her4-positive cells. We showed that the her4:Kaede reporter is expressed in a spatiotemporal pattern that faithfully recapitulates endogenous her4 expression in the retina. Due to the ability of Kaede to be irreversibly photoconverted, it is a useful tool to not only track Kaede expressing cells, but to establish a timeframe for cellular migration. Lineage tracing analysis using adult her4:Kaede; XOPS:mCFP zebrafish revealed that her4 is expressed in subsets of slowly proliferating Müller glia cells in the INL which give rise to progenitor cells that feed into the rod lineage, and that the entire process from her4 expression to rod neurogenesis takes place in under three days. We demonstrated that her4 expressing stem cells in the CMZ contribute to the lineage of Müller glia and all retinal neurons except rod photoreceptors. We established that her4 is also upregulated in response to acute light damage that results in rod and cone photoreceptor degeneration, and that the magnitude of the her4 response in the regenerating retina correlates with the amount of damage. Our results suggest that her4 and Notch-Delta signaling may play a role in a pathway that replenishes depleted progenitor cell populations by maintaining appropriate numbers of retinal stem cells.
2.2 Materials and Methods

2.2.1 Zebrafish

All zebrafish (Danio rerio) strains were bred, raised, and maintained in accordance with established animal care protocols for zebrafish husbandry (Westerfield, 1995). Adult fish, embryos, and larvae were housed at 28°C, on a 14 hr light: 10 hr dark cycle. Embryos were staged as previously described (Kimmel et al., 1995). The Tg(XRho:gap43-mCFP)q13 transgenic line, hereafter called XOPS:mCFP, has been previously described (Morris et al., 2005, Morris et al., 2011). The Tg(gfap:GFP)mi2001 line, hereafter called gfap:GFP has been previously described (Bernardos and Raymond, 2006) and was obtained from the Zebrafish International Resource Center (ZIRC, Eugene, OR). The Tg(UAS:myc-Notch1a-intra)Kca3 and Tg(hsp70i:Gal4)Kca4 lines were obtained from ZIRC and have been previously described (Scheer and Campos-Ortega, 1999). The Tg(her4:Kaede) line was generated using Tol2-mediated transgenesis (Kwan et al., 2007). Briefly, an expression clone was assembled using site-specific recombination cloning with multisite Gateway technology (Life Technologies, Carlsbad, CA). The construct contains 3.4 kb of the her4.3 promoter cloned upstream of the photoconvertible Kaede reporter, and a separate transgenesis marker consisting of the cardiac myosin light chain promoter (cmlc) cloned upstream of GFP (Ando et al., 2002). The expression clone DNA and Tol2 transposase RNA were injected into 1-cell stage zebrafish embryos at 30 and 25 ng/μl respectively. The injected embryos were screened for GFP expression and raised to adulthood, then outcrossed to identify germline transmitting F0 animals that were used to establish the her4:Kaede colony. All animal procedures were carried out in accordance with guidelines established by the University of Kentucky Institutional Animal Care and Use Committee (IUCAC).

2.2.2 Morpholino and mRNA microinjections

A translation blocking antisense synthetic morpholino oligonucleotide (tbMO) was designed to target sequence immediately upstream of the her4 start site. Another morpholino designed to block
pre-mRNA splicing (sbMO) of the single *her4* intron (which results in a non-sense mutation after the 1st exon) was also used to assess off target effects of either morpholino. Both morpholinos produced similar phenotypes, however even at low doses, injection of the tbMO resulted in unusually high mortality rates by 24 hpf. The tbMO was injected at 1 ng/embryo and the working concentration for the sbMO was 10 ng/embryo. A standard control MO, targeting a mutant variant of the human β-globin gene was used as dosage matched injection controls. All morpholinos were synthesized by GeneTools, LLC (Philomath, OR). A *her4* cDNA amplicon was generated from forward and reverse primers that contained 5’ restriction sites and cloned into pGEM-TE. Capped mRNA was synthesized using the mMessage T7 kit (Ambion, Austin, TX) according to manufacturer’s instructions and purified by phenol-chloroform extraction and ethanol precipitation. The morpholinos, mRNA (at 1.5 ng/embryo), or a combination of the morpholino and mRNA were injected at a volume of 4.18 nl/embryo in buffered solution with 0.025% dextran red (as an injection indicator) into the yolk of 1-cell stage zebrafish. All morpholino and oligo sequences are listed in (Table 2.1).

### 2.2.3 Whole mount in situ hybridization (WISH), fluorescent in situ hybridization (FISH)

WISH, FISH, and two color FISH were performed as previously described (Forbes-Osborne et al., 2013, Pillai-Kastoori et al., 2014). Antisense RNA probes were prepared by in vitro transcription of linearized plasmids containing a portion of the coding sequence of the gene of interest using T7 polymerase and digoxigenin (DIG) or fluorescein (FITC) labeling mix (Roche Applied Science, Indianapolis, IN). The *her4* and *kaede* containing plasmids were prepared by cloning PCR products into the pGEM-T-easy vector (Promega, Madison, WI). The sequences of all PCR primers used in this study are presented in (Table 2.1). Images were obtained on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments), and were exported into Adobe Photoshop for figure preparation.

### 2.2.4 Immunohistochemistry and TUNEL assay
Immunohistochemistry was performed on retinal cryosections as previously described (Fadool, 2003, Pillai-Kastoori et al., 2014, Wen et al., 2014). Enucleated adult eyes or whole embryos were collected at the indicated time points and fixed overnight in 4% PFA at 4°C. The samples were cryoprotected in 10% sucrose in PBS for at least 3 hours and in 30% sucrose overnight at 4°C. Samples were mounted in OCT Medium (Ted Pella, Redding, CA) and frozen on dry ice. 10 µm sections were cut on a cryostat (Leica CM 1850 or CM 1800, Leica Biosystems, Buffalo Grove, IL), mounted on gelatin-coated slides, and dried overnight at room temperature. Before immunolabeling, sections were rehydrated and postfixed in 1% PFA for 10 minutes at room temperature. After 2 washes in PBS, and 2 washes in PBST, sections were blocked in PBST containing 1% BSA for at least 30 minutes at room temperature. Slides were incubated with primary antibody in PBST/BSA with 5% Normal Goat serum, overnight at 4°C in a humidified chamber. The following day, slides were washed 3 times in PBST, and incubated with secondary antibody in PBST/BSA for 1 hour at room temperature in the dark. Slides were washed 2 times with PBST, counterstained with DAPI (4’, 6-diamidino-2-phenylindole, 1:10,000 dilution; Sigma-Aldrich) in PBS, and mounted in 40% glycerol in PBS. The following primary antibodies and dilutions were used: 4C12 (mouse, 1:100) generously provided by J. Fadool (Florida State University, Tallahassee, FL), which labels rod photoreceptor cell bodies; 1D1 (mouse, 1:100, J. Fadool, FSU, Tallahassee, FL), which recognizes Rhodopsin; Zpr-1 (mouse, 1:20, ZIRC), which labels red-green double cones; Zrf-1 (mouse, 1:5000, ZIRC), which labels Müller glia; HuC/D (mouse, 1:20, Invitrogen, Grand Island, NY), which recognizes retinal ganglion cells and amacrine cells; PKCα (rabbit, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes bipolar cells, Prox1 (rabbit, 1:2000, Millipore, Billerica, MA), which recognizes horizontal cells; Nr2e3 (rabbit, 1:100), generously provided by J. Nathans (Johns Hopkins, Baltimore, MD), which labels rod photoreceptor progenitor/precursor cells; anti-PCNA (mouse, 1:100, Santa Cruz Biotechnology, Dallas, TX), which labels proliferating cells; anti-BrdU (mouse, 1:500, Sigma, St. Louis, MO), which marks cells in S phase of the cell cycle; and anti-Kaede (rabbit, 1:500, MBL,
Nagoya, Japan), which labels Kaede expressing cells. Alexa fluor-conjugated secondary antibodies (Invitrogen, Grand Island, NY) and Cy-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were all used at a dilution of 1:200. Sections were counterstained with DAPI (1:10,000, Sigma, St. Louis, MO) to visualize cell nuclei. Terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed on retinal cryosections using the ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions.

2.2.5 BrdU exposure

Adult zebrafish were exposed to 0.5 % BrdU (Sigma, St. Louis, MO) in fish water overnight (16 hours) or continuously for 5 days. BrdU exposure took place in the dark, and the solution was refreshed daily. Eyes of 3 animals were dissected immediately following BrdU exposure for the indicated time, and retinal cryosections were prepared for anti-BrdU immunohistochemistry.

2.2.6 Reverse transcriptase PCR (RT-PCR) and real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from whole embryos or the eyes of adult XOPS:mCFP and her4:Kaede zebrafish using TRIzol reagent (Invitrogen, Grand Island, NY) and first-strand cDNA synthesis was performed using the GoScript Reverse Transcriptase System (Promega, Madison, WI). RT-PCR reactions were then performed using primers designed to amplify her4 and atp5h (housekeeping control) cDNA transcripts. Quantitative real-time PCR was performed using FastStart Essential DNA Green Master Mix (Roche, Indianapolis, IN) on a Light Cycler 96 Real-Time PCR System (Roche, Indianapolis, IN). For all experiments, three biological replicates and three technical replicates were analyzed and the gene expression change was determined using a relative standard curve quantification method with atp5h expression as the normalization control.

2.2.7 Photoconversion

Albino her4:Kaede; XOPS:mCFP zebrafish were briefly anesthetized with Tricaine (Sigma, St. Louis, MO) and mounted on a coverslip in a pool of dilute Tricaine in fish water. The right eye of each
animal was exposed to intense UV light for one minute using the DAPI filter on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments) with the neutral density filter at the lowest setting and at 100x total magnification. The left eye of each animal served as non-UV irradiated control. Following photoconversion, the fish were placed in fresh fish water for recovery and the eyes of three animals were dissected for cryosectioning at 24 hour increments from 0-7 days post photoconversion. A minimum of three animals were photoconverted and imaged for each time point.

2.2.8 Acute light damage

Acute light damage was performed as described previously (Vihtelic and Hyde, 2000). Adult albino her4:Kaede fish were dark adapted for 14 days in complete darkness, then immediately exposed to 18,000 lux light from four 250 W halogen bulbs centered around the tank. A bubbler, cooling fan, and water recirculation system were used to keep the temperature under 32°C. Eyes were dissected and retinal cryosections prepared at the indicated time points during and after light treatment and were processed for IHC. Eyes from three fish were analyzed for each time point.

2.2.9 Heat shock and DAPT treatment

24 hpf Tg(hsp70i:Gal4)Kca4 and Tg(UAS:myc-Notch1a-intra)Kca3 (hereafter called hsp70:Gal4 and UAS:NICD, respectively) compound heterozygote embryos and single transgenic controls were heat shocked twice for 30 minutes at 39°C and processed for WISH at 48 hpf. Pharmacological inhibition of Notch-Delta signaling in embryonic zebrafish was performed using N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a γ-secretase inhibitor that prevents proteolytic cleavage of the Notch intracellular domain from the lumen of the cell membrane (Yang et al., 2008). Wild type embryos were dechorionated and transferred to pre-warmed embryo medium containing 100 μM DAPT in 1% DMSO. Time-matched siblings in embryo medium with 1% DMSO served as carrier controls. Embryos were continuously treated from 10 until 48 hpf and then processed for RT-PCR and WISH.

2.2.10 Dual luciferase assays
HEK293 cells were transfected with varying amounts of the pcDNA3.1 mammalian expression vector (Invitrogen) containing the zebrafish *notch1a* intracellular domain (NICD) cDNA or *her4* cDNA, the pGL3 Firefly Luciferase reporter vector (Promega) containing a 3.4 kb *her4* promoter fragment driving expression of the luciferase gene, the pRL-TK vector (Promega) containing the *Renilla luciferase* gene driven by a ubiquitous tyrosine kinase promoter (to control for transfection efficiency), and balancing levels of pGEM3Z (Invitrogen) using Fugene 6 (Promega) transfecting reagent according to the manufacturer’s instructions. The total mass of DNA and molar ratios of pGL3 and pRL-TK were held constant across transfections, which were repeated a minimum of 3 times. Dose response curves were generated using NICD at 0:100, 1:20, 1:10, and 1:5 molar ratios to the *her4* reporter. Firefly and *Renilla* luciferase activity were measured at 24-36 hours post transfection using the DualGlo Luciferase Assay System (Promega). Data was analyzed as follows: Firefly luciferase (FFLuc) was baselined against untransfected control (UTC) samples (= FFLuc – UTC) and normalized using the *Renilla* luciferase (RLuc). The Relative Luciferase Activity (RLA) was calculated as (FFLuc-UTC)/(RLuc).

2.2.1 Quantification and statistical analysis

Statistical analysis was performed using a Student’s t-test with Microsoft Excel or GraphPad Prism software. For comparing the number of *her4*-positive sections and the total number of *her4*-expressing cells in the CMZ and INL, optic-nerve containing and optic-nerve adjacent sections of 6 wild type and 6 XOPS:mCFP animals were examined. For all graphs, data are represented as the mean ± the standard deviation (s.d.).

2.3 Results

2.3.1 *Her4 is downstream of the Notch-Delta signaling pathway*

To verify that *her4* is downstream of the Notch-Delta signaling pathway, the expression levels of *her4* were assessed following manipulation of the Notch pathway in zebrafish embryos. Both
Pharmacological knock-down and genetic up-regulation of Notch-Delta signaling experiments were performed, followed by semi-quantitative RT-PCR.

Pharmacological inhibition of Notch-Delta signaling in whole zebrafish embryos was accomplished by use of DAPT, a γ-secretase inhibitor that prevents proteolytic cleavage of the Notch intracellular domain from the lumen of the cell membrane (Yang et al., 2008). Zebrafish embryos were treated with DAPT at a concentration of 100 μM from 10 to 48 hpf. To upregulate the Notch pathway, expression of a constitutively active form of the NICD was induced in 24 hpf embryos by heat-shocking compound heterozygote hsp70:Gal4; UAS:NICD embryos (with single transgenic siblings used as controls). In both cases, her4 expression was assessed by RT-PCR at 48 hpf.

After treatment with DAPT, there was a noticeable reduction in her4 expression as compared to the DMSO carrier control (Figure 2.1A). In addition, the heat shocked compound heterozygote hsp70:Gal4; UAS:NICD embryos showed significantly more her4 expression when compared to the single transgenic sibling control embryos which do not ectopically express the NICD and do not have constitutively active Notch signaling (Figure 2.1B). These data indicate that expression levels of her4 can be influenced by manipulation of the Notch-Delta signaling pathway, and that her4 is positively regulated by Notch signaling.

Figure 2.1: Semi-quantitative RT-PCR for her4. A) Her4 cDNA from embryos treated with the γ-secretase inhibitor (DAPT) which knocks down the Notch-Delta signaling pathway (and DMSO carrier controls) treated from 10-48 hpf shows a decrease in her4 expression when Notch signaling is disrupted. B) NICD was induced in 24 hpf embryos by heat-shocking compound heterozygote hsp70:Gal4; UAS:NICD embryos which resulted in ectopic Notch-Delta signaling (with single transgenic siblings used as controls) shows an increase in her4 expression.
2.3.2 Morpholino mediated knock down of her4 phenocopies loss of Notch-Delta signaling in the zebrafish retina

2.3.2.1 Comparison of her4-tb morphant and Notch pathway defect retinal phenotypes

In order to determine if her4 plays a role in retinal development, expression of her4 was manipulated by anti-sense synthetic morpholino oligonucleotide mediated knockdown technology (Nasevicius and Ekker, 2000). A morpholino designed to block translation (tbMO) of her4 mRNA (Table 2.1) was injected into 1-cell stage zebrafish embryos at various concentrations to determine a usable dosage. It was immediately apparent that the mortality rate of the morphant embryos was incredibly high. At 10 ng/embryo, 100% of the morphants died before 24 hpf, and at a concentration of 5 ng, 97.4±2.4% of the embryos did not survive to 24 hpf. The dosage was further reduced to 3, 2, and 1 ng/embryo with 97.2±2.2%, 96.5±2.0%, and 94±1.5% mortality rates respectively (as compared to control morpholino injected at the same concentration). Co-injection of the apoptosis blocking p53 morpholino did not alleviate the toxicity of the her4 tbMO at any dose, and the lowest dosage (1 ng/embryo) was selected as the working concentration. Surviving embryos were collected at 36, 48, and 72 hpf (Figure 2.2). At each time point, there are apparent gross morphological defects as compared to the control morphant embryos. The her4 morphant embryos all displayed either anophthalmia or microphthalmia, pynknotic cells in the brain, altered and/or missing somites, twisted body axis, heart edema, missing or reduced pigmentation, and small body size. None of the embryos injected with the tbMO survived past 72 hpf, precluding analysis at later time points.

Many of the gross morphological defects observed in the surviving embryos were reminiscent of the defects observed when Notch-Delta signaling is disrupted, including eye defects. To determine if knock down of her4 produced a similar retinal phenotype as defective Notch signaling, I compared the morphants to embryos that had been treated with DAPT to pharmacologically inhibit the Notch pathway, as well as homozygous mind bomb (mib) zebrafish embryos. Mind bomb zebrafish have an
Figure 2.2: The her4-tbMO morphant phenotype. The her4 translation blocking morpholino results in high mortality and the surviving morphants display severe developmental defects, including anophthalmia or microphthalmia, picnotic cells in the brain, altered and/or missing somites, twisted body axis, heart edema, missing or reduced pigmentation, and small body size. Scale, 500 µm.

embryonic lethal recessive mutation in an E3-RING type ubiquitin ligase that results in complete abrogation of Notch Delta signaling through improper cycling of Delta ligands (Schier et al., 1996, Itoh et al., 2003). Morphants, DAPT-treated embryos, and homozygous mib mutants were collected at 72 hpf, and cryosectioned. Immunohistochemistry was performed to visualize ganglion cells, the first retinal cell type to differentiate from retinal progenitor cells in the retina (Figure 2.3). When compared to the control morpholino injected embryos, the her4-tbMO embryos had microphthalmia, and the retina was poorly laminated with no discernable boundaries or transition zones between the ONL, INL, and GCL, similar to what was observed in the mib<sup>−/−</sup> and DAPT treated retinas. In addition, immunostaining for GCs revealed a highly expanded number and inflated domain of ganglion cells when compared to controls.
Figure 2.3: Notch-Delta signaling disruption and the her4 morphant GC phenotype. Comparison of the first retinal neurons that differentiate in the zebrafish retina (ganglion cells) in her4 morphant and Notch-Delta knockdown embryos shows that the her4 morphant retina phenocopies disruption of Notch signaling. A-A') IHC to visualize ganglion cells in 72 hpf her4-tbMO morphant retinas show an increased number and expanded domain of GCs as compared to ctrl-MO injected controls. B-B') Mind bomb (Mib) heterozygous and homozygous retinas. C-C') DAPT treated and DMSO ctrl retinas. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; L, lens. Scale, 50 µm.
Figure 2.4: Notch-Delta signaling disruption and the *her4* morphant cone phenotype. Comparison of late born retinal neurons (cone PRCs) in *her4* morphant and Notch-Delta knockdown embryos show that the *her4* morphant retina phenocopies disruption of Notch-Delta signaling. **A-A’** IHC to visualize cone PRCs in 72 hpf *her4*-tbMO morphant retinas show an decreased number and reduced domain of cones as compared to ctrl-MO injected controls. **B-B’** Mind bomb (Mib) heterozygous and homozygous retinas. **C-C’** DAPT treated and DMSO ctrl retinas. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; L, lens. Scale = 50 µm.
When ganglion cells in the her4 morphant retinas were compared to either mib⁻/ or DAPT treated retinas where Notch signaling had been disrupted, highly similar phenotypes were observed. Immunostaining to visualize cone photoreceptors (later born retinal neurons) yielded similar results in the morphant, mib⁻/⁻, and DAPT treated retinas (Figure 2.4). Here, however, unlike the greatly expanded number of ganglion cells, the morphant, mib⁻/⁻, and DAPT treated retinas had very few cone photoreceptors compared to their respective controls. These data suggest that loss of her4 phenocopies disruption of the Notch-Delta signaling pathway in the retina. Additionally, the increase in the number and expanded domain of the first retinal cell type to differentiate (ganglion cells) happens at the expense of later born retinal neurons (such as cone PRCs) because of a prematurely depleted progenitor cell pool. These data indicate that Notch-Delta signaling, and the downstream effector her4, keep subsets of retinal progenitor cells in the cell cycle, and are required for proper retinal neurogenesis.

2.3.2.2 The her4 splice blocking morpholino results in lower mortality

To verify that the phenotypes observed in the her4-tbMO morphant retina were not artifacts of off target mRNA knockdown, a second morpholino was designed to block splicing of her4 pre-mRNA (her4-sbMO) and used to determine if it would generate the same phenotypes as previously observed (Table 2.1). In addition, use of a second morpholino that targets the same gene would lend insight into a possible explanation as to why injection of the her4-tbMO (even at relatively low concentrations) resulted in unusually high mortality rates by 24 hpf even though most DAPT treated and mib⁻/⁻ embryos (in which the entire Notch pathway is disrupted) readily survive at least 72 hpf.

A dose response curve was generated to determine the optimal concentration of the sbMO to inject per embryo. In stark contrast to the mortality observed with the tbMO, mortality at 24 hpf was very low across a range of doses (compared to control morpholino injected at the same concentrations): at 10, 5, and 2.5 ng of her4-sbMO/embryo, the mortality rates were 5.5±3.0%, 6.0±3.5%, and 4.8±4.4% respectively. Effectiveness of her4 knock down was assessed by RT-PCR to compare the relative
abundance of spliced versus non-spliced her4 mRNA transcript in the her4-sbMO and control-MO injected embryos (Figure 2.S1). Injection of 10 ng/embryo of the her4-sbMO efficiently prevented splicing of zygotic her4 pre-mRNA through 2 days post injection, but spliced her4 mRNA could be detected starting at 3 days post injection. In addition, most of the morphants (injected at the highest dose of 10 ng her4-sbMO/embryo) looked largely phenotypically normal besides varying degrees of microphthalmia and slight vertebrae curvature (Figure 2.5).

Figure 2.5: The her4-sbMO morphant phenotype. Injection of 10 ng/embryo of the her4 splice blocking morpholino resulted in minimal mortality rates (as compared to the tb-MO) and a milder overall phenotype in most of the morphant embryos. ~30% displayed moderate to severe ocular phenotypes, including microphthalmia and misshapen eye. Scale, 500 µm.
Figure 2.6: Retinal cells in the 72 hpf her4 morphant retina. The her4 morphant retina at 72 hpf displays an excess of early born retinal neurons (ganglion cells) at the expense of later born retinal cell types, and the morphant phenotype can be rescued by co-injection of 1.5 ng/embryo of her4 mRNA. A-A’’) IHC to visualize ganglion cells. B-B’’) Amacrine cells. C-C’’) Horizontal cells. D-D’’) Bipolar cells. E-E’’) Rod photoreceptor cells. F-F’’) Cone photoreceptor cells. G-G’’) Müller glia. Scale, 50 µm.

All retinal cell types were visualized in the morphant embryos by immunohistochemistry at 72 hpf. Slightly over 30% of the embryos displayed moderate to severe retinal phenotypes such as misshapen eye, poor lamination, and microphthalmia (Figure 2.6), in contrast to nearly 100% of the surviving tbMO morphants observed at the same time point. Similar to what was observed in the tbMO morphants, there was an increase in the number and an expanded domain of RGCs and a reduction or nearly complete depletion in the number of any other type of retinal cell type. To rule out off-target effects as a cause of the observed morphant phenotypes, in vitro transcribed her4 mRNA was co-
injected with the sbMO. Co-injection of capped *her4* mRNA rescued the morphant phenotype in 93.3% of sbMO injected embryos demonstrating that the observed morphant phenotypes were due to knockdown of *her4*. Injection of 1.5 ng of *her4* mRNA without morpholino resulted in no obvious changes in retinal morphology or rod, cone, and ganglion cell numbers (Figure 2.52), however injection of any higher doses of *her4* mRNA resulted in severe developmental defects and high mortality.

### 2.3.3 Her4 expression in the adult zebrafish retina

Previously, we found by microarray analysis that *her4* was upregulated in the adult XOPS:mCFP retina, which undergoes chronic rod-specific degeneration and regeneration (Morris et al., 2011). To validate the microarray data, we performed quantitative RT-PCR (qRT-PCR) on mRNA prepared from wild type and XOPS:mCFP retinas using primers designed to amplify all five *her4* tandem duplicate repeat variants (*her4.1-her4.5*) (Table S1). We found that *her4* was upregulated over 3.5 fold in the XOPS:mCFP retina compared to wild type (3.5±1.08, p<.05) (Figure 2.7A), confirming our previous results. To further investigate the possible roles of *her4*, we determined the expression pattern of *her4* in wild type and XOPS:mCFP retinas by fluorescent in situ hybridization (FISH) with antisense probes that were designed to recognize all *her4* variants. To validate our *her4* in situ probes we performed FISH in the developing retina and found that the resulting expression pattern confirmed previous studies (Figure 2.53A). From 48 hours post fertilization (hpf) to 5 days post fertilization (dpf), *her4* expression shifted from the central retina to the ciliary marginal zone (CMZ) where expression persists into adulthood (Figure 2.7B). Treatment with the Notch signaling inhibitor DAPT caused a decrease in *her4* expression (Figure 2.53B), whereas overexpression of the Notch intracellular domain (NICD) resulted in an increase in *her4* expression (Figure 2.53C), confirming that *her4* is a target of Notch signaling in the zebrafish retina.
Figure 2.7: Her4 is expressed in proliferative regions of the adult retina and is upregulated in the XOPS:mCFP background. A) qRT-PCR revealed a ~3.5 fold increase in her4 expression in the XOPS:mCFP retina. B) Fluorescent in situ hybridization (FISH) for her4 expression in the adult wild type and XOPS:mCFP central and peripheral retina demonstrated increased her4 expression in the XOPS:mCFP CMZ and INL. C) Percentage of retinal sections that contained her4-expressing cells at the CMZ (n=6 animals per group, >200 sections analyzed). D) Mean number of her4-expressing cells per CMZ. E) Percentage of sections that contained at least 1 her4-expressing cell in the INL. F) Mean her4-expressing cells per INL (n=6 animals per group, >200 INL sections analyzed). L, lens; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; CMZ, ciliary marginal zone; hpf, hours post fertilization; dpf, days post fertilization; scale bar, 50 µm; * P<0.05; ** P<0.001.

In the adult wild type retina, her4 was primarily expressed in the peripheral most areas of the CMZ (51.5±4.5% of all sections observed) (Figure 2.7B,C). Occasionally, her4 expressing cells were also
detected in the inner nuclear layer (INL) of the central wild type retina (3.1±1.8% of all INL sections observed) (Figure 2.7B,E). In the wild type background, not only were the majority of sections negative for her4 expression in the INL, but the mean number of her4 positive cells per her4 expressing INL was low (1.37±0.66). In contrast, in a background of chronic, rod-specific degeneration and regeneration (XOPS:mCFP) her4 expression was significantly elevated. The percentage of XOPS:mCFP retinal sections containing her4-positive cells in the CMZ was nearly double that of the wild type retina (92.05±2.7%), and the total number of her4-expressing cells in the CMZ was also increased in the XOPS:mCFP background (WT retina: mean 7.64±0.95; XOPS:mCFP retina 10.08±1.0) (Figure 2.7C,D). These results were unexpected because only rod photoreceptors are degenerating/regenerating in the XOPS:mCFP retina, and the CMZ is not thought to produce new rods. In addition to an increase in her4 expressing cells in the CMZ, we also observed an increase in her4-positive cells in the INL of XOPS:mCFP adult retinas compared to wild type (92.6±6.4% vs. 3.19±1.8% of sections analyzed) (Figure 2.7E). The her4 positive cells in the INL of the XOPS:mCFP retinas were widely scattered across the central retina, and the total number of her4 expressing cells per section was highly variable, ranging from zero to as many as eleven positive cells per section (Figure 2.7F). Interestingly, her4 expression was never detected in the outer nuclear layer (ONL) of the central retina, where rod progenitor cells reside in either wild type or XOPS:mCFP adults. Taken together, these results show that expression of her4 is significantly increased in both the INL of the central retina and the peripheral CMZ in response to selective rod photoreceptor degeneration.

2.3.4 Her4 is expressed in slowly proliferating subsets of Müller glia in the INL and more rapidly proliferating cells in the CMZ.

Previous studies have shown that Notch-Delta signaling regulates the proliferative response of Müller glia to acute retinal damage. Therefore, we investigated whether the her4 expressing cells in the INL of the XOPS:mCFP retinas were proliferating Müller glia. We performed FISH and immunolabeling
experiments on adult gfap:GFP; XOPS:mCFP and gfap:GFP siblings that were exposed to BrdU for either 16 hours or 5 days. The gfap:GFP line expresses the GFP reporter specifically in glial cells of the zebrafish nervous system, including Müller glia in the retina (Bernardos and Raymond, 2006). In the INL of both the single transgenic gfap:GFP and double transgenic gfap:GFP; XOPS:mCFP retina, all her4 FISH signal co-localized with the GFP reporter, indicating that her4 expression in the INL is restricted to BrdU-positive Müller cells (Figure 2.8A). In previous studies, we found that there is an increased number of proliferating rod progenitor cells in the ONL of the XOPS:mCFP when compared to the wild type retina (Morris et al., 2008b), but we did not detect a significant increase in BrdU positive cells in the INL of XOPS:mCFP retinas at 15 dpf, nor did we detect an increase in PCNA positive cells in the INL of XOPS:mCFP adults (Morris et al., 2005, Morris et al., 2008b). However, when we repeated these experiments with a greater number of samples we found a small but significant increase in the number of BrdU positive cells in the INL of the XOPS:mCFP central retina, and this increase over wild type was even larger when fish were exposed to BrdU for five days (Figure 2.8B). To determine the proportion of BrdU-positive cells in the INL that also expressed her4, we performed FISH for her4 combined with immunohistochemistry for BrdU. After 16 hours of BrdU exposure, approximately 75% of her4 expressing cells in the INL of both the wild type and XOPS:mCFP retina had incorporated the BrdU label, whereas after 5 days of BrdU exposure, this proportion reached nearly 100%. This suggests that her4 is expressed in slowly proliferating subsets of Müller glia in the INL. Interestingly, after both short and long exposures to BrdU, the total number of her4/BrdU positive cells was significantly higher in the XOPS:mCFP INL than the wild type INL (Figure 2.8C). This result suggests that chronic rod photoreceptor degeneration induces a small but significant increase in the number of slowly proliferating Müller glia in the inner retina.
Figure 2.8: *Her4* is expressed in slowly proliferating subsets of Müller glia in the INL and rapidly proliferating cells in the CMZ. **A)** *Her4* FISH with anti-GFP and anti-BrdU immunohistochemistry (IHC) in retinal sections from XOPS:mCFP; gfap:GFP zebrafish revealed that *her4* is expressed in proliferating Müller cells in the INL. **B)** Total number of BrdU-positive cells in the INL following either 16 hour or 5 day BrdU exposure in wild type and XOPS:mCFP zebrafish. There was a significant increase in the number of BrdU-positive cells in the INL with the longer BrdU exposure, and an increase of BrdU-positive cells in the XOPS:mCFP INL compared to WT (n=3 animals per group, >60 INL sections per group observed). **C)** Percentage of sections that contained n *her4*+ BrdU+ cells in the INL; insets show mean number of *her4*+ BrdU+ cells per INL section (left) and the percentage of *her4*+ cells that were also BrdU+ (right). **D)** *Her4* FISH with anti-GFP and anti-BrdU IHC in retinal sections from XOPS:mCFP; gfap:GFP. Nearly all *her4*-expressing cells in the CMZ were BrdU+. **E)** There was a small but significant increase in the number of *her4*+ BrdU+ cells in the CMZ of the XOPS:mCFP retina (top). All of the *her4*+ cells in the CMZ were also BrdU+ after 16 hours of BrdU exposure in both WT and XOPS:mCFP retinas (bottom; n=6 animals, >120
sections analyzed). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; CMZ, ciliary marginal zone; scale bar, 50 µm; * P<0.05; ** P<0.001.

As described above, we detected the expression of her4 in the CMZ of wild type adult retinas and this expression was significantly increased in the CMZ of the XOPS:mCFP line. In both the wild type and XOPS:mCFP retinas, 99% of her4-expressing cells in the CMZ had incorporated BrdU after the 16-hr exposure (Figure 2.8D,E). This result suggests that the population of cells at the her4-expressing cells in the CMZ proliferate more rapidly than the her4-expressing cells in the INL.

2.3.5 Generation of the her4:Kaede transgenic zebrafish line.

The identification of a subset of proliferating her4-expressing Müller glia in the XOPS:mCFP retina suggests that these cells may function to replenish the pool of more rapidly dividing rod progenitor cells in the ONL. To determine whether her4-expressing cells contribute to the rod photoreceptor lineage, we generated a transgenic line that expresses the photoconvertible Kaede reporter driven by a her4 promoter. A 3.4 kb region containing a segment of the 3’ UTR of her4.2 and the presumptive promoter of her4.3 (Figure 2.9A) has been used previously to generate reporter lines indicating active Notch-Delta signaling (Yeo et al., 2007). This fragment contains five Suppressor of Hairless [Su(H)] consensus binding sites, 64 E-box and 2 N-box sequences. To verify that this her4 promoter segment is responsive to Notch signaling, we carried out in vitro reporter assays using Notch1a intracellular domain (NICD) cDNA and a luciferase reporter driven by the 3.4 kb her4 promoter. Co-transfection of the NICD expression vector and the luciferase reporter into HEK293 cells significantly increased luciferase activity, demonstrating that the 3.4 kb her4 promoter segment is sufficient to respond to Notch signaling (Figure 2.54A). When a her4 expression vector was co-transfected with the her4 luciferase reporter, there was a dose-dependent decrease in luciferase activity, suggesting that her4 negatively regulates its own expression (Figure 2.54B).
Figure 2.9: *Her4:Kaede* zebrafish express the kaede transgene in a spatiotemporal pattern that recapitulates endogenous *her4* expression. **A)** Schematic representation of the *her4* locus. The 3.4 kb fragment between *her4.2* and *her4.3* contains 5 Suppressor of Hairless [Su(H)] consensus binding sites that are necessary for Notch-Delta signaling. **B)** Schematic of the *her4:Kaede* expression construct. **C)** Two-color FISH shows co-localization of *kaede* and endogenous *her4* transcript during development (red fluorescence in the lens is non-specific). **D)** Two-color FISH in the adult XOPS:mCFP retina shows co-localization of *kaede* and *her4* transcripts in the CMZ (top row) and INL of the central retina (bottom row). L, lens; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; CMZ, ciliary marginal zone; hpf, hours post fertilization; dpf, days post fertilization; scale bar, 50 μm.

An expression construct was assembled containing the 3.4 kb *her4* promoter upstream of the Kaede fluorescent reporter (**Figure 2.9B**). The *her4:Kaede* line was generated using Tol2-mediated transgenesis (Kwan et al., 2007). To determine if the *kaede* transgene demonstrated the same spatiotemporal expression pattern in the retina as endogenous *her4*, two-color FISH was performed using DIG-labeled and FITC-labeled riboprobes to detect *her4* and *kaede* mRNA respectively. In the developing retina of the *her4:Kaede* embryo, expression of the transgene overlapped with expression of
endogenous \textit{her4} at 48, 72, and 96 hpf, and both transcripts could only be detected in the CMZ by 5 dpf (Figure 2.9C). In adult \textit{her4}:Kaede; XOPS:mCFP retinas, expression of the \textit{kaede} transgene co-localized with endogenous \textit{her4} in all \textit{her4}-positive cells in the INL and the majority of \textit{her4}-positive cells in the CMZ (Figure 2.9D). These data demonstrate that expression of the \textit{her4}:Kaede transgene faithfully recapitulates expression of endogenous \textit{her4} in both the developing and regenerating retina.

The Kaede reporter is a highly stable 116 kDa homotetrameric protein that has a relatively long biological half-life compared to other fluorescent reporters (Chudakov et al., 2010). Following irradiation with ultraviolet light, the green fluorescing chromophore undergoes a $\beta$-elimination reaction that results in an irreversible photoconversion to red fluorescence (Ando et al., 2002) (Figure 2.59). We tested the feasibility of photoconverting Kaede in living tissues to establish a UV treatment paradigm and to confirm that the \textit{her4}:Kaede transgenic zebrafish would be useful for lineage tracing studies. \textit{Her4}:Kaed embryos were briefly anesthetized at 48 hpf and exposed to low intensity UV light for 1 minute. Before photoconversion, green-emitting-Kaede (gKaede) could be observed in the retina and throughout the CNS in the GFP channel only (Figure 2.55A,B). Following the short UV pulse, photoconversion into red-emitting-Kaede (rKaede) could readily be detected along with a reduction of green-emitting-Kaede (gKaede) (Figure 2.55C,D). The animals were allowed to recover and were observed intermittently for 3 weeks following the photoconversion procedure. Fluorescence of both gKaede and rKaede persisted for the entire observation period (data not shown). We then tested the photoconversion paradigm on juvenile (4-6 week old) zebrafish eyes to determine if rKaede fluorescence could be detected in the mature retina for a minimum of 1 week post photoconversion. We found that the rKaede signal was easily detectable in the central retina, and that within the 7 day window, a significant amount of new gKaede expression originating from the CMZ could be observed (Figure 2.55E). These data not only indicate that the Kaede reporter is suitable for lineage tracing studies, but also confirm that \textit{her4}-expressing cells in the CMZ proliferate to generate new retinal cells in the growing eye.
2.3.6 *Her4*-expressing Müller cells in the INL of the XOPS:mCFP retina contribute to the rod photoreceptor cell lineage.

To determine the fate of *her4*-expressing Müller glia in the XOPS:mCFP retina, we conducted lineage tracing analyses. The entire right eye of albino XOPS:mCFP; *her4*:Kaede adults was briefly exposed to intense UV light, with the left eye serving as a non-UV treated control. Eyes were dissected at various time points post photoconversion, and retinal sections were processed for immunohistochemistry. Lineage tracing analysis was restricted to the central retina to avoid Kaede-expressing cells derived from the CMZ. Because the XOPS:mCFP reporter is expressed in rods prior to degeneration, it was not possible to distinguish green fluorescing Kaede (gKaede) from mCFP fluorescence in the ONL in the non-photoconverted eye. We therefore immunolabeled retinal sections with a rod-specific antibody to distinguish gKaede-expressing non-rod cells from mCFP-expressing rods.

In the non-UV treated control eyes, all *her4*-expressing cells in the retina displayed green fluorescence, with no detectable red signal (Figure 2.10A). In contrast, when observed immediately after UV treatment, the UV-exposed eye displayed complete green to red photoconversion of all Kaede-expressing cells in the retina (Figure 2.10B). At 0 days post photoconversion (0d post PC), cells in the ONL that expressed the photoconverted rKaede reporter co-localized with markers for rod photoreceptors, indicating that cells that once expressed *her4* contribute to the rod lineage (Figure 2.10B’’’). In the INL, rKaede expressing cells with morphological characteristics of Müller glia were observed, as well as smaller round cells located in the vicinity of the presumptive rKaede-positive Müller glia. This pattern suggests that at the time of UV treatment, the lineage of *her4* expressing cells was comprised of various cells in different stages of the rod replenishing pathway.
Figure 2.4: *Her4*-expressing cells in the INL contribute to the rod photoreceptor lineage. Column 1, non-photoconverted Kaede (gKaede) and mCFP fluorescence; column 2, photoconverted Kaede (rKaede); column 3, immunolabeling for rod photoreceptors in the ONL with 4C12 antibody; column 4, merge of rKaede and 4C12. 

**A-A‴** The non-photoconverted retina (no PC) shows gKaede fluorescence in the INL, and no rKaede fluorescence. 

**B-B‴** The photoconverted retina immediately after UV treatment (0d post PC) shows only rKaede fluorescence in the INL (arrows), and co-localization of rKaede and 4C12 in the ONL (arrowheads). 

**C-C‴** At 1d post PC, new gKaede expressing cells can already be detected in the INL (arrows) along with persistent rKaede in the INL and ONL. 

**D-D‴** At 2d post PC, very little rKaede remains in the INL, but in the ONL rKaede persists in rod precursors. 

**E-E‴** By 3d post PC, only gKaede
can be detected in the INL (arrows). 3 animals and 30 sections were observed per time point. INL, inner nuclear layer; ONL, outer nuclear layer; CMZ, ciliary marginal zone; scale bar, 50 µm.

By 1d post PC, a large reduction in the number of rKaede-positive, elongated cells was observed in the INL, with an increase in the number of the smaller rounded cells in both the INL and ONL that retained rKaede signal. This same pattern was observed in the UV-treated eyes of at least three animals analyzed and in all sections examined. In addition, at 1d post PC, gKaede signal could already be detected in the INL of the same sections, indicating that her4 continued to be expressed in these cells after the UV treatment (Figure 2.10C). At 2d post PC, the majority of sections observed contained no rKaede reporter expression in the INL. The few INL sections that were rKaede positive contained no elongated rKaede positive cells (Figure 2.10D). By 3d post PC, only gKaede positive cells were found in the INL. However, numerous small rKaede positive cells were observed in the ONL (Figure 2.10E). These data suggest that the cycle of her4 expression to rod neurogenesis is completed in under 3 days.

In order to determine the identity of her4 expressing cells in the INL of the XOPS:mCFP central retina, we performed immunohistochemistry with cell-type specific antibodies on retinal sections from XOPS:mCFP; her4:Kaede adults. Elongated rKaede-positive cells in the INL co-localized with markers for Müller glia, although only a small subset of Müller glial cells expressed the Kaede reporter (Figure 2.11A). The elongated cells in the INL as well as the smaller rounded cells in the INL and ONL were also PCNA positive, indicating that subsets of Müller cells as well as their descendants were proliferating (Figure 2.11B). Furthermore, some of the small, rounded rKaede-positive cells in the INL and all of the cells in the ONL that retained rKaede fluorescence co-localized with Nr2e3, a marker for rod progenitor cells and post-mitotic rod precursors (Figure 2.11C). These data confirm that subpopulations of her4
expressing Müller glia cells proliferate in the INL and give rise to rod progenitor cells, which migrate to the ONL and differentiate into rods.

2.3.7 Her4 expressing cells in the CMZ differentiate into retinal neurons and Müller glia, but not rods.

To determine the fate of the her4 expressing cells in the CMZ, immunolabeling experiments were performed on her4:Kaede and her4:Kaede; XOPS:mCFP adult retinal sections. To rule out confounding mCFP fluorescence, double labeling was performed with anti-Kaede antibodies as well as markers for the various retinal cell types. Following the double labeling experiments, only the peripheral most areas of the central retina adjacent to the CMZ were analyzed to ensure that the Kaede positive cells we observed were derived from the CMZ.
Figure 2.12: *Her4*-expressing cells in the CMZ generate any retinal cell type except rod photoreceptors. A-A’') Kaede and amacrine cell marker (HuC/D) co-localization in the INL. B-B’’) Kaede and ganglion cell marker (HuC/D) co-localization in the GCL. C-C’’) Kaede and bipolar cell marker (PKCα) co-localization. D-D’’) Kaede and horizontal cell marker (Prox-1) co-localization. E-E’’) Kaede and red-green cone photoreceptor marker (Zpr-1) co-localization. F-F’’) Kaede and Müller glia marker (Zrf-1) co-localization. G-H’’) Kaede does not co-localize with a marker for rod photoreceptors (4C12) in the WT retina (G-G’’) or XOPS:mCFP retina (H-H’’). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OS, photoreceptor outer segments; CMZ, ciliary marginal zone; scale bar, 50 µm.

In the peripheral retina adjacent to the CMZ, we observed co-localization of the Kaede reporter with markers for Müller glia, amacrine, bipolar, and horizontal cells in the INL, as well as ganglion cells in the GCL (Figure 2.12). Interestingly, although Kaede expression was detected in each of these cell types, not all of the cells in the peripheral retina were Kaede-positive. In addition, the intensity of Kaede fluorescence decreased with increasing distance from the CMZ. Markers for cone photoreceptors in the ONL also co-localized with Kaede, but the number of these double labeled cells detected in the ONL was small compared to the number of Kaede-positive INL and GCL neurons, suggesting that perhaps the turnover rate of Kaede is higher in cones, which are highly metabolically active (Figure 2.12E).

Previous studies have suggested that rod photoreceptors are not generated from the CMZ of teleost fish (Johns and Fernald, 1981, Johns, 1982, Otteson et al., 2001). However, as described above, we observed a significant increase in the number of *her4*-expressing cells in the CMZ of the XOPS:mCFP retina, in which only rod photoreceptors degenerate. We therefore asked whether *her4*-positive cells in the CMZ could also produce rod photoreceptors. We immunolabeled retinal sections with a rod-specific antibody and searched for rods in the peripheral retina that co-localized with the CMZ-derived *her4*:Kaede reporter. In agreement with previous studies, we did not detect Kaede expression in rods adjacent to the CMZ in any wild type sections (Figure 2.12G). In addition, in XOPS:mCFP retinas we only detected one Kaede-positive rod adjacent to the CMZ in a single section (out of 160 sections examined).
These data suggest that the stem cells at the peripheral most CMZ that transiently express *her4* have the capacity to generate any retinal cell type except rods, even in the XOPS:mCFP background where the *her4*-expressing cell population in the CMZ is expanded.

2.3.8 *Her4 is upregulated in the retina following acute light damage.*

Because there was a small but significant increase of *her4*-expressing Müller glia in a background of chronic rod degeneration, we wanted to determine whether more significant damage would result in more *her4* expression. Therefore, we examined *her4*:Kaede expression following acute light damage, which results in both rod and cone photoreceptor degeneration (Vihtelic and Hyde, 2000). In contrast to the slow, chronic rod degeneration observed in the XOPS:mCFP retina, intense light damage causes synchronized photoreceptor cell death, reactive gliosis, and proliferation followed by migration and differentiation into new photoreceptor cells. Adult *her4*:Kaede albino zebrafish were dark adapted for 14 days and then exposed to 18,000 lux light for 7 days. Immunohistochemistry was performed on retinal sections to confirm that the light damage resulted in rod and cone degeneration (Figure 2.56). TUNEL and PCNA labeling confirmed that the light treatment resulted in photoreceptor apoptosis and an ensuing proliferative response (Figure 2.57). An antibody to the Kaede reporter was used to characterize the extent and timing of *her4* expression. Before the onset of light damage the Kaede reporter was not detected in the central retina (Figure 2.13A). In contrast, after 4 and 7 days of light damage (4d and 7d LD), numerous Kaede positive cells were observed in the INL and ONL of the central retina (Figure 2.13B,C). Double labeling experiments with PCNA revealed that the Kaede positive cells were proliferating (Figure 2.13H). After 2 days of recovery post light damage (2d PLD), there were very few Kaede-positive cells in the INL and only a diffuse Kaede signal in the ONL (Figure 2.13D). At 4 and 21d PLD, Kaede was not detected anywhere in the central retina (Figure 2.13E,F). QRT-PCR confirmed that *her4* was upregulated over 10-fold at 4d LD (Figure 2.13G). Interestingly, by 7d LD, the magnitude of
Figure 2.7: *Her4* expression is upregulated in the light damaged retina. A) Kaede signal is not detected in the central retina before light damage onset (0d LD). B) Kaede signal is abundant in the central retina after 4 days of light damage (4d LD) and the morphology of the ONL is highly disrupted. C) At 7d LD Kaede signal is still highly expressed, the localization is more diffuse in the ONL. D) Few Kaede positive cells were observed at 2 days post light damage (2d post LD). E-F’’ No Kaede fluorescence was observed in the central retina at 4d or 21d post LD. G. qRT-PCR confirms that *her4* is upregulated ~11 fold at 4d LD and decreases to ~5 fold over 0d LD at 7d LD. H) Kaede and PCNA double labeling shows that Kaede expressing cells are proliferating in the acute light damaged retina. INL, inner nuclear layer; ONL, outer nuclear layer; scale bar, 50 µm; * P<0.05; ** P<.001.
her4 expression change decreased to 5-fold over 0d LD, and this correlated with an increase in rod and cone labeling in the ONL, even though the light treatment was ongoing (Figure 2.56). These results suggest that after four days of intense light exposure, the zebrafish retina may adapt such that regeneration can proceed and further light damage is minimized. Taken together, these results demonstrate that the extent of her4 expression in the retina depends upon the amount of damage the retina receives.

2.4 Discussion

Morpholino mediated knockdown of her4 during embryonic retinal development resulted in a vast increase in the number and domain of ganglion cells within the retina, which came at the expense of later born retinal cell types. The pre-mature depletion of retinal progenitor cells indicates that her4 plays a role in keeping subsets of these RPCs in the cell cycle and influences cell fate decision by causing the cells that express her4 to ignore spatiotemporally regulated extrinsic cues that push non her4-expressing cells towards a particular cell fate in distinct regions of the retina at appropriate times.

Interestingly, injection of the splice blocking morpholino at a relatively high dose (10 ng/embryo) resulted in the same retinal phenotypes as injection of low dose (1 ng/embryo) translation blocking morpholino, but only in 1/3 of the embryos. The remaining embryos exhibited a range of retinal phenotypes; some of the morphants exhibited moderate to mild retinal lamination defects and comparatively less excess ganglion cells and more later born retinal cell types as compared to tbMO injected embryos. To address this discrepancy in the relative effectiveness of her4 knockdown between the tbMO and the sbMO, RT-PCR experiments were performed on 2, 4, and 48 hpf WT zebrafish embryos to assess the maternal contribution of mature her4 mRNA to the ovum (Figure 2.58). The maternal to zygotic transition of control of the transcriptome in the zebrafish embryo occurs shortly after 4 hpf (Lee et al., 2014). RT-PCR experiments showed that there is abundant maternal deposition of
mature (spliced) her4 mRNA at 2 and 4 hpf, with an expected increase in spliced her4 transcript observed at 48 hpf. Because the tbMO blocks translation of any her4 transcript (pre-mRNA or mature mRNA), no maternal effect could be observed when using it to knock down her4 expression. With the splice blocking morpholino, any maternally deposited mRNA would escape knockdown and be translated into Her4. This would result in Her4 dosage being dependent entirely upon levels of mature her4 mRNA in each ovum, regardless of how much her4-sbMO was injected.

The most striking result when knocking down her4, however, is that knockdown of only 1 of the 26 total hes/hey/her transcription factors results in a retina that phenocopies disruption of the entire Notch-Delta signaling pathway. This result was unexpected because many of the bHLH-O transcription factors expressed during zebrafish development that are known targets of Notch signaling are also expressed in the primordial eye field and later in retinal progenitor cells, and would presumably confer some redundancy in the pathway. It is unknown whether knockdown of any of the other bHLH-O transcription factors could yield a similar phenotype as knockdown of her4 alone. It is possible that there is a minimum dosage of expression hes/her/hey factors in the developing retina required to repress neurogenesis, and that her4 is simply the most highly expressed bHLH-O gene in this particular tissue.

Due to its Notch-dependent expression in most tissues, the bHLH-O transcriptional regulator her4 has been studied in the context of neurogenesis and differentiation of various components of the CNS during development (Takke et al., 1999, So et al., 2009, Forbes-Osborne et al., 2013, Radošević et al., 2014). In addition, her4 expression has been used as an indicator of active Notch-Delta signaling during regeneration of CNS tissues (Conner et al., 2014, Skaggs et al., 2014). Previous microarray data indicated that her4 was upregulated in the adult XOPS:mCFP retina, in addition to other components of the Notch pathway, including notch1a and deltaB (Morris et al., 2011). We therefore hypothesized that her4 plays a role in the regeneration of rod photoreceptors downstream of Notch-Delta signaling. In this
study, we provide support for this hypothesis by characterizing and tracking the lineage of her4-expressing cells in the XOPS:mCFP retina. Although we showed that expression of her4 decreases when Notch signaling is knocked down, and increases when components of the pathway are upregulated, it is unclear if Notch signaling activates all variants of her4, or what variants of the gene are being expressed in the retina. It is possible that only one or a few of the her4 variants are expressed in the retina downstream of Notch and other duplicates could be expressed in non-retinal tissues downstream of other pathways.

In the undamaged adult retina, her4 was primarily expressed in proliferating cells in the peripheral CMZ. In contrast, in the XOPS:mCFP retina, we observed her4 expression in a small number of slowly proliferating Müller glia cells in the INL. This result is consistent with previous work showing that in response to acute retinal damage, Müller cells in the INL proliferate to produce neuronal progenitors (Bernardos et al., 2007, Thummel et al., 2008, Montgomery et al., 2010, Gorsuch and Hyde, 2014). However, whereas acute damage induces proliferation of a large number of Müller glia, in the XOPS:mCFP line only a very small subset of Müller cells scattered throughout the INL expressed her4 and were BrdU-positive. Significantly more of these her4-expressing INL cells incorporated BrdU after exposure to BrdU for five days (compared to an overnight exposure), demonstrating that her4 is expressed in slowly proliferating Müller glia as opposed to the more rapidly proliferating rod progenitor cells at the base of the ONL. Furthermore, even though the rods are continually degenerating in the XOPS:mCFP line and there is a significant increase of rod progenitor proliferation at the base of the ONL, we did not detect expression of her4 in the rod progenitor cells. Taken together, these data suggest that the role of the her4-positive proliferating Müller glia may be to replenish the rod progenitor pool as it gets depleted by continual rod photoreceptor regeneration.

Lineage tracing analysis using our her4:Kaede transgenic line crossed onto the XOPS:mCFP background revealed that the slowly proliferating her4-positive Müller cells in the INL give rise to Nr2e3-
expressing progenitor cells. These progenitor cells then migrate to the base of the ONL where they differentiate into rod precursor cells and then rods. In contrast to the more rapid response resulting in neurogenesis following acute damage (Thummel et al., 2008, Thomas et al., 2012), we found the cycle of her4 expression to rod neurogenesis can take up to three days. As early as one day post-photoconversion, we observed new non-photoconverted Kaede in presumptive Müller glia in INL, demonstrating that the chronic rod degeneration and regeneration experienced in the XOPS-mCFP retina elicits a continual, albeit low-level, response in the INL. These data support the hypothesis that her4 upregulation in small subsets of Müller cells is associated with the need to restock the rod progenitor pool as those progenitors are continually depleted. It is possible that ONL rod progenitors have an intrinsic limit with regard to how many times they can divide to produce rod precursors, and therefore INL derived stem cells must replenish ONL progenitor numbers. The her4:Kaede transgenic line was also used to compare the her4 response in the XOPS:mCFP chronic damage model to the acute light damaged retina. We found that her4 was upregulated over 10 fold after 4 days of intense light treatment. Similarly, IHC experiments revealed that Müller cells in the INL that expressed the Kaede reporter were responsible for contributing to the proliferative response. These experiments show that the magnitude of the her4 response is correlated with the amount of damage to the retina.

In addition to upregulation of her4 in the INL of the XOPS:mCFP retina, her4 was also upregulated in the CMZ. This result was surprising because unlike other retinal neurons, rod photoreceptors are not derived from the CMZ but from populations of stem cells that reside in the INL of the central retina (Raymond et al., 2006, Morris et al., 2008a, Morris et al., 2008b). Increased mitotic activity, however, has been reported in the CMZ of the retina following acute damage, even though the cells generated do not directly contribute to the population of regenerated neurons (Negishi et al., 1982, Hitchcock and Raymond, 1992). We used the her4:Kaede transgenic line to determine what cell types her4-expressing stem cells in the CMZ have the capacity to generate. We found that cells in the
peripheral retina that retained the Kaede reporter included all retinal cell types except for rod photoreceptors. Although we cannot rule out that rods can be generated from the CMZ on the basis of lack of co-localization of rod markers with Kaede in the peripheral retina, these results are consistent with previous observations. Why we observe a significantly expanded number of her4-expressing cells in the CMZ of the XOPS:mCFP retina even though they do not contribute to rod neurogenesis remains unclear. It has been shown that acute photoreceptor damage results in release of cytokines such as ciliary neurotrophic factor (CNTF) and tumor necrosis factor alpha (TNFα), resulting in increased expression of stat3 and ascl1a and Müller glia proliferation in the INL of the central retina (Kassen et al., 2007, Kassen et al., 2009, Nelson et al., 2012, Nelson et al., 2013). It is possible that soluble factors released by apoptotic rods in the ONL diffuse to the CMZ as well as the INL of the central retina, and the stem cell niche of the CMZ is stimulated to increase her4 expression, but is still restricted as to what cell types it can produce. In addition, the specific signals that stimulate the small number of Müller glia in the INL of the XOPS:mCFP retina to express her4 and contribute to the rod lineage remain to be elucidated. Possible mechanisms include juxtacrine signaling that stimulates Notch activity, gradients of extrinsic factors generated by dying photoreceptors, intrinsic cues that sensitize subsets of Müller cells to rod degeneration, or some combination of the above.

In summary, our results demonstrate that her4 is a primary effector of the Notch-Delta signaling pathway during embryonic retinal development, and acts downstream of Notch signaling to repress pro-differentiation genes in subsets of retinal progenitor cells in a specific spatiotemporal pattern. In the adult retina, her4 is expressed in slowly proliferating subsets of Müller cells in the INL of the central retina as well as in proliferating cells of the peripheral CMZ. Previous studies concluded that there was no observable difference in the number of Müller glia in the chronic rod degeneration line (Morris et al., 2008b), and that there must be a large amount of synchronized rod cell death to initiate Müller glia proliferation in the INL (Montgomery et al., 2010). Here, we demonstrate that there is a small but
significant increase in the number of slowly proliferating subsets of Müller cells in the INL of the chronic rod degenerating XOPS:mCFP retina, as well as an increase in her4 expression in the CMZ. Why her4 expression was increased in the XOPS:mCFP CMZ even though the CMZ did not generate rods, as well as why this proliferative zone is restricted from rod neurogenesis remains unclear. In addition, the her4:Kaede transgenic zebrafish proved to be a useful tool for lineage tracing analyses due to temporal labeling via photoconversion and the robust nature of the Kaede reporter. In the future, the her4:Kaede line could be used to not only track descendants of proliferating glia in the retina, but throughout the CNS.

Author contributions
A.C.M. and S.G.W. conceived and designed experiments, S.G.W., W.W., and L.P.K. performed the experiments. A.C.M. and S.G.W. analyzed the data, and A.C.M. and S.G.W wrote the manuscript.

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### Chapter 2 Tables

Table 2.1: Chapter 2 oligo sequences and application:

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Figure 2.51: RT-PCR to assess effectiveness of the her4-sbMO. Injection of the her4 splice blocking morpholino results in loss of the spliced her4 mRNA transcript up to 3 days post injection.

**her4 mRNA**
630 bp (unspliced)
535 bp (spliced)
Figure 2.S2: Overexpression of her4 mRNA results in no apparent retinal phenotype. IHC comparing the expression of late born retinal neurons (rods and cone PRCs) to the first retinal cell type to differentiate (ganglion cells) show no difference between the retinas of 72 hpf embryos injected with 1.5 ng/embryo of her4 mRNA and uninjected controls.
Figure 2.53: Her4 expression during retinal development is downstream of Notch-Delta signaling. A) Fluorescent in situ hybridization (FISH) for her4 expression in the developing retina at 48 hpf, 72 hpf, and 5 dpf. B) Whole mount in situ hybridization (WISH) performed on 48 hpf embryos show a decrease in her4 expression in embryos treated with the γ-secretase inhibitor DAPT compared to vehicle controls (1% DMSO). C) Single transgenic hsp70:Gal4 and double transgenic hsp70:Gal4; UAS:NICD 48 hpf embryos were heat shocked at 24 hpf and processed for WISH at 48 hpf. The double transgenic embryos displayed higher levels of her4 expression upon ectopic expression of the NICD. A, anterior; P, posterior; V, ventral; D, dorsal; L, lens; scale bar, 50 µm.
Figure 2.54: The 3.4 kb *her4.3* promoter is responsive to Notch signaling. A) Dual luciferase assays were performed in HEK293 cells transfected with the pGL3-*her4* firefly luciferase reporter, the pRL-TK Renilla luciferase transfection control reporter, and varying amounts of the pCDNA3-NICD expression vector with balancing amounts of pGEM3Z. There is a dose dependent increase in luciferase activity with increasing amounts of NICD. B) Dual luciferase assays in HEK293 cells using pGL3-*her4* firefly luciferase reporter, the pRL-TK Renilla luciferase transfection control reporter, and varying amounts of the pCDNA3-*her4* expression vector show that with increasing amounts of *her4* expression there is a dosage dependent decrease in relative luciferase activity. *P*<0.05, **P*<0.001.
Figure 2.55: Her4:Kaede zebrafish express the Kaede reporter which can be stably photoconverted in living tissues. A) Non-photoconverted (gKaede) fluorescence in the brain and retina (arrowheads) and cmlc-EGFP transgenesis reporter in the heart (arrow) in the GFP channel. B) No photoconverted Kaede (rKaede) can be detected in the red channel before UV treatment. C-D) Following brief UV exposure, there is a decrease of gKaede (C) and an increase of rKaede fluorescence (D). E) Cryosection of a mature retina 1 week after UV treatment reveals rKaede fluorescence in the central retina and the contribution of new gKaede-positive cells from the CMZ. L, lens; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; CMZ, ciliary marginal zone; hpf, hours post fertilization; scale bar, 50 µm.
Figure 2.56: Acute light damage results in degeneration of rod and cone photoreceptors.

Immunohistochemistry on retinal cryosections of light damaged her4:Kaede albino zebrafish confirm degeneration of rod and cone photoreceptors at 4 and 7 days post light onset (LD). OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; scale bar, 50 µm.
Figure 2.57: Acute light damage results in apoptosis of photoreceptors and increased cell proliferation in the INL and ONL. A-C) TUNEL labeling confirms that there are apoptotic cells in the ONL at 4 and 7 days post light damage onset (LD). D-F) PCNA labeling confirms that there is a robust proliferative response from cells in the INL and ONL following light induced retinal damage. ONL, outer nuclear layer; INL, inner nuclear layer; scale bar, 50 μm.
Figure 2.58: Her4 maternal effect. RT-PCR from cDNAs generated from wild type embryos show significant maternal deposition of spliced her4 mRNA at 2 and 4 hpf (before the maternal to zygotic transition of transcription), and an increase of her4 mRNA at 48 hpf indicating zygotic transcription of her4.
Figure 2.59: The photoconvertible Kaede reporter protein. The Kaede fluorescent reporter is a 116 kDa homotetrameric protein that fluoresces green when exposed to blue light. Following exposure to UV light, the chromophore undergoes an irreversible β-elimination reaction at the amide linkage and the resulting chromophore fluoresces red when exposed to green light, with no green fluorescence when exposed to blue light.
CHAPTER 3: *Her9* acts downstream of Retinoic Acid (RA) signaling during retinal vasculogenesis

**Keywords:** Her9, HES1, Retinoic Acid, Vasculogenesis, Zebrafish, Retina

### 3.1 Abstract

A previous study demonstrated that *hairy-related 9* or *hairy/enhancer of split-related 9* (*her9*), a basic-helix-loop-helix-orange (bHLH-O) transcriptional repressor that is the zebrafish homolog of the human *HES1* gene, is among the group of genes that are upregulated in a background of chronic rod photoreceptor specific degeneration and regeneration in the zebrafish retina (Morris et al., 2011). Here we show that *her9* acts downstream of the retinoic acid (RA) signaling pathway during retinal development, and is expressed in the retina independently of Notch-Delta signaling unlike its mammalian homolog *HES1*. Knockdown of *her9* expression during embryonic ocular development resulted in a decrease in retinal vasculature, and overexpression resulted in ectopic retinal vasculature. In addition, the vasculature defect phenotype was only observed when a translation blocking morpholino was injected, and not when a splice-blocking morpholino was used. RT-PCR analysis showed that there is significant maternal deposition of spliced *her9* mRNA in the developing embryo, and that the amount of maternally deposited *her9* mRNA is sufficient to result in normal retinal vasculature.

### 3.2 Introduction

#### 3.2.1 Vasculogenesis in the zebrafish

The vasculature network in vertebrates is a highly specialized organ that is required for proper function of a number of key physiological tasks including transport of oxygen and removal of carbon dioxide, nutrient disbursement, regulation of osmotic pressure, transport of waste products and debris, immune system response, and remediation of infection. Formation of the vascular network is an
essential step in embryonic development, and is generally considered to take place primarily through two often complimentary mechanisms, vasculogenesis and angiogenesis (Patel-Hett and D'Amore, 2011). Both vasculogenesis, the de novo formation of vessels from endothelial precursor cells, and angiogenesis, the growth of new vessels from pre-existing vasculature by sprouting, are complex and highly regulated processes that are mediated by the precise coordination of multiple signaling pathways and transcription factors.

The zebrafish has recently emerged as an excellent vertebrate model for studying the various paradigms of blood and lymphatic vascular development. Due to their relatively small size, external and rapid development, optical transparency of the embryos, high fecundity, and availability of various vasculature-specific transgenic and mutant lines, the zebrafish has become a useful model for studying the formation of vasculature in vertebrates. The zebrafish have a closed circulatory system, and the anatomical and molecular features in the developing blood vessels are highly similar to those in humans and other higher vertebrates (Isogai et al., 2001). Because of their high fecundity, small size, and fast generation time, zebrafish have been utilized in large-scale forward-genetic screens in which mutations in novel genes that regulate vasculature development have been identified (Gore et al., 2012). In addition, because of their small size, the zebrafish embryos and larvae receive a sufficient amount of oxygen for the first few days post fertilization by passive diffusion alone, allowing for organogenesis and tissue development (including the retina) to proceed for a limited time in the absence of a functional cardiovascular system. This unique trait allows for the study of numerous vasculature defects in a vertebrate model system that wouldn’t be feasible in other established vertebrate models. When passive diffusion alone cannot supply adequate levels of oxygen for organogenesis and tissue development to occur before embryonic lethality (such as in mouse or chick models), the study of blood or lymphatic vessel development could not be conducted.
Similar to other vertebrates, endothelial vasculature tissues and hematopoietic cells in zebrafish embryos arise in close association with each other, and are generally considered to be derived from common precursor cells. In mammalian embryos, endothelial and hematopoietic precursor cells develop and differentiate in extraembryonic yolk sac areas, and in zebrafish, they develop in the intermediate cell mass (derived from the ventral mesoderm) after gastrulation (Detrich et al., 1995). Early hematopoietic and endothelial cells in zebrafish and mammals express a common set of genes and are controlled largely by the same regulatory networks. Early expression of stem cell leukemia (scl) and fetal liver kinase/vascular endothelial growth factor receptor 2 (flk1/vegfr2) in the ventral mesoderm have been shown to be required for endothelial and hematopoietic stem cell development in mouse and zebrafish (Kabrun et al., 1997). Additionally, forward genetic screens have identified numerous genes that are involved in retinal vasculogenesis and retinal development, and misexpression or nonsense mutations in these genes results in microphthalmia, small or missing lens, and reduced or missing retinal vasculature.

Some endothelial precursor cells become lineage restricted during mid-gastrula stages in the developing zebrafish, and they begin to express endothelial-specific genes during somitogenesis (Kimmel et al., 1990). Cells located near the animal pole of the early gastrula give rise to ectodermal fates, whereas cells located near the blastoderm margin give rise to mesodermal and endodermal fates, including vasculature endothelium in the retina. After angioblasts have been specified from the ventral mesoderm, they migrate to the embryonic midline directly above the endoderm to form the primordial axial vessels, the dorsal aorta, and the posterior cardinal vein which constitute the two major trunk axial vessels. These cells are positioned dorsal to the endodermal layer and ventral to the hypochord (Fouquet et al., 1997). Vasculogenesis of the axial vessels in the zebrafish resembles de novo assembly of vessels in other developing vertebrates with few minor differences in timing of differentiation of the dorsal aorta and posterior cardinal vein angioblasts. In all vertebrates, paired axial vessel primordia...
eventually contact each other and establish a closed circulatory network. The zebrafish has a relatively simple embryonic circulatory network that develops extremely rapidly compared to other vertebrates, however, and only contains a single dorsal aorta and posterior cardinal endothelium. In other vertebrates, persistent expression of bone morphogenetic protein (BMP) inhibitors from the midline notochord is required for the initial separation of the bilateral dorsal aorta, and it has been suggested that early absence of these BMP inhibitors during zebrafish development permits formation of a single tube at early stages (Bressan et al., 2009).

After the first axial vessels form by vasculogenesis to produce the dorsal aorta, cardinal vein, and primitive cranial vasculature, an early fate decision results in either arterial or venous identity of the forming vessel (Roman et al., 2002). Acquisition of arterial-venous (A-V) identity has been thought to be directed by physiological parameters such as blood flow, dissolved gas levels, and regional blood pressure differences that appear shortly after blood circulation begins. Recent studies, however, have demonstrated that the endothelial cells lining arteries and veins are functionally and molecularly distinct, and are regulated by pre-defined genetic programs and signaling networks (Swift and Weinstein, 2009, Patel-Hett and D'Amore, 2011). Studies performed using zebrafish have uncovered the functional roles of upstream signaling pathways and factors that result in acquisition of A-V identity. A signaling cascade consisting of sequential Hh, Vegf, and Notch-Delta signaling is required for arterial fate determination (Lawson et al., 2001), and disruption of the Notch pathway results in ectopic expansion of normally venous-restricted markers into arterial domains. Additional zebrafish studies have shown that Hh and Vegf signaling act upstream of Notch during arterial differentiation, and embryos lacking either sonic hedgehog (shh) or vegfa fail to express markers for arterial cell fate within their blood vessels, and show a similar expansion of venous-restricted markers (Lawson et al., 2002).

3.2.2 Vasculogenesis in the vertebrate retina
The zebrafish retina is considered to be particularly sensitive to the effects of reduced or ectopic expression of transcription factors that are downstream of multiple signaling pathways, partially due to the extremely rapid development of such a complex structure as the eye itself. Thus, visualization of retinal vasculature and of the eye itself allows for relatively rapid identification of genes involved in vasculogenesis, as well as small molecule screens that identify compounds that also affect this process (Lawson and Weinstein, 2002a). The retina is one of the most metabolically active tissues found in the body and has a higher rate of oxygen consumption than any other area of the CNS, including the brain (Wangsa-Wirawan and Linsenmeier, 2003). Proper nourishment of the eye and retina by vascular networks is critical for maintaining vision and visual acuity. Severe forms of vision loss and blindness in humans, including diabetic retinopathy and age relate macular degeneration (AMD) are linked with vascular abnormalities (Fruttiger, 2007).

Similar to the major axial vessels in the trunk, the cranial vasculature is initially established by de novo vasculogenesis, followed by sprouting of the more complex cranial vasculature network by angiogenesis (Lee et al., 2009). Close examination of vasculogenesis and angiogenesis in the retina reveals a highly conserved sequence of events that take place in most vertebrates, with the major difference being the rate of ocular development with respect to the rest of the CNS among the various classes in the phyla (Isogai et al., 2001).

In mammals, the mature retina is nourished by two independent circulatory systems, the choroidal and retinal vasculature networks. The choroidal vessels in the non-neural RPE are responsible for around 80% of blood flow to the retina and nourish the highly metabolically active photoreceptor cells in the ONL. The central retinal artery that emanates from the optic disc carries the remaining blood flow that nourishes cells in the INL and GCL. The retinal vessels form intraretinal capillaries that interface with cells at the inner and outer plexiform layers (Provis, 2001). During initial mammalian eye development before the inner retinal vasculature appears, oxygen is provided to the retina and lens by

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choroidal and hyaloid vessels. The hyaloid vasculature is complex of transient intraocular vessels from the optic disc that extend through the choroid fissure and partially envelop the anterior portion of the lens. The hyaloid vessels undergo progressive regression by apoptosis as the retinal vasculature network develops by angiogenesis in a synchronous fashion (Gariano and Gardner, 2005). Failure of the hyaloid vessels to regress results in ocular pathologies such as persistent foetal vasculature, which in turn leads to intraocular hemorrhages, retinal detachment, and vision loss. It has also been observed that failure of the hyaloid vessels to regress coincides with incomplete retinal vascularization, suggesting that the same regulatory pathways and signaling networks are responsible for simultaneously controlling hyaloid vessel regression and formation of the retinal vasculature network (Zhu et al., 1999).

During zebrafish retinal vasculature development, the optic artery branches out from the primitive internal carotid artery around 24 hpf and enters the eye ventrally through the optic fissure and forms the hyaloid artery. They hyaloid artery extends to the lens basket and forms a single loop and then exits the optic fissure as the hyaloid vein by 30 hpf (Alvarez et al., 2007). Endothelial cells between the lens and retina give rise to the first hyaloid vessels at 60 hpf and are tightly connected to the lens. As development continues, the vessels progressively branch and start to lose contact with the lens and adhere to the inner limiting membrane of the juvenile retina by 30 days post fertilization. Unlike mammals, the zebrafish hyaloid vessels do not regress, but simply detach from the lens and contribute to the maturing retinal vasculature network (Alvarez et al., 2007).

The retinal vasculature in the zebrafish inner limiting membrane is not organized in any defined pattern, and the majority of adult retinas have 6-7 retinal vessels branching from the central artery emerging from the optic stalk; moreover, the exact number of branches can vary from the left and right eye of the same animal. The main branches extend radially from the central artery and branch into capillary networks before connecting to a circumferential vein in the ciliary marginal zone of the retina. Unlike the human retina, the zebrafish do not possess an avascular cone rich region in the central retina.
(fovea of the macula). The zebrafish retinal vasculature extends throughout the inner limiting membrane and nourishes all GCL and INL neurons. The choroidal vasculature adjacent to the non-neural RPE of the zebrafish, similar to the choroidal vasculature in mammals, is primarily responsible for nourishment of the highly metabolically active photoreceptors.

Throughout the life of the zebrafish, the vasculature network is expanded and refined as the eye grows. In addition, the retinal blood supply is associated with a Müller glial scaffold in the retina whose end feet make direct contact with blood vessels in the inner limiting membrane and have processes that extend the entire thickness of the retina. Transgenic gfap:GFP zebrafish which express GFP in glial cells, and kdrI:mCherry or fliI:EGFP transgenic lines (which express fluorescent reporters in vasculature endothelial cells) show co-localization of reporter signal in the inner limiting membrane as well as the RPE, suggesting that it is the Müller glia cells associating with capillaries that are responsible for disbursement of oxygen and other nutrients throughout the thickness of the retina (Gore et al., 2012).

3.2.3 The retinoic acid signaling pathway

Retinoic acid (RA) is a vitamin-A derived small lipophilic molecule that acts as a morphogen. RA is a ligand for members of the nuclear RA receptor (RAR) superfamily, and binding of RA to a RAR results in activation of target genes. Vitamin-A (retinol) has long been known to be involved in the visual cycle; its derivative 11-cis retinal undergoes photoisomerization when struck by a photon of light which triggers initiation of the visual phototransduction pathway (Larhammar et al., 2009, Yau and Hardie, 2009, Fain et al., 2010). It was discovered from work in avian and rodent models that deficiency of vitamin-A as well as abnormal embryonic exposure to its metabolite retinoic acid, resulted in a wide range of developmental defects (Wilson et al., 1953, Clagett-Dame and Knutson, 2011).

After the potent teratogenic effects of RA were observed, possible functions of RA during embryonic development were inferred by global or local exposure of RA at multiple developmental stages and body/tissue locations in a wide range of species, including amphibians, reptiles, chicks,
rodents and higher mammals (Avantaggiato et al., 1996). Eventually the genes and enzymatic pathways that regulate RA synthesis in maternally deposited retinoids as well as the highly regulated process of RA catabolism were more fully characterized with the advent of modern molecular techniques. It has been shown that most elements of the RA signaling pathway (metabolic/catabolic pathways, expression patterns, and affected tissues) are highly conserved across most vertebrates (Avantaggiato et al., 1996, Rhinn and Dolle, 2012).

Retinoic acid binds to retinoic acid receptors (RARs), which are members of the highly conserved nuclear receptor superfamily that includes RARα, RARβ and RARγ. When bound to the RA ligand, RARs act in heterodimeric combinations with retinoid X receptors (RXRα, RXRβ and RXRγ). RXRs themselves cannot bind RA and act mainly as scaffolding proteins to facilitate binding of the RAR-RXR heterodimer complex to DNA (Chawla et al., 2001). There is a large degree of functional redundancy with the various permutations of RAR/RXR heterodimers, and developmental abnormalities usually only occur when two receptors are inactivated in combination or if there abnormal levels of the RA ligand (Mark et al., 2009).

After the RA ligand and RAR/RXR receptors come into contact, the heterodimer-ligand complex translocates to the nucleus where it binds to retinoic-acid-response-elements (RAREs) in the promoters of target genes. The core RARE DNA motif consists of repeats of the hexameric sequence 5′-(A/G)G(G/T)TCA-3′. Tight genetic regulation of RA signaling is accomplished by the ability of the RAR/RXR heterodimer to bind to RAREs in the absence of the RA ligand and repress expression of target genes. It is only in the presence of RA that a conformational change in the complex leads to the release of co-repressors and recruitment of co-activator transcription factors which then result in expression of target genes (Rhinn and Dolle, 2012). Some of the known co-activators include chromatin remodeling genes which decompact the chromatin and allowing for the assembly of the transcription pre-initiation complex.
The morphogen-like gradients resulting from retinoic acid production in specific areas and cell types plays a role in numerous developmental pathways; the establishment of signaling boundaries due to RA metabolism allows the RA signaling pathway to finely control the differentiation and patterning of stem and progenitor cell populations throughout the developing embryo. RA signaling and the effects of inappropriate levels of RA have been well documented in hindbrain development, where disruption of the pathway leads to numerous developmental defects (Kiecker and Lumsden, 2005). During forebrain development, RA signaling is required for proper anterior-posterior patterning and cell survival of the telencephalon. In addition, RA signaling mediates crosstalk between Hh, Wnt, and FGF to establish morphology and cell types (Halilagic et al., 2003). In some cases, excess RA in the developing zebrafish optic primordia causes a duplication of the retina along the dorsoventral axis (Hyatt and Dowling, 1997).

3.2.4 Hairy-related 9 (her9) during embryonic development

Hairy-related 9 or hairy/enhancer of split-related 9 (her9), is a basic-helix-loop-helix-orange (bHLH-O) transcriptional repressor that is the zebrafish ortholog of the human HES1 gene (see chapter 1 for an overview of the structure and function of the bHLH-O class of transcription factors). Unlike the mouse and chicken orthologs (Hes1 and c-hairy 1 respectively), the zebrafish ortholog her9 is not expressed cyclically in the presomitic mesoderm, but rather in the ectodermal tissues during early embryonic development (Leve et al., 2001). In addition, expression of her9 in all observed tissues does not appear to be sensitive to manipulation of the Notch-Delta signaling pathway, whereas the human, mouse, and chicken homologs are commonly used as indicators of active notch-delta signaling (Kitagawa et al., 2013).

The pro-neural domains in a developing embryo which give rise to primary motor, inter-, and Rohon-Beard neurons are established at the beginning of neurogenesis along the anteroposterior axis in the dorsal ectoderm. Here, pro-neural and inter-proneural domains are established by expression of numerous bHLH and bHLH-O genes, including her9. In these domains, her9 has been shown to be
downstream of Bmp signaling, and morpholino based knockdown of *her9* results in ectopic expression of pro-neural genes and a greatly expanded pro-neural domain at the expense of inter-pro-neural domains (Bae et al., 2005). These data indicated that *her9* and BMP signaling have distinct roles in neurogenesis from the Notch pathway, and *her9* functions as a prepattern gene in the early developing neuroectoderm.

To further add to the complexity of *her9* regulation in the developing zebrafish embryo, *her9* has also been implicated in floor plate development in midline precursor cells derived from the dorsal organizer during gastrulation. Expression of *her9* has been shown to regulate notochord development and promote floor plate specification, and similar to expression in other tissues, *her9* expression in early floor plate cells does not require Notch signaling (Latimer et al., 2005). Here, *her9* expression is dependent upon midline-Nodal signaling and acts downstream of the Nodal signaling pathway to inhibit notochord development.

Recent studies have also demonstrated that *her9* represses a neurogenic fate downstream of *tbx1* and retinoic acid signaling during inner ear development (Radošević et al., 2011). In the developing cranial otic placode, *her9* expression defines the posterolateral non-neurogenic field downstream of *tbx1*, and both genes are responsive to RA signaling. In addition, opposing RA and Hh signaling position the boundary between the neurogenic and non-neurogenic domains, however manipulation of Hh signaling alone does not influence *her9* expression in this tissue. In the retina, it has also been shown that an interconnected core transcriptional network consisting of *sex determining region Y-box 2 (sox2)*, *T-cell leukemia homeobox-1 (tlx)*, *GLI family zinc finger 3 (gli3)*, and *her9* transcription factors are responsible for restriction of *retina-specific homeobox gene 2 (rx2)* expression to the peripheral ciliary marginal zone where the *rx2*-positive cells behave as stem cells for both the neuro-retina and the non-neural RPE (Reinhardt et al., 2015). Although *her9* appears to be involved in a wide variety of neurogenic processes, the role that *her9* plays during retinal development, and in particular retinal vasculogenesis
remains poorly defined. In this study, we wanted to determine if her9 expression was involved in retinal vasculogenesis and to place retinal expression of her9 within the context of a gene regulatory network. We were able to demonstrate that expression of her9 in the retina is regulated by RA signaling, and that the spatiotemporal expression pattern of her9 coincides with retinal vasculogenesis. In addition, knockdown of her9 or RA signaling results in decreased retinal vasculature, while overexpression results in ectopic vascular development.

3.3 Results

3.3.1 Her9 expression in the wild type and XOPS:mCFP retina

A previous microarray analyses has demonstrated that hairy-related 9 (her9) is upregulated in the mature retinas of transgenic XOPS:mCFP zebrafish that experience constitutive rod photoreceptor degeneration and regeneration (Morris et al., 2011). These results were verified by semi-quantitative RT-PCR and quantitative PCR (Figure 3.1), and showed that her9 expression in the retina of XOPS:mCFP zebrafish is increased roughly 4-fold as compared to controls. To further understand the possible role(s) of her9, and to place her9 within the context of a retina specific gene regulatory network, it was necessary to establish which signaling pathway influenced retinal her9 expression. In order to address this question, the normal expression pattern of her9 in the retina had to be examined.

The spatiotemporal expression pattern of her9 in the wild type developing retina and in the adult XOPS:mCFP regenerating zebrafish retinas were determined by in situ hybridization using DIG-labeled RNA probes specifically designed to detect her9 transcript (Table 3.1). At 24 hpf, her9 was expressed throughout the developing wild type retina, including the lens (Figure 3.2A). From 30-36 hpf, however, her9 expression appeared to largely cease in the central retina and be restricted more towards the ciliary marginal zone, with some expression remaining in posterior regions of the central retina (from a dorsal view) (Figure 3.2B,C). By 42 hpf, retinal expression of her9 appeared to be restricted
primarily to the CMZ, with persistent expression in the posterior retina (again, from the dorsal view of a whole-mount in situ hybridization image). When viewed laterally, and with the eye dissected away from the rest of the head, it is clear that at 42 hpf that her9 is expressed at the peripheral most CMZ-lens transition area and in the choroid fissure (Figure 3.2C,D). This result was surprising because the bHLH-O genes (including the mammalian ortholog of her9, HES1) have not been previously implicated in retinal vasculature development, and as previously mentioned, these are exactly the same spatiotemporal patterns in the developing zebrafish retina where the hyaloid artery and lens basket vasculature are forming. By 48 hpf, her9 expression could not be detected anywhere in the retina, and at any later time point beyond 48 hpf, only traces of her9 expression could be observed in the hindbrain of the wild type retina (data not shown).

In the mature zebrafish retina, expression of her9 visualized by colorimetric in situ hybridization in adult retinal sections could not be detected in the central retina of the wild type zebrafish or in the CMZ (Figure 3.3A,B). In the adult transgenic XOPS:mCFP retina, however, expression of her9 could be observed adjacent to the RPE, possibly where choroidal vasculature cells are present (although it must be noted that depending on the probe used, background coloration sometimes develops in the RPE.
Figure 3.2: Expression of *her9* in the developing zebrafish retina. A-D) Whole mount in situ hybridization used to visualize the spatiotemporal pattern of *her9* expression shows that from 24 hpf to 42 hpf *her9* expression gets restricted to the peripheral retina adjacent to the lens (dorsal view). E-F) Lateral view of the retina reveals that besides expression in the peripheral most CMZ, *her9* is also expressed in the choroid fissure. L, lens; GCL, ganglion cell layer; PR, peripheral retina; CMZ, ciliary marginal zone; CF, choroid fissure; A, anterior; P, posterior; scale bar, 50 µm.
Figure 3.3: Expression of *her9* in the adult retina. *Her9* expression cannot be detected by colorimetric in situ hybridization in the adult wild type embryo, but can be detected in the choroid and CMZ of the adult XOPS:mCFP retina. A-B) Colorimetric ISH on adult wild type sections reveal that *her9* is either not expressed in the central retina, choroid, and CMZ, or that expression levels are too low to detect by this method in the wild type background. C-D) *Her9* is expressed in some cells in the choroid and CMZ of the XOPS:mCFP retina, but not in the central retina. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; CMZ, ciliary marginal zone; RPE, retinal pigmented epithelium; scale bar, 50 µm.

Even if the gene is not expressed there; during WISH experiments, however, the coloration patterns detected when using the *her9* probe is consistent with what is observed when individual or small groups of cells contain a specific transcript (Figure 3.3C). In addition, a small number of cells in the CMZ of the adult XOPS:mCFP retina also expressed *her9* (Figure 3.3D). Interestingly, in the mature XOPS:mCFP
retina, expression of her9 resembled expression of her4 in the CMZ of the chronic rod degeneration/regeneration line. Her9, unlike her4 (which is a known downstream effector of Notch-Delta signaling), was not expressed in the central neural-retina of the adult wild type or XOPS:mCFP retina, but rather adjacent to the RPE in the choroid.

3.3.2 Her9 is expressed in retinal vasculature endothelium during embryonic retinal development

To address whether expression of her9 coincides with markers for retinal vasculature, fluorescent in situ hybridization (FISH) experiments were conducted in a transgenic line of zebrafish that express EGFP specifically in vasculature endothelial cells (fli1:EGFP). At 36 hpf, expression of her9 was largely restricted to the ciliary marginal zone and the choroid fissure of the retina and overlapped with fli1 signal, especially in the choroid fissure where the hyaloid artery was forming (Figure 3.4A-D). By 42 hpf, expression of her9 in the retina could only be detected in the peripheral areas of the retina around the lens basket vasculature, and in the choroid fissure (Figure 3.4E-F). These data suggest that because of the spatiotemporally overlapping expression of her9 and fli1, that the her9 transcription factor might be playing a role in specifying precursor cells to differentiate into vascular endothelial cells, or is expressed in already differentiating vascular endothelial cells to preclude a neuronal fate in the retina.

3.3.3 Her9 is expressed downstream of RA signaling in the developing zebrafish retina

In situ hybridization and quantitative RT-PCR experiments were performed on 36 hpf embryos to determine whether her9 expression in the retina is affected by manipulation of the RA signaling pathway. 24 hpf embryos (that already had an established primordial retina and lens) were dechorionated and treated with either 1 µM retinoic acid with 0.3% DMSO, 100 µM of the RA signaling inhibitor diethylaminobenzaldehyde (DEAB) in 0.3% DMSO, or 0.3% DMSO alone as the carrier control until 36 hpf when they were processed for WISH, or heads removed and processed for qPCR. The expression pattern of her9 observed in the retina of the control group was typical of her9 expression in embryos raised in fish water, with expression at the peripheral CMZ adjacent to the lens and expression
Figure 3.4: **Her9 is expressed in retinal vasculature in the developing retina.** Fluorescent in situ hybridization (FISH) and anti-GFP immunohistochemistry (IHC) reveal *her9* is expressed in retinal vasculature. **A-H** FISH and IHC double labeling experiments in 36 and 42 hpf retinal sections of *fli1*:EGFP embryos shows co-localization of *her9* transcript and the reporter for vascular endothelial cells in the peripheral CMZ and choroid fissure in the embryonic retina during development of the retinal vasculature network. CF, choroid fissure; L, lens; scale bar, 50 µm.

In the choroid fissure (**Figure 3.5A**). Compared to the control group, the DEAB treated embryos displayed reduced *her9* expression in the retina (**Figure 3.5B**). In contrast, in the RA treated embryos there was a large increase in the amount of *her9* expression as well as an expanded domain of expression in the RA treated embryos when compared to either the control group or the DEAB treated embryos (**Figure 3.5C**). QRT-PCR experiments corroborated these findings, with the RA treated heads having a 2-fold increase in *her9* expression and the DEAB treated heads having a 3-fold decrease in *her9* expression when compared to the control group (**Figure 3.6**). The *her9* expression changes observed with treatment of both DEAB and RA were consistent and significant across all biological replicates.
Figure 3.5: Expression of her9 in the retina is downstream of retinoic acid signaling. A) WISH showing her9 expression in 36 hpf embryos treated from 24-36 hpf with the DMSO vehicle control. B) WISH showing her9 expression is decreased at 36 hpf when the embryo is treated from 24-36 hpf with the RA signaling inhibitor, DEAB. C) Expression of her9 at 36 hpf is increased when RA is added to the embryo medium from 24-36 hpf. L, lens; CF, choroid fissure.
Figure 3.6: *Her9* qPCR following RA and DEAB treatments. Quantitative RT-PCR shows *her9* expression in the whole heads of 36 hpf zebrafish embryos following drug treatments from 24-36 hpf shows that compared to DMSO controls, *her9* expression in the heads of 36 hpf zebrafish increases when RA signaling increases, and decreases when RA signaling is inhibited by DEAB. N=20 heads per biological replicate, 3 biological replicates per drug treatment. Error bars = standard deviation, *=P<0.05.

3.3.4 RA signaling influences vasculogenesis in the embryonic zebrafish retina.

Fluorescent in situ hybridization (FISH), and anti-GFP immunohistochemistry (IHC) experiments were performed on retinal sections of *fli1:EGFP* embryos that were treated with RA, DEAB, or DMSO to visualize the change in *her9* expression in sections, as well as to determine if there were any retinal vasculature defects when RA signaling is pharmacologically manipulated. As with the WISH experiments, there was an obvious decrease in *her9* expression when RA signaling was inhibited, and an increase in the RA treated retina compared to controls (Figure 3.7). IHC to visualize vascular endothelial cells revealed a decrease in retinal vasculature in the DEAB treated group, and a striking increase in the number of ectopic blood vessels in the RA treated retinas (Figure 3.7). Interestingly, the ectopic vasculature was expanded throughout the central retina, not just in the choroid. QPCR experiments
were performed on cDNA pools made from RNA from the heads of the drug treated embryos to quantify the expression levels of *vascular endothelial growth factor receptor 2* (*vegfr2*), a marker for vasculature endothelium. In agreement with the IHC data, *vegfr2* expression increased in the RA treated group, and decreased in the DEAB treated embryos when compared to controls (Figure 3.8). The expression of *vegfr2* was consistent across all biological replicates.

**Figure 3.7: Retinal vasculature following RA and DEAB treatment.** Fluorescent in situ hybridization (FISH) and anti-GFP immunohistochemistry (IHC) in the *fli1:EGFP* background reveals that along with *her9* expression, retinal vasculature development is directly affected by transient manipulation of the RA signaling pathway. A-A‴) FISH and IHC on embryos treated with DMSO control from 24-36 hpf. B-B‴) FISH and IHC on embryos treated with the RA signaling inhibitor DEAB show a decrease in *her9*
expression and a decrease in retinal vasculature. C-C”) FISH and IHC on embryos treated with RA show increased her9 expression and ectopic retinal vasculature. L, lens; CF, choroid fissure; Scale bar, 50 µm.

![Image](image.png)

**Figure 3.8: Vegfr2 qPCR following RA and DEAB treatments.** Quantitative RT-PCR shows vegfr2 expression in the whole heads of 36 hpf zebrafish embryos following drug treatments from 24-36 hpf. Quantitative RT-PCR shows vegfr2 expression in the whole heads of 36 hpf zebrafish embryos following drug treatments from 24-36 hpf demonstrating that compared to DMSO controls, vegfr2 expression in the heads of 36 hpf zebrafish increases when RA signaling increases, and decreases when RA signaling is inhibited by DEAB. These data correlate with the observed changes in retinal vasculature during the drug treatments. N=20 heads per biological replicate, 3 biological replicates per drug treatment. Error bars = standard deviation, *=P<0.05.

3.3.5 *Morpholino mediated knockdown of her9 results in reduced retinal vasculature, and overexpression results in ectopic blood vessels in the retina*

To assess the contribution of her9 to retinal and/or retinal vasculature development, a morpholino mediated knockdown strategy was used to knockdown expression of her9 in the developing zebrafish embryo (Nasevicius and Ekker, 2000). A morpholino designed to block translation (tbMO) of her9 mRNA (**Table 3.1**) was injected into 1-cell stage *fli1:EGFP* zebrafish embryos at various
concentrations to determine a usable dosage. At 3 ng/embryo, survivability of the her9 morphants was similar to the control-MO (cMO) injected embryos at the same dosage, however there was a range of morphant phenotype severity by 48 hpf (Figure 3.9A-D). Upon close examination ~30% of the embryos had mild, ~40% moderate, and ~30% severe morphant phenotypes, and the moderate category was selected for further analysis. When compared to the cMO injected embryos, the her9 morphant retinas had significantly less retinal vasculature at the same time point (Figure 3.9C’’).

![Figure 3.9: Knockdown of her9 using the her9-tbMO. A) Control embryos injected at the 1-cell stage with 3 ng/embryo of the std-ctrl morpholino. B-D) Injection of 3 ng/embryo of the her9 translation blocking morpholino results in a range of morphant phenotype severity. A’-A’’) Fli1:EGFP signal in the normal looking zebrafish control-MO retina. C-C’’) Fli1:EGFP signal in the her9 morphants ranked as “moderate” severity shows highly abnormal retinal vasculature phenotypes. L, lens; Scale bar, 50 µm.](image)

To assess the specificity of the her9-tbMO, in vitro transcribed her9 mRNA (missing the her9-tbMO binding site was co-injected at 1.5 ng/embryo with the tbMO into fli1:EGFP embryos. Addition of the capped her9 mRNA rescued the morphant phenotype (data not shown). Interestingly, however,
when injected alone, the addition of 1.5 ng/embryo of her9 mRNA resulted in ectopic and expanded retinal vasculature domains when compared to uninjected control fli1:EGFP embryos (Figure 3.10).

**Figure 3.10: Overexpression of her9.** Injection of 1.5 ng of capped her9 mRNA results in ectopic expression of vascular endothelium and expanded vasculature domains in the retina. UIC, uninjected control; L, lens; Scale bar, 50 µm.

To further verify that the observed vasculature defect phenotype observed in the her9-tbMO morphant retina, a second morpholino was used to block splicing of her9 pre-mRNA (her9-sbMO) (Table 3.1). Unlike the tbMO, however, injection of even high doses (10 ng/embryo of the sbMO) did not result in any obvious retinal defects by 48 hpf (Figure 3.11A). RT-PCR analysis was used to confirm that the her9-sbMO was capable of blocking splicing of her9 pre-mRNA (Figure 3.11B). RT-PCR analysis at 2, 4, and 48 hpf revealed the presence of significant amounts maternal deposition of spliced her9 mRNA before the onset of zygotic transcription which occurs slightly after 4 hpf (Figure 3.11C). This could explain the lack of any observable retinal vasculature defect phenotype when the sbMO was used.
Figure 3.11: The *her9*-sbMO morphant does not have a retinal phenotype. A) Unlike the *her9*-tbMO, injection of high doses (10 ng/embryo of the sbMO) did not result in any obvious retinal defects by 48 hpf. B) RT-PCR analysis was used to confirm that the *her9*-sbMO was capable of blocking splicing of *her9* pre-mRNA. C) RT-PCR analysis at 2, 4, and 48 hpf shows significant maternal deposition of spliced *her9* mRNA before the onset of zygotic transcription which occurs slightly after 4 hpf. L, lens; Scale bar, 50 µm.
3.4 Discussion

A previous microarray and subsequent validation by qPCR showed that there was around a 4-fold increase in expression of her9 in the mature XOPS:mCFP retina, which constitutively undergoes rod-specific degeneration and regeneration. These data suggested that her9 might play a role in photoreceptor regeneration or maintenance of photoreceptor progenitor cell pools similar to what was observed with her4 (HES5 in mammals) (Wilson et al., 2016). Additionally, because HES1 (the mammalian homolog of her9) is commonly used for a read-out of activated Notch-Delta signaling in mammals, we thought it could be possible that Notch signaling influences her9 expression in the retina, though it is known to be downstream of other regulatory pathways (Bmp, Nodal, Hh, RA) in other tissues during development. However, our results show that in fact retinal expression of her9 is not dependent upon the Notch pathway, but RA signaling.

In the adult wild type retina, her9 expression could not be detected anywhere in the central retina or CMZ by colorimetric in situ hybridization. There are her9 expressing cells, however, in the XOPS:mCFP CMZ as well as in discrete pockets of cells in the choroid. It is possible that the her9-expressing cells belong to the choroidal vasculature network which may be expanded in the XOPS:mCFP eye due to the high metabolic needs of constant rod photoreceptor turnover; however, further studies are needed to determine if this is the case. The purpose of the small populations of her9-expressing cells in the CMZ of the XOPS:mCFP retina also remains unclear.

In the developing retina, the spatiotemporal expression pattern of her9 overlapped with location and development of the hyaloid artery in the choroid fissure and the lens basket vasculature adjacent to the CMZ. FISH experiments performed in the fli1:EGFP zebrafish showed that expression of her9 near the CMZ and in the choroid fissure co-localized with the EGFP reporter, indicating that her9 is expressed in retinal vasculature tissue. This unexpected result suggested that her9 could be playing a role in development of the retinal vasculature network. The gene regulatory network for retinal
vasculogenesis in the zebrafish has been partially described, and this novel finding could place her9 within that network.

In order to determine what signaling pathway(s) regulate her9 expression in the retina, drugs were used to transiently knockdown candidate pathways during retinal development. Pharmacological inhibition of Notch-Delta, Hh, and Fgf signaling pathways from 24-36 hpf (after the eye field and lens primordia have already been established) did not result in altered her9 expression in the retina (data not shown). In contrast, her9 expression in the retina was sensitive to RA signaling. Addition of RA resulted in increase of her9 expression and an expansion of the expression domains in the developing retina, and inhibition of RA signaling resulted in a decrease of her9 expression. In addition, transient RA treatment or inhibition resulted not only in up- or down-regulation of her9, but greatly affected the retinal vasculature network. These data indicated that not only was her9 regulated by RA signaling in the retina, but also that exposure to retinoic acid or a drug that inhibits RA signaling during a transient window when the retinal vasculature develops in the zebrafish is enough to modulate her9 expression and influence vasculogenesis.

Morpholino mediated knockdown of her9 using a translation blocking morpholino (her9-tbMO) resulted in a decrease of vasculature endothelial cells in the retina compared to control embryos. Surprisingly, co-injection of in vitro transcribed capped her9 mRNA not only rescued the morphant phenotype, but injection of her9 mRNA alone resulted in excess retinal vasculature. These data indicate that her9, even independent of RA modulation, plays a role in development of the retinal vasculature network. In contrast to the retinal phenotype observed when using a relatively low concentration of the tbMO, injection of a splice blocking morpholino (sbMO), even at a high dose, failed to yield any appreciable retinal phenotype. Subsequent RT-PCR analysis showed that although the sbMO did indeed block splicing of her9 pre-mRNA, there was significant maternal deposition of mature her9 mRNA that can be seen before the transition to zygotic transcription. Along with the fact that her9 expression
completely subsides in the retina by 48 hpf, these data suggest that the amount of maternally deposited her9 mRNA is adequate to generate a phenotypically normal retina, even in the absence of zygotically produced mature her9 mRNA.

Taken together, these data demonstrate a novel role for her9 during zebrafish CNS development. The ability of her9 to regulate development of the retinal vasculature network downstream of RA signaling has not been previously shown, and adds another known role for this bHLH-O transcription factor. In addition the possibility that manipulation of her9 could lead to changes blood vessel development in the retina of a patient experiencing one of the several ocular diseases that lead to retinopathy and vision loss is an exciting prospect that warrants further investigation.

3.5 Materials and methods

3.5.1 Zebrafish

All zebrafish (Danio rerio) strains were bred, raised, and maintained in accordance with established animal care protocols for zebrafish husbandry (Westerfield, 1995). Adult fish, embryos, and larvae were housed at 28°C, on a 14 hr light: 10 hr dark cycle. Embryos were staged as previously described (Kimmel et al., 1995). The Tg(XRho:gap43-mCFP)q13 transgenic line, hereafter called XOPS:mCFP, has been previously described (Morris et al., 2005, Morris et al., 2011). The Tg(fli1a:EGFP)y1Tg, hereafter called fli1:EGFP has been previously described (Lawson and Weinstein, 2002b). All animal procedures were carried out in accordance with guidelines established by the University of Kentucky Institutional Animal Care and Use Committee (IUCAC).

3.5.2 Drug treatments

24 hpf wild type or fli1:EGFP embryos were manually dechorionated and treated in 6-well plates with pre-warmed 1 µM retinoic acid with 0.3% DMSO in embryo medium, 100 µM of the RA signaling inhibitor diethylaminobenzaldehyde (DEAB) in 0.3% DMSO in embryo medium, or 0.3% DMSO alone as
the carrier control in complete darkness until 36 hpf when they were processed for WISH, or heads removed and processed for qPCR.

3.5.3 RNA isolation

RNA was isolated from whole embryos at indicated developmental time points. Embryos and larvae were euthanized by rapid cooling as previously described (Wilson et al., 2009). RNA was extracted from pools of embryos using TRIzol Reagent (Life Technologies, Invitrogen), following the manufacturer’s protocol, then treated with RNase-Free DNAsel (Roche, Indianapolis, IN) to remove genomic DNA.

3.5.4 Morpholino and mRNA microinjections

A translation blocking antisense synthetic morpholino oligonucleotide (tbMO) was designed to target sequence immediately upstream of the her9 start site (table 3.1). Another morpholino designed block pre-mRNA splicing (sbMO) of the first her9 intron was also used to assess off target effects of either morpholino (table 3.1). The tbMO was injected at a concentration of 3 ng/embryo, and the sbMO was injected at a concentration of 10 ng/embryo. A standard control MO, targeting a mutant variant of the human β-globin gene (Table 3.1) was used as dosage matched injection controls. All morpholinos were synthesized by GeneTools, LLC (Philomath, OR). A her9 cDNA amplicon was generated from forward and reverse primers that contained 5’ restriction sites (Table 3.1) and cloned into pGEM-TE. Capped mRNA was synthesized using the mMessage T7 kit (Ambion, Austin, TX) according to manufacturer’s instructions and purified by phenol-chloroform extraction and ethanol precipitation. The morpholinos, mRNA (at 1.5 ng/embryo), or a combination of the morpholino and mRNA were injected at a volume of 4.18 nl/embryo in buffered solution with 0.025% dextran red (as an injection indicator) into the yolk of 1-cell stage zebrafish.

3.5.5 Reverse transcriptase PCR (RTPCR)
Following embryo collection and RNA extraction at 36 hpf, approximately 500-1000 ng of RNA derived from the heads of the embryos only was reverse transcribed into cDNA (GoScript Reverse Transcriptase System, Promega, Madison, WI) according to the manufacturer’s instructions. PCR reactions were then performed using primers designed to amplify her4 and atp5h (housekeeping control) cDNA transcripts (Table 3.1).

3.5.6 Immunohistochemistry

Whole embryos were collected at 72 hpf and fixed overnight in 4% PFA at 4°C. Fixed embryos were cryoprotected in 10% sucrose in PBS for at least 3 hours and in 30% sucrose overnight at 4°C. Samples were mounted in OCT Medium (Ted Pella, Redding, CA) and frozen on dry ice. 10 µm sections were cut on a cryostat (Leica CM 1850 or CM 1800, Leica Biosystems, Buffalo Grove, IL), mounted on gelatin-coated slides, and dried overnight at room temperature. Before immunolabeling, sections were rehydrated and postfixed in 1% PFA for 10 minutes at room temperature. After 2 washes in PBS, and 2 washes in PBST, sections were blocked in PBST containing 1% BSA for at least 30 minutes at room temperature. Slides were incubated with anti-GFP (rabbit, 1:1000, Millipore, Billerica, MA) primary antibody in PBST/BSA with 5% Normal Goat serum, overnight at 4°C in a humidified chamber. The following day, slides were washed 3 times in PBST, and incubated with secondary antibody (Alexa fluor-conjugated secondary antibodies (Invitrogen, Grand Island, NY) and Cy-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were all used at a dilution of 1:200) in PBST/BSA for 1 hour at room temperature in the dark. Slides were washed 2 times with PBST, counterstained with DAPI (4',6-diamidino-2-phenylindole, 1:10,000 dilution; Sigma-Aldrich) in PBS, and mounted in 40% glycerol in PBS. Images were obtained on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments).

3.5.7 Whole mount in situ hybridization (WISH) and fluorescent in situ hybridization (FISH)

WISH, FISH, and two color FISH were performed as previously described (Forbes-Osborne et al., 2013, Pillai-Kastoori et al., 2014). Antisense RNA probes were prepared by in vitro transcription of
linearized plasmids containing a portion of the coding sequence of the gene of interest using T7 polymerase and digoxigenin (DIG) or fluorescein (FITC) labeling mix (Roche Applied Science, Indianapolis, IN). The her9 containing plasmids were prepared by cloning PCR products into the pGEM-T-easy vector (Promega, Madison, WI). The sequences of all PCR primers used in this study are presented in (Table 3.1). Images were obtained on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments), and were exported into Adobe Photoshop for figure preparation.

Acknowledgments

I would like to thank Vince Gouge for all of the time and effort he put forth generating data for this project, and Sara Perkins for care and maintenance of zebrafish stocks.

Chapter 3 Tables

Table 3.1: Chapter 3 oligo sequences and application:

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Chapter 4: Generation and validation of zebrafish her9 mutant lines using clustered regularly interspaced short palindromic repeats (CRISPR/Cas9 nuclease) technology

KEYWORDS: Her9, HES1, CRISPR, Cas9, Zebrafish, Retina

4.1 Abstract

Previous morpholino-mediated knockdown studies have indicated that the zebrafish her9 gene plays a role in development of the retinal vasculature. However, her9 mutant lines are not available to the research community. Until recently, the only widely spread reverse genetic tools that could be used in the zebrafish with varying degrees of success have been anti-sense synthetic morpholino oligonucleotide technology. Although morpholinos allow for relatively rapid study of gene function, they only transiently knock down gene expression during early development do not cause permanent genetic lesions, which may prove to be insufficient in the study of many genes. Recently, new reverse genetic approaches have been developed in which the gene of interest is knocked out rather than transiently knocked down by inducing targeted genetic lesions, allowing not only for study of gene function during embryonic development, but throughout the life of the zebrafish. Here, we discuss using the CRISPR/Cas9 genome editing technology to generate various mutant her9 zebrafish lines, validate the mutant lines result in a predicted non-functional gene product, and describe preliminary characterization of the early her9 mutant phenotype.

4.2 Introduction

4.2.1 Forward and reverse genetic approaches in the zebrafish

Classical forward genetic screens have long been used with great success in Drosophila, however, finding mutations in vertebrates is a much slower process. One of the advantages to using the zebrafish as a model organism is that the zebrafish has remarkably high fecundity for a vertebrate. In
addition, due to the rapid external development of the optically transparent embryo, mutations are relatively easy to identify. One of the great benefits of a forward genetic screen is that it results in an unbiased mutagenesis approach that can uncover functions of novel genes (or previously undescribed cis-regulatory elements). Numerous mutant lines in which previously unknown genes have been discovered have already been made available to the zebrafish research community by use of large scale forward genetic screens (Driever et al., 1996).

With the relatively recent advance of automated DNA sequencing, large volumes of genomic sequence data have been rapidly generated from numerous types of organisms. These data sets contain sequences of genes with known functions in specific organisms that yield specific phenotypic traits, but unknown functions in other species. In addition, much of this large scale sequence data contain sequences with no known biological role. Although targeted genome editing by homologous recombination to generate a wide variety of knockout and knock-in lines has been extensively used in several different vertebrates, it requires the ability to readily culture embryonic stem (ES) cells (Babinet et al., 1989, Baribault and Kemler, 1989), something that has proven to be difficult to accomplish efficiently in zebrafish (Fan and Collodi, 2006). New targeted genome editing techniques that have been developed now allow for reverse genetic approaches to elucidate the biological function of sequences of interest in the zebrafish.

Since the early last decade, injection of synthetic antisense morpholino oligonucleotides into 1-cell stage zebrafish embryos has given researchers a quick, inexpensive, and high-throughput method of targeted gene knockdown, without tissue specific effects as observed with RNA-based interference methods (Nasevicius and Ekker, 2000). Unlike a mutant, however, morpholino mediated approach to study gene function only results in transient knockdown of the target gene during early development; the morpholino concentrations in each cell gets too diluted to be effective as the animal grows and more cell division takes place. This results in morpholino based knockdown approaches being
insufficient for the study of specific genes that function late in embryonic development or in the adult zebrafish. In addition, maternal effect (if a splice blocking morpholino is used) as well as off target effects have been shown to result in a morphant phenotype that does resemble the knockout phenotype of the same gene (Kok et al., 2015).

Zinc finger nucleases (ZFNs) were the first artificial restriction enzymes that allowed for the custom nuclease-targeted genome engineering approach to be used in the zebrafish. ZFNs have a modular architecture composed of multiple three-base recognition motifs consisting of about 30 amino acid residues that are assembled together that make up the zinc finger DNA-binding domain. The non-specific cleavage domain is from the FokI restriction endonuclease that non-specifically cuts DNA when dimerized to another FokI domain. When assembled (which is a non-intuitive and rather laborious process), the zinc finger domain binds to a target sequence in the zebrafish genome which is then cut by the FokI domain after a second ZFN (targeted to the complimentary strand) also binds and the two FokI domains dimerize. After the cleavage of the DNA at the target site, error prone double stranded break (DSB) repair pathways introduce random alterations in the DNA sequence. ZFN technology has not proven to be extremely useful for many research labs due to difficulty of production, high costs, and low efficacy in editing the zebrafish genome due to poor target recognition and off target cutting (Blackburn et al., 2013).

Transcription activator-like effector nucleases (TALENs) were built upon a DNA binding system from a group of bacterial plant pathogens and improved the custom nuclease technology by greatly expanding targeting flexibility, cutting efficiency, and target specificity. Importantly, TALEN technology is open source and relatively easy to produce, providing zebrafish laboratories with affordable genome editing tools that can be made rapidly at low cost. TALENs are made up of repeat modules of approximately 34 amino acids organized into TAL effector arrays. A two amino acid sequence within each repeat module forms the variable di-residue (RVD), and unlike the 3 base recognition ability of a
zinc finger, RVDs exhibit one-one base pairing, resulting in much higher target sequence flexibility (Bogdanove and Voytas, 2011). Like ZFNs, TALENs utilize FokI homodimers to introduce DSBs in their targets.

4.2.2 The CRISPR/Cas9 system

The newest method of targeted genome editing which has exploded in popularity within the last 2-3 years is the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated systems (Cas) (commonly referred to as CRISPR/Cas systems) which are derived from bacteria and archaea and constitute a form of adaptive immunity in these microbes (Wiedenheft et al., 2012). The CRISPR system relies on the incorporation of exogenous plasmid DNA and phage sequence into spacer regions of the bacterial genome flanked by short palindromic repeats which are cleaved and transcribed into CRISPR RNAs (crRNAs). The crRNAs contain sequences that are complimentary to the target sequence on the invading phage, and when the crRNAs form a complex with Cas DNA nucleases and the target DNA sequence, DSBs are generated. There are three categories of CRISPR/Cas systems found in bacteria and archaea (Wiedenheft et al., 2012).

The type II CRISPR system is the most useful for genome editing because it involves a transactivating CRISPR RNA (tracrRNA) and crRNA that readily assemble into a complex with the Cas9 endonuclease (Deltcheva et al., 2011). The crRNA contains a spacer sequence that determines the target DNA sequence and binds on either strand next to a NGG-trinucleotide protospacer adjacent motif (PAM) that is required for recognition of the target sequence. Cas9 then cuts both strands of DNA within the target sequence resulting in DSBs and subsequent error prone DSB repair (Jinek et al., 2012).

Similar to the type II CRISPR system found in nature, the CRISPR/Cas9 genome editing method only requires a dual-RNA structure consisting of a tracrRNA and crRNA duplex (hereafter referred to as guide-RNA or gRNA) that has been designed to target a gene of interest as well as Cas9 mRNA to be synthesized. After injection of the gRNA and Cas9 mRNA into an embryo, the sequence in the gRNA that
is complimentary to the target site recognizes the target sequence adjacent to the PAM site. After binding, the gRNA associates with Cas9 which results in DSB usually 4–6 bp upstream of the PAM site (Jinek et al., 2012).

The CRISPR/Cas9 genome editing system has been show to work with very high efficiency and good specificity in a wide variety of organisms, including zebrafish, at a very low cost and rapid development rate. In addition, the system has quickly matured to where libraries of zebrafish specific donor plasmids and zebrafish codon optimized Cas9 plasmids are available to the research community. Compared to other techniques available, the Cas9/gRNA system is considerably easier to develop and implement. Unlike the comparatively complicated assembly of the TAL effector array in the TALEN system, in which a more sophisticated recombination based cloning method must be used to make the expression construct, the only synthesis involved is of specific gRNA oligo pairs of ~100 nucleotides (which are then easily ligated into existing donor vectors) and a Cas9 expression plasmid. Many groups have been very successful at creating a variety of genetic lesions using this system (Jao et al., 2013, Ni et al., 2014, Hisano et al., 2015).

In this study, I designed two gRNAs that target *her9* in order to make stable *her9* mutant lines that would result in loss of gene product in order to study the function of *her9* in the developing retina as well as the adult. In addition, I developed a CRISPR/Cas9 based method to knock-in a mCherry reporter to the endogenous *her9* locus (which would also result in loss of *her9* gene function).

4.3 Results

4.3.1 Design and generation of CRISPR/Cas9 constructs

In order to use the CRISPR/Cas9 targeted genomic editing system to knockout *her9*, I designed two single-stranded guide RNAs (gRNAs) that were complimentary to specific sequences within the *her9* coding region hereafter referred to as *her9* CRISPR 1 and *her9* CRISPR 2 (Figure 4.1). *Her9* CRISPR 1 was
Figure 4.1: CRISPR targets in the *her9* genomic locus. The *her9* locus contains 3 exons spanning 1786 bp on linkage group 23. The first CRISPR target site is 54 bp 3’ of the translation start site, and 46 bp upstream of the beginning of the bHLH domain. The second CRISPR target site is just after the end of the Orange domain in exon 3.

designed to target a region of exon 1 ~50 bp 3’ of the start codon and before the basic DNA binding domain in the bHLH motif. CRISPR 2 was designed to target a site near the middle of the coding region, just after the Orange domain. CRISPR gRNAs were synthesized by direct in vitro transcription (IVT) from a restriction digest fragment produced from a donor vector that contained the synthetic oligos. Zebrafish codon optimized and nuclear localized cas9 mRNA was also made by IVT.

To verify that the cas9 mRNA was effective (as well as to demonstrate that we could make gRNA properly), we first tested the efficiency of CRISPR/Cas9 targeted gene editing in our hands by creating mosaic “sandy” zebrafish by targeting the *tyrosinase* gene, which encodes an enzyme required for melanin synthesis and normal pigment formation (Jao et al., 2013). Injection of 50 pg/embryo of the *tyrosinase* gRNA and 150 pg/embryo of nls-zCas9-nls mRNA resulted in a range of “sandy” zebrafish with various degrees of pigment loss in a mosaic fashion, and genomic lesions in the *tyrosinase* allele were also verified by high resolution melting analysis (HRMA) and sequencing (data not shown).

4.3.2 Injection of *her9* gRNA and Cas9 results in *her9* gene disruption in F0 embryos
CRISPR/Cas9 mutagenesis of \textit{her9} should result in different mixtures of mutant and wildtype alleles in the mosaic F0 embryos regardless of whether the gRNAs are injected together or independently. The small amplicons generated during single embryo F0 HRMA analysis will contain wild type and various different mutant sequences that were amplified from different cells, resulting in heteroduplex DNA which will melt at a lower temperature than wild type homoduplex DNA due to improper base pairing. Whenever heteroduplex DNA was detected in F0 embryos by HRMA, pools of the PCR product from that single embryo (using the same HRMA primers) were ligated into a sequencing vector and sequencing was performed on a minimum of 10 colonies. Because half of the HRMA amplicons are expected to be wild type from heteroduplex DNA (and these were not F1 heterozygote embryos but F0 mosiacs, so the ratio will be much lower than 50% mutant to wild type amplicons) sequencing multiple samples from the same single embryo increased the chances of detecting mutant variants of \textit{her9} from sequencing).

\textbf{Figure 4.2: HRMA analysis in CRISPR injected F0 embryos.} A-A’) PCR products (HRMA amplicons) from whole embryo genomic DNA extracts of CRISPR 1 gDNA + nls-zCas9-nls mRNA injected embryos show a
variety of melting curves due to mosaicism compared to uninjected controls. **B-B'** HRMA showing melting curves generated when using CRISPR 2 + nls-zCas9-nls mRNA compared to uninjected controls. **C** Genetic lesions in the *her9* CRISPR 1 target site recovered from F0 mosaic embryos. **D** Only 1 genetic lesion was recovered from CRISPR 2 F0 mosaic embryos (10 sequencing reactions total).

Injection of 50 pg/embryo of the *her9* gRNA and 150 pg/embryo of nls-zCas9-nls mRNA into 1-cell stage *fli1*:EGFP embryos (which express the EGFP reporter in vasculature endothelial cells) resulted in mosaic expression of numerous *her9* alleles that harbored various genetic lesions when using CRISPR 1 (as demonstrated by HRMA analysis and sequencing), but few mutations were observed when CRISPR 2 was injected (**Figure 4.2A-D**). In addition, retinal vasculature defects were observed in the F0 mosaic embryos injected with CRISPR 1, but only a small percentage of the F0 CRISPR 2 injected embryos had any phenotypic abnormalities at all at 24 hpf (**Figure 4.3A-B**), and the few (~5% compared to uninjected controls) CRISPR 2 embryos observed that were delayed could be attributable to the injection/rearing process itself rather than mosaic knock out of *her9*. By 48 hpf, the CRISPR 1 F0 embryos still had ocular defects like micropthalmia and coloboma as well as decreased retinal vasculature and the CRISPR 2 embryos looked phenotypically normal (**Figure 4.3C-D**). In addition to the retinal vasculature phenotype observed in the CRISPR 1 F0 embryos, ~15-20% had defective vascular networks in the tail that looked like bulbs of expanded vasculature tissue at 48 hpf (**Figure 4.4**). Blood cells could be easily observed pooling up in the bulb region, however, blood was still flowing through the tail region and the moving cells were either bumping off of or flowing around the anterior portion of the pooled blood cells. This defect was not observed in any of the CRISPR 2 F0 embryos.
Figure 4.3: Retinal phenotypes observed in F0 mosaic embryos. A-A’’) Brightfield images of uninjected control (UIC) and her9 CRISPR injected embryos at 24 hpf show ocular defects in the CRISPR injected embryos. B-B’’) Fli1 reporter shows abnormal retinal vasculature in the CRISPR 1F0 but not in the CRISPR 2 F0 compared to the UIC. C-C’’) The 48 hpf CRISPR 1 F0 eye is microphthalmic and has coloboma, while the CRISPR 2 F0 eye is only slightly smaller than the UIC eye. D-D’’) Fli1 signal shows that the CRISPR 1 F0 eye has abnormal retinal vasculature at 48 hpf while vasculature in the CRISPR 2 eye closely resembles the vasculature in the UIC eye.
Figure 4.4: Vasculature defects in the her9 CRISPR F0. The 48 hpf her9 CRISPR 1 F0 displays defective tail vasculature in ~15% of the F0 embryos observed. The circulatory system was still functional, but bulbs of expanded vasculature endothelial cells held pools of blood cell near the tail.

When considering the contrast in the observed F0 mosaic phenotypes between the two CRISPRs, these data suggest that CRISPR 1 was more effective at creating genetic lesions in the her9 allele than CRISPR 2. This could simply be due to the higher binding affinity of gRNA 1 to the target
sequence than gRNA 2. Due to this, for the remainder of the study, only her9 CRISPR 1 was used for mutagenesis of the her9 gene.

4.3.4 Identification of F0 founder zebrafish that transmit mutant her9 alleles in the germline

To determine if CRISPR/Cas9 generated genetic lesions in her9 were heritable, putative F0 founders were screened to not only determine if they were transmitting a mutant her9 allele in their germline, but to also characterize the type of mutant allele being transmitted. Since these were mosaic fish, a single F0 could be transmitting several different types of mutant her9 alleles (or only wild type).

Putative F0 founders were outcrossed to fli1:EGFP fish (to maintain this useful reporter in the mutant background), and single embryos were then used for HRMA analysis. It has been reported that the CRISPR/Cas9 mutagenesis approach typically yields 1-10% germline transmittance of targeted mutant alleles (Blackburn et al., 2013). Unlike the HRMA analysis in F0 mosaics, the majority of the embryos were expected to be wild type and have a similar melting curve as control groups (all homoduplex DNA), and when heteroduplex amplicons were detected in single embryos, likely the other mutant embryos in that group would share the same melting curve as they would likely be sharing the same mutation. Although the majority of the putative F0 founders screened did not transmit any mutant variants of her9 in the germline (36/46 total F0s screened at ~100 embryos from each potential founder), 10 F0 fish were recovered that transmitted a her9 mutation in the germline. Nine of the founders transmitted 1 mutant her9 allele, and 1 founder was found by HRMA to transmit 2 different mutant her9 alleles in the germline (Figure 4.5).

After identifying a founder by HRMA, the genomic DNA of the F1 embryo used for the HRMA experiments was used as a template for PCR. The PCR amplicons were ligated into sequencing vectors and a minimum of 6 clones were sequenced from each HRMA positive embryo to identify the wild type and mutant her9 sequences from each HRMA positive F1. Interestingly, the first 7 germline transmitting mutant her9 sequences recovered were all deletions in multiples of 3 (ranging from 6-36 nucleotides)
Figure 4.5: HRMA identifies mutant her9 alleles transmitted in the germline to F1 embryos. A-B) Normalized melting peaks (A) and difference plot (B) of the her9 CRISPR 1 HRMA amplicon from individual F1 embryos shows the wild type her9 curve (blue arrow) and a single her9 mutant allele passed in the germline from the F0 founder to several F1 embryos. C-D) Normalized melting peaks (A) and difference plot (B) of her9 CRISPR 1 HRMA amplicon from individual F1 embryos shows a grouping of the wild type allele (blue arrow), however this F0 transmits two mutant her9 alleles in the germline (red arrows).

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Figure 4.6: Sequence of the target site of CRISPR 1 F1 her9 carrying mutants. A) The first seven germline transmitted her9 mutant alleles recovered were all in-frame deletions that would lead to a slightly truncated protein, but not affect any of the functional domains and likely result in functional her9 gene product. B) The next three mutant her9 alleles recovered that were transmitted in the germline produce early frameshift deletion and truncated proteins.
This early result raised the concern that the frame-shifting mutations in her9 might result in haploinsufficiency (and would therefore not be recovered) given that there is a less than 0.05% chance that the first seven germline transmitting mutants identified would all have in-frame deletions. However, the next three mutations uncovered were a 1 bp deletion, a 1 bp insertion, and a 10 bp insertion in three different F0 fish that resulted in early frameshift mutations of the her9 gene (Figure 4.6).

4.3.5 Identification of germline transmitting F1 and F2 her9 mutant zebrafish

The frameshift mutant F0 founders were then repeatedly outcrossed to the fli1:EGFP line and the resulting F1 embryos were raised to adulthood. Tail clips were performed on individual F1 fish and genomic DNA was extracted for genotyping. The 1 bp deletion mutation in the her9 gene (progeny of the F532.4 F0 founder) can be identified by restriction fragment length polymorphism (RFLP) analysis due to a BsaJI restriction site being introduced into the gene by the 1 bp deletion mutation. Fortunately, the F532.4 F0 founder not only had the highest fecundity of the 3 out-of-frame her9 mutation carrying fish, but also the highest germline transmission rate. Seven heterozygous her9 mutant F1 fish were recovered that harbored the 1 bp deletion and were used to generate ~100 F2 fish from both incrosses and outcrosses of F1s (as of this writing, only 1 heterozygous F1 fish carrying the 1 bp insertion mutation has been recovered; numerous F2 fish from that line have been generated, but not screened). The remainder of this chapter will focus on the 1 bp deletion line.

Another benefit of using RFLP rather than the more expensive HRMA analysis method to screen for mutants, is that although HRMA analysis is very good at distinguishing between homo- and heteroduplex amplicons, this method is not very sensitive when distinguishing between homoduplex (1 bp deletion) mutant and homoduplex (wild type) amplicons of the same length. Although the sequences between the two PCR products are different at one nucleotide, their respective melting curves are much closer than that of heteroduplex DNA. On the other hand, RFLP analysis, when possible, is the far better
choice when screening F2 embryos or adults resulting from heterozygous F1 (Figure 4.7A). As shown in figure 4.7B, when F2 embryos resulting from F1 incrosses were screened by HRMA alone, the majority of the time the homozygous mutant and homozygous wild type embryos grouped together due to highly similar melting curves, while only the heterozygous carriers with heteroduplex amplicons grouped differently (Figure 4.7B).

![Figure 4.7: RFLP and HRMA analysis to identify 1 bp deletion her9 mutant F1 and F2 fish. A) 556 bp PCR amplicons generated from genomic DNA template of F1 and F2 zebrafish digested with BsaI. The her9 homozygous wild type, homozygous mutant and heterozygous fish are all easily distinguishable from each other by RFLP analysis. B) Heterozygous fish are easily identified by HRMA due to the heteroduplex DNA, but the homozygous wild type and homozygous mutant fish grouped very closely together when performing HRMA analysis on F2 larvae and were nearly indistinguishable.](image)
4.3.6 Her9 is required for survival past larval stages in the zebrafish

Due to the presence of maternal pre-spliced her9mRNA deposited into the ovum, which masked the effect of the her9 splice-blocking morpholinos during embryonic development (discussed in chapter 3), I wanted to generate homozygous her9 mutant lines that I could then incross to observe the true phenotype of complete loss of her9 during early embryonic development. In particular, I was curious if the maternal zygotic mutant phenotype would recapitulate the various vasculature defect phenotypes that were observed in embryos that were injected with the her9translation blocking morpholino.

To address this question and to generate to her9 homozygous mutant lines, F1 or F2 heterozygote her9 mutant zebrafish (at the time only heterozygote F2 mutants had been recovered, or homozygous mutants would have been used) were repeatedly incrossed to various members of different families and the resulting offspring were grown to a stage where they could be safely tail-clipped and genotyped by the RFLP screening procedure mentioned previously. Some of the individual zebrafish were adults when screened, but most were juveniles (3-6 weeks old). Surprisingly, after screening a total of 74 zebrafish derived from heterozygous incrosses, zero homozygous mutants were recovered, and instead the population of F2 juveniles and adults contained 46 heterozygous carriers, and 25 homozygous wild type fish. Assuming the her9 mutant allele would follow the simple 1:2:1 Mendelian ratio for a heterozygous incross, I would have expected around ~18 homozygous wild type, ~37 heterozygous, and ~18 homozygous mutants; and this obviously wasn’t the case ($\chi^2 < p=0.0001$). However, numerous homozygous mutant embryos were previously detected during the screening of initial F1 heterozygous incrosses conducted at 2-3 dpf. These results suggested that her9 homozygous mutant offspring were dying some time between 3 dpf and the juvenile stage.

During the normal rearing process of the zebrafish offspring, there is usually some degree of death at 24 hpf (primarily due to improper fertilization of the egg). In general, if an embryo survives past
24 hpf and into later larval stages, the survival rate is reasonably high (Westerfield, 1995). It has been observed, however, that some mortality occurs in all wild type strains around 13-14 dpf due to complete exhaustion of the yolk and starvation, usually of the larval zebrafish with the slowest grow rate. Whereas the offspring of her9 heterozygous mutant incrosses experienced approximately normal rates of survival at 24 hpf I noticed that there seemed to be unusually high mortality around ~10-14 dpf as compared to other lines our lab commonly uses.

To determine at what stage the her9 homozygous mutant larvae were dying, multiple her9 heterozygous incrosses were set up using different families of het carriers. At 24 hpf, there were 132 surviving embryos that all looked phenotypically normal. By 5 dpf, there were 116 surviving larvae (consistent with normal rearing of other lines). From this point on, the larvae were separated into groups of 10 in 6 well plates with embryo medium refreshed daily (and normal feeding routines). Any larvae that looked phenotypically abnormal (no swim bladder, misshapen body/head, small body size, etc.) or displayed abnormal behavior (laying on their side at the bottom of the plate, not responding to touch stimuli, etc.) were removed and processed for gDNA extraction/genotyping. Using these criteria, 40 out of 116 total progeny (34.5%) of her9 heterozygous incrosses were classified as phenotypically abnormal. The abnormal larvae collected at 5 dpf had a curved body, excess yolk, heart edema, and were microphthalmic compared to normal looking larvae collected at the same time point (Figure 4.8A-B). In addition, the abnormal looking larvae had severe vasculature defects including abnormal intersegmental vessel formation and incomplete connections of vasculature throughout the trunk (Figure 4.8B’-C’’). All larvae with abnormal phenotypic or behavioral characteristics were found to be her9 homozygous mutants, whereas the normal looking larvae collected at the same tie point were either homozygous wild type or heterozygous. The heads were dissected from the first batch of larvae that displayed the aforementioned abnormal characteristics at 5 dpf (after imaging and cataloging), then sectioned to closely visualize the retina (with the rest of the body processed for gDNA extraction), and
**Figure 4.8: The her9 mutant phenotype.** A) 5 dpf wild type zebrafish larvae. A’-A”) *Fli1* signal (vasculature endothelial cell reporter) showing normal intersegmental vessels for this stage. B) Compared to the wild type control, the 5 dpf *her9* mutant larvae displays microphthalmia, increased yolk sac size, loss of swim bladder, and body curvature. B’-B”) *Fli1* signal shows severe vasculature defects including abnormal intersegmental vessel formation and incomplete connections of vasculature throughout the trunk. C-C”) Retinal sections of the same 5 dpf wild type larvae from A shows normal lamination and retinal vasculature for that stage. D-D”) The 5 dpf *her9* retina has poorly laminated and has numerous morphological defects, including almost complete absence of retinal vasculature.

processed for IHC along with wild type larvae to serve as controls (Figure 4.8C-D). The retinas of the homozygous mutant larvae were poorly laminated and had numerous morphological defects, including almost complete absence of retinal vasculature.

After the initial 5 dpf collection, any abnormal larvae were collected daily and processed for genomic DNA until 14 dpf, when all of the remaining larvae were collected for DNA extraction. Following the final collection and DNA extraction, RFLP analysis was conducted on all of the samples to determine genotype. Interestingly, abnormal looking larvae were only observed prior to 10 dpf, with *her9* homozygous mutants accounting for 67% (n=27/40) of the embryos collected before this time point. From 10-14 dpf, no abnormal animals were observed, and 100% of the remaining larvae were either heterozygous or homozygous wild type (n=76). In summary, from a pool of 116 larvae at 5 dpf, 23% of the larvae were homozygous *her9* mutants, 57% were heterozygous, and (20%) were homozygous wild type. Interestingly, of the 76 embryos collected after 10 dpf (that all looked normal), not a single homozygous mutant was found out of the group. These data strongly suggest that homozygous mutation of *her9* is lethal in zebrafish during the larval stage (between 5 and 10 dpf).
4.4 Discussion

As described in Chapter 3 of this dissertation, morpholino mediated knockdown of the zebrafish bHLH-O transcription factor her9 (ortholog of the mammalian HES1) resulted in striking retinal vasculature defect phenotypes. However, due to the transient nature of gene knockdown with morpholinos, it was impossible to study later occurring phenotypes that might result from loss of her9 gene function. Therefore, we concluded that it would be highly beneficial to study the loss of her9 gene function in a genetic knockout zebrafish model. In this study, we demonstrate generation of various her9 knockout lines using the recently developed CRISPR/Cas9 genome editing tools.

HRMA analysis followed by sequencing confirmed various genetic lesions were created in F0 embryos when injected with gRNA targeting the first exon of her9 along with Cas9 mRNA. In addition, various vasculature defect phenotypes could be observed in the F0 mosaic embryos, including loss of retinal vasculature and abnormal vasculogenesis in the tail resulting in bulbs of blood vessels in a portion of the embryos observed. This early data was a good indication that the loss of her9 might not only play a role in development of the retinal vasculature network, but in vasculogenesis throughout the body.

Numerous F0 embryos were raised to adulthood and screened for germline transmission of mutant her9 alleles. Although the first CRISPR mutants generated harbored only in-frame deletions, the next three germline transmitted her9 mutant alleles recovered from F0 founders contained indels that resulted frameshift mutations, and 2 of the 3 germline transmitting mutations are now being analyzed in subsequent generations.

Initially, heterozygous her9 mutant zebrafish were incrossed and the resulting embryos were raised to adulthood to screen for both heterozygous and homozygous F2 fish. Interestingly, although homozygous her9 mutants could be detected in embryos at Mendelian ratios, not a single her9-/- adult fish was ever recovered. Subsequent analysis revealed that all of the homozygous mutant larvae die.
before 14 dpf and display a wide range of phenotypic abnormalities, including severe vasculature
defects throughout the body. Similar to what was observed when using a splice blocking morpholino to
to knockdown her9 expression, it is reasonable to assume that the maternally deposited mRNA is sufficient
to permit survival of her9-/- embryos at least to the 5 dpf larval stage. However, as the pool of maternal
wild type her9 is depleted, numerous morphological and vascular defects start to appear that ultimately
lead to death of the larvae at later stages due to loss of her9 function.

Taken together, we have successfully utilized the CRISPR/Cas9 targeted genome editing
approach to induce genetic lesions in the zebrafish her9 allele resulting in stable germline transmitting
mutants. In addition, we confirmed previous morpholino data that loss of her9 gene function results in a
wide range of vascular defects during development, and that maternal deposition of her9 mRNA is
sufficient for embryonic survival to early larval stages, but additional Her9 is required for further
development. It is possible that the high mortality observed in the her9 homozygous mutant larvae is
due to vasculature defects, and that at mid- to late-larval stages, enough oxygen and nutrients are not
available to tissues because they are not properly vascularized. Although it will be impossible to
generate maternal zygotic her9 mutant embryos to study early developmental phenotypes due to the
inability to recover any homozygous her9 mutant adults, there are numerous zygotic phenotypes
observed in the larval stage that could give much insight into these novel functions of her9. The retinas
of her9 homozygous mutants were poorly laminated, and had a greatly expanded GCL domain. In
addition, the retinal vasculature network was largely absent in these larvae, suggesting that her9 not
only plays a role in proper neurogenesis, but formation of retinal vasculature. It may be possible, that in
conjunction with translation blocking morpholinos to inhibit any maternal effect, a facsimile of a true
maternal zygotic her9 homozygous mutant zebrafish could be generated to study both early embryonic
and late larval defects resulting from loss of her9.
4.5 Materials and Methods

4.5.1 Zebrafish

All zebrafish (*Danio rerio*) strains were bred, raised, and maintained in accordance with established animal care protocols for zebrafish husbandry (Westerfield, 1995). Adult fish, embryos, and larvae were housed at 28°C, on a 14 hr light: 10 hr dark cycle. Embryos were staged as previously described (Kimmel et al., 1995). The Tg(*fli1a*:EGFP)y1Tg, hereafter called *fli1*:EGFP has been previously described (Lawson and Weinstein, 2002b). All animal procedures were carried out in accordance with guidelines established by the University of Kentucky Institutional Animal Care and Use Committee (IUCAC).

4.5.2 Generation of guide RNA oligonucleotides

The two target sites for CRISPR/Cas9 based genome editing within the her9 gene were selected to be in the first and third exons. First target site was 54 bp 3’ of the translation start site, and 46 bp upstream of the beginning of the bHLH domain (Table 4.1). The second target site is immediately after the coding region that corresponds to the Orange domain of *her9*. Single strand DNA oligonucleotides used to generate the guide RNA (gRNA) donor vector were designed using the ZiFit online tool (http://zifit.partners.org/ZiFiT/), selecting the T7 promoter to generate a 100-115 bp amplicon. Oligos were annealed at a concentration of 100 µM (5 minutes at 95-100°C, then slowly cooled to room temperature for 5 hours) and ligated into BsaI (NEB: R0535S) digested pDR274 vector (Addgene: 42250). Recombinant vectors containing the gRNA insert sequence were digested with DraI (NEB: R0129S) and the insert was amplified by PCR using DraI primer. The PCR product was (QIAquick PCR purification kit) and in vitro transcribed using Ambion-MEGAscript T7 Transcription Kit (Life Technologies#AM1334) then purified by phenol:chloroform extraction followed by ethanol precipitation. A gRNA targeting the tyrosinase gene was used as a positive control for the study. pT7tyrgRNA (Addgene: 46761) plasmid was linearized using BsmHI (NEB: R3136T) and in vitro transcribed using Ambion-MEGAscript T7
Transcription Kit (Life Technologies: AM1334) then purified by phenol:chloroform extraction followed by ethanol precipitation.

4.5.3 Cas9 mRNA

The pT3TS-nls-zCas9-nls (Addgene: 46757) expression vector that contains a zebrafish codon optimized Cas9 coding sequence flanked by nuclear localization sequence was linearized with XbaI (NEB: R0145S) and purified by phenol:chloroform extraction and ethanol precipitation. Capped mRNA was generated using Ambion mMESSAGE mMACHINE T3 Transcription Kit (Life Technologies:AM1304) and purified by phenol:chloroform extraction and ethanol precipitation.

4.5.4 Microinjections

Cas9 mRNA and gRNA were injected at 150-300 and 50-100 ng/embryo respectively at a volume of 4.18 nl/embryo in buffered solution with 0.025% dextran red (as an injection indicator) into the yolk of 1-cell stage zebrafish.

4.5.5 High resolution melting analysis (HRMA)

Genomic DNA was isolated from individual 24-72 hpf embryos or from adult tail clips by placing the sample in 20 µl Thermopol buffer and incubating at 95°C for 10 minutes, then adding 5 µl of proteinase-K (10 mg/ml), incubating again at 55°C for 2 hours followed by a 10 minute 95°C proteinase-K denaturation step. HRMA was performed using LightCycler 480 High Resolution Melting Master (Roche) according to manufacturer’s instructions on a LightCycler 96 Real-Time PCR System (Roche). Primer sequences used for HRMA study are listed in (Table 4.1).

4.5.6 Restriction fragment length polymorphism (RFLP) analysis

The mutant her9 allele carrying a 1 bp deletion could be identified by RFLP analysis due to the introduction of a BsaJI (NEB: R0563S) restriction endonuclease site created by the CRISPR mutagenesis based deletion. Genomic DNA from whole embryos or from tail clips were amplified by PCR using the primers listed in (Table 4.1).
Acknowledgments

I would like to thank Vince Gouge for all of the time and effort he put forth in assisting with this project, and Sara Perkins for care and maintenance of zebrafish stocks.

Chapter 4 Tables

Table 4.1: Chapter 4 oligo sequences and application

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Chapter 5: Summary and Discussion: Her4 and Her9 bHLH-O transcription factors in the developing and regenerating vertebrate retina

5.1 Summary and Discussion

For proper visual function, a myriad of diverse cell types, tissue structures, and refined interconnections between numerous neurons and glia must be formed and maintained with near precision. The ability to see, or detect photons of light emanating from our environment, is enabled in part by the retina, with further processing of visual information taking place in the brain. The retina itself is a highly organized multi-layered tissue that results from complex interactions of regulatory networks and spatiotemporally controlled gene expression events during development. Because the ability to see is a critical function of our own biology, the extrinsic and intrinsic signals as well as the gene regulatory networks that control neurogenesis of the retina have been intensely studied. However, there are still many questions about how the retina develops, is maintained, and in the case of some cold-blooded animals, regenerates following damage.

The retina is the external most part of the central nervous system, and as such, is more tractable to study and experimental manipulation (including possible treatment) than other parts of the CNS such as the brain. The mammalian CNS, including the retina, is unable to repair damage from various types of acute trauma or chronic disease. Damage to the mammalian retina results in glial scarring, apoptosis of retinal cells, various degrees of vision loss, and in many cases, complete blindness. The zebrafish retina, however, undergoes a proliferative response that results not only in new retinal cells of the correct type positioned in the proper location, but also functional integration of these cells the existing neural networks and full restoration of vision after the retina has been damaged. The long term goal for many teams who study zebrafish visual system biology is to understand exactly how and why many cold-blooded animals such as the zebrafish are capable of retinal regeneration, what gene regulatory networks and cell types enable and regulate this phenomenon, and if it is possible to recapitulate or
emulate these processes in human patients with visual system disorders. Our current understanding of retinal neurogenesis, including both development and regeneration, are too incomplete to be able to design and implement all but the most rudimentary types of therapies for patients suffering from vision loss. A greater understanding of the gene regulatory networks and cellular mechanisms that enable retinal neurogenesis will enhance future therapeutic efforts to maintain or restore functional vision to patients suffering from visual impairment or vision loss.

In this dissertation, the goals were to determine the functions of the basic-helix-loop-helix-orange (bHLH-O) transcription factors *her4* and *her9* during development and regeneration of the vertebrate retina using the zebrafish as a model organism (Figure 5.51). The roles of these two genes in retinogenesis have not been previously described in zebrafish, nor have the functions of their mammalian orthologs (*HES5* and *HES1* respectively) during retinal development been elucidated. *Her4* and *her9*, as well as many of the other bHLH-O paralogs are known to be involved in numerous developmental processes including regulation of the segmentation clock during somitogenesis, neural tube closure, floor plate development, and establishment of cranial sensory placodes. In most cases, genes of this class tend to restrict differentiation of cells from adopting a typically pro-neural fate and keep progenitors in the cell cycle so that they can give rise to later born neurons and glia downstream of the Notch-Delta signaling master regulatory pathway.

Both *her4* and *her9* have been shown to be upregulated in a background of chronic, rod-specific degeneration and regeneration in the zebrafish retina, and are primarily expressed in proliferative areas of the developing as well as the mature retina. Thus, the initial hypothesis was that these genes play a role in the maintenance of pools of proliferating stem and progenitor cells that give rise to rod photoreceptors. The experiments detailed in Chapter 2 of this dissertation placed *her4* directly downstream of the Notch-Delta signaling pathway, and the primary function of Notch signaling has been widely considered to be repression of cells from differentiating into neurons, and to keep cells in the cell...
cycle. Consistent with this idea, and perhaps one of the most important insights into the function of her4 with respect to retinal neurogenesis, is that morpholino mediated knockdown of her4 results in a retina that phenocopies disruption of the entire Notch-Delta signaling pathway. This evidence suggests that her4 is a primary effector of Notch-Delta signaling during retinal neurogenesis, even though there are numerous other genes that are Notch responsive and are also expressed in the retina.

Why is it that knockdown of this single transcription factor phenocopies Notch pathway defects, when there are over 20 Notch-responsive bHLH-O genes expressed in the zebrafish? One possible explanation is that the genes directly regulated by her4 in the retina have unknown cis-regulatory elements that preclude binding of any other bHLH-O protein besides Her4. Another possibility is that there could be heterodimerization partners for Her4 that are only expressed in the retina, and it is unique combinations of her gene expression that leads to repression of target genes. A third possibility is that there is a minimum threshold of Notch-responsive bHLH-O genes that must be expressed during retinal development to see the effects of Notch-Delta signaling, and her4 is simply one of the more highly abundantly expressed bHLH-O genes in the retina. To further explore this unexpected result, additional experiments would need to be performed. One possible approach would be to try to rescue the morphant phenotype with a complimentary bHLH-O gene (Notch-responsive, WRPW tetrapeptide C-terminal motif). If this other bHLH-O transcription factor was able to rescue the her4 morphant phenotype, it would indicate that there is nothing particularly special about her4, and that the observed knockdown phenotype is simply due to a decrease in the dosage of bHLH-O factors in the retina. Another approach could be to try to knock down expression of every bHLH-O gene that is expressed in the retina, independently of her4. If knockdown of any of the other genes phenocopies the her4 morphant, that would be strong evidence that the function of her4 in the retina is dependent upon unique heterodimerization partners.
Additional experiments detailed in Chapter 2 showed that in the adult retina, *her4* is primarily expressed in the CMZ, and that in a background of rod degeneration/regeneration (XOPS:mCFP), *her4* was upregulated in both the CMZ, and central retina. The expression patterns of *her4* here were unexpected because in the XOPS:mCFP retina, it is the rod progenitor cells at the base of the outer nuclear layer that are rapidly proliferating in response to rod degeneration, and *her4* was not expressed in these cells. Instead, we found that *her4* was upregulated in slowly proliferating subsets of Müller glia in the INL of the central retina in addition to the increased *her4* expression at the CMZ. Previous studies have concluded that there was no significant difference in the number of Müller cells in the central XOPS:mCFP retina compared to wild type controls, and this appeared to be the case, but the number of *her4*-expressing Müller glia that have re-entered the cell cycle is increased in the transgenic background. We therefore hypothesized that it is this population of cells that feed into the rod photoreceptor lineage, and also suspected that there might be some contribution to the rod lineage from the CMZ as well.

The transgenic *her4*:Kaede zebrafish was generated to conduct lineage tracing experiments in order to determine the fate of *her4* expressing cells in the retina. We demonstrated that expression of the transgene recapitulates expression of the endogenous *her4* transcript, even though the *her4.3* promoter segment used to drive expression of Kaede only represented one of the five possible *her4* promoters in the *her4* loci. Lineage tracing analysis using the *her4*:Kaede line crossed onto the XOPS:mCFP background revealed that the slowly proliferating *her4*-positive Müller glia in the inner nuclear layer give rise to Nr2e3-expressing progenitor cells. These progenitor cells then migrate to the base of the outer nuclear layer where they differentiate into rod precursor cells and then rods. In contrast to the comparatively more rapid response of Müller cells following acute damage (Thummel et al., 2008, Thomas et al., 2012), the cycle of *her4* expression to rod neurogenesis can take up to three days. As early as one day post photoconversion, new non-photoconverted Kaede in presumptive Müller
cells in the inner nuclear layer could already be observed, demonstrating that the chronic rod degeneration and regeneration experienced in the XOPS:mCFP retina elicits a continual, albeit low-level, response in the inner nuclear layer. These data support the hypothesis that upregulation of her4 in small subsets of Müller cells spread throughout the central retina is associated with the need to restock and replenish the rod progenitor pool as those progenitors are continually depleted. In addition, acute light damage that lead to widespread synchronized rod and cone photoreceptor cell death showed that the magnitude of the her4 response is correlated with the amount of damage the retina receives. Similar to the XOPS:mCFP retina, it is her4-expressing Müller cells in the inner nuclear layer that mediate the proliferative response following injury.

As mentioned previously, in addition to upregulation of her4 in the central retina, her4 was also upregulated in the CMZ. This result was surprising because unlike other retinal neurons, rod photoreceptors are not derived from the CMZ but from populations of stem cells that reside in the INL of the central retina (Raymond et al., 2006, Morris et al., 2008a, Morris et al., 2008b). The her4:Kaede line was used to determine what cell types the CMZ had the capacity to generate, and consistent with previous observations, the lineage of her4-expressing cells from the CMZ includes all retinal cell types except for rod photoreceptors. Why there is a significantly expanded number of her4-expressing cells in an area of the retina that does not give rise to rod photoreceptors in the XOPS:mCFP background remains unclear. It has previously been demonstrated that acute photoreceptor damage results in the release of cytokines such as CNTF and TNFα which in turn result in increased expression of stat3 and ascl1α and Müller glia proliferation in the INL of the central retina (Kassen et al., 2007, Kassen et al., 2009, Nelson et al., 2012, Nelson et al., 2013). It is possible that soluble factors released from apoptotic rod cells diffuse throughout the retina, and the cells in the stem cell niche at the CMZ are stimulated to increase her4 expression but the CMZ itself is still restricted as to what cell types it can produce. In addition, the signals that stimulate small numbers of Müller cells in the INL to express her4 and
contribute to the rod lineage remain unknown, as well as why only small subset of Müller cells respond to these signals. Possible mechanisms include juxtacrine signaling that stimulates Notch activity, gradients of extrinsic factors generated by dying photoreceptors, intrinsic cues that sensitize subsets of Müller cells to rod degeneration, or some combination of the above.

Unlike its paralog her4, her9 is not responsive to Notch-Delta signaling, even though the mammalian homolog of her9 (HES1) is commonly used as a readout for activated Notch signaling. Her9 is a particularly interesting bHLH-O transcription factor because it is an effector of multiple distinct signaling pathways and regulatory networks in a tissue dependent manner (as discussed in detail in Chapter 3). For example, it is downstream of Bmp signaling and involved in pre-patterning of the early neuroectoderm, and downstream of midline Nodal signaling during notochord development (Bae et al., 2005, Latimer et al., 2005).

Drug screens to transiently inhibit Notch-Delta, Hh, and Fgf signaling pathways did not result in altered her9 expression in the retina. Manipulation of the retinoic acid (RA) signaling pathway, however, did change the expression levels and expression domain of her9 in the retina, suggesting that in retinal tissue, her9 is downstream of RA signaling. Analysis of the presumptive upstream cis-regulatory elements of her9 show that there are three predicted Su(H) binding sites in the promoter region of her9, suggesting that it could be a Notch responsive gene. However, these Su(H) sites are also flanked by multiple retinoic acid responsive elements (RARs), including several RARs within the coding region of the gene as well. It is possible that the RAR elements are blocking activation of her9 by Notch mediated signaling, and that this lead to sub-functionalization of this gene in the retina, however, further studies must be completed to fully understand the contribution of the cis-regulatory elements that play a role in the expression of her9 in the retina.

Interestingly, the spatiotemporal expression pattern of her9 in the retina coincides with development of the retinal vasculature. During early embryonic retinal development, her9, like her4, is
expressed throughout the proliferating neuroepithelialial cells of the primordial eye, and expression is restricted to the peripheral CMZ as the retina matures. Unlike her4, however, her9 is expressed in the choroid fissure and expression of her9 here persists until angiogenesis of the hyaloid artery is completed. Fluorescent in situ hybridization of experiments to visualize her9 expression along with immunohistochemistry to visualize vasculature endothelial cells showed co-localization of her9 transcript with retinal vasculature in both the choroid artery and vein, as well as the lens basket vasculature. These data suggested that her9 could play a previously undescribed role in vasculogenesis downstream of the RA signaling pathway in the retina.

Morpholino mediated knockdown of her9 using a translation blocking morpholino resulted in a decrease of vasculature endothelial cells in the retina compared to controls. The morphant phenotype could not only be rescued by co-injection of in vitro transcribed capped her9 mRNA, but injection of her9 mRNA alone resulted in excess retinal vasculature. These data coincide with the excess of retinal vasculature cells when the embryo was transiently exposed to excess RA from 24-36 hpf and a decrease in retinal vasculature when RA signaling was inhibited during the same time period, further supporting the observations from in situ hybridization and qPCR experiments that her9 appeared to be an effector of RA signaling during retinal vasculature development. These exciting results demonstrate that her9 has a novel function in development of the retinal vasculature network.

To better understand the role of her9 during retinal vasculogenesis, her9 mutants were generated using the CRISPR/Cas9 targeted genome editing technique (Varshney et al., 2015). High resolution melting analysis (HRMA) followed by sequencing of the her9 allele showed that the CRISPR/Cas9 based genome editing efficiently created genomic lesions in the her9 allele, and that these mutations were heritable. Interestingly, a variety of vasculature defect phenotypes could be detected even in the mosaic F0 embryos that were injected with the her9 CRISPRs, including loss of retinal
vasculature and deformed trunk blood vessels that resulted in bulbs of vasculature endothelial cells that inhibited normal blood flow in the tail of the developing larvae.

When zebrafish heterozygous for a mutant frame-shift allele of her9 were incrossed, and the subsequent generation raised to adulthood, her9-/- zebrafish were never recovered. Subsequent analysis showed that the homozygous her9 mutant larvae were dying before 14 dpf and possessed a wide range of phenotypic abnormalities, including severe vasculature defects throughout the body such as intersegmental vessel defects and severe retinal vasculature and lamination defects. So although it will probably be impossible to ever recover homozygous her9 mutant adults that could be used to generate maternal zygotic mutant embryos, zygotic phenotypes in homozygous mutants resulting from heterozygous incrosses can be studied.

Similar to what was observed when using splice blocking morpholinos rather than translation blocking morpholinos to knockdown her9 expression, it is reasonable to assume that the maternally deposited mRNA is sufficient to permit survival of her9 homozygous mutant embryos at least to the 5 dpf larval stage. However, as the pool of her9 is depleted, and no endogenous her9 is generated to replace it, numerous morphological and vascular defects start to appear that may lead to death of the larvae at later stages.

This phenomenon could prove to be a major disadvantage of using genetic knockouts (either generated through tilling, TALENs, CRISPRs or otherwise) when studying early developmental phenotypes as compared to the morpholino mediated transient knockdown approach. It has already been documented that the mutant phenotypes resulting from lesions in numerous zebrafish genes do not necessarily recapitulate the morphant phenotypes observed when those same genes are knocked down (Kok et al., 2015). Presumably, this is in part due to the significant effects of maternal deposition of spliced mRNA within the ovum, and also due to the fact that the zebrafish develops extremely rapidly compared to other vertebrate model systems. As with many questions in biology, there is no perfect
solution, and best available technique to study the function of a particular gene during early development might include both use of knockout lines as well as translation blocking morpholinos if the use of maternal zygotic mutants is not feasible.

The numerous bHLH-O genes expressed in the zebrafish play a wide variety of roles during development as well as in various tissues of the adult zebrafish. This dissertation describes some of the functions of two of these bHLH-O transcription factors (\textit{her4} and \textit{her9}) in the retina. Given that the one of the primary functions of the Notch-Delta signaling pathway is to regulate neurogenesis by keeping stem- and progenitor cells in the cell cycle, it stands to reason that a downstream effector of the Notch pathway would suppress expression of pro-neural genes. Only a few targets of \textit{her4} mediated repression have been identified in the retina, including \textit{neuroD}, \textit{ath5}, \textit{ascl1a}, and \textit{insm1a}, and all of these genes play a pro-neuronal function during retinal neurogenesis. To better understand the role of \textit{her4} during retinal development and regeneration, a more complete description of the \textit{her4} gene regulatory network will have to be elucidated. One of the ways that this can be accomplished would be to generate mutant zebrafish lines in which \textit{her4} is knocked out. This may prove to be difficult because \textit{her4} is encoded on five tandem duplicate repeats (all of which produce the same gene product), so every iteration of the \textit{her4} gene would need to either be independently knocked out or the entire \textit{her4.1-her4.5} allele removed altogether. Fortunately, with new genome editing technologies available in the zebrafish, this might be feasible. In addition to completely removing the \textit{her4} allele, it would be beneficial to have the ability to conditionally knockout the allele in a tissue and temporally dependent manner to study the effects of loss of all \textit{her4} variants not only during embryonic retinal development but also during regeneration. To accomplish this, CRISPR/Cas9 mediated genome editing techniques could be used to knock-in LoxP sites that flank the allele, and these transgenic fish could be outcrossed to \textit{rx2-CRE} or \textit{rx2-CreER}\textsuperscript{T2} lines to generate conditional knockouts and temporally controlled conditional knockouts respectively (Hans et al., 2011). This would enable a more complete understanding of the
contribution of her4 to the processes of retinal development and retinal regeneration independently, and would give valuable insight into the gene regulatory networks that control retinal neurogenesis downstream of her4.

A similar approach could be undertaken to differentiate between the roles of her9 during development and its role during regeneration. Her9, unlike her4, is not Notch-dependent and is regulated by different signal transduction pathways in a tissue dependent manner. The experiments outlined in Chapters 3 and 4 of this dissertation provide evidence that her9 plays a role in development of not only the retinal vasculature network, but also blood vessels throughout the trunk of the zebrafish. This novel finding adds to the known roles of her9 during the segmentation clock during somitogenesis, floor plate development, and establishment of non-neural identity during cranial sensory placode development. It is interesting that one gene functions in such diverse physiological processes in different tissues, and can be activated by numerous signaling pathways. This leads me to believe that one of the primary functions of her9 in the zebrafish might be to mediate cross-talk between regulatory networks during development so that cell- or tissue-specific events take place with proper coordination. Given that a her9 homozygous mutant adult zebrafish cannot be recovered (thus precluding the study of maternal zygotic mutants), a conditional knockout approach to study the various functions of her9 would be highly desirable. At this point, it is clear that her9 is involved to some degree in vasculogenesis, but why is it upregulated in the retina of zebrafish that experience rod-specific degeneration and regeneration remains unknown. It is possible that the function of her9 at the CMZ could be redundant with the function of her4, but her9 appears to play no role in rod neurogenesis. Her9 expression in the choroid, however, could be indicative of the need for additional vasculature to meet the metabolic demand of constant rod photoreceptor turnover. If this were the case, then a conditional knockout of her9 in a background of chronic rod degeneration and regeneration could result in a more severe retinal
phenotype in the XOPS:mCFP eye, and could possibly lead to secondary loss of other retinal neurons besides rod photoreceptors.

There could be numerous long term implications of this work, including possible roles in gene therapy in human patients who have experienced various degrees of retinal degeneration and vision loss from ophthalmic disorders. Induction of HES5 (her4 in zebrafish) expression in a diseased human retina, for example, could be part of a possible treatment that would induce resident Müller glia to dedifferentiate and re-enter the cell cycle resulting in progenitor cells that could be further directed to differentiate as replacement neurons. Knockdown of HES1 (her9 in zebrafish) could possibly be used to suppress excess vasculogenesis during retinopathy, and overexpression could possibly provide a mechanism to generate new vasculature tissue de novo not only in a diseased retina, but throughout the body. For these possibilities to become a reality a much more thorough understanding of the exact mechanisms of these genes within the context of larger gene regulatory networks would need to be realized. In addition, there are numerous other factors and pathways that have yet to be described that enable regeneration in the zebrafish but preclude regeneration in mammals.

Taken together, the projects detailed in this dissertation provide a detailed examination and description of novel functions of two bHLH-O transcription factors during development and regeneration of the zebrafish retina. This dissertation includes detailed descriptions of the expression patterns and knockdown and/or knockdown phenotypes observed with her4 and her9 and shows the importance of these genes during embryonic retinal development and regeneration of the mature zebrafish retina. It is my hope that these studies could contribute in some way towards a greater understanding of development and regeneration of the visual system which could in turn lead to the development or deployment of therapies that could meaningfully delay or reverse vision loss resulting from a variety of diseases that lead to visual impairment in humans.
Figure 5.S1: Her4 and her9 during retinal development and regeneration. The bHLH-O transcription factors her4 and her9 are upregulated during zebrafish rod degeneration/regeneration. The her4 transcription factor plays a role in retinal neuron neurogenesis, while her9 plays a role in development of the retinal vasculature network.


Dyer MA (2003) Regulation of proliferation, cell fate specification and differentiation by the homeodomain proteins Prox1, Six3, and Chx10 in the developing retina. Cell cycle (Georgetown, Tex) 2:350-357.


Gorsuch RA, Hyde DR (2014) Regulation of Muller glial dependent neuronal regeneration in the damaged adult zebrafish retina. Experimental eye research 123:131-140.


Sonic hedgehog, secreted by amacrine cells, acts as a short-range signal to direct differentiation and laminating in the zebrafish retina.


Leve C, Gajewski M, Rohr K, Tautz D (2001) Homologues of <small>c-hairy1</small> (<small>her9</small>) and <small>lunatic fringe</small> in zebrafish are expressed in the developing central nervous system, but not in the presomitic mesoderm. Development genes and evolution 211:493-500.


Ng Chi Kei J, Dudczig S, Currie PD, Jusuf PR (2016) Feedback from each retinal neuron population drives expression of subsequent fate determinant genes without influencing the cell cycle exit timing. The Journal of comparative neurology.


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