The Role of Sox4 in Regulating Choroid Fissure Closure and Retinal Neurogenesis

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Dr. Ann C. Morris, Major Professor
Dr. David F. Westneat, Director of Graduate Studies
THE ROLE OF SOX4 IN REGULATING CHOROID FISSURE CLOSURE AND RETINAL NEUROGENESIS

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

Wen Wen
Lexington, Kentucky

Director: Ann C. Morris, Associate Professor of Biology
Lexington, Kentucky

2016

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The development of the vertebrate eye is tightly controlled by precise genetic regulations. From a single ocular primordium to bilateral eyes with complex structures and cell types, it requires intensive proliferation and migration for cells in both the ectoderm and mesoderm to accomplish ocular morphogenesis, and during this process cell differentiation and interaction takes place to establish the complex composition of ocular cell types and cellular connections. Genetic defects can lead to severe abnormalities in eye morphogenesis and cell differentiation during ocular development. A tremendous amount of work has been done to identify both intrinsic and extrinsic factors that regulate ocular development. However, much more work is needed to fully understand this complex process.

Sox4 is known as a transcription activator that regulates cell survival and differentiation in multiple embryonic tissues during development. Evidence of its requirement during ocular development has recently emerged, but the mechanism by which Sox4 regulates ocular development is far from elucidated. Chapter 1 of this dissertation provides an overview of different stages in embryonic eye development and known genetic interactions during each stage. It also reviews recent knowledge about SoxC proteins and their roles in ocular development. Chapter 2 presents data characterizing the expression profile of the zebrafish sox4 co-orthologs, sox4a and sox4b, in the developing eye. Additionally, it presents data from morpholino-mediated sox4 knockdown in zebrafish, which indicate that Sox4 deficiency leads to defects in choroid fissure closure through elevation in the Hedgehog (Hh) signaling pathway. Sox4 knockdown causes upregulation of the Hh ligand Indian Hedgehog b (ihhb), which alters the proximal-distal boundary of the optic vesicle and inhibits choroid fissure closure. Chapter 3 presents data reporting the generation of sox4 mutant zebrafish lines using the CRISPR/Cas9 genome editing system. Characterization of one sox4a maternal zygotic (MZ) mutant line confirms Sox4’s role in negative regulation of Hh signaling and reveals new evidence that maternal and zygotic sox4 are both critical for ocular development. Chapter 4 presents data demonstrating that sox4 is required for rod photoreceptor neurogenesis. Rod photoreceptor terminal differentiation is delayed in both sox4 morphants and sox4 CRISPR mutants, while rod progenitor and precursor cells are properly specified. In Chapter 5, the roles of Sox4 in regulating ocular development are summarized based on the results, and implications of the results are discussed to expand our understanding of the genetic regulation of ocular morphogenesis and retinal neurogenesis.

KEYWORDS: Sox4, Eye development, Coloboma, Hedgehog signaling, Rod photoreceptor, CRISPR/Cas9

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April 26, 2016
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THE ROLE OF SOX4 IN REGULATING CHOROID FISSURE CLOSURE AND RETINAL NEUROGENESIS

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To Mama, Baba, and Qi
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CHAPTER 1: KEEPING AN EYE ON SOXC PROTEINS: VERTEBRATE EYE DEVELOPMENT AND SOXC TRANSCRIPTION FACTORS

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1.1 Abstract
The formation of a mature, functional eye requires a complex series of cell proliferation, migration, induction among different germinal layers, and cell differentiation. These processes are regulated by extracellular cues such as the Wnt/BMP/Hh/Fgf signaling pathways, as well as cell intrinsic transcription factors that specify cell fate. In this review article we provide an overview of stages of embryonic eye morphogenesis, extrinsic and intrinsic factors that are required for each stage, and pediatric ocular diseases that are associated with defective eye development. In addition, we focus on recent findings about the roles of the SOXC proteins in regulating vertebrate ocular development and implicating SOXC mutations in human ocular malformations.

1.2 Introduction
When we were enjoying afternoon tea in the backyard, and watching a ray of sunlight thrust through the wooden fence and paint every little thing a warm golden hue; when we were hiking in the mountain trails wishing to capture in our eyes all the maniacal red from those autumn leaves; when we were lying on the ground staring at the Milky Way and counting the blinking little stars, we absolutely adore the beauty of nature. Yet there is one more important thing to appreciate that
allows us to be able to enjoy those lovely moments: the eye. As a delicate camera, the eye captures light and processes it into electronic signals that can be decoded by the brain to give us a perception of the world.

For those born with healthy eyes, it might be difficult to imagine how life will be affected if the eyes do not function normally. However, more than one hundred million people worldwide suffer from visual impairment. And according to estimates by the NIH National Eye Institute, the population affected by common eye diseases will double from 2010 to 2050. Visual impairment and blindness are among the most significant factors contributing to reduced quality of life. To develop better therapeutic treatments for sight-threatening diseases, we must better understand how the eye develops. This review will focus on the major steps during vertebrate eye development, introducing how the eye takes its shape and how different parts of the eye specify their fates. It will also highlight some of the known genes and cell signaling pathways that regulate these developmental stages. Additionally, we will provide an overview of the functions of SoxC transcription factors, including an emerging role in regulating vertebrate eye development.

Throughout the review, we will follow the established nomenclature. All gene names will be italicized and protein names will not be italicized. For both gene and protein names, all letters capitalized are for humans, first letter capitalized are for rodents, and all letters lowercase are for other vertebrates such as zebrafish (\textit{Danio Rerio}) and \textit{Xenopus}.

\textbf{1.3 Origin and morphogenesis of the vertebrate eye}

1.3.1 Optic primordium specification

The formation of vertebrate eye begins with the induction of the optic primordium from the anterior neural plate. As an extension of the brain, the optic primordium is part of the embryonic
forebrain, which is composed of the telencephalon, the optic primordium, and the diencephalon (Figure 1.1A) (Rubenstein and Beachy, 1998). Early in the gastrulation stage of embryonic development, cells contributing to these three parts of the forebrain are intermixed. Through cell migration and cell fate determination, the optic primordium cells converge together and form a clear boundary away from the telecephalic precursors rostrally and laterally and from the hypothalamus caudally (Figure 1.1B) (Inoue et al., 2000). The specification and separation of the eye primordium from the rest of the forebrain is a process that requires regulation of both extrinsic cell signaling from surrounding tissues and intrinsic expression of transcription factors in optic progenitor cells.

Decades of research have identified cell signaling pathways that regulate eye primordium formation, among which are the bone morphogenetic proteins (BMP), fibroblast growth factor (FGF), and wingless (Wnt) signaling pathways. Inhibition of BMP, Wnt, and FGF signaling primes the neural tissues, including the optic primordium, to commit to an anterior neural plate fate. Neural induction and optic primordium specification require inhibition of BMP signaling in the anterior neural tube by BMP antagonists such as Chordin, Noggin, Follistatin, and Cerberus (Barth et al., 1999) and a decreased gradient of Wnt and FGF signaling from the posterior to anterior neural tube (Zuber, 2010). Local activation of FGF signaling before gastrulation represses cell migration in the anterior neural plate and prevents cells from taking a retinal fate. This inhibition is partly through the FGF modulation of Ephrin B1 which encodes a transmembrane protein involved in cell adhesion (Moore et al., 2004). Low levels of Wnt/β catenin activity are required for telencephalic specification, whereas high levels promote diencephalic identity. Residing in between the telencephalon and diencephalon, does optic primordium specification require an intermediate level of Wnt/β catenin activity? It turns out to be more complicated. Both canonical and non-canonical Wnt activities are required for the formation of optic primordium. Wnt8- and Fz8-mediated activation of canonical Wnt/β catenin
signaling inhibits optic primordium formation, whereas Wnt11- and Fz5-mediated activation of non-canonical Wnt signaling stimulates optic primordium specification by promoting the convergence of the eye field cells and inhibiting the canonical Wnt/β catenin activity (Cavodeassi et al., 2005). Other Wnt modulators and targets have been identified to regulate optic primordium specification and separation. Please refer to these review articles for a more comprehensive summarization (Chuang and Raymond, 2002; Esteve and Bovolenta, 2006; Wilson and Houart, 2004).

In addition to receiving extrinsic cell signaling from the surrounding tissues, the progenitor cells in the optic primordium also express specific eye field transcription factors (EFTF) that define the fate of the optic primordium. The vertebrate EFTFs include otx2, rx (rax), pax6, six3/six6 (optx2), lhx2, ET (tbx3), hesx1, and nr2e1 (tlx) (Chow and Lang, 2001; Graw, 2010; Shaham et al., 2012; Zuber, 2010; Zuber et al., 2003). Combinations of these EFTFs were necessary and sufficient to induce formation of an ectopic eye in Xenopus embryos (Zuber et al., 2003). Although we will focus on the vertebrate eye field specification, it is very important to acknowledge studies that have been done on eye development in Drosophila. Such work greatly inspired the identification of genes that are involved in vertebrate eye field specification. Many vertebrate EFTFs are homologs of the Drosophila eye specific genes (Erclik et al., 2009; Kumar, 2001). For example, Pax6 in vertebrates is a homolog of Drosophila twin of eyeless (toy) and eyeless (ey) (Quiring et al., 1994) and Six3 and Six6 in vertebrates are homologs of Drosophila sine oculis (so) and optix7 (Loosli et al., 1998).

EFTFs are expressed in overlapping domains in the optic primordium and regulate optic primordium specification by mediating the outcome of cell signaling, regulating cell migration, maintaining cell proliferation, and cross-regulating each other’s expression. For example, local activation of BMP4 in the anterior neural plate leads to a repression of eye formation due to...
reduced expression of *pax6*, *otx2*, and *rx* (Hartley et al., 2001). *Six3* is required for eye formation by negatively regulating BMP4 expression (Gestri et al., 2005) and Wnt signaling in the optic primordium, and by positively regulating Hh signaling in the diencephalon (Geng et al., 2008). The intense cell movement regulated by FGF, Wnt, and ephrin signalings can also be modulated by EFTFs. *Rx3* is required to set up the complementary expression pattern of *eph* and *ephrin* in the anterior neural plate that facilitates the formation of optic primordium (Cavodeassi et al., 2013).

To ensure the optic primordium reaches an appropriate size before neurogenesis, EFTFs *Rx*, *Pax6*, *Six3*, *Six6*, and *Lhx2* maintain the proliferation of eye progenitor cells (Agathocleous and Harris, 2009). EFTFs also regulate each other’s expression and cooperatively modulate optic primordium specification. For example, *Pax6*, *Rx*, and *lhx2* can induce *Six6* expression in the optic primordium in *Xenopus* and mice (Terada et al., 2006; Tetreault et al., 2009).

1.3.2 Eye field segregation

During neurulation, in response to secreted signals from the ventral midline, the telencephalic progenitor cells migrate dorsally over the optic primordium toward the midline, the ventral diencephalic progenitor cells move anteriorly and ventrally forming the hypothalamus, and cells in the optic primordium migrate laterally and split into two bilateral optic vesicles (OV) (Figure 1.1C) (Chuang and Raymond, 2001, 2002; Li et al., 1997; Varga et al., 1999). The prechordal mesendoderm located ventral to the anterior neural plate was found to be the source of the secreted factors that are critical for eye field segregation, as removal of it from the developing vertebrate embryo results in cyclopic eyes due to a failure in the separation of the eye field (Adelmann, 1936; Li et al., 1997; Pera and Kessel, 1997). Ligands for Nodal/TGF-β and Hedgehog (Hh) signaling pathways expressed in the prechordal mesendoderm are identified as critical secreted factors that signal to the anterior neural tube to induce eye field segregation and
pattern the proximodistal axis of optic vesicles (Ekker et al., 1995; Pei and Feldman, 2009; Schier and Shen, 2000). Mutations in Nodal-related ligands including Squint/ndr2 (Sqt) and Cyclops/ndr1 (Cyc), as well as Nodal mediator One-eyed pinhead (Oep) result in variable degrees of cyclopia and holoprosencephaly (Gritsman et al., 1999; Pei and Feldman, 2009). Hh signaling expressed in the neuroectoderm acts downstream of the Nodal/TGF-β signaling to facilitate the segregation of the eye field (Muller et al., 2000). Mutations of Sonic hedgehog (Shh) in humans and mice are associated with cyclopia and holoprosencephaly (Belloni et al., 1996; Chiang et al., 1996; Roessler et al., 1997). Zebrafish Shh mutant sonic-you (syu) does not exhibit cyclopia due to the functional redundancy of another Hh ligand tiggy-winkle hedgehog (twhh) (Ekker et al., 1995).

Driven by the migration of individual eye field progenitor cells, the separated eye fields continue evaginating from the neural tube and form bilateral optic vesicles (OV) with a bi-layered ball-like shape (Brown et al., 2010). Some EFTFs also contribute to the evagination of the eye field. For example, mutation in rx3 disrupts the migration and proliferation of the progenitor cells in the eye field and leads to defects in the evagination of the eye field (Loosli et al., 2003; Rembold et al., 2006; Stigloher et al., 2006).
Figure 1.1. Origin of the vertebrate eye and the morphological changes of the anterior neural tube. Schematic drawing showing the dorsal view of the anterior neural tube (A), migration of the telencephalon, eye field, and diencephalon (B), and the formation of the optic vesicle (C). Transcription factors associated with each stage of eye development are indicated in purple boxes on the bottom of the figure. t, telencephalon; ef, eye field; d, diencephalon; h, hindbrain; ty, thalamus; n, notochord. A, anterior; P, posterior; D, dorsal; V, ventral.
1.3.3 Optic cup and optic stalk formation

Once the OV reach the surface ectoderm, a series of morphological transformations and cell fate determinations shape the OV into an optic cup (OC) (Figure 1.2A). A combination of advanced microscopy techniques and transgenic animal models have allowed in vivo time lapse observations of OC morphogenesis (Ivanovitch et al., 2013; Keller et al., 2008; Kwan et al., 2012; Martinez-Morales et al., 2009). The distal side of the OV enlarges in size and folds along the dorso-ventral and anterior-posterior axes to form the OC. The OC is a bi-layered structure in which the inner layer becomes the future neural retina and the outer layer becomes the retina pigmented epithelium (RPE). The proximal side of the OV that connects to the diencephalon attenuates and becomes the optic stalk, which will encompass bundles of the retinal ganglion cell axons. The expression of Pax2-Pax6 and Vax-Rx in the optic stalk and optic cup, regulated by Hh signaling, is critical for the proximo-distal differentiation of the developing eye (Chiang et al., 1996; Ekker et al., 1995; Take-uchi, 2003; Varga et al., 2001). Because of the folding towards the ventral side, an optic fissure is formed along the OC and optic stalk, which eventually fuses at the completion of ocular morphogenesis (Martinez-Morales and Wittbrodt, 2009; Sinn and Wittbrodt, 2013). The transient opening of the optic fissure allows the mesoderm-derived periocular mesenchymal cells to enter the eye and form the hyaloid vessels, which supply nutrients to the retina, lens, and iris during early ocular development (Figure 1.3). At the same time as the OV makes contact with the surface ectoderm, lens induction is initiated. The surface ectoderm adjacent to the OV differentiates into the lens placode, which thickens, invaginates and detaches from the surface ectoderm, eventually becoming the lens vesicle (Figure 1.2A) (Lang, 2004).
Figure 1.2. Differentiation and morphological changes of the optic cup. Schematic drawing showing the developing lens and optic cup (A), the anterior segments, retina, and the optic nerve (B). Transcription factors associated with each process are indicated in purple boxes on the bottom of the figure. lp, lens placode; oc, optic cup; os, optic stalk; r, retina; L, lens; RPE, retinal pigmented epithelium. D, dorsal; V, ventral.
Figure 1.3. Choroid fissure closure and ocular vascular formation. A schematic representation of the migration of periocular mesenchymal cells through the choroid fissure (A) and the formation of ocular vascular vessels while the choroid fissure closes (B). Transcription factors associated with each processes are indicated in purple boxes on the bottom of the figure. r, retina; L, lens. D, dorsal; V, ventral.
Along with the formation of the OC, ocular progenitor cells in distinct regions of the OC undertake different fates by expressing different sets of transcription factors. Actually, the dorso-ventral and naso-temporal identities in the eye are already apparent at the optic vesicle stage (Figure 1.1C) (Picker et al., 2009). The topography of the OC is critical for the future differentiation of the vertebrate eye, as it determines the sequential neurogenesis in the retina, the projection of retinal ganglion cell axons to the brain, and the differentiation of multiple structures in the eye. The dorso-ventral axis of the OC is set up by the collaboration of different signaling activities (Chuang and Raymond, 2002; Peters, 2002). BMP4 expressed in the dorsal OC is critical for cells there to take the dorsal fate by expressing \( \text{tbx2}, \text{3}, \text{and 5} \), and repressing ventral markers \( \text{vax} \) and \( \text{pax2} \) (Behesti et al., 2006; Holly et al., 2014; Koshiba-Takeuchi et al., 2000). Retinoic acid (RA), Hh, and Fgf signaling activities are required for the specification of the ventral fate (Lupo et al., 2005). RA deficiency resulted in a loss of the ventral OC and optic stalk fate, but did not affect the expression of dorsal-ventral markers such as \( \text{pax2}, \text{pax6}, \text{tbx6}, \text{vax2}, \text{raldh1}, \text{and raldh3} \) (Maden et al., 2007). Hh signaling specifies the ventral OC and optic stalk fate by regulating \( \text{pax2} \) and \( \text{vax} \) gene expression (Take-uchi, 2003). The naso-temporal axis of the OC (mainly the neural retina) is also determined by the activity of multiple cell signaling pathways. Fgf signaling from the dorsal forebrain and olfactory epithelium specify the nasal identity of the OC while Hh signaling in the ventral forebrain is necessary for the temporal identity of the OC (Hernandez-Bejarano et al., 2015; Picker et al., 2009). In addition, \text{fgf19} \) is expressed in the nasal retina in zebrafish and \text{fgf19} \) deficiency results in reduction in nasal retina markers \text{ephrin A3}, \text{ephrin A5a}, and \text{foxg1}; temporal marker \text{eph A4b} on the other hand is expanded to the entire retina (Nakayama et al., 2008).

### 1.4 Cellular differentiation of the vertebrate eye

#### 1.4.1 The anterior segment
The anterior segment of the vertebrate eye includes the cornea, lens, iris, and ciliary body. It is very important for focusing light onto the retina and regulating intraocular pressure (Figure 1.2B) (Gould et al., 2004). The cornea and lens are transparent and refract light into the eye. The iris controls the size of the pupil and how much light can enter the eye. The ciliary body secretes aqueous humor which nourishes the lens and cornea, and contributes to the generation of intraocular pressure that supports the shape of the eye. The establishment of the anterior segment involves cell interaction from the surface ectoderm, neural crest derived mesenchymal tissues, and the neuroectoderm (Gage et al., 2005; Soules and Link, 2005). The lens placode is derived from the thickening and invagination of the surface ectoderm. As it detaches from the surface ectoderm, mesenchymal cells start migrating in between the lens vesicle and the surface ectoderm, and differentiate into the corneal stroma and corneal endothelium. The iris and ciliary body differentiate from the peripheral edges of the neural retina and RPE, together with another group of mesenchymal cells.

Transcription factors expressed in the developing lens and mesenchymal tissues including Maf, Foxe3, Foxc1, Pitx2, and Pitx3, are critical for the development of the anterior segment (Jamieson et al., 2002; Kozlowski and Walter, 2000; Semina et al., 2001; Semina et al., 2000). Mutations of these genes in humans are usually related to diseases with anterior segment defects such as Peter’s anomaly (central corneal opacity with adhesions of the iris, cornea, Descemet's membrane, corneal endothelium, and lens) and Axenfeld-Rieger syndrome (thin iris, off-center or multiple pupils, glaucoma). In addition, as a key regulator of vertebrate eye development, PAX6 is also critical for anterior segment differentiation. Pax6 is required for the expression of many anterior segment genes including Six3, Sox2, c-Maf, MafA/L-Maf, Proxl, crystallins, and cell adhesion molecules (Cvekl and Tamm, 2004; Lang, 2004; Takamiya et al., 2015). Mesenchymal cell derived BMP and cross-talk of RA and canonical Wnt signaling through pitx2 are also required for anterior segment development (Chang et al., 2001; Gage and Zacharias, 2009).
1.4.2 The retina and the retinal pigmented epithelium (RPE)

The inner layer of the OC becomes the neural retina and the outer layer becomes the RPE. The RPE and retina fate determination in the OC is already established during the evagination stage with the expression of the earliest retinal marker *Vsx2* and RPE marker *Mitf* (Green et al., 2003; Horsford et al., 2005; Tachibana, 2000). The adult vertebrate retinal cell types that are differentiated from a single pool of retinal progenitor cells are six types of neurons [retinal ganglion cell (RGC), amacrine cell, horizontal cell, bipolar cell, cone photoreceptor, rod photoreceptor] and one type of residential glial cell (the Müller cell). These cells are organized into three layers in the retina: the ganglion cell layer (GCL) that contains cell bodies of the RGCs, the inner nuclear layer (INL) that contains cell bodies of the amacrine, horizontal, bipolar, and Müller glia cells, and the outer nuclear layer (ONL) that contains cell bodies of rod and cone photoreceptor cells. The three nuclear layers are separated by the two plexiform layers containing retinal neuron synaptic connections. All the retinal cell types are generated from a single pool of multipotent retinal progenitor cells, whose proliferative state is controlled by the Notch signaling effectors *Hes1* and *Hes5* prior to neurogenesis (Hatakeyama et al., 2004; Jadhav et al., 2006).

Retinal neurogenesis in most vertebrates follows a conserved temporal and spatial sequence. It initiates with the retinal region most adjacent to the optic stalk, and the RGCs are the first to differentiate, followed by the amacrine cells, horizontal cells and cone photoreceptors. Bipolar cells, rod photoreceptors and the Müller cells are generated last (Bassett and Wallace, 2012).

The differentiation of retinal cell types is determined by the change of the retinal progenitor cell competence, which is accomplished by changes in the expression of intrinsic transcription factors and responsiveness to extrinsic signaling pathways over time (Alexiades and Cepko, 1997; Cepko et al., 1996; Harris, 1997; Livesey and Cepko, 2001). RGC differentiation is initiated by the expression of a basic helix-loop-helix (bHLH) transcription factor *Ath5* in response to Hh and Fgf signaling activities, and maintained by the expression of a POU domain transcription factor.
The differentiation of retinal interneurons (amacrine cells, bipolar cells, and horizontal cells) require a combined expression of several bHLH and homeobox transcription factors, including *Mash1*, *Math3*, *Vsx*, *NeuroD*, *Six3*, *Prox1*, *Pax6*, *Ptf1a* and *Poxn4*. Please see (Ohsawa and Kageyama, 2008; Stenkamp, 2007) for a comprehensive review. Photoreceptor cells are determined by the expression of the homeobox gene *Crx* and *Otx*, and the bHLH gene *NeuroD* (Hennig et al., 2008; Nishida et al., 2003). The expression of distinct subsets of transcription factors within the photoreceptor progenitor cell population further facilitates the differentiation of rod or cone photoreceptors, with *TRβ2*, *RXRγ*, *RORβ*, and *NR2F1* for cones; and *Nrl*, *Nr2e3*, and *Mash1* for rods (Swaroop et al., 2010). The Müller cell is the only type of glial cell in the retina that originates from the retinal progenitor cells. Notch activity and the expression of *Hes1*, *Hes5*, *Sox2*, and *Sox9* are key regulators for Müller cell differentiation (Bernardos et al., 2005; Jadhav et al., 2009; Muto et al., 2009).

RPE cells differentiate from the outer layer of the OC; they express melanin and are indispensable for eye development and phototransduction. The RPE not only absorbs light, nourishes and recycles the outer segment discs of photoreceptor cells, and converts all-trans retinal to 11-cis retinal, but it also participates in the development of anterior segment, in choroid fissure closure, and in photoreceptor differentiation [reviewed in (Bharti et al., 2006; Chow and Lang, 2001; Strauss, 2005)]. The differentiation of RPE cells requires the expression of *Mitf*, *Otx2*, and *Pax6* and the presence of extraocular mesenchymal cells (Capowski et al., 2014; Fuhrmann et al., 2000; Martinez-Morales et al., 2003). Although the mechanism of how the periocular mesenchymal cell acts on the specification of RPE cells is not clear, evidence has suggested that *activin A* in the BMP signaling pathway is one of the candidate molecules that can induce RPE differentiation through RPE and periocular mesenchymal interaction (Fuhrmann et al., 2000). Wnt/β catenin activity is also required for RPE differentiation, as conditional Wnt-
deficiency mice exhibit a loss of Mitf and Otx2 expression and a transdifferentiation of RPE to the neural retina (Fujimura et al., 2009). In addition, Hh signaling activity in the RPE and adjacent tissues is also critical for RPE differentiation. Hh ligands including shh, twhh, and ihh are expressed in the developing RPE (Dakubo et al., 2008; Perron, 2003; Stenkamp et al., 2000). Inhibition of Hh signaling in the chick causes a reduction in RPE specific gene expression and a transdifferentiation of RPE to the retina (Zhang and Yang, 2001b).

1.4.3 Ocular vasculature

As one of the most metabolically active organs, the eye has a high demand for nutrition during embryonic genesis and oxygen for its function in phototransduction when mature. The nutrition supply to the eye is provided by the ocular vasculature system in most vertebrates (not include birds). This system is initiated with two extra-retinal vascular systems: the choroidal vascular system that nourishes the outer retina and the hyaloid system that supplies the intraocular tissues including the anterior segment and the retina. The choroidal vascular vessels sprout from the internal carotid artery through angiogenesis. During development, they spread and encircle the entire optic cup at the basement membrane outside of the RPE and form a circular vessel, the annular vessel, at the anterior optic cup. The angiogenesis of the hyaloid vascular system is initiated by the entry of the hyaloid artery into the eye through the choroid fissure. It then extends through the vitreous to the anterior optic cup and forms the tunica vasculosa lentis (TVL) posterior to the lens, which connects with the annular vessel and hence links the hyaloid vascular system to the choroidal vascular system. During mammalian ocular development, the retina thickens over time due to proliferation of retinal progenitor cells and retinal neurogenesis. The choroidal vascular system persists to nourish the outer retina while the hyaloid vascular system is not sufficient to nourish the inner retina. As a result, the hyaloid vascular vessels retract and are replaced by the intraretinal vascular system. Zebrafish hyaloid vessels, on the other hand, do not regress. The intraretinal vascular vessels are derived from the base of the hyaloid artery and form
three plexi within the retina (the superficial, intermediate, and deep plexus) by the guidance of astrocytes (which are differentiated from neural crest cells and migrated into the retina) and Müller glia in the retina. [Reviewed by (Dorrell et al., 2007; Ruhrberg and Bautch, 2013; Saint-Geniez and D'Amore, 2004).]

Proper ocular angiogenesis requires vascular endothelial growth factor (VEGF) expressed by the RPE, lens, and astrocytes in the retina (Alvarez et al., 2007; Ikeda et al., 2006). VEGF and bFGF factors secreted by the RPE cells are critical for choroidal vasculature development (Rousseau et al., 2003; Sakamoto et al., 1995). VEGF expression in the lens is thought to affect hyaloid vessel formation, especially the TVL (Gogat et al., 2004). The superficial and deep retinal vasculature angiogenesis are induced by the expression of VEGF in the astrocytes and Müller glia (Stenzel et al., 2011; Stone et al., 1996; Stone et al., 1995; West et al., 2005). Mutations of VEGF receptor molecules, such as the tyrosine kinase FLK1 (VEGFR2) and neuropilin 1 (NRP1), result in failure of vascular outgrowth in the retina and the brain (Gerhardt et al., 2003; Gu et al., 2003). Notch signaling is also required for ocular vasculature development and is associated with choroidal neovascularization (CNV) observed in the wet form of age-related macular degeneration (Ahmad et al., 2011; Shawber and Kitajewski, 2004). In addition, Wnt signaling is critical for the regression of the hyaloid vasculature; mutation of the Wnt ligand norrie disease protein (NDP) and its receptor frizzled 4 lead to defects in retinal vascular development and a persistent hyaloid vasculature (Xu et al., 2004).

1.5 Ocular coloboma

Diseases with photoreceptor loss can directly affect vision. One example is retinitis pigmentosa (RP), that initiates from loss of rod photoreceptors followed by a progressive loss of cone photoreceptors (Hartong et al., 2006). Defects in ocular vasculature formation can also cause vision loss, such as the wet form of age-related macular degeneration (AMD) that is caused by an
abnormal outgrowth of the choroidal vascular vessels (van Lookeren Campagne et al., 2015). In addition, many vision-threatening ocular diseases are related to abnormalities during embryonic eye development [reviewed in (Morris, 2011)]. For example, improper optic primordium segregation can lead to holoprosencephaly (HPE) with cyclopia as the most severe form, which affects 1 in 100,000 live births (Dubourg et al., 2007; Geng et al., 2008; Orioli et al., 2011). Defects in the anterior segment development can lead to a variety of ocular abnormalities such as congenital glaucoma and cataracts, and anterior segment dysgenesis (ASD) (Gould et al., 2004; Reis and Semina, 2011; Roche et al., 2006; Sowden, 2007).

One of the most common causes for congenital blindness associated with ocular developmental defects is ocular coloboma, which accounts for 5-10% of child blindness cases worldwide and affects about 7.5 in 10,000 live births worldwide (Chang et al., 2006; Cheong et al., 2007). Ocular coloboma is due to the failure in choroid fissure closure. It usually affects the inferonasal quadrant of the eye. Based on the location and timing of the defect during ocular morphogenesis, it can affect many parts of the eye from anterior to posterior including the cornea, iris, ciliary body, retina, RPE, optic nerve, and the choroid (Chang et al., 2006; Gregory-Evans et al., 2004). Ocular coloboma is often associated with other developmental defects, either in the eye, such as microphthalmia and anophthalmia, or systematically, such as CHARGE syndrome (coloboma, heart anomaly, choanal atresia, retardation, genital and ear anomalies), renal-coloboma syndrome (hypoplastic kidney, coloboma, malformation of the optic nerve), and Coffin-Siris syndrome (absence of the fifth finger and toe nails, characteristic facial features, mental retardation) (Fleck et al., 2001; Lalani et al., 1993; Schimmenti, 2011; Schrier Vergano et al., 1993; Williamson and FitzPatrick, 2014).

Ocular colobomas are very heterogeneous. More than 20 known genes have been associated with coloboma, and the inheritance can be autosomal dominant, autosomal recessive, and X-linked
A coloboma gene network (CGN) has been proposed based on human and mouse model studies, demonstrating the regulatory network of known coloboma causative genes including transcription factors, cell signaling molecules, and cell cycle regulators (Gregory-Evans et al., 2004). Many ocular coloboma cases are associated with mutations in genes that affect early optic cup patterning and morphogenesis. Moreover, ocular coloboma can also be caused by environmental factors, including consumption of alcohol and maternal vitamin A deficiency during pregnancy.

Hedgehog signaling is a central node in the coloboma gene regulatory network. Mutations in \textit{Shh} are associated with ocular coloboma in both humans and mice (Schimmenti et al., 2003). Mutations in Hh target genes including \textit{pax2} and \textit{vax1/2} also lead to ocular coloboma (Barbieri et al., 2002; Eccles and Schimmenti, 1999; Macdonald et al., 1995; Take-uchi, 2003). In addition, the zebrafish \textit{blowout (blw)} mutant, which harbors a mutation in \textit{patched1} (a Hh receptor and negative regulator of Hh signaling), also causes coloboma (Lee et al., 2008).

Another coloboma-related signaling pathway involves retinoic acid (RA) signaling. RA regulates the expression of several coloboma-causing genes, including \textit{shh}, \textit{patched1}, \textit{vax}2, and \textit{retinoid binding protein gene (RBP4)} (Busch et al., 2014; Chou et al., 2015; Helms et al., 1997; Manolescu et al., 2010; Seeliger et al., 1999; Sen et al., 2005). In addition, RA also influences choroid fissure closure by regulating the migration and gene expression of the neural crest-derived periocular mesenchymal cells (Lupo et al., 2011; Matt et al., 2008).

In addition to defects related to ocular morphogenesis and optic cup patterning, ocular coloboma can also be caused by impaired dissolution of the choroid fissure basal lamina and reduced cell adhesion molecules (Gregory-Evans et al., 2011; Moosajee et al., 2008; Tsuji et al., 2012). Several zebrafish mutants carrying mutations in extracellular matrix and cell adhesion molecules
exhibit ocular coloboma, such as the zebrafish pac mutant that encodes a mutation in N-cadherin and the laminin beta 1 (lamb1) and laminin gamma 1 (lamc1) mutants (Lee and Gross, 2007; Masai et al., 2003). Conditional knockout of the adherens junctions (AJs) component α-catenin in the mouse eye also causes coloboma (Chen et al., 2012). Defects in retina folding and choroid fissure closure in vitamin A deficient (VAD) mice embryos are also associated with reduced cell adhesion molecules including N-cadherin and beta-catenin (See and Claggett-Dame, 2009).

1.6 Hedgehog (Hh) signaling and vertebrate eye development

Cell-cell signaling plays a critical role in embryonic development. Hh signaling is one of the most extensively studied cell signaling pathways during development and adult homeostasis. As a morphogen, Hh responding cells that receive different concentrations of Hh ligands will undertake distinct cell fates. One such example is the dorsoventral patterning of the vertebrate neural tube. Also functioning as a mitogen, Hh signaling regulates the proliferation of progenitor cells during organogenesis and the maintenance of adult stem cell homeostasis (Briscoe and Therond, 2013; Fuccillo et al., 2006; Ingham and McMahon, 2001). Hh was discovered from a genetic screen for genes that disrupt the patterning of the Drosophila embryo in 1980; mutant larvae exhibited a ‘lawn’ of denticles instead of denticle stripes on each body segment (Nusslein-Volhard and Wieschaus, 1980). The appearance of the mutant larva was reminiscent of a hedgehog, which inspired the name of the gene. In the early 1990s, Hh was identified as a secreted protein and Hh genes in Drosophila and vertebrates were cloned (Echelard et al., 1993; Krauss et al., 1993; Lee et al., 1992; Mohler and Vani, 1992; Riddle et al., 1993; Tabata et al., 1992). The Drosophila genome has a single hh gene, while mammals and chick have three Hh genes due to genome duplication: Desert Hedgehog (Dhh), Sonic hedgehog (Shh), and Indian hedgehog (Ihh). Vertebrate Dhh is a closer homolog to Drosophila hh than Shh or Ihh (Kumar et al., 1996). Zebrafish has five Hh genes with further duplication within the Shh and Ihh family: Dhh, Shha, Shhb (tiggy-winkle hedgehog), Ihha, and Ihhb (echidna hedgehog) (Currie and
Ingham, 1996; Ekker et al., 1995; Krauss et al., 1993). Since its discovery, many studies have revealed the function of Hh signaling in the development of neural tissues, limb buds, muscles, bones, cartilage, and reproductive tissues; and its correlation with congenital diseases, the maintenance of adult progenitor cell proliferation, and cancers (Briscoe and Therond, 2013).

1.6.1 Vertebrate Hh signaling transduction

Like many other cell signaling pathways, Hh signaling functions in a non-cell-autonomous fashion, which requires ligand-secreting cells and signal-receiving cells. After being translated in the signal-secreting cell, the Hh molecule is cleaved into a ~19kDa amino-terminal peptide (N-HH) and a ~ 25kDa carboxy-terminal peptide (C-HH) by autoproteolysis. The N-HH is responsible for all Hh signaling activities while the C-HH acts as a cholesterol transferase. Before secretion, N-HH undergoes two covalent modifications: a cholesterol group is added to the C-terminus (mediated by the C-HH); and a palmitic acid group is added to the N-terminus (mediated by membrane-bound O-acyltransferase (MBOAT) proteins) (Briscoe and Therond, 2013).

The modifications of N-HH are necessary for its biological function, as well as its intracellular trafficking and extracellular distribution distance (Kohtz et al., 2001; Lewis et al., 2001). Once it arrives at the plasma membrane of the secreting cell, modified Hh molecules can be released to the extracellular space either as a monomer (facilitated by the transmembrane protein dispatched (DISP) and SCUBE2), or as multimers (Tukachinsky et al., 2012; Zeng et al., 2001). Modified Hh molecules can also interact with the heparan sulphate chains of glypian and can be assembled with lipophorin into lipoproteins and released from the cell (Panakova et al., 2005). In addition to the above mechanisms, where Hh molecules are released in a soluble form, Hh can also be transported through tissues by extracellular vesicles (exovesicles) and through specialized filopodia to achieve long-distance transportation (Sanders et al., 2013; Vyas et al., 2014).
Hh receiving cells express two transmembrane Hh receptors: Patched (Ptch1 or Ptch2), a twelve transmembrane protein, and Smoothened (Smo), a seven transmembrane G protein-coupled receptor (GPCR) (Nakano et al., 1989; van den Heuvel and Ingham, 1996). The binding of Hh ligand with Ptch in vertebrates is facilitated by several co-receptors including low-density lipoprotein receptor-related protein 2 (LRP2), growth arrest-specific 1 (GAS1), CAM-related/downregulated by oncogenes (CDO) and brother of CDO (BOC) (Allen et al., 2011; Briscoe and Therond, 2013). Vertebrate Hh signaling activity requires the presence of the primary cilia of a cell (Huangfu and Anderson, 2005). In the absence of Hh ligand, Ptch is present at the ciliary base and negatively regulates the activity of Smo, inhibiting the movement of Smo into the cilia (Milenkovic et al., 2009; Rohatgi et al., 2007; Wang et al., 2009). Upon Hh and Ptch binding, Ptch moves out of the cilia while Smo is relieved from Ptch repression, accumulates in the cilium, and is activated by phosphorylation (Chen et al., 2004).

The phosphorylated Smo triggers the activation of downstream effectors, the zinc finger containing glioma-associated oncogene family (Gli) proteins. Vertebrates possess three Glis, Gli1, Gli2, and Gli3, which are homologs of the Drosophila Cubitus interruptus (Ci). All three Gli molecules have a DNA binding domain composed of tandem zinc fingers and a transcription activation domain at the C-terminus. Gli2 and Gli3, but not Gli1, also have a N-terminal transcription repressor domain. The outcome the Hh signaling activity is through the dynamic balance between the activator form (GliA) and the repressor form (GliR) of Glis. In the absence of Hh ligands, Gli2/3 are held in the primary cilia microtubule by a protein complex containing the Suppressor of used (Sufu) and kinesin Kif7, which promotes the phosphorylation of Gli2/3 by Protein kinase A (PKA), casein Kinase 1 (CKI) and glycogen synthase Kinase 3 (GSK3) (Liem et al., 2009; Robbins et al., 2012). The phosphorylation and subsequent ubiquitylation of Gli2/3 lead to the proteolytic cleavage of the C-terminus, generating the repressor form of Gli2/3R. In the presence of Hh ligands, the proteolytic cleavage of Gli2/3 is blocked, so that they remain as the
activator form. Through translocation into the cell nucleus, GliR and GliA represses or activate target genes, respectively. In mammals, Gli2 is the main Hh activator while Gli3 is mainly responsible for the target gene repression when the pathway is inactive (Hui and Angers, 2011; Sasaki et al., 1999). Gli1 plays a minimal role in mammalian Hh signal transduction, but its transcription is positively regulated by Hh signaling; thus, it is often used as a readout of Hh activity (Park et al., 2000). Unlike mammals, zebrafish and *Xenopus* Gli1 is an essential activator in response to Hh activation (Karlstrom et al., 2003; Lee et al., 1997).

1.6.2 The role of Hh in vertebrate optic vesicle patterning

Hh signaling from the ventral midline plays a critical role in optic field separation and the proximodistal and dorsoventral patterning of the optic vesicles. As described previously, the optic primordium splits into two bilateral optic vesicles along with the anterior migration of the diencephalon. The separation of the optic primordium largely depends on the expression of Hh from the ventral midline tissues. Shh loss of function in human and mice causes severe defects in the development of anterior neural tube and failure in optic primordium segregation, resulting in cyclopia (Chiang et al., 1996; Roessler et al., 1996). *Shha* mutant zebrafish do not exhibit cyclopia due to functional redundancy with *shhb* (Currie and Ingham, 1996; Etheridge et al., 2001; Schauerte et al., 1998). Both *shha* and *shhb* in zebrafish have to be knocked down to affect optic primordium separation (Nasevicius and Ekker, 2000). Gain-of-function studies in zebrafish and *Xenopus* have suggested that the Hh-dependent optic primordium separation is achieved by the Hh regulation of *pax2* and *pax6* expression in the distal and proximal optic vesicle, respectively. Hh overexpression promotes expanded expression of *pax2* and reduces the expression of *pax6*, and is often associated with ocular coloboma (Ekker et al., 1995; Macdonald et al., 1995; Pillai-Kastoori et al., 2014; Wen et al., 2015). Loss of midline derived Hh during early development leads to reduced or no expression of *pax2*, resulting in failure in optic primordium separation (Amato et al., 2004; Ekker et al., 1995). Overexpression of *shha* in zebrafish cyclops mutant,
which has a loss of \textit{shha} and \textit{shhb}, promotes the expression of \textit{pax2} and rescues the cyclopic phenotype (Macdonald et al., 1995). The reciprocal regulation of \textit{pax2} and \textit{pax6} further defines the boundary between the optic vesicle and the optic stalk (Macdonald et al., 1995; Schwarz et al., 2000).

The distinct expression domains of \textit{pax2} and \textit{pax6} also establish the proximodistal pattern of the developing eye. \textit{Pax2} expression is critical for the specification of the optic stalk and the formation of the optic nerve. \textit{Pax6} expression in the optic vesicle, on the other hand, is required for proper retinal neurogenesis, RPE formation, and lens induction (Baumer et al., 2003; Oron-Karni et al., 2008; Perron, 2003; Raviv et al., 2014; Richardson et al., 1995; Torres et al., 1996).

In addition, Hh signaling activity in the ventral midline also promotes the establishment of the dorsoventral axis of the eye by inducing expression of \textit{vax} in the ventral eye, while Bmp signaling induces \textit{tbx5} expression in the dorsal eye (Huh et al., 1999; Lupo et al., 2005; Sasagawa et al., 2002; Zhao et al., 2010). Zebrafish \textit{smo} mutant exhibits a loss of Hh activity and a significant reduction of \textit{vax} expression in the ventral eye (Takeuchi, 2003; Varga et al., 1999).

Hh overexpression in zebrafish can rescue the loss of ventral identity in the Nodal mutant eye and restore the expression of \textit{vax} genes in the ventral eye (Takeuchi, 2003). The mutual inhibition between \textit{pax2-pax6} and \textit{vax-tbx} guarantees the proper patterning of the proximodistal and dorsoventral axes of the developing eye.

1.6.3 The role of Hh during the cellular differentiation of vertebrate eye

In addition to regulating the bilateral segregation of optic vesicles, Hh signaling activity is also involved in retinal and RPE cell differentiation (Choy and Cheng, 2012; Wallace, 2008). In the \textit{Drosophila} imaginal eye disc, \textit{shh} expression in the morphological furrow induces the expression of a bHLH transcription factor \textit{atonal (ato)} in a strip of progenitor cells ahead of the furrow. \textit{ato} induces the differentiation of new photoreceptors and they in turn express new \textit{shh}, thus forming
a self-propagating ‘sequential-induction’ wave (Jarman et al., 1995; Kumar, 2001). In most vertebrates, a similar wave of Shh expression in the ganglion cell layer is observed and it coordinates with the expression of the fly atonal homolog ath5 and the wave of retinal ganglion cell differentiation from retinal regions adjacent to the optic stalk, eventually spreading to the entire retina (Jensen and Wallace, 1997; Masai et al., 2000; Neumann, 2000; Wang et al., 2005; Zhang and Yang, 2001a). The expression of Shh is required for its own spread through the retina, as observed in the zebrafish shh mutant sonic-you (syu), in which Shh expression in the retina is initiated in the first differentiated RGCs, but fails to spread (Neumann, 2000). Ath5 expression is required for the differentiation of RGCs, as the zebrafish lakritz mutant that harbors a mutation in the ath5 locus exhibits a complete loss of RGCs (Kay et al., 2001). Evidence shows some conserved requirement of Shh and Ath5 for RGC differentiation between flies and vertebrates. However, the ‘sequential-induction’ model does not apply to vertebrates, since the expression wave of ath5 in zebrafish requires neither the presence of differentiated RGCs nor intraretinal Shh expression (Kay et al., 2005). In fact, using a pharmacological approach, Kay et al found that the midline-derived Shh activity prior to retinal neurogenesis is required for the timing of Ath5 expression and RGC differentiation (Kay et al., 2005). Moreover, the midline Hh activity prior to retinal neurogenesis is also required for the RGC axon pathfinding within the retina (Stacher Horndli and Chien, 2012) and confines the growth cone of the optic nerve during optic chiasm formation (Troussse et al., 2001). Intriguingly, Hh as a morphogen actually plays a dual function in regulating RGC differentiation. Receiving low levels of Hh, retinal progenitor cells in front of the differentiation wave would exit the cell cycle and differentiate into RGCs. On the other hand, Hh concentration is higher behind the differentiation wave and the remaining progenitor cells there would retain their progenitor character to adopt later cell type fates (Zhang and Yang, 2001a).

Soon after the expression in the ganglion cell layer, another wave of Shh expression is initiated in the differentiating amacrine cells in the inner nuclear layer of the zebrafish and mouse retina.
(Jadhav et al., 2006; Shkumatava et al., 2004). The second wave of Shh expression is independent of the first wave in the RGCs, because it still occurs in ath5 mutant fish that have no RGCs (Shkumatava et al., 2004). Transplantation of WT cells into ath5 mutant embryos reveals that shh activity is required for the differentiation of inner nuclear layer retinal cell types as well as photoreceptors (Shkumatava et al., 2004). In addition, in vitro and in vivo experiments have shown that Müller glia differentiation also requires Hh activity. Overexpression of N-HH in embryonic mouse retinal explants results in an increased number of Müller glia cells, and a similar increase of Müller cells is also observed in Xenopus when Hh is overexpressed (Amato et al., 2004; Jensen and Wallace, 1997).

Additionally, Hh expression is also observed in the RPE in many vertebrates. Shh is expressed in the zebrafish and Xenopus RPE (Perron, 2003; Stenkamp et al., 2000). Ihh and Dhh are expressed in the RPE and cells adjacent to the RPE in mice, and they are required for maintaining the expression of the Hh effector gene Gli in the periocular mesenchymal tissues (Dakubo et al., 2008; Levine et al., 1997; Wallace and Raff, 1999). Inhibition of Hh activity either by anti-Shh antibody or cyclopamine treatment in chick and Xenopus results in a hypopigmentation in the ventral eye, suggesting a requirement of Hh activity in RPE cell differentiation (Stenkamp et al., 2002; Zhang and Yang, 2001b).

Several studies in zebrafish Hh mutant lines suggest that Hh activity is also required for photoreceptor differentiation. Reduced numbers of photoreceptors and abnormal retina lamination are observed either in the sonic-you (syu, mutation of shh), you-too (mutation of gli2), and slow muscle-omitted (smu, mutation of smo) zebrafish mutants, or in morpholino-mediated shha and shhb deficient zebrafish (Stenkamp and Frey, 2003; Stenkamp et al., 2000). Reduced expression of one of the photoreceptor progenitor genes rx1 is observed in the zebrafish smu mutant, suggesting rx1 might be regulated by Hh signaling activity (Stenkamp et al., 2002). Conversely,
Hh overexpression in the rat retina results in an increased number of photoreceptors, suggestive of a conserved role of Hh in photoreceptor differentiation in mammals and fish (Levine et al., 1997). Interestingly, overactivation of Hh signaling in mice retinal explants expressing a constitutively active Smo reduces the number of photoreceptors in a non-cell autonomous fashion, indicating that the differentiation of photoreceptor cells is sensitive to optimal levels of Hh signaling (Wallace, 2008; Yu et al., 2006). There is still a debate about whether the Hh activity from the inner nuclear layer or from the RPE is important for photoreceptor development and more experiments need to be conducted.

1.7 Zebrafish as a model for studying eye development

The zebrafish, Danio rerio, is a tropical freshwater teleost fish that has become a favorite model for studying eye development because of its ease of breeding, large numbers of offspring, external and rapid development, and its optical transparency during embryonic morphogenesis (Grunwald and Eisen, 2002). Taking advantage of microscopy techniques and transgenic zebrafish lines, zebrafish embryonic development has been thoroughly documented (Kwan et al., 2012; Li et al., 2000; Schmitt and Dowling, 1994). The zebrafish optic primordia evaginate from the developing forebrain around 12 hours post fertilization (hpf). From 12 to 18 hpf, the optic primordia undergoes evagination, reorientation, invagination and rotation. Lens induction is initiated at around 16 hpf. By 24 hpf, the zebrafish bi-layered optic cup and the detached lens vesicle are well formed. The choroid fissure in the ventral eye is also visible by then. At 48 to 60 hpf, the two lips of the choroid fissure in the retina fuse together and form a continuous retina (Schmitt and Dowling, 1994).

The zebrafish retinal neurogenesis is under precise spatiotemporal control. The first cells to exit the cell cycle differentiate into ganglion cells starting around 32 hpf in the ventronasal retina (Schmitt and Dowling, 1996). Retinal lamination and cell differentiation are completed around 96
hpf (Stenkamp, 2007) and the first visual response can be detected by ERG as early as 72 hpf (Branchek, 1984). Similar to other vertebrates, the zebrafish retina is also arranged into three nuclear layers and two plexiform layers, but unlike mammals, the zebrafish retina displays continual neurogenesis throughout life and has the ability to regenerate any type of retinal neuron in response to injury. New retinal neurons are generated by proliferation and differentiation of progenitor cells in the ciliary marginal zone (CMZ) at the retinal periphery (Raymond et al., 2006; Wan et al., 2016). In addition, new rod photoreceptors are generated from Müller glia-derived rod progenitor cells located in the outer nuclear layer (ONL) to retain visual sensitivity (Johns and Fernald, 1981) [please refer to chapter 4 for more detail]. In response to acute injury, a subset of zebrafish Müller glia cells re-enter the cell cycle and de-differentiate, producing clusters of neurogenic progenitor cells, which can differentiate into any type of retinal neuron (Bernardos et al., 2007).

Many genes related to eye development were identified in zebrafish by large-scale forward genetics screens using chemical mutagenesis or retroviral integration (Fadool et al., 1997; Gross et al., 2005; Malicki et al., 1996; Neuhauss et al., 1999). In addition, reverse genetic techniques are also broadly applied in the identification of genes that are critical for eye development in zebrafish (Huang et al., 2012). These techniques include antisense morpholino, zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system (please refer to chapter 3 for detailed review).

1.8 SoxC and eye development

1.8.1 Sox Subfamilies in vertebrates

The SOX proteins are transcription factors named after a shared motif called the SRY box, a high-mobility-group (HMG) DNA binding domain homologous to the DNA-binding domain of
the mammalian sex determining region on the Y chromosome (SRY). SOX proteins are found extensively throughout the animal kingdom, including nematodes, arthropods, birds, fish, reptiles, amphibians, and mammals (Bowles et al., 2000). In vertebrates about 30 SOX genes have been identified that are placed into 8 subgroups (A-H) on the basis of sequence similarity and genomic arrangements (Bowles et al., 2000; Schepers et al., 2002). Two new subgroups I and J have been introduced to include *Xenopus sox31* and a new invertebrate nematode (*Caenorhabditis elegans*) sox gene. The HMG box allows SOX proteins to recognize a specific linear DNA sequence 5’-(A/T)(A/T)CAA(A/T)G-3’in the minor grove of the DNA helix (Ferrari et al., 1992).

By regulating target gene transcription or posttranslational modification, SOX proteins are involved in multiple developmental processes (Wegner, 2010), including embryonic eye development. Mutations in SOX2, which belongs to the SOXB subfamily, result in anophthalmia or microphthalmia (Faivre et al., 2006). Sox2 is also required for the induction of the first lens specific gene δ-crystallin (Kamachi et al., 2001). Sox9, a SOXE family member, is required for expression of several retinal genes (such as calb2a, calb2b, crx, neurod, rx1, sox4a and vsx1) and for the differentiation of Müller glia and photoreceptors (Yokoi et al., 2009). In addition, Sox9 can also regulate visual cycle gene expression in the RPE (Masuda et al., 2014), and a recent study in Sox9−/− mice demonstrates that Sox9 and Sox10 are both required for the formation of lacrimal gland (Chen et al., 2014).

### 1.8.2 SoxC proteins and their known target genes

Recently, a role for the SOXC proteins in regulating eye development has emerged from studies in animal models and in humans. The SOXC family in invertebrate animal species consists of a single member (Cremazy et al., 2001). In most vertebrates, the SOXC family includes three intronless genes: *SOX4* [MIM: 184430], *SOX11* [MIM: 600898], and *SOX12* [MIM: 601947] (Penzo-Mendez, 2010). SOXC proteins are known as transcriptional activators. Apart from the
HMG domain in the N-terminus, a transactivation domain (TAD) in the C-terminus is present among the SOXC members. Like other SOX members, the HMG domain of SOX4, SOX11, and SOX12 is greater than 50% identical to the corresponding domain of SRY, both at nucleotide and amino acid level. Within the subgroup, SOXC proteins share a high degree of similarity within the HMG box domain as well as flanking sequences and the TAD domain (Figure 1.4A) (Bhattaram et al., 2010; Koopman et al., 1991), and are strongly conserved across vertebrate species. Among SOXC proteins, SOX4 is the most efficient in DNA binding due to the lack of the acidic domain and SOX11 has the highest transactivation activity because of its continuous α-helical structure (Dy et al., 2008; Hoser et al., 2008; Penzo-Mendez, 2010; van de Wetering et al., 1993; Wiebe et al., 2003).

Although they are essential and play multiple roles in embryonic development and tissue differentiation, very few SOXC target genes have been identified (Figure 1.4B). One known target gene is classIII β-tubulin (also known as Tubb3 or Tuj1). It is a pan-neuronal gene expressed specifically in the nervous system and is critical for neurogenesis (Memberg and Hall, 1995). Sox4 and Sox11 can transactivate the lacZ reporter driven by the Tubb3 promoter and directly interact with the sequence upstream of Tubb3 in DNA-binding gel shift assays (Bergsland et al., 2006). Sox12 can also bind to Tubb3 but the transactivation activity is relatively low compared to Sox4 and Sox11 (Hoser et al., 2008). Another SoxC target gene is Tead2, a downstream mediator of the Hippo signaling pathway. Several SoxC binding motifs are present in the promoter region and first exon of Tead2, and direct binding has been detected both in vitro and in vivo by EMSA and chromatin immunoprecipitation assay (ChIP) (Bhattaram et al., 2010). Sox4 can bind to the T-cell specific enhancer at the 3’ end of the CD2 gene and transactivate CD2 (Wotton et al., 1995). Moreover, SoxC proteins also interact with protein partners to regulate target gene transcription. For example, SoxC proteins synergize with POU domain transcription factors Brn1 and Brn2 to activate Nestin expression in the neural tube (Tanaka et al., 2004). In
addition to these transcriptional targets, SoxC proteins are also involved in target protein stability. For example, in response to DNA damage, Sox4 can directly bind to p53 and enhance its acetylation, thus inhibiting Mdm2-mediated p53 ubiquitination (Pan et al., 2009).

SoxC family members are extensively expressed in the vertebrate nervous system during development, redundantly regulating neuronal and mesenchymal progenitor survival and neuronal cell fate determination (Bergsland et al., 2011; Bhattaram et al., 2010; Dy et al., 2008). They are strongly expressed in immature neurons that have already exited the cell cycle in the ventricular region of the brain (Bhattaram et al., 2010). SoxC expression decreases while the nervous system matures. SoxC family member expression is limited to the forebrain and the caudal spinal cord (Jankowski et al., 2006).
Figure 1.4. Schematic of the Human SOX4 protein and known functions of Sox4 in regulating tissue differentiation. (A) The Human SOX4 protein has 474 amino acids. It has a high-mobility-group (HMG) DNA binding domain at the N terminus and a transactivation domain (TAD) at the C terminus. (B) Sox4 regulates the differentiation of multiple cell types during embryonic development. Target genes known for some processes are listed. Sox4 promotes neuron differentiation through activation of Tubb3 and synergistic cooperation with POU domain transcription factors Brn1 and Brn2 to induce Nestin expression.
1.8.3 SoxC expression in the developing vertebrate eye

In the embryonic vertebrate eye, SoxC genes are expressed in a partially overlapping pattern in both time and space (Cizelsky et al., 2013; Dy et al., 2008; Maschhoff et al., 2003; Pillai-Kastoori et al., 2014; Uy et al., 2014; Wen et al., 2015). They are expressed in the neuroepithelium of the optic cup, in the cells of the surface ectoderm and lens placode, and also in the surrounding neural crest derived mesenchymal cells (Table 1.1). Their expression is initiated in the retina in the first group of cells that exit the cell cycle to differentiate into RGCs, and subsequently spreads throughout the ganglion cell layer and into the inner nuclear layer, along with the progression of retinal cell differentiation (Cizelsky et al., 2013; Jiang et al., 2013b; Pillai-Kastoori et al., 2014; Usui et al., 2013b; Wen et al., 2015). Expression of Sox4 and Sox11 coincides with the onset of expression of the ganglion cell marker Brn3b and amacrine cell marker Islet1 (Jiang et al., 2013b; Usui et al., 2013b). In addition, sox11 is also expressed in the developing lens (Pillai-Kastoori et al., 2014). As retinal neurogenesis progresses, SoxC expression is downregulated in mature retinal neurons. Very little expression is detected in the mammalian adult eye. In vertebrates that display persistent retinal neurogenesis, such as Xenopus and zebrafish, sox4 and sox11 continue to be expressed in the retinal progenitor cell niche called the ciliary marginal zone (CMZ) throughout adulthood (Cizelsky et al., 2013; Pillai-Kastoori et al., 2014; Wen et al., 2015).
### Table 1.1. SoxC expression in the developing vertebrate eye.

<table>
<thead>
<tr>
<th>Eye (in general)</th>
<th>Lens</th>
<th>GCL</th>
<th>INL</th>
<th>ONL</th>
<th>CMZ</th>
<th>POM</th>
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</thead>
<tbody>
<tr>
<td><strong>Mouse</strong></td>
<td>Sox4</td>
<td>Sox11</td>
<td>Sox4</td>
<td>Sox4</td>
<td>Sox11</td>
<td>ND</td>
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<tr>
<td></td>
<td>Sox11</td>
<td>Sox12</td>
<td>Sox11</td>
<td></td>
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<tr>
<td><strong>Xenopus</strong></td>
<td>sox4</td>
<td>sox11</td>
<td>sox4</td>
<td>SOx4</td>
<td>ND</td>
<td>sox4</td>
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<td>sox11</td>
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<td>sox11</td>
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<tr>
<td><strong>Zebrafish</strong></td>
<td>sox4</td>
<td>sox11b</td>
<td>sox4b</td>
<td>sox4a</td>
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<td></td>
<td>sox11</td>
<td>sox11a</td>
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<td>sox11b</td>
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<tr>
<td><strong>Chick</strong></td>
<td>Sox4</td>
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</tr>
<tr>
<td><strong>Lamprey</strong></td>
<td>Sox4</td>
<td>Sox11</td>
<td>Sox11</td>
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</tbody>
</table>

GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; CMZ, ciliary marginal zone; POM, periocular mesenchyme; ND, not described.
1.8.4 Functional studies of SoxC proteins in animal models during eye development

In mice, global knockout of *Sox11* and/or *Sox4* is lethal due to common trunk defect in the ventricular chamber of the heart. However, *Sox12* knockouts are viable and fertile without obvious developmental abnormalities (Dy et al., 2008; Hoser et al., 2008; Schilham et al., 1996). Compound *Sox11*\(^{+/−}\); *Sox4*\(^{+/−}\) heterozygote embryos also die at birth, suggesting that optimal dosage of *Sox4* and *Sox11* is required for the development of cardiac tissues in mice. *Sox11*\(^{−/−}\) homozygous mutant mice embryos exhibit a range of ocular abnormalities, including Peter’s anomaly, microphthalmia, coloboma, and open eyelids (Sock et al., 2004; Wurm et al., 2008). No eye defects were reported in *Sox4*\(^{−/−}\) mutant mice, however this may be due to the early embryonic lethality of this model.

A persistent lens stalk and delayed lens maturation are also observed in SoxC knockout mice as well as in zebrafish and *Xenopus soxC* morphants (Cizelsky et al., 2013; Pillai-Kastoori et al., 2014; Wurm et al., 2008). Immunohistochemical analysis of *Sox11*\(^{−/−}\) embryos during lens invagination stage revealed reduced cell proliferation in the lens placode, suggesting the requirement of *Sox11* for the lens vesicle separation from the surface ectoderm (Wurm et al., 2008). A recent study using lens-specific *Pax6* conditional knockout mice suggests that as lens development progresses, *Pax6* is required to suppress the expression of *Sox11* in the lens via *miR-204* (Shaham et al., 2013).

In addition to ocular morphogenesis and lens defects, SoxC deficiency also leads to defects in RGC differentiation in the vertebrate retina. *Sox4, Sox11*, and *Sox4/Sox11* conditional knockout mice were generated using a *Six3-Cre* line, knocking out *Sox4* and/or *Sox11* by E9 in the eye field and ventral forebrain (Jiang et al., 2013b). Only a modest reduction of RGCs was detected in *Sox4* or *Sox11* single knockouts, but a complete loss of RGCs and significant reductions in other
retinal cell types were observed in the Sox4/Sox11-double knockout retina (Jiang et al., 2013b). In agreement with the observations in mice, the expression of the RGC marker pouf4f1 was absent in the retinas of both Sox4- and Sox11-deficient Xenopus embryos, indicating a defect in RGC differentiation (Cizelsky et al., 2013). The mechanism of how SoxC proteins regulate retinal ganglion cell neurogenesis is still to be investigated, but Usui et al reported a change of histone modification status of several proneural genes including Ath5, NeuroD, and Fbox in Sox4 and Sox11 conditional knock-out retinas, suggesting that SoxC proteins may epigenetically influence retinal progenitor cell competence and differentiation (Usui et al., 2013b).

Sox4 and Sox11 are also potential regulators of vertebrate photoreceptor differentiation, as their expression levels were both upregulated in a chronic rod photoreceptor degeneration and regeneration zebrafish model, suggesting a role for SoxC factors in rod photoreceptor differentiation (Morris et al., 2011a). In contrast, in vitro SoxC gain-of-function analysis on mouse retinal explants showed an increased numbers of cone photoreceptors at the expense of numbers of rod photoreceptors and Müller glia, suggesting that SoxC factors inhibit rod photoreceptor differentiation (Usui et al., 2013b). These seemingly conflicting observations suggest that maybe SoxC factors have distinct roles for photoreceptor differentiation among different species, or that photoreceptor differentiation requires an optimal SoxC expression level, such that too much or too little significantly compromises terminal differentiation.

1.9 SoxC and human ocular defects

One of the most common syndromes among coloboma patients is CHARGE syndrome. Mutations in Chromatin remodeler chromodomain-helicase-DNA-binding protein 7 (CHD7) are identified in 65% of patients with CHARGE syndrome (Chang et al., 2006; Gage et al., 2015; Vissers et al., 2004). CHD7 regulates neurogenesis by epigenetically changing the chromatin structure of target genes and modifying their transcription activity (Kim and Roberts, 2013). Loss
of CHD7 in mouse neural stem cells results in a significant reduction in neurogenesis as well as a loss of SoxC gene expression (Feng et al., 2013). Computational analysis of gene expression profiling data from the Cancer Genome Atlas Project (TCGA) revealed that SOX4 and SOX11 display the strongest correlation with CHD7 expression, and overexpression of SOX4/SOX11 can rescue neural differentiation defects in cultured CHD7 mutant neural stem cells (Feng et al., 2013), suggesting that SOX4 and SOX11 are direct targets of CHD7. Given the coloboma phenotype present in Sox11 mutant mice and Sox4/11-deficient zebrafish, it is tempting to speculate that altered expression of SOX4 and SOX11 also underlies the coloboma observed in CHARGE syndrome patients. In fact, a de novo 6.5 Mb genome duplication at 2p25 was detected recently in a patient with CHARGE syndrome and SOX11 along with 23 other genes were duplicated in this chromosome region (Sperry et al., 2016).

Additionally, two patients with Coffin-Siris syndrome that displayed visual defects were reported to have heterozygous mutations in the HMG domain of SOX11 (Tsurusaki et al., 2014). Genetic sequencing also revealed two novel heterozygous SOX11 variants from 79 patients with MAC (microphthalmia, anophthalmia, and coloboma) (Pillai-Kastoori et al., 2014).

1.10 Conclusions and perspective
Along with the development of forward and reverse genetic analysis methods and the generation of animal models, more and more regulatory mechanisms for ocular development have been unveiled. Several cell signaling pathways are required for the development of eye in a tissue-specific and timely manner by regulating progenitor cell proliferation and specification. Intrinsic expressions of transcription factors are not only consequences of different cell signaling activities, but also prime the competence of ocular progenitor cells to respond to extrinsic cell signaling. Although SoxC transcription factors are well known for their pro-differentiation functions in many developmental processes including neuron differentiation and survival in the brain,
surprisingly little is known about their transcriptional targets. Even less is understood for the function of SoxC factors in ocular development. Future studies will likely focus on the investigation of SoxC target genes, and exploration of the mechanisms of SoxC genes in regulating ocular development.

1.11 Rationale, Hypothesis and Specific Aims

Previous studies have identified SoxC as indispensable transcription factors for cell survival and differentiation in the developing as well as adult nervous system (Bhattaram et al., 2010; Chen et al., 2015a; Cheung et al., 2000; Mu et al., 2012; Potzner et al., 2010). Their peak expression in postmitotic neuronal progenitor cells suggests that SoxC act as a critical link between the self-renewal and terminal differentiation of progenitor cells. Although they are highly expressed in the developing vertebrate eye in a spatiotemporal-specific pattern and SoxC loss-of-function affects retinal neurogenesis, their target genes and mechanisms in regulating the differentiation of ocular tissues are still in a shadow (Cizelsky et al., 2013; Jiang et al., 2013b; Usui et al., 2013a).

The aim of this dissertation was to determine the role of Sox4 during vertebrate ocular morphogenesis and retinal neurogenesis. Based on previous knowledge on Sox4 expression pattern in the developing eye and its upregulation in a chronic rod photoreceptor degeneration and regeneration zebrafish model (Jiang et al., 2013b; Morris et al., 2011a), the initial hypothesis was that Sox4 is required for proper eye morphogenesis and rod photoreceptor differentiation. Presented in this dissertation is evidence of the requirement of Sox4 for proper ocular morphogenesis, specifically during the process of proximo-distal patterning of the optic vesicle and choroid fissure closure. And for the first time we reported a negative regulatory relationship between the Sox4 and the Hh signaling ligand \textit{indian Hedgehog b (ihhb)} during ocular morphogenesis in zebrafish. Additionally, efforts were put into the generation and characterization of Sox4 mutant zebrafish lines using CRISPR/Cas9-mediated genome editing.
system. A delay in rod photoreceptor differentiation was observed in the mutant, providing evidence for the requirement of Sox4 during rod photoreceptor neurogenesis.

This work will be laid out in the following aims:

**Specific Aims**

I. Characterize the expression pattern of *sox4a* and *sox4b* in the developing eye.
   • Using whole mount and fluorescent in situ hybridization, describe the expression of *sox4a* and *sox4b* during different stages of ocular development.

II. Characterize the phenotypes of *sox4a* and *sox4b* misexpression in the developing eye at the morphological and cellular level.
   • Use morpholino-mediated knockdown and mRNA overexpression to generate *sox4a/b* loss-of-function and gain-of-function zebrafish embryos.
   • Analyze morphology changes of the developing eye in *sox4a/b* loss-of-function and gain-of-function embryos.
   • Analyze cellular changes of the developing eye in *sox4a/b* loss-of-function and gain-of-function embryos, including differentiation of retinal cell types and levels of apoptosis and proliferation.

III. Based on *sox4* misexpression phenotypes, test the hypothesis that Hh signaling contributes to ocular morphogenesis defects displayed in *sox4* loss-of-function and gain-of-function eyes.
   • Evaluate the expression levels of Hh target genes *pax2a* and *pax6a* in control and *sox4* morphants by fluorescent in situ hybridization and quantitative real time PCR (qPCR) at different stages of eye development.
   • Test the contribution of Hh signaling to the abnormal ocular phenotypes in *sox4* morphants by using pharmacological inhibition of Hh signaling on *sox4* morphant embryos.
   • Evaluate the effect of *sox4* knockdown on the expression of Hh receptor gene *patched2* using a transgenic zebrafish Tg(GBS-pch2:nlsEGFP) at early stages of eye development.
• Analyze the expression levels of Hh pathway ligands, receptors, and downstream effectors in control and sox4 morphants by qPCR. Confirm changes of expression at protein level by western blot.
• Evaluate the contribution of ihhb ligand to the abnormal ocular phenotypes displayed by sox4 morphants by co-knockdown of both ihhb and sox4 in developing zebrafish embryos.
• Test the expression level of ihhb in sox4 overexpressing embryos.
• Identify potential intermediate molecules between sox4 and ihhb by examining expression levels of known Hh regulators in sox4 morphants.

IV. Determine whether Sox4 and Sox11 function redundantly to regulate ocular morphogenesis.
• Analyze the phenotypes of sox4 and sox11 double knockdown embryos and compare it with sox4 or sox11 single knockdown embryos.
• Test whether sox11 mRNA injection can rescue sox4 morphant phenotypes.

V. Generation of sox4 mutants using CRISPR/Cas9 genome editing system.
• Generate stable sox4a and sox4b mutant lines by CRISPR/Cas9 for future studies of the requirement of Sox4 in embryonic and adult retinal neurogenesis and regeneration.
• Characterize mutants’ phenotypes at morphological, cellular, and molecular levels at embryonic and juvenile stages.

VI. Examine the requirement of Sox4 in rod photoreceptor neurogenesis.
• Confirm the upregulation of sox4a/b in adult Tg(XRho:gap43-mCFP) q13 transgenic zebrafish retina by FISH and RT-PCR.
• Analyze rod photoreceptor differentiation in sox4 morphant embryos
• Analyze rod photoreceptor differentiation in Sox4 mutant embryos and juveniles.

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

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2.1 Abstract

SoxC transcription factors play critical roles in many developmental processes, including neurogenesis, cardiac formation, and skeletal differentiation. In vitro and in vivo loss-of-function studies have suggested that SoxC genes are required for oculogenesis, however the mechanism was poorly understood. Here, we have explored the function of the SoxC factor Sox4 during zebrafish eye development. We show that sox4a and sox4b are expressed in the forebrain and periocular mesenchyme adjacent to the optic stalk during early eye development. Knockdown of sox4 in zebrafish resulted in coloboma, a structural malformation of the eye that is a significant cause of pediatric visual impairment in humans, in which the choroid fissure fails to close. Sox4 morphants displayed altered proximo-distal patterning of the optic vesicle, including expanded pax2 expression in the optic stalk, as well as ectopic cell proliferation in the retina. We show that the abnormal ocular morphogenesis observed in Sox4-deficient zebrafish is caused by elevated Hedgehog (Hh) signaling, and this is due to increased expression of the Hh pathway ligand Indian
hedgehog b (ihhb). Consistent with these results, coloboma in sox4 morphants could be rescued by pharmacological treatment with the Hh inhibitor cyclopamine, or by co-knockdown of ihhb. Conversely, overexpression of sox4 reduced Hh signaling and ihhb expression, resulting in cyclopia. Finally, we demonstrate that sox4 and sox11 have overlapping, but not completely redundant, functions in regulating ocular morphogenesis. Taken together, our data demonstrate that Sox4 is required to limit the extent of Hh signaling during eye development, and suggest that mutations in SoxC factors could contribute to the development of coloboma.

**Highlights**

- Sox4 is required for choroid fissure closure, and Sox4 deficiency causes ocular coloboma.
- Knockdown of Sox4 alters proximo-distal patterning of the optic stalk and vesicle.
- Knockdown of Sox4 results in elevated Hh signaling.
- Sox4 negatively regulates expression of the Hh ligand gene Indian Hedgehog (ihhb).

**2.2 Introduction**

Ocular coloboma is a developmental disorder that occurs when the choroid fissure, a transient opening that forms in the ventral portion of the optic cup, fails to properly close, causing a cleft in the inferonasal quadrant of the eye. Depending on the timing and location of the closure defect, coloboma can affect multiple regions of the developing eye, including the cornea, iris, ciliary body, retina, pigmented epithelium, and the optic nerve (Chang et al., 2006). Coloboma is often observed in conjunction with other ocular abnormalities, such as microphthalmia, anophthalmia, or anterior chamber defects, and may also be associated with more complex syndromes affecting multiple systems (Chang et al., 2006; Eccles and Schimmenti, 1999; Guirgis and Lueder, 2003; Morrison et al., 2000; Porges et al., 1992). Although coloboma represents an important cause of pediatric visual impairment, contributing to 3-11% of childhood blindness worldwide (Hornby et
al., 2000), the molecular and cellular mechanisms underlying choroid fissure closure and the genetic etiology of coloboma remain poorly understood. This is partly because ocular coloboma is both phenotypically and genetically heterogeneous, and although numerous coloboma-causing genes have been identified, mutations in these genes account for only a minority of cases. Based on data from patient studies and from animal models, a coloboma gene network has been proposed, which highlights the complex relationships between several genes implicated in coloboma or associated ocular malformations (Gregory-Evans et al., 2004). The members of this network include eye field specific transcription factors (EFTFs), cell cycle regulators, and cell signaling molecules that collectively control cell proliferation, cell migration, cell fate specification, and cell death.

One of the central hubs of the coloboma gene network is the secreted molecule Sonic Hedgehog (Shh), one of the ligands for the evolutionarily conserved Hedgehog (Hh) signaling pathway. Hh signaling is critical for the correct morphogenesis, growth, and patterning of several embryonic tissues and organs, and also plays a key role in tissue regeneration, stem cell maintenance, and tumorigenesis (Briscoe and Therond, 2013; Ingham and McMahon, 2001). In the development of the visual system, Hedgehog (Hh) signaling is required at several stages. Early in development, Hh signals emanating from the ventral midline regulate the segregation of a single eye field into two bilateral optic primordia. As the optic vesicles evaginate from the forebrain, Hh signaling controls proximo-distal patterning of the optic vesicle into optic stalk and optic cup domains, respectively (Ekker et al., 1995). And after optic cup formation, Hh signaling within the developing retina regulates neurogenesis by controlling retinal progenitor cell proliferation and differentiation (Marti and Bovolenta, 2002). Reflecting its central role in oculogenesis, mutations in Shh are associated with several ocular defects, including cyclopia, microphthalmia, anophthalmia, and coloboma (Amato et al., 2004; Gongal et al., 2011; Schimmenti et al., 2003). Moreover, the downstream targets of Shh include several coloboma-causing genes (Sanyanusin et
al., 1995; Slavotinek et al., 2012). However, less is known about the factors that lie upstream of Hh ligand expression – such genes may also contribute to the pathogenesis of coloboma.

We recently demonstrated that the SoxC transcription factor Sox11 is one of the upstream regulators of Hh signaling, and that in the zebrafish, Sox11 regulates choroid fissure closure as well as retinal neurogenesis by inhibiting the expression of Shh (Pillai-Kastoori et al., 2014). The Sox proteins are named for a shared motif called the SRY box, a high-mobility-group (HMG) DNA binding domain homologous to the DNA-binding domain of the mammalian sex-determining gene SRY. Based on amino acid identity within the HMG domain, Sox proteins are divided into 8 groups (A to H). The SoxC group includes Sox4, Sox11 and Sox12 (Bowles et al., 2000). SoxC family members display overlapping expression domains in several embryonic tissues, including neuronal and mesenchymal progenitor cells (Dy et al., 2008). The SoxC factors function redundantly in the development of some tissues, such as the nervous system, but also have distinct roles in the development of other tissues, such as the heart (Bergsland et al., 2006; Bhattaram et al., 2010; Paul et al., 2013; Penzo-Mendez, 2010). Moreover, Sox11 null mice survive several days longer than Sox4 null mice, suggesting non-redundant roles for these two proteins in early embryogenesis.

In this study, we examined the function of Sox4 in zebrafish ocular development. Like Sox11, Sox4 is expressed in multiple embryonic tissues, including the retina (Dy et al., 2008; Pillai-Kastoori et al., 2014; Usui et al., 2013a). In the mouse, Sox4 expression initiates in the central retina near the optic nerve at E11.5. As retinal development proceeds, Sox4 is expressed in the ganglion cell layer (GCL) and the neuroblastic layer (NBL). Constitutive Sox4 null mice die at E14.5 due to severe cardiac malformation and defects in B lymphocyte differentiation (Schilham et al., 1996), precluding a more thorough assessment of Sox4’s role in ocular development in this model. A conditional Sox4 knockout mouse in which Sox4 is deleted in the developing retina
displayed a modest reduction in ganglion cell number, but no other ocular defects were reported, and the effect of Sox4 deletion on Hh signaling was not investigated (Jiang et al., 2013b).

Zebrafish possess two co-orthologs of the mammalian Sox4 gene: sox4a and sox4b. Previously, microarray analysis of retinal mRNA from a zebrafish model of chronic rod photoreceptor degeneration and regeneration revealed that sox4a and sox4b mRNA levels are up-regulated in response to rod photoreceptor loss (Morris et al., 2011a), suggesting they function in rod regeneration. However, the role of sox4a/b in embryonic zebrafish ocular development was not known. In this study, we show that Sox4 is required for choroid fissure closure and proper proximo-distal patterning of the optic vesicle in zebrafish. We also demonstrate that loss of Sox4 affects retinal progenitor cell proliferation. Furthermore, we show that similar to loss of Sox11, the ocular phenotypes of Sox4-deficient zebrafish are caused by elevated Hh signaling. However, in contrast to Sox11, we found that Sox4 primarily regulates expression of the Hh signaling ligand Indian Hedgehog (Ihh), rather than Shh. Therefore, our data demonstrate that Sox4 and Sox11 together control levels of Hh signaling during ocular development by regulating expression of distinct Hh ligands, and suggest that SoxC factors may be additional members of the coloboma gene network.

2.3 Results

2.3.1 Sox4a and sox4b are expressed in periocular tissues and the developing retina

We performed fluorescent in situ hybridization (FISH) with antisense probes for sox4a and sox4b to determine their expression patterns in the forebrain and the eye during zebrafish embryonic development. Previous studies reported expression of sox4 as early as the 5 somite stage (approximately 12 hours post fertilization, hpf) in the lateral plate mesoderm and mid-trunk endoderm of zebrafish embryos, respectively (Mavropoulos et al., 2005). Using FISH, we detected sox4a expression in the dorsal forebrain and around the optic stalk at 12 hpf, (Figure
2.1A-A’’). Sox4b was not detectable at this time point. At 18 hpf, during optic vesicle invagination to form the bi-layered optic cup, expression of sox4a was detected in cells on either side of and directly adjacent to the optic stalk (Figure 2.1B). More posteriorly, sox4a expression was observed in periocular cells in the dorsal diencephalon (Figure 2.1B’-B’’). Expression of sox4b was also occasionally observed in this region, although not in all embryos analyzed (Figure 2.1C’-C’’). Some of the sox4a-positive periocular cells were of neural crest origin, as they co-localized with GFP+ cells in the sox10:EGFP transgenic line (Hoffman et al., 2007) (Figure 2.1F, arrows). At 24 hpf, expression of both sox4 paralogs was observed in the ventral diencephalon adjacent to the retina (Figure 2.1D-E’’), and sox4a expression persisted in the dorsal sox10:EGFP+ periocular mesenchyme (Figure 2.1H). Expression of sox4a and sox4b was first observed in the ventronasal retina at 36 hpf (Figure 2.1J-K), coinciding with the onset of retinal neurogenesis. These sox4+ cells did not co-localize with PCNA, a marker for proliferating cells, suggesting that they were postmitotic (Figure 2.1P-U). At 48 hpf, sox4a expression was detected in the inner half of the inner nuclear layer (INL), whereas sox4b was expressed in both the ganglion cell layer (GCL) and the inner portion of the INL (Figure 2.1L-M). Some of the sox4a/b-positive cells co-localized with the ganglion and amacrine cell marker HuC/D at 48 hpf (Figure 2.1W-X’’). At 72 hpf, scattered expression of sox4a and sox4b was observed in the outer half of the INL, and expression of sox4a/b was also detected in the ciliary marginal zone (CMZ), the persistently neurogenic region at the periphery of retina (Figure 2.1N-O). The sox4a/b-positive cells in the CMZ were located in the central CMZ and mostly did not co-localize with PCNA (Figure 2.1T-U), suggesting that sox4a/b marks postmitotic neuronal precursors in this region. In the distal INL, rare sox4b-positive cells co-localized with the horizontal cell marker Prox1, however the sox4a-positive cells did not co-localize with horizontal, bipolar, or Müller cell markers (Figure S2.1). Instead, we found that some sox4a-positive cells in the outer INL also expressed crx (Figure 2.1Y-Y’’), suggesting that sox4a may be expressed in some photoreceptor progenitors (Nelson et al., 2008). Taken together, the early expression of sox4 adjacent to the
optic stalk and evaginating optic vesicle, and later expression within the developing retina, suggests that it functions during zebrafish ocular development.
Figure 2.1. Expression of *sox4a* and *sox4b* during ocular development. (A-A'') Fluorescent in situ hybridization (FISH) for *sox4a* performed on transverse cryosections at 12 hpf. (B-D'') FISH for *sox4a* (B-B'' and D-D'') or *sox4b* (C-C'' and E-E'') performed on transverse cryosections taken at the level of the anterior, middle, and posterior optic vesicle at 18 or 24 hpf. (F-I) FISH with *sox4a* and *sox4b* probes performed on sections from *sox10:EGFP* transgenic embryos at 18 and 24 hpf. (J-O) FISH for *sox4a* or *sox4b* performed on transverse retinal cryosections at 36, 48, and 72 hpf. (P-U) FISH combined with immunohistochemistry for the proliferation marker PCNA at 24, 48, or 72 hpf. (V-V'') Control FISH with a sense probe for *sox4a* and *sox4b* at 12 and 72 hpf. (W-X'') FISH combined with immunohistochemistry for HuC/D, which labels ganglion and amacrine cells, at 48 hpf. (Y-Z'') Two-color FISH with *sox4a/b* and *crx* probes performed at 72 hpf. Arrows indicate co-localization, arrowheads indicate *sox4a/b*+ cells that did not co-localize with the indicated marker. All scale bars equal 50 µm. D, dorsal; V, ventral; B, brain; OV, optic vesicle; L, lens; R, retina; P, peripheral CMZ; C, central CMZ; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; ON, optic nerve.
Figure S2.1. Co-localization of *sox4a/b* with cell specific markers at 72 hpf. (A-B’’) FISH for *sox4a* and *sox4b* followed by immunostaining for the ganglion and amacrine cell marker HuC/D. Whereas *sox4a* did not co-localize with HuC/D (A-A’’), some *sox4b*-positive cells co-localized with HuC/D (B-B’’, arrows). (C-D’’) FISH for *sox4a* and *sox4b* followed by immunostaining for the horizontal cell marker Prox1. *Sox4a* did not co-localize with Prox1 (C-C’’), however some *sox4b*-positive cells in the outer half of the INL co-localized with Prox1 (D-D’’, arrows). (E-F’’) FISH for *sox4a* and *sox4b* followed by immunostaining for the bipolar cell marker PKC-α. No co-localization with PKC-α was detected. (G-H’’) FISH for *sox4a* and *sox4b* was performed in embryos transgenic for *gfp:GFP* which labels Müller glial cells. No co-localization with Müller cells was observed. All scale bars equal 25 µm. GCL, ganglion cell layer; INL, inner nuclear layer.
2.3.2 Knockdown of sox4 causes ocular coloboma

Translation blocking morpholinos (MOs) targeting the 5’-UTR upstream of the translation start site were used to knock down sox4a/b expression in zebrafish embryos. The efficiency of each sox4 MO was assessed by co-injecting an EF1α-EGFP plasmid containing the MO binding site. At 24 hpf, 91.94± 1.93% of the control MO injected embryos displayed GFP expression; in contrast, none of the sox4 MO injected embryos showed any GFP expression, indicating that the sox4 MOs are highly efficient in blocking target mRNA translation (Figure S2.2A-E).

Morpholino-injected embryos were scored for ocular phenotypes at 24, 48, and 72 hpf by light microscopy. Since some toxicity was observed in sox4 morphants, a p53 MO was co-injected to inhibit non-specific cell death. Knockdown of sox4a resulted in ocular coloboma in 12.23± 2.89% of embryos (n= 17/139), whereas knockdown of sox4b caused coloboma in 9.24±2.79% of embryos (n= 11/119). Knockdown of both sox4a/b simultaneously caused coloboma in 47.84 ± 7.32% of embryos (n= 133/278), suggesting a synergistic effect on the phenotype (Figure S2.2F).

In all subsequent experiments, sox4a and sox4b MOs were co-injected, and sox4a/b MO-injected embryos are referred to as sox4 morphants. Occasionally, pericardial edema and a curved tail were also observed in sox4 morphant embryos (data not shown). However, as these phenotypes can be produced by non-specific activity of MOs, we did not analyze them further. We observed a higher incidence of coloboma (76.92%, n= 130/169) in sox4 morphants without the p53 MO, suggesting that cell death may also contribute to the coloboma phenotype (data not shown).

We performed three separate experiments to confirm the specificity of the sox4 morphant coloboma phenotype. First, a second set of non-overlapping sox4 MOs was used, which produced a similar proportion of choroid fissure closure defects to the first set (Figure S2.2G-J). Second, sox4a and sox4b mRNAs (lacking the MO binding site) were co-injected with the sox4 MOs, and the embryos were scored for coloboma at 48 hpf. Co-injection of sox4a/b mRNA significantly reduced the incidence of coloboma in sox4 morphants to 16.49% ± 3.37% (Fisher’s exact test, P<0.0001; Figure 2.2A’’-D’’, 2.2G), suggesting that this phenotype resulted from specific
knockdown of *sox4*. Finally, we utilized the CRISPR/Cas9 system (Hruscha et al., 2013; Hwang et al., 2013; Jao et al., 2013; Talbot and Amacher, 2014) to target mutations to the *sox4a* and *sox4b* genomic loci. For each gene, single guide RNAs (sgRNAs) were designed to target two different sites within the coding region, neither of which overlapped with the MO target sequence. In founders injected with Cas9 mRNA and *sox4a* or *sox4b* sgRNAs, we observed coloboma in 31.19± 6.40% (n= 13/44) and 20.91± 8.14% (n= 9/48) of injected embryos, respectively (Figure S2.2M-O). In contrast, none (n= 0/201) of the uninjected controls (UIC) displayed coloboma, and only 1.33± 2.31% (n= 1/89) of embryos injected with a *tyrosinase* (*tyr*) sgRNA/Cas9 exhibited coloboma, although *tyr* sgRNA/Cas9 was very effective at inducing pigmentation defects, as previously described (Jao et al., 2013) (Figure S2.2K-L, S2.2O). High resolution melt curve analysis (HRMA) and sequencing of the *sox4a* and *sox4b* target regions in individual injected embryos confirmed that short indels were generated in the *sox4* gRNA/Cas9 injected individuals with high efficiency (Figure S2.2P-Q and data not shown), with the mutation frequency ranging from 52.78% to 94.44%. These data suggest that the CRISPRs induced bi-allelic gene inactivation in mosaic injected embryos, as has been reported previously in zebrafish and mice (Jao et al., 2013; Yen et al., 2014; Zhang et al., 2014), resulting in ocular phenotypes similar to those seen in the *sox4* morphants. Taken together, these results strongly support the specificity of the *sox4* morphant coloboma phenotype.

The first sign of an ocular defect in *sox4* morphants was detected at the 8-10 somite stage (12-14 hpf), when the horizontal crease (formed during the evagination of the optic vesicle), becomes visible. Compared to the control morphants, the *sox4* morphants that developed ocular coloboma at 48 hpf always displayed a “crooked” horizontal crease at 12-14 hpf (Figure 2.2A’, arrow), suggesting that the coloboma resulted from an earlier ocular morphogenesis defect. At 24 hpf, the two lips on either side of the choroid fissure in the ventral retina were either wide open or bent backwards in *sox4* morphants (Figure 2.2B’). The persistently open choroid fissure was more
obvious at 48 and 72 hpf, because this region of the ventral retina appeared to lack pigmentation of the underlying retinal pigmented epithelium (RPE) (Figure 2.2C’). Ventral views of sox4 morphant embryos revealed a hole in the posterior RPE through which the retinal tissue extruded into the brain, which accounts for the apparent lack of pigmentation in this region when viewed laterally (Figure 2.2D’). Histological sections of 72 hpf control and sox4 morphant embryos clearly demonstrated the extrusion of colobomatous tissue through the open choroid fissure (Figure 2.2F, asterisk). Sox4-deficient retinas also displayed less mature lamination compared to control retinas, however all three retinal cell layers were present.

We immunolabeled control and sox4 morphant embryos with an antibody that recognizes laminin, which is present in the basement membrane surrounding the optic cup. As the nasal and temporal lips of the retina on either side of the choroid fissure fuse together, laminin is degraded and disappears. In control morphants at 48 hpf, laminin immunostaining revealed that the nasal and temporal lobes of the choroid fissure were closely apposed, but not yet fused (Figure 2.3A-A’). In contrast, the choroid fissure was much wider in sox4 morphants at this time (Figure 2.3B-B’). By 72 hpf in control morphants, only faint expression of a single layer of laminin was visible at the now closed choroid fissure; however, in sox4 morphants the choroid fissure remained open with two distinct layers of laminin outlining the unfused lips of the nasal and temporal retina (Figure 2.3C-D’).
Figure 2.2. Knockdown of sox4 causes coloboma. Representative images of control MO-injected embryos (A-D), sox4 MO-injected embryos (A’-D’), and embryos injected with both sox4 MO and sox4 mRNA (A’’-D’’). A minimum of 5 embryos were imaged for each group. Images in A-C’’ are lateral views; images in D-D’’ were taken from the ventral side of the embryo. Insets show the whole body of the same embryo in the larger panel. Arrow in A’ indicates the horizontal crease; arrows and brackets in B-C’’ indicate the choroid fissure. (E-F) Transverse DAPI-stained sections of control and sox4 morphants at 72 hpf. The coloboma in the sox4 morphant retina is prominent (F, asterisk). (G) Quantification of the coloboma phenotype at 48 hpf. In control morphants, 0.61± 0.49% of embryos displayed coloboma (n= 2/329). In sox4 morphants, 45.01± 4.43% of embryos displayed coloboma (n= 257/571). Co-injection of sox4 mRNA significantly reduced the incidence of coloboma to 16.49± 3.37% (n= 31/188; Fisher’s exact test, P< 0.0001). Scale bars for the insets in B-C’’ equal 500 µm.
Figure S2.2. Efficiency and specificity of sox4 MOs and sox4 CRISPR phenotype. (A-D’)
EF1α-EGFP plasmids containing the sox4a or sox4b MO binding site were co-injected with either control MO or sox4 MO. At 24 hpf, control MO injected embryos showed ubiquitous GFP expression (A-B’), whereas sox4 MO injected embryos showed no GFP expression (C-D’). (E) Quantification of the percentage of injected embryos with GFP expression at 24 hpf. In control MO injected embryos, 91.94± 1.93% had GFP expression (n= 107/116). In sox4 MO injected
embryos, none of them showed any GFP expression (n=0/227). (F) Sox4a and sox4b are both required for choroid fissure closure. Two different doses of sox4a and sox4b MO were injected into zebrafish embryos either separately or combined. A synergistic effect on the incidence of coloboma was observed when sox4a and sox4b MO were co-injected at both doses (Fisher’s exact test, P< 0.0001). (G-J) A second set of non-overlapping sox4a/b MOs also resulted in coloboma at similar proportions to sox4 MO1 (40.85± 8.39%, n= 183/448). Bracket and arrows in (H) and (I) indicate the open choroid fissure and extrusion of the retina at the coloboma region. (K-L) Representative images of eyes from uninjected control (UIC) and tyrosinase (tyr) sgRNA/Cas9 injected embryos at 48 hpf. The tyr sgRNA/Cas9 injected embryos displayed a reduction in retinal pigmentation but did not have coloboma. (M-N) Representative images of eyes from sox4a and sox4b sgRNA/Cas9 injected embryos at 48 hpf that displayed coloboma (asterisk). (O) Quantification of the percentage of embryos with coloboma at 48 hpf. (P-Q) HRMA analysis detected the presence of mutant alleles in individual sox4a/b sgRNA/Cas9 injected embryos (arrows), which were confirmed by sequencing (not shown).
2.3.3 Proximo-distal patterning of the optic vesicle is altered in Sox4-deficient zebrafish

Coloboma can result from an abnormal or enlarged optic stalk, which may arise when there is a shift in the cell fate boundary between the optic stalk and the optic vesicle (Chang et al., 2006). We performed FISH on control and sox4 morphant sections to examine the expression of pax2a and pax6a, which mark the optic stalk and optic vesicle domains, respectively. In control morphants at 18 hpf, pax2a expression was restricted to a wedge of cells located between the optic vesicle and the brain, and pax6a expression was evident throughout the optic vesicle (Figure 2.3E). In sox4 morphants however, the region of pax2a expression was expanded into the optic vesicle, and there was a corresponding retraction of pax6a expression (Figure 2.3F). The expansion of pax2a expression in sox4 morphants persisted at 48 and 72 hpf, where it was particularly prominent in the ventral retina around the open choroid fissure (Figure 2.3G-H and data not shown). The changes in pax2a and pax6a expression in sox4 morphants were confirmed by quantitative RT-PCR (qPCR), which showed that in sox4 morphant heads at 18 hpf pax2a was significantly up-regulated (1.69±0.25 fold) while pax6a was significantly down-regulated (2.0±0.08 fold; Figure 2.3I). This result indicates that the proximo-distal patterning of the optic vesicle was disrupted in Sox4-deficient embryos.
Figure 2.3. Persistence of laminin expression at the choroid fissure and altered proximo-distal patterning of the optic vesicle in sox4 morphants. (A-D’) Laminin immunostaining on control and sox4 morphant embryos at 48 and 72 hpf. Representative images from 15-20 individuals analyzed for each group are shown. (E-H) Double FISH for pax2a and pax6a at 18 and 48 hpf. (I) qPCR analysis revealed a 1.69-fold increase in pax2a expression and a 2.0-fold decrease in pax6a expression in sox4 morphant heads at 18 hpf (Student’s t-test, P<0.05). All scale bars equal 50 µm. Asterisks in A’ and C’ indicate the closing or fused choroid fissure in control morphants; brackets in B’ and D’ indicate the open choroid fissure in sox4 morphants.
2.3.4 Sox4-deficient retinas display ectopic cell proliferation

Altered retinal progenitor cell proliferation has been observed in coloboma models (Kim et al., 2007; Liu et al., 2012). We used an antibody to phosphohistone H3 (PH3) to label mitotic cells in control and sox4 morphant eyes. At 18 and 24 hpf, no apparent difference was observed between control and sox4 morphants (Figure 2.4A-E). At 36 and 48 hpf, the total number of PH3+ cells in the retina was significantly reduced in sox4 morphants compared to controls. Interestingly, some PH3+ cells were ectopically located in the inner retina of sox4 morphants, whereas in controls PH3+ cells were all aligned at the apical border of the retina (Figure 2.4A, 2.4F-I). At 72 hpf, when retinal differentiation is largely complete, PH3+ cells in control morphant retinas were confined to the peripheral CMZ (Figure 2.4J). Sox4 morphant retinas possessed PH3+ cells at the CMZ as well, but we also observed ectopic PH3+ cells in the GCL, which clustered with PCNA-positive cells (Figure 2.4K). These cells did not express the retinal ganglion cell marker Zn8, or the retinal progenitor cell gene pax6a (Figure S2.3). We also observed PCNA-positive cells within the colobomatous tissue protruding from the sox4 morphant retinas (Figure 2.4K). Taken together, these data suggest that overproliferation does not contribute to the coloboma phenotype of sox4 morphants, since no difference in cell proliferation was observed at 18 or 24 hpf, by which time abnormal ocular morphogenesis was evident. However, sox4 does appear to influence cell proliferation in the retina, as knockdown caused an overall reduction in the number of dividing cells as well as ectopic cell proliferation in the GCL.

We performed a TUNEL assay to determine whether apoptosis was elevated in sox4 morphant retinas. No apparent difference was observed at 18, 24, and 72 hpf in the number of TUNEL-positive cells in the optic vesicle/retina between control and sox4 morphant eyes (Figure 2.4L). However, at 18 hpf the number of TUNEL-positive cells was significantly higher in the optic stalk region of sox4 morphants compared to controls (Figure 2.4N, arrows; Fig. 2.4W, Student’s t-test, P< 0.05). Also, at 48 hpf sox4 morphant retinas possessed a highly variable, but
significantly greater number of TUNEL-positive cells than control retinas (Figure 2.4L, 2.4S-T).

Moreover, as mentioned previously we observed an increased proportion of embryos with
coloboma when the p53 MO was omitted from the injection (data not shown). Taken together,
these data suggest that apoptosis, particularly within the optic stalk region may contribute to the
severity of the coloboma phenotype.
Figure 2.4. *Sox4* knockdown causes ectopic cell proliferation in the retina. (A) Quantification of PH3-positive cells in control and *sox4* morphant retinas from 18 to 72 hpf. 8-10 individuals were analyzed in each time point (Student’s *t*-test, *P*< 0.01). (B-K) Representative transverse sections through the optic vesicle and retina of control and *sox4* morphants immunolabeled for PH3 at the indicated time points. Ectopic PH3-positive cells are indicated by arrows. At 72 hpf, the ectopic PH3-positive cells in the GCL also clustered with PCNA-positive cells (K, arrow). (L) Quantification of TUNEL-positive cells in control and *sox4* morphant optic vesicle and retina from 18 to 72 hpf. 10-20 individuals were analyzed for each time point (Student’s *t*-test, *P*=0.017). (M-V) Representative transverse sections of control and *sox4* morphant retinas labeled for TUNEL-positive cells from 18 to 72 hpf. (W) Quantification of TUNEL-positive cells in control and *sox4* morphant optic stalk at 18 hpf. Arrows in N indicate increased TUNEL-positive cells in the optic stalk of an 18 hpf *sox4* morphant. All scale bars equal 50 µm.
Figure S2.3. *sox4* knockdown causes ectopic proliferation in the ganglion cell layer. (A-B) Clusters of PNCA- and PH3-positive cells were observed in the GCL of *sox4* morphant retinas at 72 hpf (B, dashed line), whereas in control morphants proliferative cells were only observed in the CMZ. (C-D) *Pax6a* was expressed in the GCL and inner half of the INL in control and *sox4* morphants at 72 hpf, however a dense clump of extra cells in the GCL of *sox4* morphants was *pax6a*-negative (D, dashed line). (E-F) Immunostaining of control and *sox4* morphant retinas with the ganglion cell marker Zn-8. Clusters of Zn-8-negative cells were present in the GCL of *sox4* morphants (F, dashed line). D, dorsal; V, ventral; L, lens; CMZ, ciliary marginal zone; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; ON, optic nerve.
2.3.5 Hh signaling is elevated in Sox4-deficient zebrafish

As mentioned previously, we have shown that knockdown of Sox11 causes coloboma due to an elevation of Hh signaling (Pillai-Kastoori et al., 2014). Moreover, the coloboma phenotype in both sox11 and sox4 morphants resembles other animal models of overactive Hh signaling (Bassett et al., 2010; Lee et al., 2012; Lee et al., 2008; Zhang et al., 2013). Therefore, we asked whether cyclopamine, a pharmacological Hh inhibitor, could reduce the incidence of coloboma in sox4 morphants. We treated sox4 morphants with 2 µM cyclopamine, a concentration which did not produce any ocular defects on its own (Figure 2.5E), and which was shown previously to rescue the coloboma phenotype in zebrafish blowout mutants (which carry a mutation in the ptch2 receptor) and sox11 morphants (Lee et al., 2008; Pillai-Kastoori et al., 2014). Sox4 morphants were exposed to either cyclopamine or an equivalent amount of ethanol vehicle from 5.5 to 13 hpf, and then analyzed at 48 hpf for the presence of coloboma. In vehicle-treated sox4 morphants, 57.78±2.28% of embryos (n= 104/180) displayed coloboma at 48 hpf, consistent with our previous results. In contrast, cyclopamine treatment suppressed the incidence of coloboma to 15.87±2.20% (n= 26/165; Fisher’s exact test, P<0.0001; Figure 2.5A-E). Treatment of sox4 morphants with cyclopamine from 10 to 24 hpf also significantly decreased the proportion of embryos with coloboma, however the effect was not as strong as with the earlier treatment period (data not shown).

To confirm that Hh signaling was elevated in response to sox4 knockdown, we injected sox4 MOs into heterozygous ptch2:EGFP transgenic embryos, which report on Hh signaling by expressing GFP in cells that activate the Hh target gene ptch2 (Shen et al., 2013). Elevation of GFP in the ventral forebrain was observed in sox4 morphants as early as 12 hpf and through 72 hpf in sox4 morphants (Figure 2.5F-I and data not shown), confirming that Hh signaling activity was indeed upregulated in Sox4-deficient embryos. To quantify the elevation of endogenous ptch2 expression in sox4 morphants, we performed qPCR on mRNA from control and sox4 morphant embryos at 8,
10, and 12 hpf. We found that \textit{ptch2} transcript was upregulated in \textit{sox4} morphants by more than 2-fold at all time points (Figure 2.5J). Taken together, these data indicate that knockdown of Sox4 results in an elevation in Hh signaling in the early embryo that leads to ocular morphogenesis defects and coloboma.
Figure 2.5. Hh signaling is elevated in Sox4-deficent zebrafish. (A-D’) Representative eye and whole body images of vehicle-treated and cyclopamine-treated sox4 morphants at 24 and 48 hpf. (E) Quantification of the percentage of coloboma in vehicle- and cyclopamine-treated control and sox4 morphants (control morphants + vehicle: 1.14± 0.96% coloboma, n= 2/175; control morphants + cyclopamine: 0.49± 0.79% coloboma, n= 1/205; sox4 morphants + vehicle: 57.78± 2.28% coloboma, n= 104/180; sox4 morphants + cyclopamine: 15.87± 2.20% coloboma, n= 26/165; Fisher’s exact test, P<0.0001). (F-I) Representative eye and body lateral images (F-G’) and transverse sections (H, I) of ptch2:EGFP(+/−) transgenic embryos injected with control (F-F’, H) or sox4 MO (G-G’, I) at 12 hpf. (J) qPCR analysis of ptch2 expression in embryos injected with control or sox4 MO at 8, 10, and 12 hpf. Relative transcript abundance was normalized to level of atp5h (three biological replicates; Student’s t-test, P<0.01).
2.3.6 Sox4 negatively regulates expression of Indian Hedgehog b (ihhhb)

In Sox11-deficient zebrafish, elevation of Hh signaling is caused by an increase in expression of the Shh gene \[shha; (Pillai-Kastoori et al., 2014)\]. To determine whether Sox4 also regulates expression of \textit{shha} or some other component of the Hh signaling pathway, we performed qPCR on mRNA prepared from the control and \textit{sox4} morphant embryos at 8, 10, and 12 hpf, and from the heads of control and \textit{sox4} morphant embryos at 18 and 24 hpf. Intriguingly, we found that expression of \textit{shha} was not significantly different in \textit{sox4} morphants compared to controls at any time point. However, the transcript level of the Hh ligand \textit{ihhhb} was significantly upregulated in \textit{sox4} morphants as early as 8 hpf and was strongly upregulated at 18 and 24 hpf (Figure 2.6A, Figure S2.4, Student’s \textit{t}-test, \(P<0.01\)). We also detected a more variable increase in expression of \textit{ptch1}, \textit{ptch2}, and \textit{gli1} in \textit{sox4} morphants at 18 and 24 hpf (Figure S2.4). We confirmed the upregulation of \textit{ihhhb} transcript in \textit{sox4} morphants at 12 and 24 hpf by whole mount in situ hybridization (WISH), which revealed that \textit{ihhhb} expression in \textit{sox4} morphants was upregulated in the ventral midline at 12 hpf (Figure 2.6C), and at 24 hpf \textit{ihhhb} expression was upregulated in the hypothalamus and ventral diencephalon, and had also expanded into regions of the telencephalon and dorsal diencephalon that did not express \textit{ihhhb} in controls (Figure 2.6E). However, the increase in \textit{ihhhb} expression in \textit{sox4} morphants was not ubiquitous, as \textit{ihhhb} expression in the posterior notochord was similar to controls at 24 hpf (Figure 2.6G). We also confirmed that Ihhhb protein levels were elevated in 24 hpf \textit{sox4} morphant heads by Western blot (Figure 2.6H). Moreover, qPCR of mRNA prepared from cyclopamine-treated \textit{sox4} morphants revealed that expression of \textit{ihhhb} returned to control levels following cyclopamine treatment (Figure 2.6I).

Taken together, these results demonstrate that knockdown of Sox4 causes an increase in Hh signaling primarily through upregulation of \textit{ihhhb} expression.

Next, we co-injected an \textit{ihhhb} MO together with the \textit{sox4} MOs and quantified the incidence of coloboma at 48 hpf. Co-injection of the \textit{ihhhb} MO significantly reduced the incidence of coloboma.
in sox4 morphants in a dosage dependent manner: 19.33± 2.52% (n= 14/75) of sox4 morphant embryos displayed coloboma when 0.5 ng of ihhb MO was injected (compared to 51.24± 2.70% with sox4 MO alone), whereas injecting 1.0 ng of ihhb MO suppressed coloboma to 10.67± 2.08% (n= 9/91; Figure 2.6J). We also co-injected a previously described shha MO (Nasevicius and Ekker, 2000) with the sox4 MOs to determine whether it could rescue the coloboma phenotype. When 2.0 ng of shha MO was injected the proportion of embryos with coloboma was not significantly different from that observed with sox4 MO alone (Figure 2.6J). However, 3.0 ng of shha MO did significantly reduce coloboma to 34.39 ± 5.03% (n= 79/229; Fisher’s exact test, P=0.0001), although the extent of rescue was not as strong as with the ihhb MO. Together, these results confirm that Sox4 negatively regulates the expression of ihhb and that elevated ihhb expression in Sox4-deficient zebrafish causes coloboma.
Figure 2.6. Sox4 negatively regulates ihhb expression. (A) *ihhb* and *shha* qPCR of mRNA from control and *sox4* morphant whole embryos at 8, 10 and 12 hpf, and heads at 18 and 24 hpf. Relative transcript abundance was normalized to level of *atp5h* or *gapdh*. Y axis represents the
average ratio of normalized \textit{sox4} morphant to control expression (three biological replicates; Student’s \textit{t}-test, \(P<0.01\)). (B-G) \textit{Ihhb} expression in control and \textit{sox4} morphants at 12 and 24 hpf detected by WISH. Representative images are presented from 15-20 individuals analyzed. Lateral images in B-E focus on the midline, therefore the eye is not visible. Arrows in B and C mark the ventral forebrain at the level of the optic vesicle. (H) Western blot analysis of protein lysates from control or \textit{sox4} morphant heads at 24 hpf. Two biological replicates were performed (sets 1 and 2) (I) qPCR of mRNA from vehicle- and cyclopamine-treated \textit{sox4} morphants (Student’s \textit{t}-test, \(P<0.01\)). (J) Quantification of the proportion of embryos with coloboma at 48 hpf in \textit{sox4} morphants co-injected with two different doses of either \textit{ihhb} MO or \textit{shha} MO (\textit{sox4} MO alone: 51.24± 2.7%, \(n= 209/404\); \textit{sox4} MO + 0.5 ng/embryo \textit{ihhb} MO: 19.33± 2.52%, \(n= 14/75\); \textit{sox4} MO + 1.0 ng/embryo \textit{ihhb} MO: 10.67± 2.08%, \(n= 9/91\); \textit{sox4} MO + 2.0 ng/embryo of \textit{shha} MO: 41.84± 5.62%, \(n= 53/125\); \textit{sox4} MO + 3.0 ng/embryo of \textit{shha} MO, 34.39± 5.03%, \(n= 79/229\)) Fisher’s exact test, \(P<0.0001\) for \textit{sox4} MO + \textit{ihhb} MO; \(P= 0.1676\) for \textit{sox4} MO + 2.0 ng/embryo of \textit{shha} MO; \(P=0.0001\) for \textit{sox4} MO + 3.0 ng/embryo of \textit{shha} MO. f, forebrain; tel, telencephalon; di, diencephalon, hy, hypothalamus; mhb, midbrain-hindbrain boundary; n, notochord.
Figure S2.4. Upregulation of Hh signaling pathway genes in sox4 morphants. qPCR of mRNA from control and sox4 morphant heads at 18 and 24 hpf. Relative transcript abundance was normalized to levels of gapdh at 18 hpf and atp5h at 24 hpf. Y axis (log-scale) represents the average ratio of normalized sox4 morphant to control expression (three biological replicates; Student’s t-test, P<0.01).
Since knockdown of Sox4 caused an elevation in ihhb expression and Hh signaling, we predicted that overexpression of Sox4 would result in a corresponding reduction in Hh activity. We injected in-vitro synthesized control or sox4a/b mRNA into wild type embryos, and scored the injected embryos at 24 hpf for the presence of cyclopia, a phenotype associated with reduced Hh activity from the midline (Ekker et al., 1995). Whereas only 0.78±1.34 % of embryos (n= 1/128) injected with a control Td-Tomato mRNA exhibited cyclopia, we detected cyclopia in 36.54 ± 5.10% (n= 38/104) of the sox4 mRNA-injected embryos (Figure 2.7A-B). In addition, we found that the cyclopic embryos often possessed a truncated body and U-shaped somites (Figure 2.7C-F), a phenotype that is also observed with reductions in Hh signaling (Currie and Ingham, 1996; Schauerte et al., 1998; Wolff et al., 2003). To confirm that the cyclopic phenotype was specific for overexpression of sox4, we injected the same total amount of mRNA containing different ratios of control Td-Tomato and sox4 mRNA. We observed that both the occurrence and severity of cyclopia increased with increasing concentration of sox4 mRNA (Figure 2.7G), strongly suggesting that the cyclopic phenotype was not an artifact of mRNA injection. qPCR performed on mRNA prepared from the heads of injected embryos confirmed that ihhb expression was reduced in the embryos overexpressing sox4 at 18 and 24 hpf, although the reduction was not statistically significant at 24 hpf (Figure 2.7H; Student’s t-test, P= 0.0565). Surprisingly, although sox4 knockdown did not affect the expression level of shha, sox4 overexpression significantly reduced the expression of shha in the head. Taken together, these data suggest that sox4 can negatively regulate the expression of both ihhb and shha, and that overexpression of sox4 causes a reduction in midline Hh activity, leading to cyclopia.
Figure 2.7. Overexpression of sox4 inhibits Hh ligand expression and causes cyclopia. (A-F)
Representative images of embryos injected with control tdTomato or sox4 mRNA. (A-B)
Representative images of the head were taken at 72 hpf from the ventral side. (C-F) Lateral views of body showing the somites. Insets in (C) and (D) were enlarged in (E) and (F). (G)
Quantification of the percentage of cyclopia in embryos injected with different combinations of control and sox4 mRNA. 1.0 ng/embryo Td-tomato: 0.76± 1.31%, n= 1/108; 0.2 ng sox4a/b + 0.6 ng Td-tomato/embryo: 9.35± 1.17%, n= 13/139; 0.3 ng sox4a/b + 0.4 ng Td-tomato/embryo: 17.03± 3.69%, n= 11/67; 0.4 ng sox4a/b + 0.2 ng Td-tomato/embryo: 25.56± 2.20%, n= 25/97. (H) qPCR analysis of ihhb and shha expression in control and sox4 overexpressing heads at 18 and 24 hpf. In sox4 mRNA injected embryos, ihhb expression was significantly decreased by 1.61-fold at 18 hpf (Student’s t-test, P<0.05) and 1.51-fold at 24 hpf (Student’s t-test, P=0.057). Shha expression was also significantly reduced following sox4 mRNA injection at both 18 and 24 hpf (by 1.82-fold and 3.57-fold, respectively; Student’s t-test, P<0.05).
2.3.7 Bmp7 is a potential intermediate between sox4 and ihhb

Our results support the hypothesis that Sox4 is a negative regulator of ihhb expression. However, SoxC transcription factors are known to act as transcriptional activators rather than repressors (Bergsland et al., 2006; van de Wetering et al., 1993). Therefore, we hypothesized that Sox4 indirectly inhibits ihhb expression by activating an intermediate repressor. One candidate Hh repressor is Bmp7. Bmp7 is known to modulate Hh signaling (both positively and negatively), was previously shown to be significantly down-regulated in Sox11-deficient zebrafish and Sox11 null mice, and bmp7 mRNA can partially rescue coloboma in sox11 morphants (Bastida et al., 2009; Duench and Franz-Odendaal, 2012; Manning et al., 2006; Pathi et al., 1999; Pillai-Kastoori et al., 2014; Seki and Hata, 2004). Moreover, Bmp7 mutant mice display ocular malformations such as microphthalmia and optic fissure defects (Morcillo et al., 2006). We performed qPCR for bmp7b transcript levels in controls and sox4 morphants at 8, 10, 12, 18 and 24 hpf. This analysis revealed that bmp7b expression was significantly downregulated at all time points in sox4 morphants (Figure 2.8A, Student’s t-test, P< 0.01). We then injected bmp7b mRNA along with the sox4 MO and found that this partially rescued the coloboma phenotype of sox4 morphants (Figure 2.8B, Student’s t-test, P< 0.01). These data suggest that Sox4 may negatively regulate Ihhb at least in part through the activation of Bmp7b. We also analyzed the expression of four additional regulators of Hh signaling: two Hh inhibitors (fgfr2 and kras), and two Hh activators (lhx8 and nkx6.1; (Cai et al., 2000; Flandin et al., 2011; Mukhopadhyay et al., 2013). Fgfr2 was downregulated in sox4 morphant heads at 18 hpf and lhx8 was upregulated in sox4 morphants at 12 hpf, suggesting that these genes may also function downstream of Sox4 and upstream of Hh signaling during eye development (Figure S2.5, Student’s t-test, P<0.05).
Figure 2.8. *Bmp7b* expression is reduced in *sox4* morphants. (A) qPCR analysis revealed a significant reduction in *bmp7b* expression in *sox4* morphant embryos at 8, 10, 12 hpf and heads at 18 and 24 hpf (Student’s *t*-test, *P*<0.01). (B) *bmp7b* mRNA injection significantly reduced the proportion of embryos with coloboma in *sox4* morphants. Control MO only: 0.74± 1.28%, *n*= 1/110; *sox4* MO only: 55.66± 8.32%, *n*= 59/106; *sox4* MO+ *bmp7b* mRNA: 35.56± 10.02%, *n*= 29/80 (Student’s *t*-test, *P*< 0.05).
Figure S2.5. Expression of Hh regulators in sox4 morphants. qPCR of mRNA from control and sox4 morphant embryos at 12 hpf or heads at 18 hpf. Relative transcript abundance was normalized to atp5h (three biological replicates; Student’s t-test, P<0.05).
2.3.8 Sox4 and sox11 have overlapping functions in regulating choroid fissure closure

Our previous work demonstrated that Sox11-deficient zebrafish, similar to sox4 morphants, exhibit ocular coloboma and elevated Hh signaling. Moreover, sox4 mRNA injection can significantly reduce the incidence of coloboma in sox11 morphants (Pillai-Kastoori et al., 2014). To further investigate the functional overlap between Sox4 and Sox11, we injected sox4 and sox11 MOs, either alone or together, at half the usual dose. When a half-dose of sox4 MO was injected, only 11.05± 3.15% (n= 16/145) of morphants exhibited coloboma at 48 hpf. Similarly, when a half-dose of sox11 MO was injected, 22.31± 4.00% (n= 16/71) of the morphants exhibited coloboma. However, co-injection of half-doses of sox4 and sox11 MOs together resulted in 72.47± 2.95% (n= 51/70) of embryos with coloboma (Figure 2.9A-H, 2.9I), suggesting that knockdown of the two SoxC factors simultaneously produced a synergistic effect on the coloboma phenotype. Furthermore, injection of sox11a/b mRNA into sox4 morphants significantly suppressed the incidence of coloboma from 41.09±7.36% (n= 113/275) to 10.14± 3.09% (n= 28/276, Figure 2.9J). We did not detect abnormalities in lens development in sox4 morphants (Figure 2.9C, arrow). Moreover, the proportion of embryos with a malformed lens was not increased by addition of the sox4 MO (data not shown). Taken together, these results indicate that Sox4 and Sox11 have partially overlapping roles in regulating choroid fissure closure and can functionally compensate for one another during ocular morphogenesis, but are not functionally redundant with respect to lens development.
Figure 2.9. Synergistic effect of sox4 and sox11 knockdown on coloboma. (A-H)
Representative images of embryos injected with control MO (A, E), a half-dose of sox4 MO (B, F), a half-dose of sox11 MO (C, G), and half-doses of sox4 and sox11 MOs (D, H). (A, E) Half the normal dose of sox4 MO caused a low incidence of mild coloboma. (C, G) Half the normal dose of sox11 MO resulted in low incidence of coloboma and lens malformations at 24 hpf (C, arrow). (D, H) Injection of a half-dose of both sox4 and sox11 MO significantly increased the incidence of coloboma, and the coloboma phenotype was more severe compared to sox4 or sox11 morphants alone. (I) Quantification of the proportion of embryos with coloboma at 48 hpf (half-dose sox4 MO, 11.05± 3.15%, n= 16/145; half-dose sox11 MO, 22.31± 4.0%, n= 16/71; half-dose of both sox4 and sox11 MO, 72.47± 2.95%, n= 51/70; Fisher’s exact test, P=0.0001). (J) Sox11 can compensate for the loss of Sox4. Injection of sox11 mRNA into sox4 morphants significantly reduced the incidence of coloboma from 41.09±7.36% (n= 113/275) to 10.14± 3.09% (n= 28/276; Fisher’s exact test, P<0.0001). All scale bars equal 100 µm.
### Table 2.1. Primer sequences used in Chapter 2.

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2.4 Discussion

Although many genes have been identified that contribute to coloboma (Chang et al., 2006), the molecular mechanisms of choroid fissure closure have not been completely elucidated. It has been shown in animal models as well as in human patients that alterations in Hh signaling can cause coloboma (Lee et al., 2012; Lee et al., 2008; Schimmenti et al., 2003; Takeuchi, 2003). However, not much is known about how proper levels of Hh activity are maintained during eye development. In this study, we demonstrated that successful choroid fissure closure requires the activity of Sox4 to limit both the level and extent of expression of the Hh pathway ligand Ihh, permitting proper proximo-distal patterning of the optic stalk and vesicle. Together with our previous work demonstrating a role for Sox11 in controlling expression of Shh during ocular development (Pillai-Kastoori et al., 2014), our studies firmly place the SoxC transcription factors upstream of the Hh signaling pathway in the complex genetic network that regulates vertebrate choroid fissure closure.

The involvement of both Sox4 and Ihh in choroid fissure closure is a novel result, but is consistent with previous work demonstrating that related family members (Sox11 and Shh, respectively) also participate in this process. Although coloboma was not reported in the Sox4 null mutant mouse (Cheung et al., 2000; Schilham et al., 1996), the early lethality of that model likely precluded a thorough characterization of any ocular phenotypes. Coloboma was also not reported in a conditional mouse mutant lacking Sox4 expression within the developing eye (Jiang et al., 2013b). However, our cyclopamine rescue data suggest that the coloboma phenotype of sox4 morphants is caused by disruption in early, midline-derived Hh signaling, likely due to loss of sox4 expression in the forebrain and/or periocular mesenchyme (POM). Therefore, alterations to Sox4 expression within the optic vesicle may not have been sufficient to cause coloboma.

Finally, 65% of patients with CHARGE syndrome, a genetic disorder characterized by coloboma and cardiac abnormalities in addition to other birth defects, carry a mutation in CDH7 (Chang et
al., 2006), which directly activates Sox4 and Sox11 (Feng et al., 2013). Therefore, it is possible that altered expression of Sox4 (and/or Sox11) underlies the coloboma phenotype observed in CHARGE syndrome patients.

Our data strongly suggest that knockdown of Sox4 results in an early elevation of midline-derived Hh signaling, which ultimately results in defects in ocular morphogenesis and coloboma. How might this occur? We observed that the expression of the optic stalk marker pax2a was significantly expanded into the optic vesicle of sox4 morphants. As Hh signaling has a well-established role in specifying the optic stalk region through regulation of Pax2 expression, our results suggest that the elevation in Hh signaling from the midline expands the region of the optic vesicle that is fated to become optic stalk tissue. The decrease in bmp7b expression in sox4 morphants may also contribute to the defect in proximo-distal patterning of the optic stalk and vesicle, as others have shown previously that Bmp7 is required prior to Hh signaling for proper expression of Pax2 in the optic disk (Morcillo et al., 2006). In conjunction with (or as a result of) reduced Bmp7 and expanded Pax2, we observed an increase in apoptosis in the optic stalk region at 18 hpf. Therefore, we propose that misspecification of the optic vesicle cells as optic stalk physically interferes with proper closure of the choroid fissure, resulting in coloboma, and that the abnormally specified optic stalk cells ultimately die by apoptosis.

Although many studies in animal models and humans have demonstrated the critical role of Shh in the morphogenesis and patterning of the developing eye (Amato et al., 2004), there are much less data on the role of Ihh, which is better known for its function in regulating skeletal and intestinal development (Kosinski et al., 2010; Seki and Hata, 2004; St-Jacques et al., 1999). However, some previous studies are consistent with our finding that Ihh levels influence ocular development. For example, overexpression of murine Ihh in zebrafish embryos caused expansion of pax2 expression in the optic stalk (Hammerschmidt et al., 1996), and in the mouse, Ihh was
shown to be required for proper development of the RPE and peri-ocular tissues (Dakubo et al., 2008). Interestingly, although we observed a significant elevation (approximately 4-fold) of ihhb expression at 8-12 hpf in sox4 morphants, this increase was even more pronounced at 18 and 24 hpf (approximately 15-fold). This suggests that ihhb is itself a target of Hh signaling, resulting in a feedback loop of enhanced expression when Hh signaling is increased. A similar feedback loop with respect to Hh signaling and shha expression has been observed previously (Pillai-Kastoori et al., 2014), suggesting that this may be a general phenomenon of Hh signaling and Hh ligand expression.

Similar to other studies of SoxC family members, our results indicate that Sox4 and Sox11 have overlapping, but not completely redundant, functions in eye development. Thus, although sox4 and sox11 were able to compensate for each other when ectopically overexpressed, we observed distinct differences in their knockdown phenotypes. First, although knockdown of either sox11 or sox4 resulted in coloboma, abnormal lens development was only observed following knockdown of sox11. Second, the penetrance of the coloboma phenotype was higher in Sox11-deficient zebrafish than in Sox4-deficient zebrafish [~70% versus ~50%, respectively; this study and (Pillai-Kastoori et al., 2014)]. And third, whereas both sox11 and sox4 morphants displayed elevated Hh signaling, this occurred through increased expression of distinct Hh ligands (shha and ihhb, respectively).

These phenotypic differences may be explained by the differing expression patterns of sox11 and sox4. For example, whereas both sox4 and sox11 are expressed in the diencephalon adjacent to the optic vesicle at 18 and 24 hpf, sox4 (but not sox11) is also expressed in the periocular mesenchyme (POM). As the POM was previously shown to be a target of Ihh but not Shh signals in the mouse eye (Dakubo et al., 2008), sox4 expression in the POM may function in a negative feedback mechanism to limit the level or duration of Ihh signaling there. Similarly, sox11, (but
not sox4) is expressed in the developing zebrafish lens, which may account for the unique lens phenotype in sox11 morphants. Finally, the differing penetrance of the coloboma phenotype between the two knockdown models may be due to differences in their transactivation properties, as previous work has shown that Sox11 is a stronger transcriptional activator than Sox4 (Dy et al., 2008).

Targeted disruption of Sox4 or Sox11 in the developing mouse retina results in a reduction in retinal ganglion cell (RGC) number and optic nerve thickness (Jiang et al., 2013b). Although we detected expression of both sox4 and sox11 in the zebrafish GCL during retinal development, we did not observe a loss of ganglion cells in either sox4 or sox11 morphants (data not shown). It is possible that we failed to detect an early delay in RGC differentiation due to the much shorter window of retinal neurogenesis in the zebrafish compared with mouse (Cepko et al., 1996; Stenkamp, 2007). Alternatively, it is possible that knockdown of both sox4 and sox11 is required to alter RGC formation in the zebrafish.

Although we did not detect a loss of RGCs, we did observe ectopic cell proliferation in the GCL of the sox4 morphant retina. This phenotype has been observed in other models of overactive Hh signaling, such as zebrafish and mouse Ptc receptor mutants (Bibliowicz and Gross, 2011; Moshiri and Reh, 2004), and is consistent with the known role of the Hh pathway in promoting retinal progenitor cell proliferation. However, we also found that the total number of proliferating cells was reduced in the retinas of sox4 morphants at 36 and 48 hpf, whereas it was elevated at 72 hpf. These seemingly contradictory results could be explained by alterations in the cell cycle kinetics of RPCs, either via elevated Hh signaling [as described by (Locker et al., 2006)] or through a Hh-independent role of Sox4 in regulating cell cycle exit. Alternatively, the ectopically located proliferating cells in sox4 morphant retinas could indicate disruptions in the organization of the retinal neuroepithelial cells. Finally, it is not clear whether the altered cell proliferation in
the retina is due to loss of *sox4* expression (and the subsequent elevation in Hh signaling) from
the midline, or from within the retina itself. Future studies may be able to separate the early and
later functions of Sox4 in eye development using photo-morpholinos or inducible transgenes to
control the timing of *sox4* inactivation and rescue.

2.5 Materials and methods

2.5.1 Zebrafish strains and maintenance

All zebrafish (*Danio rerio*) strains were bred, raised, and maintained at 28.5°C on a 14 hour
light:10 hour dark cycle according to established protocols (Westerfield, 2000). The
*Tg(gfap:GFP)mi2001* transgenic line (Bernardos et al., 2007), hereafter called *gfap:GFP*, was
obtained from the Zebrafish International Resource Center (ZIRC, Eugene, OR). The *Tg(GBS-
ptch2:nlsEGFP)* transgenic line (Shen et al., 2013), hereafter called *ptch2:EGFP*, was kindly
provided by R. Karlstrom (University of Massachusetts, Amherst, MA). The
*Tg(−7.2sox10:EGFP)zf77* transgenic line (Hoffman et al., 2007), hereafter called *sox10:EGFP*, was generously provided by B.A. Link (Medical College of Wisconsin, Milwaukee, WI).

Embryos were staged according to established developmental hallmarks (Kimmel et al., 1995).
All animal procedures were carried out in accordance with guidelines established by the
University of Kentucky Institutional Animal Care and Use Committee.

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

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 SP6, T7, or T3 polymerase and
digoxigenin (DIG) or fluorescein (FITC) labeling mix (Roche Applied Science, Indianapolis, IN).
The *sox4a* plasmid was 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 given in Table
2.1. The *sox4b* plasmid (Mavropoulos et al., 2005) was generously provided by Bernard Peers
(Université de Liège, Sart Tilman, Belgium). The *ihhb* plasmid (Chung et al., 2013) was generously provided by H.C. Park (Korea University, Ansan, Gyeonggido, Republic of Korea). The *crx* and *pax6a* plasmids (Ochocinska and Hitchcock, 2007; Shen and Raymond, 2004) were kindly provided by Y.F. Leung (Purdue University, West Lafayette, IN). The *pax2a* plasmid (Lee et al., 2008) was kindly provided by J.M. Gross (University of Texas, Austin, TX). WISH and FISH were performed as previously described (Forbes-Osborne et al., 2013; Pillai-Kastoori et al., 2014). Images were obtained on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments).

2.5.3 Morpholino and mRNA microinjection

All morpholinos (MOs) were obtained from Gene Tools, LLC (Philomath, OR) and injected into zebrafish embryos at the one- to two-cell stage. The following MOs were used in this study:

- **standard control MO**: 5’-CCTCTTACCTCAGTTACAATTTATA-3’;
- **sox4a MO1**: 5’-GCGCTAAGAGTCTTTCTTCTTCACT-3’;
- **sox4b MO1**: 5’-ACGCGCCTTCAGTTCTCTTCTTCTACT-3’;
- **sox4b MO1**: 5’-ACCGCGCCTTCCAGTTCTTCTTCTCTACT-3’;
- **ihhb MO**: 5’-
- **shha MO**: 5’-CAGCACTCTCGTCAAAAGCCGCATT-3’

The specificity of the *sox4* morphant phenotypes was confirmed with a second, non-overlapping set of *sox4* morpholinos (*sox4a MO2*: 5’-CAACAGTCTCAACTTTTAATTGCGC-3’; *sox4b MO2*: 5’-GAGACTCAGTCTGATTTAGTCGACAC-3’). Embryos were injected with 5.3 ng each of *sox4a MO1* and *sox4b MO1*, 10.5 ng each of *sox4a MO2* and *sox4b MO2*, or 10.5 ng of standard control MO. Both *sox4* MO1 and MO2 generated similar phenotypes. Unless stated otherwise, all data presented in this study were from embryos injected with *sox4a MO1* and *sox4b MO1*. A p53 morpholino (Robu et al., 2007) was co-injected (at 1.5-fold the amount of *sox4* MOs) to suppress cell death (p53 MO 5’-GCGCCATTGTCTGTCCAAGAATTG-3’).
To determine the efficiency of the *sox4* MOs, PCR fragments corresponding to the 5’ UTRs of *sox4a* and *sox4b* mRNA and containing the *sox4a* MO and *sox4b* MO target sequence, respectively, were amplified and cloned upstream and in frame with the GFP gene in the *pEF1α:GFP* plasmid (Addgene, Cambridge, MA). One-cell stage zebrafish embryos were injected with 50 pg/embryo of *pEF1a-sox4a MO:GFP* and 50 pg/embryo of *pEF1a-sox4b MO:GFP* plasmids in the presence or absence of *sox4a MO1* and/or *sox4b MO1*. GFP expression in injected embryos was analyzed by fluorescence microscopy at 24 hpf.

For mRNA rescue and overexpression experiments, the zebrafish *sox4a/b* and *sox11a/b* coding sequences (lacking the MO target sites) were PCR amplified from 48 hpf complimentary DNA (cDNA) and cloned into the pGEM-T-easy vector (Promega, Madison, WI). The capped mRNAs were synthesized with the mMessage mMACHINE transcription kit (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Control *Td-tomato* mRNA was synthesized from *pRSET-B-td-Tomato* (kindly provided by D.A. Harrison, University of Kentucky, Lexington, KY). For mRNA rescue experiments, 0.5 ng/embryo of *sox4a* and *sox4b* mRNA or 1.0 ng/embryo of *sox11a* and *sox11b* mRNA was co-injected with the *sox4a/b* MOs. For mRNA overexpression experiments, 0.5 ng/embryo of *sox4a* and *sox4b* mRNA or an equimolar amount of *Td-tomato* mRNA was injected into wild type embryos. Alternatively, a total of 1 ng of mRNA containing different ratios of control *Td-Tomato* and *sox4* mRNA was injected. *Bmp7b* mRNA was synthesized from a *pCRII-bmp7b* plasmid (a kind gift from Dr. S. Fabrizio, The Novartis Institutes for Biomedical Research, Cambridge, MA). 1.0 ng/embryo of *bmp7b* mRNA was injected into *sox4* morphants.

2.5.4 CRISPR sgRNA and *Cas9* mRNA synthesis and injection

*Sox4a* and *sox4b* CRISPR target sites were identified and the corresponding sgRNA oligos were designed using the ZiFiT online software (www.zifit.partners.org/ZiFiT/; Table 2.1). Oligo pairs
(100µM) for each sgRNA were mixed with NEBuffer4 (New England Biolabs, Ipswich, MA), incubated in boiling water for 5 minutes, followed by 2 hours annealing at room temperature, and then ligated with linearized pDR274 vector (Addgene, Cambridge, MA) at 16°C overnight. Recombinant plasmid was digested with DraI to drop out the sgRNA template, followed by PCR amplification (Table 2.1) using the KOD Hot Start Master Mix (Millipore, Billerica, MA) and purification using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). sgRNA was generated using the MEGAscript T7 Transcription Kit (Life Technologies, Carlsbad, CA). To prepare the tyr sgRNA, pT7tyrgRNA (Addgene, Cambridge, MA) was linearized with BamHI and sgRNA was synthesized using MEGAscript T7 Transcription Kit (Life Technologies). To generate the Cas9 mRNA, pCS2-nCas9n plasmid (Addgene, Cambridge, MA) was linearized with NotI and capped Cas9 mRNA was synthesized using the mMESSAGE mMACHINE SP6 Transcription Kit (Life Technologies). To confirm the quality of sgRNA and Cas9 mRNA, RNA was mixed with formamide, heated at 72°C for 5 minutes and run on a 1% (wt/vol) agarose gel. The following sgRNA and Cas9 mRNA doses were microinjected into embryos at the one-cell stage: 100 pg/embryo of sox4a or sox4b sgRNA + 200 pg/embryo of Cas9 mRNA; 50 pg/embryo of tyr sgRNA + 150 pg/embryo of Cas9 mRNA.

2.5.5 HRMA analysis
To isolate genomic DNA from uninjected or sgRNA/Cas9 injected individual embryos, 24 hpf dechorionated embryos were placed into individual wells of a 96-well plate containing 20 µl of 1X ThermoPol Buffer (New England Biolabs, Ipswich, MA). The plate was placed in a PCR cycler at 95°C for 10 minutes, after which 5 µl of 10 mg/ml Proteinase K (Sigma, St. Louis, MO) was added to each well and the plate was incubated at 55°C for 1 hour and 95°C for 10 minutes. HRMA analysis was performed on a LightCycler 96 Real-Time PCR System (Roche, Indianapolis, IN) using LightCycler 480 High Resolution Melting Master (Roche), following the manufacturer’s instructions. Primer sequences used for HRMA are listed in Table 2.1.
2.5.6 Immunohistochemistry and TUNEL assay

Immunohistochemistry was performed on cryosections or whole zebrafish embryos as previously described (Fadool, 2003a; Forbes-Osborne et al., 2013). Images were obtained on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments) using the 20X objective. The following primary antibodies and dilutions were used: anti-PCNA (mouse, 1:100, Santa Cruz Biotechnology, Dallas, Texas), which labels cells in G1/S phase; anti-PH3 (rabbit, 1:500, Millipore, Billerica, MA), which labels cells in G2/M phase; anti-Zn-8 (mouse, 1:10, ZIRC, Eugene, OR), which labels ganglion cells; anti-Prox1 (rabbit, 1:1000, Acris, San Diego, CA), which recognizes horizontal cells; anti-PKCα (rabbit, 1:100, Santa Cruz Biotechnology, Dallas, Texas), which labels bipolar 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. For laminin immunostaining, 48 and 72 hpf zebrafish embryos were hybridized with anti-laminin (mouse, 1:60, Sigma, St. Louis, MO), and imaged on a laser scanning confocal microscope (Leica TCS SP5). TUNEL assay was performed on retinal cryosections using the ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions.

2.5.7 Real-time quantitative RT-PCR

Total RNA was extracted from the whole body of control and sox4 morphant embryos at 8, 10, and 12 hpf or the heads of control and sox4 morphant embryos, or TdTomato and sox4 mRNA injected embryos, at 18 and 24 hpf using TRIzol reagent (Invitrogen, Grand Island, NY). RNA was reverse-transcribed using the GoScript Reverse Transcriptase System (Promega, Madison, WI). Real time PCR was performed using Maxima SYBR Green qPCR master mix (Thermo Scientific, Waltham, MA) on an iCycler iQ Real Time PCR Detection system (Bio-Rad, Hercules, CA), or using FastStart Essential DNA Green Master (Roche, Indianapolis, IN) on a
LightCycler 96 Real-Time PCR System (Roche, Indianapolis, IN). For all experiments, three biological replicates were analyzed, and relative transcript abundance was normalized to expression of the housekeeping genes \textit{gapdh} or \textit{atp5h}.

2.5.8 Cyclopamine treatments

Cyclopamine (Sigma, St. Louis, MO) was resuspended at 1mM in 100% ethanol and diluted with fish water to a final concentration of 0.2 µM. For vehicle controls, 0.2% ethanol in fish water was used. Embryos injected with standard control MO or \textit{sox4} MOs were exposed to cyclopamine or ethanol from 5.5 to 13 hpf and then placed into fresh fish water until 24 hpf.

2.5.9 SDS PAGE and western blots

Protein was extracted from pools of 80-100 24 hpf control and \textit{sox4} morphant heads. Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Ten µg of total protein per sample was diluted 1:1 with Laemmli buffer and 2.5% β-mercaptoethanol, boiled for 10 minutes, and then separated by SDS-PAGE on 10% polyacrylamide gels. Resolved proteins were transferred to nitrocellulose membranes and blocked in 1X TBS/0.05% TWEEN/5% BSA for 1 hour at room temperature prior to incubation with either anti-IHH antibody (rabbit polyclonal, 1:800, Sigma, St. Louis, MO), or anti-β-Actin (rabbit polyclonal, 1:2000, Abcam, Cambridge, MA) as a loading control. The IHH antibody immunogen sequence partially overlaps with zebrafish Ihha, Ihhb and Shhb protein sequences. Membranes were washed and incubated in goat anti-rabbit-peroxidase secondary antibody (1:3000, Sigma, St. Louis, MO). Blots were developed using the Pierce ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions.
2.5.10 Statistical analyses
Significance was calculated using a two-tailed Student’s \( t \)-test or Fisher’s exact test, with \( P<0.05 \) being considered significant. For all graphs, data are represented as the mean ± the standard deviation (s.d.).

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CHAPTER 3: GENERATION AND CHARACTERIZATION OF SOX4A AND SOX4B MUTANT ZEBRAFISH LINES USING CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS (CRISPR)/ CRISPR-ASSOCIATED-9 (CAS9) SYSTEM.

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3.1 Abstract
Morpholino-mediated gene knockdown demonstrated that Sox4 regulates ocular morphogenesis through inhibition of Hh signaling. To investigate the long-term consequences of loss of Sox4 activity, we generated targeted mutations in sox4a and sox4b gene loci using CRISPR/Cas9 genome editing system. Sox4a and sox4b mutant lines and sox4a/b double mutant lines were successfully generated. Sox4aC2 maternal zygotic (MZ) mutant that results in a frame-shift and premature termination codon upstream of the sox4a transactivation domain exhibits microphthalmia/coloboma, tail curvature, and heart edema. It also has an increase in the expression of Hh signaling pathway genes ihhb and ptch2, and a reduction in the expression of bmp4. Taken together, sox4 CRISPR mutant lines provided us useful tools to further analyze the requirement of sox4 during ocular development and regeneration, and the negative regulatory relationship between Sox4 and Hh signaling pathway was supported.

3.2 Introduction
Morpholino-mediated gene knockdown is a quick and efficient method in generating loss-of-function phenotypes. However, due to fast cell proliferation in developing zebrafish embryos, the
efficiency of morpholino can reduce significantly by 4 dpf, hindering further analysis during later developmental stages as well as during adult retina neurogenesis. In addition, because *sox4a* and *sox4b* are single exon genes, morpholino interference with splicing is not applicable. The morpholinos we designed are translation-blocking morpholinos, which inhibit the translation of both maternal and zygotic *sox4a* and *sox4b* mRNA. As a result, we cannot separate the requirement and function of maternal from zygotic *sox4*. Furthermore, the non-specific activation of p53-mediated apoptosis and potential for off-target effects are drawing researchers’ preferences away from using morpholinos. To overcome the disadvantages of using morpholino mediated-gene knockdown, it is necessary to explore the requirement of *sox4* in a genetic null mutant. The early embryonic lethality of *Sox4*−/− null mice prior to retinal neurogenesis greatly reduces its value for studying retinal development. Together with the lack of an animal model with proper conditional Sox4 knockout in the eye, it would be very beneficial to generate a Sox4 mutant in zebrafish.

A number of precise genome editing tools have been developed in recent years, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) (Bill et al., 2009; Dahlem et al., 2012; Ekker, 2008; Hruscha et al., 2013; Huang et al., 2012; Hwang et al., 2013; Jao et al., 2013; Nasevicius and Ekker, 2000). Using these technologies, double strand breaks (DSB) can be generated in a target gene, which stimulates error-prone non-homologous end joining (NHEJ) repair. ZFNs and TALENs are based on DNA-protein interaction by engineering nucleases to recognize specific DNA sequences. Both of them can efficiently modify genes in zebrafish, but they do have limitations such as target sequences that are difficult to design, extended production time, and high costs. The recently emerged CRISPR/Cas system is more versatile for zebrafish genome editing applications due to its ease in identifying target sequences guided by RNA instead of protein, quicker production times, low costs, and relatively
higher efficiency in genome editing even at multiple loci simultaneously (Blackburn et al., 2013; Hwang et al., 2013; Jao et al., 2013; Talbot and Amacher, 2014).

3.2.1 CRISPR/Cas adaptive immunity

Bacteria and archaea are the most abundant organisms on earth. In order to survive various genetic invasions from viruses and foreign plasmids, they have evolved an adaptive immune mechanism mediated by the CRISPR/Cas system. Present in most prokaryotic genomes (Sorek et al., 2008), the CRISPR/Cas system is composed of a series of repeat-spacer-repeat sequences and independent genes coding for different types of Cas proteins. Previously known as short-sequence DNA repeat (SSR), the repetitive sequence was first described from the *Escherichia coli* genome in 1987 as an array of 29 base pair (bp) nucleotide repeats with spacer sequences in between (Ishino et al., 1987). It was named CRISPR in 2002 by Jansen (Jansen et al., 2002), whose group also identified the presence of *Cas* genes adjacent to the CRISPR loci. The spacer sequences are now known to be derived from invading exogenous DNA and integrated into the host genome (Barrangou et al., 2007; Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). It is now well known that CRISPR/Cas mediated immunity is accomplished in three steps. First is the adaptation stage. A short invading virus or plasmid sequence with a protospacer adjacent motif (PAM) is recognized and inserted into the host’s CRISPR locus through the activity of Cas1 and Cas2 proteins (Brouns et al., 2008; Garneau et al., 2010; Mojica et al., 2009). The second stage is expression. The CRISPR locus is transcribed into a long primary CRISPR RNA (pre-crRNA) through an AT-rich leader sequence located at the 5’ end, which serves as a promoter (Jansen et al., 2002; Pul et al., 2010). It is then processed into CRISPR-derived RNAs (crRNAs) by endoribonuclease activity from either a single Cas protein or CRISPR associated complex for antiviral defense (Cascade). The third stage is interference. Each crRNA contains a spacer sequence complementary to the protospacer of the foreign DNA. It recognizes and binds to the
target, and recruits Cas proteins to cleave and silence the foreign sequence by endonuclease activity (Jinek et al., 2012).

Based on the phylogenetic relationships of Cas proteins and functional analysis including differences in protein complex composition, pre-crRNA processing, and target cleavage mechanisms during the three stages, CRISPR/Cas systems are classified into three types (Makarova et al., 2011). Cas1 and Cas2 proteins are associated with the insertion of a new spacer sequence into the CRISPR locus, and are present in all three types of the CRISPR/Cas system. For the adaptation stage, type I and type II, but not type III, require PAM for spacer recognition (Mojica et al., 2009). PAM sequence varies corresponding to different CRISPR types (Mojica et al., 2009). A typical PAM sequence for the type II CRISPR/Cas system is NGG. For the transcription stage, type I pre-crRNA is processed by the Cascade complex, while type II pre-crRNA is complementarily bound by a trans-activating crRNA (tracrRNA) and cleaved by RNaseIII in the presence of Cas9 (Deltcheva et al., 2011). Type III pre-crRNA is processed by Cas6 and then passed to two types of protein complexes, Csm or Cmr (Rouillon et al., 2013; Staals et al., 2013). For the interference stage, type I crRNA remains a complex with Cascade proteins to recognize target sequences, and triggers their cleavage by Cas3 activity. Type II system target recognition and cleavage is facilitated by the Cas9-tracrRNA:crRNA complex (Jinek et al., 2012). Cas9 protein is a dual RNA-guided endonuclease, which has two catalytic domains homologous to the RuvC and HNH endonuclease. Each domain cuts one strand of the target DNA (Jinek et al., 2012). The type III system can be divided into two subtypes: IIIA and IIIB. crRNA in IIIA is bound by the Csm complex and recognizes DNA as a target (Marraffini and Sontheimer, 2008), while in IIIB subtype, crRNA binds to the Cmr complex and recognizes RNA as a target (Hale et al., 2009).

3.2.2 Application of CRISPR/Cas9 system in zebrafish genome editing
The type II CRISPR/Cas system has been broadly utilized for genome editing as it only requires a single Cas9 protein for pre-rRNA maturation and target cleavage, while type I and type III CRISPR/Cas systems require multiple Cas protein complexes. The most well studied type II CRISPR/Cas is the CRISPR/Cas9 system from *Streptococcus pyogenes* (Jinek et al., 2012), which has been reported to successfully edit the genome in yeast, nematodes, fruit flies, zebrafish, and plants (Bao et al., 2015; Bassett et al., 2013; Friedland et al., 2013; Hwang et al., 2013; Jiang et al., 2013a). The prokaryotic CRISPR/Cas9 has been modified in order to function efficiently in eukaryotic cells. A nuclear localization signal (NLS) such as SV40 NLS is added to either the C terminus or both sides of the *Streptococcus pyogenes* Cas9 gene (*SpCas9*) allowing it to enter the eukaryotic cell nucleus (Cong et al., 2013; Hwang et al., 2013; Mali et al., 2013). In addition, in order to increase the efficiency of *SpCas9* translation in-vitro in mammalian and human cell lines or in-vivo in zebrafish embryos, the natural *SpCas9* sequence is codon-optimized to those that are preferred by humans or zebrafish (Cong et al., 2013; Gonzales and Yeh, 2014; Hwang et al., 2013; Mali et al., 2013). As described previously, tracrRNA:crRNA duplex is required for proper genome editing. In order to simplify the synthesis and delivery of the tracrRNA:crRNA duplex, an alternative single strand guide RNA (sgRNA) has been designed. It contains the target sequence followed by another sequence that mimics the hairpin secondary structure in the tracrRNA:crRNA duplex and shows similar genome targeting and editing abilities (Hwang et al., 2013; Jinek et al., 2012). Zebrafish optimized plasmids for in-vitro generation of Cas9 mRNA and sgRNA are commercially available (http://www.addgene.org/CRISPR/).

Several CRISPR/Cas9 mediated genome editing strategies have been utilized in zebrafish. The most common way to disrupt target gene function is to introduce indel mutations (insertion or deletion) by NHEJ, which may result in a frameshift of the downstream sequence and abolish target gene function. The efficiency of frameshift mutation varies depending on the nature of target sequence and gene function. If a linearized donor DNA fragment is provided, it may be
integrated into the target locus by homology-independent insertion (Auer et al., 2014). To facilitate a precise predetermined mutation, such as a point mutation or an insertion of a small tag into the gene locus, donor DNA containing homology arms flanking the sequence in the form of either single stranded oligonucleotides (ssODNs) or linearized plasmid DNA can be inserted into the gene locus by homologous recombination (Hisano et al., 2015). If two sgRNAs targeting the same chromosome are delivered simultaneously, a large fragment of chromosome between the two sgRNA may be deleted (Ota et al., 2014). Two sgRNAs targeting different chromosomes that lead to chromosomal translocation have been reported in mouse, human cell culture, and C. elegans (Blasco et al., 2014; Chen et al., 2015b; Lagutina et al., 2015; Torres et al., 2014), but has not yet been successfully generated in zebrafish (Ota et al., 2014).

In this study, the sox4a and sox4b loci were disrupted using the CRISPR/Cas9 system. They were each targeted with two different sgRNA molecules recognizing distinct sites within the coding region of sox4a and sox4b. These mutant zebrafish lines permit us to not only confirm the sox4 morphant phenotypes but also to determine the requirement for sox4 during adult neurogenesis as well as under injury and regeneration conditions.

3.3 Results

3.3.1 Generating sox4a and sox4b mutant lines using CRISPR/Cas9 system

3.3.1.1 Single strand guide RNA (sgRNA) design

As described previously, Sox4 proteins have two critical domains, the DNA-binding HMG domain and the transactivation TAD domain. To generate mutant alleles with disruption of the coding sequence for these two domains, two sgRNA molecules (sox4a C1 and C2) were designed to target sox4a and another two sgRNAs (sox4b C1 and C2) were designed to target sox4b (Figure 3.1). C1 sgRNAs contain a customized sequence that targets upstream of the HMG domain while C2 sgRNAs contain target sequence between the HMG domain and the TAD. The
experimental design was as follows: C1 injection will possibly lead to frameshift and interruption of both HMG and TAD domains; C2 injection will interrupt the coding of the TAD domain while the HMG domain is intact; C1+C2 injection will result in a genomic DNA deletion between C1 and C2 loci, interrupting both HMG and TAD domains. Although it might have a similar effect as C1 injection, C1 and C2 coinjection bypasses the possibility of an alternative translation start site that might rescue a frameshift mutation caused by C1 alone.
Figure 3.1. Schematic representation of sox4 genes and their CRISPR target sites. (A) sox4a C1 sgRNA targets 5’ of the sox4a HMG domain on the forward strand. Sox4a C2 sgRNA targets between the HMG and TAD domain on the forward strand. (B) Sox4b C1 sgRNA targets 5’ of the sox4b HMG domain on the forward strand. Sox4b C2 sgRNA targets between the HMG and TAD domain on the reverse strand. sgRNA, single strand guide RNA; C, CRISPR. Sequences in grey, CRISPR target sequences; sequences in bold, PAM sites.
3.3.1.2 Sox4a and Sox4b gene disruption in F0 sgRNA and Cas9 mRNA injected zebrafish

All four sgRNA molecules and the Cas9 mRNA were in-vitro transcribed. sgRNA C1 and C2 were either individually or simultaneously injected with Cas9 mRNA into F0 embryos at the 1-cell stage. A concentration of 300 pg/embryo Cas9 mRNA and 100 pg/embryo sgRNA was used to generate a high percentage of mutagenesis without interrupting normal embryonic development. If genome editing occurs, a F0 injected embryo will be a mosaic of cells containing Sox4 WT alleles or mutant alleles. Genomic DNA from individual uninjected control (UIC) embryos and injected F0s was extracted and regions encompassing the C1 or C2 locus were PCR amplified with primers listed in Table 2.1. Mutagenesis was analyzed by high resolution melting analysis (HRMA), since the PCR product from injected mosaic mutant embryo will form heteroduplex DNA that melts at a lower temperature compared to UIC homoduplex DNA (Figure 3.2A-D). For C1+C2 injected F0s, mutagenesis was simply detected by PCR amplification of the whole coding region following electrophoresis to determine whether smaller than expected amplicons were produced due to genomic deletion (Figure 3.2E, F). The rate of successful mutagenesis ranged from 50% to 92% in the F0 injected embryos, as shown by HRMA (data not shown). Various mutant alleles for Sox4a and Sox4b generated in the F0 embryos were detected by HRMA or PCR and confirmed by Sanger sequencing (Figure 3.2).
Figure 3.2. Mutagenesis in F0 sgRNA/Cas9 injected embryos. (A, B) HRMA analysis detected the presence of mutant alleles in individual sox4a C1 sgRNA/Cas9 and C2 sgRNA/Cas9 injected embryos, which were confirmed by sequencing. The net base pair changes of each indel mutation was noted at the right of each sequence (+, insertion; –, deletion). (C) Electrophoresis of sox4a whole coding region PCR product revealed genomic deletion in sox4a C1+C2 sgRNA/Cas9 injected embryos. Six individual embryos were showed here. Individual 1, 2, 4, and 6 were WT, while individual 3 and 5 contain mutant allele that was shorter than the WT allele. (D, E) HRMA analysis detected the presence of mutant alleles in individual sox4b C1 sgRNA/Cas9 and C2 sgRNA/Cas9 injected embryos, which were confirmed by sequencing. The net base pair changes of each indel mutation was noted at the right of each sequence (+, insertion; –, deletion). (F) Electrophoresis of sox4b whole coding region PCR product revealed genomic deletion was detected in sox4b C1+C2 sgRNA/Cas9 injected embryos. Six individual embryos were showed here. Individual 1, 2, and 4 were WT, while individual 3, 5, and 6 contain mutant allele that was shorter than the WT allele. UIC, uninjected control; arrows, mutant allele HRMA melting curve; sub, substitution; C, CRISPR; sequences in blue, sgRNA target sites; sequences in red, mutations.
3.3.1.3 Establishment of stable sox4a and sox4b mutant lines

To establish stable sox4a and sox4b mutant lines, F0s were raised to adulthood and screened for germline transmission by out-crossing with WT adult zebrafish. F1 embryos were screened for the inheritance of sox4 mutant alleles using HRMA. Once the F1 embryos of a F0 founder were confirmed to carry a mutant allele, more F1 embryos, which were a mixed population of WT and different types of heterozygous mutants, were raised to adulthood. To identify heterozygous individuals, these F1s were tail clipped for genomic DNA extraction and the sgRNA target sites were PCR amplified and sequenced. F1s that were confirmed to be heterozygous for an inherited frameshift mutant allele were kept for further out-crossing with WT to generate F2s. F2s were incrossed to generate F3s from which homozygous mutant fish were identified by sequencing or RFLP analysis (if a restriction enzyme recognition site was introduced or destroyed by the mutation). The strategy of this three-generation crossing is summarized in Figure 3.3. Once sox4a and sox4b mutant lines were established, they were crossed with each other to generate a sox4a/b double mutant.
Figure 3.3. Schematic flowchart for generating homozygous mutant using CRISPR/Cas9 system. sgRNAs and the Cas9 mRNA were microinjected into fertilized zebrafish embryos (F0) at the one-cell stage. F0s were raised to adulthood and screened for germline transmissions by outcrossing with WT. F1s were screened for inheritance of *sox4* mutant allele using HRMA and sequencing. F1 heterozygous mutants were raised to adulthood and outcrossed with WT to generate F2s. F2s were incrossed to generate F3s, of which 25% were expected to be homozygous mutant for *sox4*. Different mutant alleles were color coded.
3.3.1.4 Identification of sox4 mutants: molecular defect and predicted effect on mRNA and protein

Three sox4a mutant lines (sox4aC1, sox4aC2, sox4aC1+C2) and three sox4b mutant lines (sox4bC2.1, sox4bC2.2, sox4bC1+C2) were established. Details of mutant DNA sequences and the corresponding protein sequences are listed in Figure 3.4. Two sox4a/b double mutant lines were generated by crossing sox4aC1 with sox4bC2.1 and sox4aC2 with sox4bC2.2, respectively. The sox4aC1 mutant allele has a 10 base pair (bp) deletion around the PAM site and a 3 bp insertion downstream of the target site, resulting in a frame-shift and premature termination codon upstream of the HMG domain. This mutant mRNA is expected to be translated into 31 amino acids compared to 363 amino acids in WT. This truncated protein has only the first 5 amino acids identical to WT and lacks both the HMG and TAD domain (Figure 3.4A, 3.4B).

The sox4aC2 mutant allele has 1 bp insertion and 7 bp substitutions, changing from the WT sequence 5'-CGGAAAGGA-3' to the mutant sequence 5'-GAAGACGCGG-3'. This change in genomic sequence results in a frame-shift and premature termination codon upstream of the sox4a transactivation domain. The mutant mRNA is expected to be translated into a truncated protein lacking the TAD domain, with only 292 amino acids compared to 363 amino acids in WT (Figure 3.4A, 3.4B). The first 255 amino acids are identical to WT.

The sox4aC1+C2 mutant allele was generated by simultaneous injection of C1and C2 sgRNAs/Cas9. C1 and C2 sgRNA target sites are 724 bp apart. The mutant allele has the first 427 bp deleted and the last 297 bp inverted, resulting in a premature termination codon upstream of the HMG domain. This mutant mRNA is expected to be translated into 68 amino acids compared to 363 amino acids in WT. This truncated protein has only the first 6 amino acids identical to WT and lacks both the HMG and TAD domain (Figure 3.4A, 3.4B).
The *sox4bC2.1* mutant allele has a 2 bp deletion at the PAM site, resulting in a frame-shift and premature termination codon upstream of the *sox4b* TAD domain. This mutant mRNA is expected to be translated into a truncated protein lacking the TAD domain while the HMG domain is intact. The total length of this mutant Sox4b protein is 169 amino acids compared to 342 amino acids in WT, and the first 156 amino acids are identical to WT (Figure 3.4C, 3.4D).

The *sox4bC2.2* mutant allele has a 4 bp deletion around the PAM site, resulting in a frame-shift and premature termination codon upstream of the *sox4b* TAD domain. This mutant mRNA is expected to be translated into 316 amino acids compared to 342 amino acids in WT, lacking the TAD domain while the HMG domain is intact (Figure 3.4C, 3.4D). The first 155 amino acids are identical to WT.

The *sox4bC1+C2* mutant allele was generated by simultaneous injection of C1+C2 sgRNA/Cas9. C1 and C2 sgRNA target sites are 307 bp apart. The mutant allele has a 407 bp deletion, resulting in a premature termination codon upstream of the HMG domain. This mutant mRNA is expected to be translated into 128 amino acids compared to 342 amino acids in WT. This truncated protein has the first 51 amino acids identical to WT and lacks both the HMG and TAD domain (Figure 3.4C, 3.4D).

The generation and screening of *sox4aC1+C2* and *sox4bC1+C2 F3 -/-* lines are still in progress. So we were not able to characterize the phenotypes of maternal zygotic (MZ) mutants from these two lines yet.

Among the four *sox4* single mutant lines, we observed a variety of developmental defects in *sox4aC2* MZ mutants, including ocular microphthalmia/coloboma. A detailed characterization of the mutant phenotypes is described in section 3.3.2.
Surprisingly, no obvious morphological defects in the eye or elsewhere were found in the
sox4aC1 MZ mutant embryos (data not shown). This may be due to one of several possibilities: a) an alternative translation start codon may be present in the mutant allele, which generates another mutant protein that is truncated at the N terminus but retains the functional domains of Sox4a. Indeed, the sox4aC1 mutant allele has genomic disruption very early (immediately after the codon of the 5th amino acid) and an alternative in-frame translation start codon is present at the 32nd amino acid, which is upstream of the HMG domain. This may result in the translation of a mutant Sox4a protein that retains both the HMG and TAD domain and retains protein function; b) the presence of Sox4b may compensate for the loss of Sox4a function in sox4aC1 MZ mutants. This is possible, but we think a complete functional redundancy is unlikely, as defects were observed in sox4aC2 MZ mutants.

In addition, no obvious morphological defects were observed in either sox4bC2.1 or sox4bC2.2 MZ mutants. Both lines have genomic disruption downstream of the HMG domain coding region. As a result, it is unlikely that an alternative translation start site will generate another functional Sox4b protein. Then why do the mutant embryos look normal? It is possible that a) compared to Sox4a, Sox4b is unnecessary for embryonic development. We do not favor this explanation because sox4b morpholino injection generated an even higher percentage of embryos with ocular coloboma compared to a sox4a morpholino injection (Figure S2.2); b) the presence of Sox4a compensates for the loss of Sox4b in these mutants. We have detected a mild increase of sox4a expression by RT-PCR and qPCR in sox4b C2.1 mutant embryos at 24 hpf (data not shown), which suggests there might be an upregulation of sox4a in sox4b mutants that could compensate for Sox4b function.

The establishment of homozygous sox4aC1; sox4bC2.1 and sox4aC2; sox4bC2.2 double mutant lines should allow us to analyze whether a loss of both sox4a and sox4b will cause a more severe
phenotype than single mutants alone. However, sox4aC1; sox4bC2.1 MZ mutant embryos did not exhibit any obvious morphological defects. This was not totally unexpected, since, as described above, we suspect that the sox4aC1 mutation might be rescued by an alternative translation start site downstream of the sox4aC1 target site. For the other double mutant line (sox4aC2; sox4bC2.2) only one female and one male homozygous adult fish have been recovered to date. Unfortunately, although they are both sexually mature, they have not yet generated any viable offspring from incrosses. They have been out-crossed with WT fish on a weekly basis, but the female always generates a majority of unfertilized or nonviable eggs (with occasionally very few fertilized ones, n=8/224) and the male has not successfully mated with any WT females yet. These data may suggest that sox4a/b double mutants have defects in gametogenesis or their progeny die very early in development prior to the gastrula stage; more adult double mutants must be generated and tested to determine whether this is indeed the case.
Figure 3.4. Schematic representation of \textit{sox4} mutants’ genomic DNA loci and predicted protein sequences. (A) Genomic DNA sequence of \textit{sox4a} in WT and three established \textit{sox4a} mutant lines: C1, C2, and C1+C2. (B) Sox4a WT protein sequence and predicted mutant protein sequences based on genomic DNA sequence. Numbers represent the number of amino acids. (C) Genomic DNA sequence of \textit{sox4b} in WT and three established \textit{sox4b} mutant lines: C2.1, C2.2, and C1+C2. (D) Sox4b WT protein sequence and predicted mutant protein sequences based on genomic DNA sequence. Numbers represent the number of amino acids. Sequences in grey represent identical sequences; sequences in blue were sgRNA target sites; sequences in red were mutations. bp, base pair; inv, inversion; del, deletion.
3.3.2 Characterization of sox4aC2 MZ mutant phenotype

Since the sox4aC2 MZ mutant line was the first established and has the most obvious
developmental defects, I mainly focused on characterizing the mutant phenotypes of this line.

3.3.2.1 Sox4a mRNA was significantly reduced in sox4aC2 MZ mutants

To determine whether the sox4aC2 mutant allele affected sox4a mRNA transcription, RT-PCR
and quantitative real-time PCR were performed on cDNAs reverse transcribed from 24 hpf
sox4aC2 MZ mutant embryo total RNA. The housekeeping gene gapdh and two other sox genes
(sox4b and sox11b) were also amplified as controls. Interestingly, while all the control genes
displayed no change in expression between WT and sox4aC2 mutants, sox4a transcripts were
significantly reduced in sox4aC2 MZ mutants (Figure 3.5). It was only weakly detected in RT-
PCR and almost undetectable in qPCR. This observation suggests that there might be a nonsense-
mediated mRNA decay (NMD) of sox4a in sox4aC2 MZ mutants. Although not tested, we
assume that due to the significant reduction of sox4a mRNA, the levels of mutant Sox4a protein
were also greatly reduced or absent.
Figure 3.5. *Sox4a* mRNA was significantly reduced in *sox4aC2 MZ* mutants. (A) Electrophoresis of *sox4a, sox4b, sox11b*, and *gapdh* RT-PCR products for WT and *sox4aC2 MZ* mutant embryos at 24 hpf. (B) *sox4a, sox4b* and *sox11b* qPCR of mRNA from WT and *sox4aC2 MZ* mutants at 24 hpf. Relative transcript abundance was normalized to level of *gapdh*. Y axis represents the average ratio of mutant to WT expression (three biological replicates; Student’s *t*-test, *P*<0.01).
3.3.2.2 High percentage of sox4aC2 MZ mutant eggs were unfertilized due to loss of maternal sox4a

We noticed that approximately 50% of sox4aC2 MZ embryos were dead by 24 hpf. To determine whether this was due to embryonic lethality or unfertilized eggs, we took light microscopy images of those embryos at 4 hpf during the blastula stage. For WT embryos at 4 hpf, the sphere of cells were well organized at the animal pole of the fertilized egg (Figure 3.6A). Very few eggs were unfertilized in WT crosses (3.6%, n=2/56). In contrast, approximately 40%-60% of eggs were unfertilized in sox4aC2 homozygous mutant incrosses. Unfertilized eggs did not have a distinguishable blastodisc and the animal pole lacked clearly defined cells (Figure 3.6A). The blastodisc surface of the unfertilized egg was always uneven and bubbles were often observed inside and on top of the animal pole (Figure 3.6A). Sox4a and sox4b were both expressed maternally, as detected by RT-PCR at 3 hpf in the fertilized egg during blastulation stage (Figure S3.1). To determine whether the unfertilized eggs were caused by defects in oogenesis or spermatogenesis, homozygous and heterozygous sox4aC2 females and males were crossed with each other or with WT fish and the percentage of unfertilized eggs from each cross was recorded (Figure 3.6B). We found two interesting phenomena: 1) No matter what was the genotype of the male, as long as the female was a homozygous mutant, the resulting cross always had higher rate of unfertilized eggs (first three crosses shown in Figure 3.6B); 2) When the progeny had the same zygotic genotype (as shown in figure 3.6B cross 2 and 6, cross 3 and 5), maternal loss of sox4a resulted in higher rate of infertility. Based on these observations, we conclude that the loss of sox4a in female fish causes defects in oogenesis that lead to a high rate of unfertilized eggs.

In addition, we observed a reduced sex ratio of females to males in the progeny of sox4aC2 MZ in-crosses from approximately 1:1 to 1:9. It is possible that Sox4a is required for female survival, such that not all female embryos survive to adulthood, or Sox4a may be involved in female sex determination such that loss of Sox4a results in a biased differentiation to male.
**Figure S3.1.** Maternal expression of *sox4a* and *sox4b* in 3 hpf WT embryos. RT-PCR performed on pooled 3 hpf WT embryos (n=50). Two biological repeats were shown. *gapdh* was used as a housekeeping control.

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Figure 3.6 Maternal loss of sox4a causes high rate of unfertilized eggs. (A) Representative images of fertilized WT and sox4aC2 MZ embryos and unfertilized sox4aC2 MZ embryo at 4 hpf. The blastodisc is on the dorsal side of the embryo. (B) The percentage of unfertilized eggs was calculated from six different types of crosses (data of each type of cross was collected from three biological replicates). The maternal and zygotic genotype and the parental genotypes are labeled beneath each column. Crosses that represent loss of maternal sox4 are highlighted in the red rectangle (cross 1-3). Two pairs of reciprocal crosses (cross 3 and 5, 2 and 6) are indicated with red lines. Mix, a mixed population of +/-, +/-, and -/-; question mark, either + or -.
3.3.2.3 Developmental defects in sox4aC2 MZ mutant embryos

The morphology of surviving sox4aC2 MZ mutant embryos was observed and recorded from 24 to 72 hpf. About 20-30% of mutant embryos exhibit apparent developmental defects, including a short and twisting tail, and thin anterior neural tissue at 24 hpf (Figure 3.7A, 3.7B). By 72 hpf, these defective embryos display varying severity of tail truncation and curvature, heart edema, and microphthalmia (Figure 3.7C-H). A small portion of these embryos also have coloboma and hypopigmentation in the ventral eye (Figure 3.7H), which were identical to the sox4a/b morphant phenotypes. Although the incidence of coloboma in the sox4aC2 MZ mutants (< 10%) was much lower than that of the sox4 morphants (~50%) (Wen et al., 2015), this may be due to the presence of wild type sox4b transcripts that compensate for the loss of sox4a (Figure 3.5).

The tail curvature, pericardial edema and microphthalmia/coloboma phenotypes were highly correlated with the maternal loss of sox4a, since very few embryos were observed with these defects in homozygous progenies generated from heterozygous incrosses (Figure 3.7I, cross 6) or from a heterozygous female crossed with a homozygous male (Figure 3.7I, cross5). Nevertheless, comparison among progenies from crosses of homozygous sox4aC2 female fish with homozygous, heterozygous, and WT males revealed that zygotic loss of sox4a also contributed to the mutant phenotypes (Figure 3.7, cross1, 2, and 3). We observed the highest incidence of mutant phenotypes when both maternal and zygotic sox4a were mutated.
Figure 3.7. 20%-30% sox4aC2 MZ mutants exhibit tail curvature, heart edema, and microphthalmia/coloboma. Representative images of WT embryos (A, C, E, G) and sox4aC2 MZ embryos (B, D, F, H). A minimum of 5 embryos were imaged for each group. A and B show embryos at 24 hpf. C-H show embryos at 72 hpf. Arrow in F indicates the tail curvature in the mutant; arrows in H indicate the microphthalmia/coloboma and hypopigmentation in the mutant eye and the mutant heart edema. (I) The percentage of mutant phenotypes was quantified from six different types of crosses (data for each type of cross was collected from three biological replicate crosses). The maternal and zygotic genotype and the parental genotypes are labeled beneath each column. Scale bars for the insets in B-C equal 500 µm.
3.3.2.4 Microphthalmia is observed in 70% of sox4aC2 MZ mutants

Although approximately 70% of sox4aC2 MZ mutant embryos were grossly comparable to WT with respect to morphology and no obvious developmental defects were observed, we noticed a significant reduction in eye size in sox4aC2 MZ mutant embryos at 5 dpf (Figure 3.8C, 3.8D). To determine whether this eye size reduction was due to an overall decrease in body size, we measured the ratio of eye size to body length in WT and sox4aC2 MZ mutants. This analysis demonstrated that whereas the average body length of sox4aC2 mutants was similar to WT, the eye size of sox4aC2 MZ mutants was significantly reduced.
Figure 3.8. 70% *sox4aC2* MZ embryos exhibit microphthalmia. WT embryos at 5 dpf (A, C) were compared with *sox4aC2* MZ embryos at 5 dpf (B, D). (E) Box plot of WT and mutant eye size at 5 dpf. Mutant embryos display a significantly smaller eye size. (F) Box plot of WT and mutant body length from nose to tail tip at 5 dpf. Mutant and WT body length were comparable. (G) Ratio of eye size to body length. Mutant eyes were significantly smaller than WT even after normalized over body length. 20 larvae were measured for WT and mutant. Student’s t-test, p<0.05. Blue lines, mean value.
3.3.2.5 Sox4aC2 MZ mutant embryos display increased cell death

Sox4 is required for neuronal and mesenchymal tissue survival (Bhattaram et al., 2010; Jiang et al., 2013b; Thein et al., 2010). To determine whether the sox4aC2 mutant allele affected cell survival during development, 24 hpf WT and sox4aC2 MZ mutant embryos were stained with acridine orange (AO). A few dead cells were observed in the WT embryo at this time point, in the developing lens and sporadically scattered along the neural tube (Figure 3.9A, 3.9B arrow). However, we observed a significantly increased number of AO positive cells in the mutant around the brain and periocular tissue (Figure 3.9D, 3.9E). To further examine whether sox4aC2 mutant allele affects cell survival at later stages in the retina, age matched WT and sox4aC2 MZ mutants were collected at 5 dpf, 2 weeks, and 4 weeks, cryosectioned and immunolabeled with an antibody against activated-Caspase 3, which labels apoptotic cells. Very few Caspase 3-positive cells were observed in either WT or mutant retinas, and no significant differences were seen in any of the age groups (Figure 3.9C, 3.9F, 3.9G, student’s t-test, p<0.05).

To test if the increase in cell death observed at 24 hpf in sox4aC2 mutant embryos was contributed by p53-mediated apoptosis, we injected a p53 morpholino into the sox4aC2 MZ mutant embryos. However, it failed to rescue cell death in the mutant embryos (data not shown), suggesting cell death in the mutants might be mediated through a different mechanism. This also provided a possible explanation why we did not observe many activated-Caspase 3 positive cells in mutants. However, since we did not examine the number of activated-Caspase 3 positive cells at time points earlier than 5 dpf, we cannot rule out the possibility that there might be more apoptotic cells in the mutant eye at earlier stages.
Figure 3.9. *Sox4aC2* MZ mutant embryos display increased cell death. (A, B) WT 24 hpf embryos were stained with acridine orange (AO). A few AO positive cells were observed in lens (white arrow in B) and scattered along the body. n=10. (C) Activated-Caspase 3 positive cells (yellow) were observed in WT 5 dpf retina. n=6 (D, E) *Sox4aC2* MZ 24 hpf embryos were stained with AO. Increased AO positive cells were observed in the brain and periocular tissues. n=10. (F) Activated-Caspase 3 positive cells (yellow) were observed in *sox4aC2* MZ 5 dpf retina. n=7. (G) Quantification of activated-Caspase 3 positive cells in WT and *sox4aC2* MZ mutants at 5 dpf, 2 weeks, and 4 weeks. Student’s t-test, p<0.05.
3.3.2.6 Hh and BMP signaling are altered in sox4aC2 MZ mutants

Previously, we reported a significant upregulation of Hh signaling in sox4 morphants, due to an increased expression of *indian hedgehog b* (*ihhb*) (Wen et al., 2015). To determine whether Hh signaling was also elevated in the sox4aC2 MZ mutants, we compared the expression of several Hh signaling genes in WT and mutants at 24 hpf by qPCR. Based on the morphology, mutant embryos were divided into normal and abnormal groups and RNAs were extracted separately from the two groups. We examined the expression level of two Hh ligands (*shha* and *ihhb*), and two Hh receptors (*ptch1* and *ptch2*). Intriguingly, we detected a significant upregulation of *ihhb* (~2-fold) and *ptch2* (~18-fold) expression in the abnormal sox4aC2 MZ embryos, consistent with the elevation of Hh signaling in sox4 morphants (Figure 3.10A, student’s t-test, p< 0.01).

However, in the morphologically normal mutants, none of the examined Hh genes were significantly increased, suggesting that the normal looking mutant individuals may somehow avoid Hh elevation by other regulatory mechanisms.

Bmp signaling is able to modulate Hh signaling and was reduced in sox4 morphants (Wen et al., 2015). Interestingly, the tail curvature phenotype in sox4aC2 MZ mutant embryos was very similar to several zebrafish Bmp mutants, such as *snailhouse* (*bmp7*), *swirl* (*bmp2*), and *piggytail* (*smad5*) (Dick et al., 2000; Kramer et al., 2002; Schmid et al., 2000). To test whether Bmp signaling was altered in sox4aC2 MZ mutants, we analyzed the expression of three Bmp ligands (*bmp4*, *bmp7a*, and *bmp7b*) and three downstream molecules (*smad1*, *smad5*, and *smad9*) in WT and sox4aC2 MZ mutants by qPCR. Again, analysis was performed separately on mutants that were morphologically normal or abnormal. We found a significant downregulation of *bmp4* in the abnormal mutants at 24 hpf (Figure 3.10B, student’s t-test, p< 0.01). In addition, most of the analyzed *bmp* genes were decreased in both normal and abnormal mutants, although the difference was not significant due to high variability (Figure 3.10B). The downregulation of *bmp4* in the mutants was confirmed by WISH at 6 and 24 hpf (Figure 3.11). At 6 hpf, *bmp4* was
strongly expressed in the ventral region and prechordal plate of the WT embryos (Figure 3.11C); in contrast, in the mutant embryos $bmp4$ expression was greatly (Figure 3.11D). At 24 hpf in WT embryos, $bmp4$ expression in the eye was observed in a patch of retinal cells dorsal to the lens (Figure 3.11E, 3.11G, black arrow); this expression was absent in a subset of mutants (n=3/10, Figure 3.11F, 3.11H, black arrow). Interestingly, $bmp4$ expression in the olfactory placode was not affected in those mutant embryos (Figure 3.11G, 3.11H, white arrow). $Bmp4$ was also expressed in the tail of 24 hpf WT embryos (Figure 3.11I, dashed line). Normal mutant individuals exhibited comparable $bmp4$ expression in the tail (Figure 3.11J, dashed line). However, in mutants with tail curvature, the $bmp4$ expression pattern was altered, being mostly constricted to the ventral part, and appeared to have a higher expression intensity (Figure 3.11K, dashed line).

In summary, the elevation of Hh signaling and downregulation of Bmp signaling in $sox4aC2$ MZ mutants was consistent with what we have observed in $sox4$ morphants.
Figure 3.10. Upregulation of Hh signaling pathway genes and downregulation of Bmp signaling pathway genes in sox4aC2 MZ mutants. qPCR of mRNA from WT and sox4aC2 MZ mutant embryos at 24 hpf. Relative transcript abundance was normalized to levels of gapdh. Y axis (log-scale) represents the average ratio of normalized sox4aC2 MZ to WT expression (two biological replicates; Student’s t-test, P<0.01).
Figure 3.11. Bmp4 was downregulated in sox4aC2 MZ mutants. Bmp4 expression in WT and sox4aC2 MZ mutants was detected by WISH at 6 hpf (C-D) and 24 hpf (E-K). For each time point, at least 10 embryos were analyzed. (A, B) Control gapdh expression was not changed in WT (A) and mutant (B) embryos at 6 hpf. (C, D) Bmp4 expression was at a much lower level in mutant (D) compared to WT (C). (E, F) Lateral view of 24 hpf embryos demonstrated the expression of bmp4 in WT dorsal retina (E, arrow), which was absent in mutants (F, arrow). (G, H) Dorsal view of the 24 hpf embryo heads demonstrated expression of bmp4 in WT dorsal retina (G, black arrow), which was absent in mutants (H, black arrow). Bmp4 expression in the olfactory placode was not affected (G and H, white arrows). (I-K) Lateral view of the tail showed bmp4 expression in the tail fin in WT (I, dashed line) and normal sox4aC2 MZ (J, dashed line) embryos at 24 hpf. Bmp4 expression in the mutant with tail curvature showed an incomplete expression pattern (K, dashed line).
3.4 Discussion

CRISPR/Cas9 genome editing tool is a revolutionary technique that allows targeted genome editing in a large variety of model organisms. In this study, we successfully applied the CRISPR/Cas9 genome editing system to generate \textit{sox4a} and \textit{sox4b} mutant zebrafish lines for the first time. The efficiency of mutagenesis in F0s was as high as 92\%, and through the process of founder fish screening, we also observed a high rate of germ line transmission ranging from 75\% (3 in 4 fish) to 100\% (2 in 2 fish). This robust targeted genome editing allowed us to generate six \textit{sox4} single mutant lines and two \textit{sox4a/b} double mutant lines in a time period of 16 months. We were able to target each \textit{sox4} gene with a single sgRNA molecule or two sgRNA molecules simultaneously. All the single mutant lines were fertile and were able to stably pass the mutant allele to the next generation, providing us a valuable opportunity to study the function of Sox4a and Sox4b during embryonic ocular development and adult ocular regeneration.

Similar to Sox4-deficient embryos generated by morpholino injection, coloboma was also observed in the \textit{sox4aC2} MZ mutants, and in agreement with our previous hypothesis that the ocular defects were contributed by elevation in Hh signaling, altered Hh and Bmp signaling were observed in \textit{sox4aC2} MZ mutants as well, indicating a) the morphant phenotypes were true phenotypes of Sox4 deficiency; b) loss of \textit{sox4a} alone is sufficient to affect ocular morphogenesis and Hh signaling. We have noticed that although the phenotypes of \textit{sox4} morphants and \textit{sox4aC2} mutants were similar, the incidence of coloboma was much lower in the mutants and the upregulation of \textit{ihhb} in mutants was not as high as in morphants. A possible explanation is that the morphants are deficient in both \textit{sox4a} and \textit{sox4b}, but the \textit{sox4aC2} MZ mutant still has a functional Sox4b. Due to overlapping spatiotemporal expression and functional redundancy, the presence of Sox4b may partially compensate for the loss of Sox4a, resulting in the milder ocular phenotype in \textit{sox4aC2} mutants.
In \textit{sox4} morphants, we carefully controlled the morpholino dosage to produce ocular defects with minimal systematic abnormality to reduce the chance of off-target phenotypes. Although pericardial edema was occasionally observed in \textit{sox4} morphants, we did not analyze its correlation with Sox4-deficiency (Wen et al., 2015). In the \textit{sox4aC2} MZ mutant, heart edema was observed in a significant number of embryos by 72 hpf (~30%), indicating that \textit{sox4a} is required for proper heart development. This is in agreement with studies in mice, which have shown that Sox4 is essential for heart development. \textit{Sox4} null mice die from circulatory failure at embryonic day 14 (E14) due to defects in endocardial ridge development, which prevents proper formation of the semilunar valves (Schilham et al., 1996).

The \textit{sox4aC2} mutant phenotype also supports our hypothesis that Sox4 is required for periocular and ocular tissue survival. Similar to what we have observed in \textit{sox4} morphants, we detected a significant increase in cell death in \textit{sox4aC2} mutants at early developmental stages when \textit{sox4} is highly expressed in the periocular tissues. Sox4 is closely associated with the regulation of apoptosis, although its exact role is controversial. On the one hand, Sox4 acts as a pro-apoptotic factor in many cancer cells. A glycine-rich region in human \textit{SOX4}, named \textit{CD}, is identified as a pro-apoptotic domain to induce apoptosis (Hur et al., 2004). In response to DNA damage in many cancer cells, \textit{Sox4} expression is upregulated and it stabilizes p53 protein by blocking Mdm2-mediated p53 ubiquitination and degradation; hence Sox4 promotes p53-mediated apoptosis in DNA damage response (DDR)-associated cancers (Pan et al., 2009). In addition, Sox4 also promotes apoptosis by inducing PUMA-mediated apoptosis in a p53-independent fashion (Jang et al., 2013). On the other hand, Sox4 can promote cell survival in some other types of cancer cells. Reduced expression of \textit{Sox4} in adenoid cystic carcinoma (ACC) cells leads to an increase in apoptosis (Pramoonjago et al., 2006). \textit{Sox4} overexpression in hepatocellular carcinoma (HCC) cells represses the expression of p53-mediated pro-apoptotic gene \textit{Bax} and inhibits apoptosis (Hur et al., 2010). During normal embryonic development, Sox4 also acts as a cell survival factor, such
that reduced Sox4 expression results in increased cell death in the developing spinal cord, retina, and pro-B cells (Cizelsky et al., 2013; Sun et al., 2013; Thein et al., 2010).

A novel sox4aC2 mutant phenotype that we observed is the reduced percentage of fertilization from eggs produced by homozygous females. We also observed a reduced number of adult female sox4aC2 MZ homozygous mutant fish compared to male homozygous mutant fish. In addition, sox4aC2; sox4bC2.2 double mutant females produced a significant number of nonviable eggs and the double mutant male was not able to stimulate spawning behavior from females. We cannot rule out that these phenomena are special individual cases that are not relevant to the loss of sox4a and/or sox4b. However, it still draws our attention to the possibility that Sox4 might affect the differentiation and maturation of female reproductive tissues in zebrafish.

Although it is not clear what role Sox4 plays in the development of reproductive tissues, it has been reported to be strongly expressed in mouse ovary, mammary glands and uterus at pubertal and adult stages (Hunt and Clarke, 1999; van de Wetering et al., 1993), and in human breast cells that are normal or malignant (Graham et al., 1999). Its expression is positively regulated by progesterone and inhibited by estrogen (Graham et al., 1999; Hunt and Clarke, 1999). The control of sox4 expression by sex hormones suggests changes in sox4 expression may be involved in reproductive tissue development during different stages of the estrus cycle. A mouse Sox4 mutant allele with flox sites in the 5’ and 3’ UTR causes a significant reduction in the number of female Sox4\textsuperscript{flox/flox} mice compared with male Sox4\textsuperscript{flox/flox} mice, suggesting a disruption of Sox4 may affect female survival or sex determination (Wiles et al., 2014). In teleosts, sox4 expression in reproductive tissues has not been reported. However, sox3, sox8b and sox9b are expressed in the oocytes of sea bream (Zapater et al., 2013) and sox24 (which also belongs to the SoxC family) is expressed in the oocytes of rainbow trout (Kanda et al., 1998). In zebrafish, sox4b is expressed in the developing pituitary and is required for the differentiation of gonadotrope cells by inducing the expression of a zinc finger transcription factor gata2 (Quiroz et al., 2012). Gonadotrope cells
in the anterior pituitary are endocrine cells that secret gonadotrophins such as follicle stimulating hormone (FSH) and luteinizing hormone (LH). As a result, sox4 loss of function mutations may affect female reproductive tissue development and maturation.

It seems that the unfertilized egg phenotype was highly correlated with maternal loss of sox4a while the microphthalmia/coloboma, tail curvature, and heart edema phenotypes were contributed by both the maternal and zygotic loss of sox4a. It is highly likely that maternal expression of sox4 is required for both proper oogenesis in female fish and for proper ocular development in fertilized zebrafish embryos (Figure 3.12). Based on the fact that not all eggs from sox4aC2 homozygous females were unfertilized, and not all sox4aC2 MZ mutants exhibit mutant phenotypes, it is reasonable to hypothesize that the maternal loss of sox4a in the surviving embryos may be compensated for by other genes (e.g. sox4b and/or sox11) or through alterations in cell signaling. If the requirement for sox4a in oogenesis and ocular morphogenesis share overlapping mechanisms, is it possible that those embryos that bypassed the defects in oogenesis may also exhibit milder or no ocular defect phenotypes.

In the future, we can separate the effects of maternal loss of sox4 and zygotic loss of sox4 by generating tissue-specific sox4 mutant zebrafish lines. Successful tissue-specific knockout using CRISPR/Cas9 system has been reported in zebrafish (Ablain et al., 2015). One challenge of this strategy is to choose an appropriate promoter to drive Cas9 expression, which can knockout sox4 spatiotemporally in the correct tissue and during the critical time period in the eye. As we have reported previously, sox4a and sox4b are expressed in multiple regions in the anterior of the embryo, including the forebrain, the diencephalon, and different retinal cells during retinal neurogenesis (Wen et al., 2015). To knock out sox4 in the entire embryonic eye field, one candidate promoter to drive Cas9 expression is the promoter of the rx3 gene. Rx3 is one of the earliest eye field markers that regulate eye field specification and it is expressed in the eye field as early as the 70% epiboly stage during gastrulation, when eye field segregation is initiated.
(Stigloher et al., 2006). However, Cas9 expression driven by the \textit{rx3} promoter cannot affect \textit{sox4} expression in the forebrain and the diencephalon since \textit{rx3} is not expressed in these tissues. In addition, \textit{sox4a} is also expressed in the periocular mesenchymal tissues around the eye and adjacent to the optic stalk (Wen et al., 2015). To knock out \textit{sox4} expression in the periocular cells, a mesenchymal cell promoter such as \textit{sox10} may be appropriate (Hoffman et al., 2007).
Table 3.1. Primer sequences used in Chapter 3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>sox4aC1</td>
<td>TAGGGCACAGACCTGGCTATGG</td>
<td>AAACCCATAGCCAGGTCTGTGC</td>
<td>sgRNA synthesis</td>
</tr>
<tr>
<td>sox4aC2</td>
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<td>AAACTTCCGCTCGAGGCAAGCGGTG</td>
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</tr>
<tr>
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<td>AAACCTGGCCGTTTTCGACCAG</td>
<td>sgRNA synthesis</td>
</tr>
<tr>
<td>sox4bC2</td>
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<td>sgRNA synthesis</td>
</tr>
<tr>
<td>dr274</td>
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<td>AGCACCCGACTCGGTCCAC</td>
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<td>HRMA</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>TGGCATTTTGGGCGATCAGAG</td>
<td>qPCR</td>
</tr>
</tbody>
</table>
3.5 Materials and methods

3.5.1 Zebrafish strains and maintenance

All zebrafish (*Danio rerio*) strains were bred, raised, and maintained at 28.5°C on a 14 hour light:10 hour dark cycle according to established protocols (Westerfield, 2000). The Tg(XIRho:EGFP)^{fl1} transgenic line (hereafter referred to as XOPS:GFP) was a generous gift from J.M. Fadool (Florida State University, Tallahassee, FL) and has been previously described (Fadool, 2003b). Embryos were staged according to established developmental hallmarks (Kimmel et al., 1995). All animal procedures were carried out in accordance with guidelines established by the University of Kentucky Institutional Animal Care and Use Committee.

3.5.2 sgRNA and Cas9 mRNA synthesis and injection

*Sox4a* and *sox4b* CRISPR target sites were identified and the corresponding sgRNA oligos were designed using the ZiFiT online software (www.zifit.partners.org/ZiFiT/; Table 3.1). Oligo pairs (100µM) for each sgRNA were mixed with NEBuffer4 (New England Biolabs, Ipswich, MA), incubated in boiling water for 5 minutes, followed by 2 hours annealing at room temperature, and then ligated with linearized pDR274 vector (Addgene, Cambridge, MA) at 16°C overnight. Recombinant plasmid was digested with DraI to drop out the sgRNA template, followed by PCR amplification (Table 3.1) using the KOD Hot Start Master Mix (Millipore, Billerica, MA) and purification using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). sgRNA was generated using the MEGAscript T7 Transcription Kit (Life Technologies, Carlsbad, CA). To prepare the tyr sgRNA, pT7tyrgRNA (Addgene, Cambridge, MA) was linearized with BamHI and sgRNA was synthesized using MEGAscript T7 Transcription Kit (Life Technologies). To generate the *Cas9* mRNA, pCS2-nCas9n or pTST3-nCas9n plasmid (Addgene, Cambridge, MA) was linearized with NotI or XbaI respectively, and capped Cas9 mRNA was synthesized using the mMESSAGE mMACHINE SP6 or T3 Transcription Kit respectively (Life Technologies). To confirm the quality of sgRNA and *Cas9* mRNA, RNA was mixed with formamide, heated at
72°C for 5 minutes and run on a 1% (wt/vol) agarose gel. The following sgRNA and Cas9 mRNA
doses were microinjected into embryos at the one-cell stage: 100 pg/embryo of sox4a or sox4b
sgRNA + 300 pg/embryo of Cas9 mRNA; 50 pg/embryo of tyr sgRNA + 150 pg/embryo of Cas9
mRNA.

3.5.3 Genomic DNA extraction
To isolate genomic DNA from embryos, 24 hpf dechorionated embryos were placed into
individual wells of a 96-well plate containing 20 µl of 1X ThermoPol Buffer (New England
Biolabs, Ipswich, MA). To extract genomic DNA from adult zebrafish, tail fins were cut using a
razor blade and placed into individual wells of a 96-well plate containing 20 µl of 1X ThermoPol
Buffer (New England Biolabs, Ipswich, MA). The plate was placed in a PCR cycler at 95°C for
10 minutes, after which 5 µl of 10 mg/ml Proteinase K (Sigma, St. Louis, MO) was added to each
well and the plate was incubated at 55°C for 1 hour and 95°C for 10 minutes.

3.5.4 HRMA analysis
HRMA analysis was performed on a LightCycler 96 Real-Time PCR System (Roche,
Indianapolis, IN) using LightCycler 480 High Resolution Melting Master (Roche), following the
manufacturer’s instructions. Primer sequences used for HRMA are listed in Table 3.1.

3.5.5 Sanger sequencing
CRISPR target sites were amplified from embryo or adult tail genomic DNA. Purified PCR
products were either sequenced directly or cloned into pGEM®-T Easy Vector (Promega,
Madison, WI). Samples were sequenced bi-directionally using the same primers used in the PCR
reaction. Sequencing results were analyzed using Clustal Omega
(http://www.ebi.ac.uk/Tools/msa/clustalo).
3.5.6 Restriction Fragment Length Polymorphism (RFLP)

The *sox4aC1* mutant allele contains mutations that destroy the BstNI recognition site in the WT allele. The *sox4aC2* mutant allele contains mutations that destroy the BsrBI recognition site in the WT allele. These regions were PCR amplified and digested with BstNI or BsrBI (NEB, Ipswich, MA) respectively. Products were resolved with 2% agarose gel electrophoresis and visualized with EtBr staining.

3.5.7 Reverse transcript PCR and real-time quantitative RT-PCR

Total RNA was extracted from the whole body of WT or mutant embryos at 24 hpf using TRIzol reagent (Invitrogen, Grand Island, NY). RNA was reverse-transcribed using the GoScript Reverse Transcriptase System (Promega, Madison, WI). PCR was performed using Taq DNA polymerase with thermoPol buffer (NEB, Ipswich, MA). Real time PCR was performed using FastStart Essential DNA Green Master (Roche, Indianapolis, IN) on a LightCycler 96 Real-Time PCR System (Roche, Indianapolis, IN). For all experiments, three biological replicates were analyzed, and relative transcript abundance was normalized to expression of the housekeeping gene *gapdh*. Primers used for this experiment were listed in Table 3.1.

3.5.8 Acridine orange staining

Twenty four hpf WT and mutant embryos were incubated in the dark with 5 ug/mL acridine orange (Sigma) for 10 minutes, then rinsed in fish water for 30 seconds. Embryos were then transferred to a glass bottom Petri dish with 1% low melting temperature (LMT) agarose+ MS 222 to hold them in position. Images were obtained on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments) using the 10X objective with GFP filter.
3.5.9 Immunohistochemistry and cell counts

Immunohistochemistry was performed on cryosections or whole zebrafish embryos as previously described (Fadool, 2003a; Forbes-Osborne et al., 2013). Images were obtained on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments) using the 20X objective. The following primary antibodies and dilutions were used: anti-activated-Caspase 3 (rabbit, 1:500, Abcam, Cambridge, MA). 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. Samples were counterstained with DAPI (1:10,000, Sigma, St. Louis, MO) to visualize cell nuclei.

3.5.10 Whole mount in situ hybridization (WISH)

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 SP6, T7, or T3 polymerase and digoxigenin (DIG) labeling mix (Roche Applied Science, Indianapolis, IN). The gapdh plasmid was prepared by cloning PCR products into the pGEM-T-easy vector (Promega, Madison, WI). Primers used for PCR are listed in Table 3.1. The bmp4 plasmid (Dick et al., 2000) was generously provided by Matthias Hammerschmidt (University of Cologne, Koeln, Germany). WISH was performed as previously described (Forbes-Osborne et al., 2013; Pillai-Kastoori et al., 2014). Images were obtained on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments).

3.5.11 Statistical analyses

Significance was calculated using a two-tailed Student’s t-test or Fisher’s exact test, with P<0.05 being considered significant. For all graphs, data are represented as the mean ± the standard deviation (s.d.).
3.6 Acknowledgments

I would like to thank Sara Perkins for zebrafish care, Abirami Krishna for mutant fish screening and breeding assistance, and the laboratory of Dr. Jakub Famulski and Dr. Ashley Seifert for technical assistance. I also thank Dr. Hammerschmidt from Cologne University Institute of Developmental Biology for kindly sharing their plasmids for generating BMP signaling molecule WISH probes.
CHAPTER 4: LOSS OF SOX4 DELAYS ROD PHOTORECEPTOR DIFFERENTIATION

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KEYWORDS: rod photoreceptor; Sox4

4.1 Abstract
Rod photoreceptors are generated from a lineage of committed progenitor cells. The genetic regulation of rod lineage differentiation is not fully elucidated. We found that sox4a and sox4b were both upregulated in a chronic rod degeneration and regeneration transgenic adult retina, suggesting their potential involvement in rod neurogenesis. Using morpholino-mediated sox4 knockdown and sox4 CRISPR mutant zebrafish, we observed a significant reduction in rod photoreceptor number, specific to Sox4 deficiency. Embryonic rod photoreceptor neurogenesis was delayed while the expression of rod lineage cell markers was not affected by sox4 knockdown, indicating that Sox4 was not required for rod lineage cell specification but may be required for the terminal differentiation of rod photoreceptors. The sox4aC2 MZ mutant retina exhibited a similar reduction in rod neurogenesis at embryonic and early juvenile stages while late juvenile and adult rod photoreceptor population was not affected. These findings indicate that Sox4 regulates rod photoreceptor neurogenesis during embryonic stages, but is not required for the persistent rod neurogenesis in adult zebrafish.

4.2 Introduction
Rod photoreceptors are light-sensitive neurons located in the posterior part of the retina that can detect dim light and which play important roles in both day and night vision. Defects in rod photoreceptor function can lead to severe vision loss. For example, patients with retinitis
pigmentosa suffer from night blindness and tunnel vision or complete vision loss, which is caused by the loss of rod photoreceptors and a subsequent degeneration of cone photoreceptors as well (Hartong et al., 2006). Proper rod photoreceptor neurogenesis requires intrinsic cell fate determination, extrinsic regulation of cell-cell and soluble molecule contact, and massive cell migrations and connections (See (Brockerhoff and Fadool, 2011; Stenkamp, 2011) for a comprehensive review of rod neurogenesis and regeneration). Our understanding of the genetic networks that regulate rod photoreceptor development is far from complete. The long-term goal of our research is to fill in gaps of the genetic network for retinal neurogenesis and provide valuable data for potential therapeutic treatments for patients with photoreceptor disorders.

4.2.1 Persistent retinal neurogenesis in zebrafish

As a diurnal vertebrate with conserved retinal structure, the zebrafish is a favorable model to study retinal development and retinal related human diseases. Consistent with mammals, the zebrafish retina contains six types of neurons and one type of glia arranged into three layers. Unlike mammals, the zebrafish retina continues to grow postembryonically. There are two sources of retinal stem cells in the postembryonic zebrafish retina: a) Multipotent stem cells reside in the ciliary marginal zone (CMZ) at the periphery of the retina. The CMZ is a remnant of the embryonic retinal neuroepithelium. Stem cells in the CMZ proliferate continuously throughout the life of zebrafish and differentiate into new neurons (except for rods) which are added to the peripheral retina in an annular fashion like the growth rings in a tree (Johns, 1977; Raymond, 1986). b) The second source of retinal stem cells is the Müller glia (Bernardos et al., 2007); a subset of Müller glia, seeded into the retina during embryonic retinal neurogenesis, contributes exclusively to rod photoreceptor neurogenesis during normal retinal growth. The density of rod photoreceptors decreases as the retina grows in size. To preserve visual sensitivity, new rod photoreceptors are generated from a lineage of cells (the rod lineage) derived from the Müller glia and are inserted into the outer nuclear layer of the mature retina. Müller glia cells are
also the source of progenitor cells in response to retinal damage and regeneration (discussed in detail in 4.2.3).

4.2.2 Rod lineage in zebrafish

The presence of precursor cells in the ONL of the teleost retina that differentiate specifically to rod photoreceptors was discovered more than thirty years ago. When $^3$H-thymidine was injected intraocularly into adult goldfish or cichlids to label proliferating cells, labeled cells were found in the ONL and eventually integrated into the retina exclusively as rod photoreceptors (Johns, 1982; Johns and Fernald, 1981). Several following studies further suggested that these rod precursors in the ONL were derived from proliferative cells in the INL (rod progenitors) and migrated to the ONL along the Müller glia processes (Julian et al., 1998; Otteson et al., 2001; Raymond and Rivlin, 1987). Recently, the rod progenitor cells in the INL were proved to be derived from Müller glia (Bernardos et al., 2007; Morris et al., 2008a; Morris et al., 2008b; Nelson et al., 2008).

During normal retinal neurogenesis, Müller glia cells slowly divide in the INL and produce fusiform shaped daughter cells, the rod progenitor cells, which form clusters and migrate to the ONL along the Müller cell processes to become rod precursors. The rod precursors further divide and undergo terminal differentiation to become rod photoreceptors.

The differentiation of the rod lineage cells is under precise genetic control. Rod progenitors are derived from $pax6^+$ Müller glial cells (Bernardos et al., 2007). Once committed to the rod lineage, these cells cease expression of $pax6$ and start to express transcription factors such as neuroD, $rx1$, and $crx$ (Liu et al., 2001b; Nelson et al., 2008; Ochocinska and Hitchcock, 2007). The expression of an orphan nuclear receptor $nr2e3$ in the rod lineage cells initiates when cells are in the distal region of INL. Once in the base of ONL, rod precursors become molecularly homogeneous and
express neuroD, rx1, crx, and nr2e3. Terminally differentiated rod photoreceptors that are post-mitotic express all these transcription factors as well as rod opsin (Nelson et al., 2008).

4.2.3 Rod regeneration in response to rod photoreceptor loss in zebrafish

Damaged mammalian retina undergoes Müller glia hypertrophy that results in retina disorganization and scarring with poor retinal regeneration (Bringmann et al., 2006). In contrast, zebrafish retina is capable of regenerating any retinal cell type in response to mechanical, chemical, and genetic injuries. Zebrafish Müller glia cells act as multipotent retinal stem cells under retinal injury conditions. In response to retinal injury or chronic retinal neuron degeneration, zebrafish Müller glia re-enter the cell cycle and produce retinal progenitor cells that migrate to the appropriate location corresponding to retinal injury, and differentiate into proper retinal neurons that restore retinal function by integrating into the surrounding retina (Bernardos et al., 2007; Fausett et al., 2008; Yurco and Cameron, 2005). Notch-Delta signaling activity, pax6 and ascl1a expression are up-regulated in Müller glia during retinal regeneration (Fausett et al., 2008; Thummel et al., 2010; Wilson et al., 2015; Yurco and Cameron, 2007).

Several retinal regeneration models specific to the loss of rod photoreceptors suggested a distinct rod photoreceptor regeneration pathway separate from the regeneration of other retinal neurons. When rod photoreceptors were pharmacologically ablated in goldfish by tunicamycin, only the rod precursor cells in the ONL but not INL rod lineage cells underwent cell proliferation and differentiation into new rod photoreceptors (Braisted and Raymond, 1993). Another model was the Tg(XRho:gap43-mCFP) q13 transgenic zebrafish line (XOPS:mCFP) that expresses a membrane-targeted CFP in rod photoreceptors driven by the Xenopus rod opsin promoter (Morris et al., 2005). This transgenic line was a chronic rod degeneration and regeneration model. Rod photoreceptors died shortly after the initiation of rod opsin expression and cell proliferation was limited only in the ONL. An acute rod photoreceptor degeneration model was the Tg(zop:nfsB-EGFP) (nt20) transgenic zebrafish (Montgomery et al., 2010). A subset of rod photoreceptors of this
transgenic line expressed a fused protein of the *E. coli* nitroreductase and GFP driven by the zebrafish rod opsin promoter. Exposure of metronidazole induced these rod photoreceptors to synthesize cytotoxin, which killed them. And a specific proliferation of rod precursors in the ONL was stimulated. Interestingly, in a similar transgenic line Tg(zop:nfsB-EGFP)(nt19) that killed all the rod photoreceptors, cell proliferation was both stimulated in rod precursor cells in the ONL and Müller glia in the INL, suggesting that the genetic ablation of all rod photoreceptors in this transgenic line not only stimulates the proliferation of rod precursor cells, but also triggers dedifferentiation of Müller glia cells to generate rod progenitor cells.

4.2.4 SoxC and rod photoreceptor neurogenesis

Although there is a lack of data for rod neurogenesis in SoxC-deficient mouse models due to their prenatal death prior to the differentiation of the majority of rod photoreceptors, evidence has emerged for the requirement of proper levels of SoxC expression in rod neurogenesis. Several rod lineage specific genes were reduced in the Sox11 null mouse retina at E16 (Usui et al., 2013b). Conditional Sox4/11 double knockout in the mouse retina resulted in a dramatic loss of retinal neurons including photoreceptors (Jiang et al., 2013b). In vitro culture of *Sox4* and *Sox11* overexpression mice retina at E17 for two weeks revealed a significantly decreased number of rod photoreceptors (Usui et al., 2013b). Early expression of *Sox4* and *Sox11* contributed to precocious rod neurogenesis and subsequent rod degeneration in mice with a retinoblastoma (Rb) mutation (Benavente et al., 2014). In addition, *sox11* was reported to regulate rod neurogenesis in zebrafish and knockdown of *sox11* in zebrafish embryonic retina caused significant reduction in the number of mature rod photoreceptors (Pillai-Kastoori et al., 2014). These data indicate that proper expression level and proper expression timing of SoxC proteins are both important for rod photoreceptor neurogenesis.
Previously, *sox4a* and *sox4b* were both reported to be upregulated about 3-fold in the XOPS:mCFP adult zebrafish retina by microarray analysis (Morris et al., 2011a), suggestive of their potential relationship with rod photoreceptor degeneration and regeneration. The negative regulation of Hh activity also suggests a potential requirement of *sox4* for rod photoreceptor differentiation, since proper levels of the extraretinal and retinal Hh signaling were required for retinal neurogenesis, including rod photoreceptor differentiation and survival (Stenkamp and Frey, 2003; Stenkamp et al., 2000; Yu et al., 2006). As a result, we hypothesize that *sox4* may also be involved in rod photoreceptor cell neurogenesis. Here, we examined the effect of loss of *sox4* on zebrafish retinal neurogenesis, especially on the differentiation of rod photoreceptors using *sox4* morphants as well as CRISPR mutants.

### 4.3 Results

4.3.1 *Sox4* is upregulated in Tg(XRho:gap43-mCFP) q13 chronic rod degeneration and regeneration of transgenic adult zebrafish retinas

To confirm the previous microarray analysis, RT-PCR was performed on age matched WT and XOPS:mCFP adult retinas to examine the expression level of *sox4a* and *sox4b*. Both *sox4a* and *sox4b* were upregulated in the XOPS:mCFP retina (Figure 4.1E). This upregulation still needs to be confirmed by qPCR and in-situ hybridization. Preliminary in-situ hybridization analysis revealed that *sox4a* was only expressed in a few cells at the CMZ in the WT adult retina (Figure 4.1A, arrow), but not detected in the central WT retina (Figure 4.1C). *Sox4a* expression was observed in more cells in the XOPS:mCFP adult retina CMZ (Figure 4.1B, arrows). Intriguingly, *sox4a* was also detected in a few cells located at the base of the ONL in the XOPS:mCFP central retina, where the rod precursors reside (Figure 4.1D, arrows); however, *sox4a*-positive cells in the ONL were not observed in all retinas examined. In addition, some *sox4a* positive cells were found at the base of XOPS:mCFP INL (Figure 4.1B, 4.1D, arrowheads), which might be microglia based on their small and round morphology, though more confirmation needs to be
done. Taken together, these preliminary results suggest that Sox4 is upregulated in response to rod photoreceptor loss and it may play a role in rod photoreceptor regeneration.
Figure 4.1. Upregulation of *sox4a* and *sox4b* in the XOPS:mCFP adult retina. (A-B) Expression of *sox4a* in the adult WT (A) and the XOPS:mCFP (B) peripheral retina detected by FISH. (C-D) Expression of *sox4a* in the adult WT (C) and the XOPS:mCFP (D) central retina detected by FISH. (E) Levels of *sox4a* and *sox4b* mRNA in the WT and the XOPS:mCFP adult retina detected by RT-PCR. *β-actin* serves as a housekeeping gene. Arrows, expression in retinal neurons; arrowheads, expression in potential microglia cells; CMZ, ciliary marginal zone; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars equal to 50 µm.
4.3.2 Sox4 knockdown delays embryonic rod photoreceptor neurogenesis

4.3.2.1 Sox4 morphants exhibit fewer rod photoreceptors in the embryonic retina

The upregulation of sox4a and sox4b in the XOPS:mCFP adult retina suggested that they might be involved in rod photoreceptor regeneration. Since regeneration partially recapitulates the process of development, we first tested the requirement for sox4 in rod photoreceptor neurogenesis during embryonic development. Sox4 deficient embryonic retinas were generated by sox4a and sox4b morpholino injection. To minimize the secondary effect of coloboma on retinal neurogenesis, we divided the sox4 morphants into groups that displayed coloboma and those without coloboma. Control and sox4 morphant embryos at 72 hpf were cryosectioned and labeled with retinal specific cell markers by immunohistochemistry. In sox4 morphants with coloboma, we observed a severe disruption in retinal lamination and neurogenesis (Figure 4.2A, 4.2B). Occasionally, we also observed ectopically located rod photoreceptors in the INL of these morphants, usually clustered together and forming a rosette structure (Figure 4.3F, S4.1). In sox4 morphants with no coloboma, all the other retinal neurons were comparable with WT in terms of cell numbers and cell morphology except for rod photoreceptors (Figure 4.2A, 4.2C). Rod photoreceptors on the other hand were significantly reduced, revealed by the XOPS:GFP transgene or 4C12 antibody labeling. A few rods were present in the ventral patch of the retina in sox4 morphants, but were significantly reduced or completely absent in the dorsal and central retina (Figure 4.2B). The reduction of rod photoreceptors in sox4 MOs was confirmed by qPCR for the mature rod marker rhodopsin, which was significantly reduced in sox4 morphants (Figure 4.2D).
Figure 4.2. *Sox4* morphants exhibit fewer rod photoreceptors in the embryonic retina. (A) Immunohistochemistry labeling of different retinal neurons including retinal ganglion cells, amacrine cells, horizontal cells, bipolar cells, cone photoreceptors, and Müller glia in control and *sox4* morphants retina with or without coloboma at 5 dpf. (B) Representative images of rod photoreceptors visualized by the XOPS:GFP transgene in control and *sox4* morphants retina with or without coloboma at 3 dpf. (C) Numbers of each cell type were counted in control and *sox4* morphants retina without coloboma at 5 dpf. The number of rod photoreceptors was significantly reduced in *sox4* morphants (Student’s t-test, P<0.01). Only sections containing the optic nerve were used for counting. At least 10 embryos were analyzed for each category. (D) Quantitative RT-PCR revealed a significant reduction in *rhodopsin* gene expression in *sox4* morphants. *n*=30/category. Three biological replicates were performed for each group. (Student’s t-test, P<0.01). All scale bars equal 100 µm.
Figure S4.1. Displaced rod photoreceptors in sox4 morphants with coloboma. Representative images of control MOs and sox4 MOs with coloboma with ectopic rod photoreceptors. Rod precursor gene *nr2e3* and *rhodopsin* were labeled by two colored FISH. In control MOs, mature rod photoreceptors were organized in the ONL and colocalized with *nr2e3*. In sox4 MOs with coloboma, very few mature rod photoreceptors were present in the ONL. In addition, ectopic clusters of rod photoreceptors were displaced in the INL and appeared as a rosette (arrow). Scale bar equals 100 µm.
4.3.2.2 Rod photoreceptor terminal differentiation is delayed in the sox4 morphant retina

To examine whether the reduction of rod photoreceptors in sox4 MOs was caused by defects in rod lineage cell specification or rod terminal differentiation, we tested the expression of the rod lineage specific genes neuroD, crx, and nr2e3 in WT and sox4 morphants at 72 hpf by FISH. Interestingly, all three genes were normally expressed in sox4 morphants (Figure 4.3A-I). In addition, qPCR also confirmed that nr2e3 expression level was comparable between control and sox4 morphants (Figure 4.3J). These results suggested that rod lineage cells were properly specified in sox4 morphants with or without coloboma and the reduction of rods in sox4 MOs probably was due to defects in rod photoreceptor terminal differentiation.
Figure 4.3. *Sox4* morphants exhibit reduced numbers of mature rod photoreceptors. (A–C) Representative images of two colored FISH labeling neuroD and rhodopsin in WT (A), *sox4* morphants without coloboma (B), and *sox4* morphants with coloboma (C) at 72 hpf. (D–F) Representative images of two colored FISH labeling crx and rhodopsin in WT (D), *sox4* morphants without coloboma (E), and *sox4* morphants with coloboma (F) at 72 hpf. (G–I) Representative images of two colored FISH labeling *nr2e3* and rhodopsin in WT (G), *sox4* morphants without coloboma (H), and *sox4* morphants with coloboma (I) at 72 hpf. (J) Quantitative RT-PCR performed on mRNA from control and *sox4* morphants heads at 72 hpf revealed that *nr2e3* transcript levels were comparable between control and *sox4* morphants. Relative transcript abundance was normalized to *gapdh*. n=30/category. Three biological replicates were performed for each category (Student’s *t*-test, *P*<0.01). Scale bar equals 100 µm.
To further examine whether rod photoreceptor terminal differentiation was arrested or just delayed in sox4 morphants, we reexamined the number of mature rods in WT and sox4 morphants at 72 hpf and added a later developmental time point at 96 hpf. Only sections that crossed the optic nerve (ON) were chosen and the numbers of rods were counted ventral and dorsal to the ON. Consistent with previous observation, the number of rods at 72 hpf was significantly reduced in sox4 morphants with or without coloboma at both the ventral retina and dorsal retina (Figure 4.4A-C, 4.4G). However, by 96 hpf, numbers of rods were comparable between control and sox4 MOs with no coloboma, suggesting that rod terminal differentiation has caught up in sox4 MOs without coloboma (Figure 4.4D-E, 4.4G, student’s t-test, p<0.05). Alternatively, the increase in rod cell number in sox4 morphants at 96 hpf could be due to dilution and loss of effectiveness of the morpholino. The number of rods in sox4 morphants with coloboma was also increased by 96 hpf, although it did not reach the number of rods in controls, especially in the ventral retina (Figure 4.4F, 4.4G). This probably was due to secondary effects of the coloboma in the ventral eye that inhibited neurogenesis.

Taken together, these data suggest that Sox4 deficiency leads to a delay in rod photoreceptor terminal differentiation while rod lineage specification is not affected.
Figure 4.4. Recovery of rod photoreceptor numbers in sox4 morphants at 96 hpf. (A-C) Representative images of two colored FISH labeling neuroD and rhodopsin in control (A), sox4 morphants without coloboma (B), and sox4 morphants with coloboma (C) at 72 hpf. (D-E) Representative images of two colored FISH labeling neuroD and rhodopsin in control (D), sox4 morphants without coloboma (E), and sox4 morphants with coloboma (F) at 96 hpf. (G) Quantification of the number of rod photoreceptors in control and sox4 morphants at 72 and 96 hpf. The numbers of rod photoreceptors dorsal and ventral to the optic nerve were counted. n=15 individuals per category. Student’s t-test, p<0.05. Scale bar equals 100 µm.
4.3.3 *Sox4aC2* MZ mutants exhibit fewer rods in both embryonic and early juvenile retinas

4.3.3.1 Comparison of embryonic rod photoreceptors among *sox4* CRISPR mutant lines

Given that *sox4* MOs showed a delay in rod photoreceptor neurogenesis, we were curious to know whether rod neurogenesis was affected in *sox4* mutants. We took advantage of the *sox4* mutant zebrafish lines generated by CRISPR/Cas9 to determine the requirement for Sox4 in rod neurogenesis at embryonic and juvenile stages. We first examined the presence of rod photoreceptors in established *sox4a* and/or *sox4b* CRISPR mutant lines at 5 dpf when the embryonic retina was fully laminated. Embryos at 5 dpf were collected from WT and six different *sox4* mutant lines, including *sox4aC2* MZ, *sox4aC2* Z, *sox4b C2.2* MZ, *sox4bC2.2* Z, *sox4aC2 Z*, *sox4bC2* Z double mutant, and *sox4bC1+C2* Z. Since all the lines were in the XOPS:GFP background, in which mature rod photoreceptors express GFP, rod photoreceptors were visualized by whole-mount confocal imaging of the GFP signal (Figure 4.5).

In WT, rod photoreceptors were observed to be densely packed in the ventral patch of the retina where they initially differentiate and scattered throughout the dorsal and central retina (Figure 4.5A). Among all the *sox4* mutant lines, the *sox4aC2* MZ mutant retina had the most noticeable reduction in rod photoreceptors. Images of rod photoreceptors were obtained from both normal looking and abnormal looking *sox4aC2* MZ mutant individuals. All *sox4aC2* MZ individuals exhibited smaller eye size and reduced rod photoreceptors compared to WT (Figure 4.5B, 4.5C). Some abnormal individuals also showed an open choroid fissure and a few rod photoreceptors were observed around the coloboma area (Figure 4.5C). For the other five *sox4* mutant lines, the presence of rod photoreceptors seemed to be comparable to WT (Figure 4.5 D-G), except for *sox4bC1+C2* Z, in which some individuals appeared to have fewer rods around the optic nerve region compared to WT (Figure 4.5H). It is possible however, that more precise quantification of rod photoreceptor number in the other mutant lines will reveal a subtle reduction in those retinas as well.
Figure 4.5. Comparison of embryonic rod photoreceptors among sox4 CRISPR mutant lines on XOPS:GFP background. Representative image of the overall organization and appearance of rod photoreceptors in WT (A), maternal zygotic sox4aC2 mutant with microphthalmia but no coloboma (B), maternal zygotic sox4aC2 mutant with coloboma (C), zygotic sox4aC2 mutant (D), maternal zygotic sox4bC2.2 mutant (E), zygotic sox4bC2.2 mutant (F), zygotic sox4aC2 and sox4bC2.2 double mutant (G), and zygotic sox4bC1+C2 mutant (H), visualized by expression of the transgene XOPS:GFP at 5 dpf. ON, optic nerve; D, dorsal; V, ventral. Scale bar equals 100 µm.
4.3.3.2 The sox4aC2 MZ mutant retina has a specific reduction in the number of rod photoreceptors

We further examined rod photoreceptor neurogenesis in sox4aC2 MZ mutants in a longer time period spanning from embryonic to juvenile and adult stages. Retinas of sox4aC2 MZ mutants (abnormal looking) with heart edema, tail curvature, and microphthalmia/coloboma were severely underdeveloped (Figure S4.2) and those individuals never survived past 5 dpf. As a result we did not further examine their retinas. Hereafter, sox4aC2 MZ mutant retina only refers to mutants without systematic developmental defects (normal looking). Sox4aC2 MZ mutant zebrafish eyes were collected and cryosectioned at 5 dpf, and 2, 4, and 8 weeks post fertilization (wpf).

We first compared the number of rod photoreceptors in WT and sox4aC2 MZ mutant retinas at all the time points. GFP positive cells were counted on sections containing the optic nerve (ON). Five dpf and 2 wpf sections were counted from dorsal to the ON (D) and ventral to the ON (V), while 4 wpf and 8 wpf were counted from three 100 µm areas [two dorsal to the optic nerve (D1 and D2), one ventral to the optic nerve (V)]. At 5 dpf, the sox4aC2 MZ mutant had significantly fewer rods than WT, especially in the dorsal retina (Figure 4.6A, 4.6B, 4.6I). At 2 wpf, the reduction of rods in the mutant was even more obvious, in that both the ventral and dorsal retina possessed fewer rods than WT (Figure 4.6C, 4.6D, 4.6I). Considering that the reduction of the number of rods in mutants may be due to the previously described decrease in eye size, the density of rod photoreceptors was calculated by dividing the dorsal rod number by the length of the retina from the optic nerve to the dorsal retinal margin and the ventral rod number by the length of the retina from the optic nerve to the ventral retinal margin. The number of rods per 100 µm retina length at 5 dpf and 2 wpf was plotted (Figure 4.6J). Interestingly, the number of rods was still significantly reduced in sox4aC2 MZ mutants even after normalization for eye size (Figure 4.6J). At 5 dpf, there was a 25.6% decrease in rod density in mutant dorsal retina compared to WT. At 2 wpf, the mutant had a 35.4% decrease in rod density in the dorsal retina.
and a 31% decrease in the ventral retina. At 4 wpf, although the number of rods in the three counted areas was comparable between WT and mutants (Figure 4.6E-F, 4.6I), we noticed that in WT rod photoreceptors were densely packed with each other while gaps of rod photoreceptors were often observed in the mutant retina ventral to the ON, which was likely to be established during embryonic retinal neurogenesis (Figure 4.6E’-F’, arrowheads). By 8 wpf, no difference was observed between WT and mutant (Figure 4.6G-I). These observations suggest that loss of sox4a may affect rod neurogenesis during embryonic and early juvenile stages, but the persistent rod neurogenesis in late juvenile and adult stages does not require sox4a function.
Figure S4.2. Retinal neurogenesis is severely impaired in the sox4aC2 MZ mutant with microphthalmia and coloboma. Immunohistochemistry labeling of rod photoreceptors, retinal ganglion cells, amacrine cells (A, C), and cone photoreceptors, bipolar cells (B, D) of 5 dpf WT (A, B) and sox4aC2 MZ abnormal retinas (C, D). L, lens; on, optic nerve; b, brain. Scale bar equals 50 µm.
Figure 4.6. Reduction of rod photoreceptor in *sox4aC2 MZ* mutant retina. Representative images of the transverse section from the WT retina (A, C, E’, G, G’) or the *sox4aC2 MZ* mutant retina (B, D, F’, H, H’) at 5 dpf (A, B), 2 wpf (C, D), 4 wpf (E-F’), and 8 wpf (G-H’), showing rod photoreceptors with the expression of the XOPS:GFP transgene. E’, F’, G’ and H’ show the retina region ventral to the optic nerve, enlarged from E, F, G and H. (I) Quantification of the numbers of rod photoreceptors counted from dorsal or ventral to the optic nerve at 5 dpf and 2 wpf, or from the three selected 100 µm regions (white boxes) at 4 wpf and 8 wpf. *Sox4aC2 MZ* retina had significantly reduced numbers of rod photoreceptor at 5 dpf and 2 wpf. *Sox4aC2 MZ* retina still display significantly reduced numbers of rod photoreceptor cells (Student’s t-test, p<0.05). (J) Quantification of the numbers of rod photoreceptors at 5 dpf and 2 wpf retina normalized by the size of the retina. *Sox4aC2 MZ* retinas still display significantly reduced numbers of rod photoreceptor cells (Student’s t-test, p<0.05). All scale bars equal 100 µm.
The next question we asked was whether loss of *sox4a* affects rod photoreceptor neurogenesis specifically or if it also affects retinal neurogenesis of other cell types. To answer this question, we examined *sox4aC2* MZ retinal neurogenesis by labeling retinal neurons with antibodies against red-green cone photoreceptors (Zpr-1); Müller glia (Zrf1); and a subset of retinal ganglion cells, amacrine cells and bipolar cells (Islet-1). Retinal neurogenesis was compared between WT and *sox4aC2* MZ mutant retina at all the time points from 5 dpf to 8 wpf. No apparent difference was observed in the Islet1 labeling (Figure 4.7A). The number of cones and Müller glia cells was counted. Cones were counted dorsal and ventral to the optic nerve at 5 dpf and 2 wpf, while in 4 and 8 wpf retinas cones were counted from three 100 µm areas (V1, V2, and D). We found that at 5 dpf and 2 wpf, the number of cone photoreceptors was slightly but significantly reduced in *sox4aC2* MZ mutant retinas and no difference was observed at 4 and 8 wpf (Figure 4.7B, 4.7D). Since we noticed that the mutant eyes were smaller than WT at 5 dpf and 2 wpf, we also calculated the density of cone photoreceptors the same way as we did for the rod photoreceptors. In contrast to what we found for rod photoreceptor density, cone density was comparable between WT and mutants at 5 dpf and 2 wpf after it was normalized for eye size (Figure 4.7E). Müller glia cells in the whole retina were counted and no difference was observed between WT and mutants at any time point examined (Figure 4.7C, 4.7D). These observations suggest that with the exception of rods, differentiation of other retinal neurons during late embryonic (5 dpf) and juvenile stages (2 wpf to 8 wpf) were not affected by loss of Sox4a.
Figure 4.7. Retinal neurogenesis for other cell types was not affected in the sox4aC2 MZ mutant retina. Representative images of the WT and the sox4aC2 MZ mutant retina transverse sections at 5 dpf, 2 wpf, and 4 wpf, immunolabeled with Islet1 antibody for retinal ganglion cells, amacrine cells and bipolar cells (A), Zpr1 antibody for cone photoreceptors (B), and Zrf1 antibody for Müller glia cells (C). (D) Quantitative analysis of the numbers of cone photoreceptors and Müller glia cells in WT and sox4aC2 MZ mutant retina at 5 dpf, 2 wpf, 4 wpf, and 8 wpf. Cone photoreceptors were significantly reduced in sox4aC2 MZ mutant retina at 5 dpf and 2 wpf, while Müller glia cells were comparable between WT and the mutant at all the time points analyzed (Student’s t-test, p<0.05). (E) Quantification of the numbers of cone photoreceptors at 5 dpf and 2 wpf retina normalized to the size of retina. The number of cone
photoreceptors per 100 µm retina region between WT and sox4aC2 MZ retinas were comparable (Student’s t-test, p<0.05).

4.4 Discussion

In this study, we examined the requirement for Sox4 in retinal neurogenesis, particularly in rod photoreceptor neurogenesis during embryonic and juvenile to adult stages. We found that Sox4 deficiency resulted in a specific delay in rod photoreceptor neurogenesis. Rod neurogenesis was delayed at 72 hpf when sox4a and sox4b were knocked down by morpholino injection. Rod lineage genes crx, neuroD, and nr2e3 expression were not affected by sox4 knockdown, suggestive of a normal rod lineage cell specification. The recovery of rod cells by 4 dpf in sox4 morphants without coloboma indicated that there was only a delay rather than an arrest in embryonic rod neurogenesis, with the caveat that the effectiveness of morpholino is significantly reduced after 4 dpf, so it could be due to the recovery of Sox4 function. Previously, deficiency in Sox11, another SoxC family transcription factor, was also reported to cause reduction in rod photoreceptors in zebrafish embryonic retinas (Pillai-Kastoori et al., 2014). Similar to what we found for Sox4 deficiency, Sox11 knockdown by morpholino also led to reduced rod numbers while rod lineage genes were not affected. This indicates that Sox4 and Sox11 are both required for rod photoreceptor terminal differentiation. In addition, we observed ectopically located rod photoreceptors in the INL in sox4 morphants with coloboma. Interestingly, displaced rod photoreceptors were also observed in Xenopus retina injected with sox4 morpholino (Cizelsky et al., 2013). The displacement of rod photoreceptor indicates there might be either a defect in the migration of rod progenitor cells or a deregulation of proper retinal progenitor cell differentiation such that cells that should become interneurons in the INL instead differentiated into rod photoreceptors.

The generation of sox4 CRISPR mutants provides us a valuable opportunity for the first time to examine the requirement of Sox4 in rod neurogenesis during a long period from embryonic to
adult neurogenesis. Consistent with the sox4 morphant phenotype, sox4 mutant zebrafish also exhibit a delay in rod neurogenesis. Among the different sox4 mutant lines, we observed the strongest reduction of rod numbers in the sox4aC2 MZ mutant from 5 dpf to 2 wpf. Smaller eye size was also observed for most sox4aC2 MZ mutant embryos at these two time points. However, the number of rod photoreceptors was still significantly reduced even after controlling for eye size. For juvenile and adult retinas at 4 and 8 wpf, although the numbers of rod photoreceptors within the counted areas were statistically similar between the WT and sox4aC2 MZ mutant, the ventral region of the mutant retina had noticeable gaps within the rod photoreceptor layer at 4 wpf. The analysis of sox4 morphants together with sox4aC2 MZ mutant retinas suggest that Sox4 deficiency causes delayed rod photoreceptor neurogenesis, while persistent rod neurogenesis in the adult retina is not affected.

One of the advantages of the sox4aC2 MZ zebrafish mutant is that the majority of individuals survive to adulthood, unlike Sox4 null mice that die prenatally at E14 (Bhattaram et al., 2010; Schilham et al., 1996). Since more than 70% of mouse rod photoreceptors are differentiated postnatally (Young, 1985), the prenatal death of Sox4 null mice largely precludes studies of the requirement for Sox4 in rod photoreceptor neurogenesis. For this reason, very few studies of the function of Sox4 in rod neurogenesis have been conducted in mouse models. Not until recently has evidence emerged that altered expression of Sox4 in the mouse retina affects rod neurogenesis (Benavente et al., 2014; Jiang et al., 2013b; Usui et al., 2013b).

The mechanism of how Sox4 regulates rod neurogenesis remains unclear. Considering our previous discovery that Sox4 negatively regulates the level of Hh signaling during early embryonic development in the anterior neural tube (Wen et al., 2015), we think that elevation in Hh signaling activity could be one of the reasons for the delayed rod neurogenesis in Sox4 deficient retina. Ectopic Hh activation in perinatal mouse retina explants reduced rod
photoreceptor neurogenesis (Yu et al., 2006). In addition, extraretinal Hh signaling in the
prediordal plate was also reported to affect the progression of rod photoreceptor differentiation in
zebrafish (Stenkamp and Frey, 2003; Stenkamp et al., 2000). We have shown that sox4
knockdown significantly elevated the level of Hh activity in the ventral midline of the zebrafish
anterior neural tube. Hh signaling there was known to be required for the initiation of retinal
neurogenesis (Kay et al., 2005). As a result, we hypothesize that Sox4 is required for proper rod
neurogenesis through regulating Hh signaling activity.

4.5 Materials and Methods

4.5.1 Zebrafish strains and maintenance

All zebrafish (Danio rerio) strains were bred, raised, and maintained at 28.5° C on a 14 hour
light:10 hour dark cycle according to established protocols (Westerfield, 2000). The Tg
(XLRho:EGFP)fl1 transgenic line (hereafter referred to as XOPS:GFP) and the Tg(XRho:gap43-
CFP)q13 line (hereafter referred to as XOPS-mCFP) were generous gift from J.M. Fadool
(Florida State University, Tallahassee, FL) and has been previously described (Fadool, 2003b;
Morris et al., 2005). Embryos were staged according to established developmental hallmarks
(Kimmel et al., 1995). All animal procedures were carried out in accordance with guidelines
established by the University of Kentucky Institutional Animal Care and Use Committee.

4.5.2 Fluorescent in situ hybridization (FISH)

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 SP6, T7, or T3 polymerase and
digoxigenin (DIG) or fluorescein (FITC) labeling mix (Roche Applied Science, Indianapolis, IN).
The sox4a and sox4b antisense probes have been previously described (Wen et al., 2015). The
neuroD antisense probes have been previously described (Morris et al., 2011b). The crx, and
nr2e3 probes have been previously described, and plasmids for generating these probes were
kindly provided by Y.F. Leung (Purdue University, Indiana). FISH was performed as previously described (Pillai-Kastoori et al., 2014). Images were obtained on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments).

4.5.3 RT-PCR
Total RNA was extracted from the retinas of age matched adult XOPS:GFP and XOPS:mCFP zebrafish using TRIzol reagent (Invitrogen, Grand Island, NY). RNA was reverse-transcribed using the GoScript Reverse Transcriptase System (Promega, Madison, WI). Sox4a and sox4b were amplified by PCR with standard buffer and taq polymerase with primers listed in Table 2.1. For all experiments, three biological replicates were analyzed.

4.5.4 Immunohistochemistry
Immunohistochemistry was performed on cryosections of 3 dpf, 4 dpf, 5 dpf, 2 week, 4 week, and 8 week zebrafish retinas, or whole zebrafish embryos at 5 dpf, or whole mount adult zebrafish retinas as previously described (Fadool, 2003a; Forbes-Osborne et al., 2013). Images were either obtained on an inverted fluorescent microscope (Eclipse Ti-U; Nikon Instruments) or on a laser scanning confocal microscope (Nikon C2 plus). The following primary antibodies and dilutions were used: anti-Zn-8 (mouse, 1:10, ZIRC, Eugene, OR), which labels ganglion cells; anti-Proxl (rabbit, 1:1000, Acris, San Diego, CA), which recognizes horizontal cells; anti-PKCa (rabbit, 1:100, Santa Cruz Biotechnology, Dallas, Texas), which labels bipolar cells; anti-Zpr-1 (mouse, 1:20; ZIRC, Eugene, OR), which labels red-green cones; anti-Nr2c3 (rabbit, 1:100). 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. Samples were counterstained with DAPI (1:10,000, Sigma, St. Louis, MO) to visualize cell nuclei.
4.5.5 Statistical analyses

Significance was calculated using a two-tailed Student’s $t$-test, with $P<0.05$ being considered significant. For all graphs, data are represented as the mean ± the standard deviation (s.d.).

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CHAPTER 5: SUMMARY AND DISCUSSION: RESEARCHING TODAY, SEEING TOMORROW

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For most congenital and age-related ocular diseases, our understanding of their etiologies are still very limited. Ocular coloboma is a good example: although it is one of the most common vision-threatening conditions among congenital ocular disorders, only a handful of causative genes have been identified, and they account for less than 10\% of clinically reported ocular coloboma cases. Due to the complexity of ocular development, it is very challenging to completely understand its detailed regulatory network at the molecular level. However, it is essential to continue identifying new genes that are required for eye development or are causal for ocular diseases, because they may provide potential new therapies and new pharmacological targets that can improve patients’ vision and quality of life.

Proper eye development requires precise genetic control that coordinates ocular cell proliferation, migration, differentiation, and survival. Although our current knowledge about ocular morphogenesis and neurogenesis is very poor, thanks to molecular and cellular biological techniques and the development of new animal models, we have gained knowledge about some critical intrinsic and extrinsic genetic factors that regulate the process of eye development. Promisingly, an increasing number of genes for ocular development are being identified based on loss-of-function phenotypes in human patients and animal models studies. However, our understanding for most of their functions and mechanisms in regulating ocular development are still like scattered pieces of puzzles. A regulatory gene network remains to be built, especially between cell signaling pathways and intrinsic expression of transcription factors.
Sox4 is one such transcription factor that has been valued as a potential ocular regulatory gene. The goal of this dissertation was to determine the requirement of Sox4 in ocular morphogenesis and retinal neuron differentiation, and to identify target genes or cell signaling pathways that were regulated by Sox4 during ocular development. Our general hypothesis originally was that Sox4 was required for ocular development and loss-of-function of Sox4 should result in ocular defects. This hypothesis was tested and supported in Chapter 2, Chapter 3, and Chapter 4. Morpholino-mediated sox4 deficiency caused a range of ocular defects, including ocular coloboma, altered proximodistal patterning of the optic vesicle, increased periocular cell death during early eye developmental stages, ectopic cell proliferation in the retinal ganglion cell layer, and delayed rod photoreceptor neurogenesis. These multiple developmental defects together with the spatiotemporal expression pattern of sox4 in the developing eye suggested that Sox4 was involved in multiple ocular developmental processes. Based on the coloboma phenotype in Sox4 deficient embryos, in Chapter 2 we tested our second hypothesis that Sox4 negatively regulates Hh activity and that the coloboma phenotype was caused by elevated Hh activity in Sox4 deficient embryos. Consistent with our hypothesis, we reported a significantly increased expression of the Hh ligand indian hedgehog b (ihhb) in Sox4 deficient embryos and inhibition of Hh activity or ihhb expression rescued the coloboma phenotype. In chapter 3, we further tested and confirmed our first two hypotheses on a sox4a mutant background generated by the CRISPR/Cas9 genome editing system. Mosaic knockout of sox4a or sox4b in F0 founders already phenocopies the coloboma phenotype. In the F3 maternal zygotic sox4a homozygous mutant, ocular abnormalities including coloboma and severe microphthalmia were exhibited in about 25% of the embryos and the rest of embryos had mild microphthalmia. The penetrance of ocular defects in sox4a mutant is lower than other defects such as heart edema and tail curvature, this is probably due to the expression of sox4b in the eye that partially compensates for the loss of sox4a. In addition, we proposed that BMP signaling that limits Hh activity was intermediate between Sox4 and Hh, since several BMP signaling molecules were downregulated in sox4a.
mutants. In Chapter 4, we focused on the requirement of Sox4 in rod photoreceptor neurogenesis based on the observation that sox4 expression was upregulated in a chronic rod photoreceptor degeneration and regeneration transgenic zebrafish retina. Although rod progenitor and precursor cells were well defined in Sox4 deficient retina, we observed a delay in rod maturation in both the sox4 morphants and sox4a mutants, indicating that Sox4 might be involved in rod photoreceptor terminal differentiation.

It is likely that Sox4 plays different roles during ocular development based on its expression time and location. As characterized in chapter 2, sox4a and sox4b are not expressed within the retina when all the retinal progenitor cells are actively proliferating prior to 32 hpf. During early ocular morphogenesis stages, sox4 expression can only be detected in the forebrain and ventral diencephalon. Expression patterns of sox4a and sox4b are partially overlapping, with sox4a expressed earlier in the forebrain than sox4b. In addition, sox4a is also expressed in the periocular mesenchymal cells adjacent to the optic stalk, where sox4b expression cannot be detected. Coincidently, the ventral diencephalon is close to the source of Hh signaling in the prechordal plate and sox4 expression region in the ventral diencephalon partially overlaps with the expression region of Hh ligands. The periocular tissues are also sensitive to Hh activity by expressing high levels of Hh receptor genes. Moreover, the optic stalk expresses pax2, which is a target gene of Hh signaling. The close spatial relationship between extraretinal sox4 expressing tissues and Hh signal sending and responding tissues provides possible explanations for how Sox4 may regulate Hh signaling, by either cell-autonomously regulating Hh ligand transcription or limiting the short-range diffusion of the Hh ligands.

Later on in development, sox4a and sox4b expression within the retina follows the same pattern: they are expressed mostly in immature neurons that have already exited the cell cycle but have not yet undergone terminal differentiation. Towards the end of retinal neurogenesis when the
majority of retinal neurons are terminally differentiated, the expression of sox4 is restricted to the regions that are adjacent to the persistently proliferating CMZ. Its expression in the adult retina is also limited to the CMZ, and not detected elsewhere. However, in the chronic rod photoreceptor degeneration and regeneration adult retina, sox4 expression in the CMZ is upregulated and is also detected in the outer nuclear layer in a few cells that may be differentiating rod precursor cells. Their expression in immature neurons are not limited only to the retina, but are also active in the developing brain (Bergsland et al., 2006; Chen et al., 2015a; Cheung et al., 2000). Analysis using the chick spinal cord revealed that sox4 expression was limited to post-mitotic maturing neural cells and Sox4 can promote the expression of pan-neuronal genes (Bergsland et al., 2006). The requirement of Sox4 in the brain to promote neuronal gene expression in maturing neurons suggests that sox4 in the retina may also exert similar functions for the terminal differentiation of neurons.

Reduction of Sox4a/b proteins during development results in ocular coloboma that is most obvious at 48 hpf when the RPE cells are pigmented. However, several lines of evidence indicate that the ocular coloboma in Sox4-deficient embryos results from a combination of developmental defects that occur much earlier. Firstly, an abnormal distortion of the horizontal crease in the optic vesicle was observed around 10 to 12 hpf in sox4 morphants. The horizontal crease is the lateral appearance of the connection between the optic vesicle and the optic stalk. The distortion indicates that the morphology of the optic stalk is already abnormal at this developmental stage. Secondly, even with inhibited p53 activity by p53 MO co-injection, an increased number of apoptotic cells were still observed in sox4 morphants at 18 hpf around the optic stalk region, where sox4 is strongly expressed. As discussed in Chapter 3, Sox4 is known for its requirement for neuronal and mesenchymal cell survival in the central nervous system, and sox4 knockdown without inhibition of p53 caused a much higher proportion of embryos with ocular coloboma (Bhattaram et al., 2010; Thein et al., 2010; Wen et al., 2015). Excessive periocular and retinal cell
death has been reported to be closely related to ocular coloboma (Gregory-Evans et al., 2011; Moosajee et al., 2008). As a result, it is reasonable to postulate that ectopic cell death early in the optic stalk region may contribute to the ocular coloboma phenotype at later developmental stages.

Thirdly, we detected elevation in Hh activity at very early developmental time points. The elevation of Hh activity was supported by four independent experimental results: 1) During ocular development, midline Hh signaling promotes the expression of Pax2 and Pax6 in the optic stalk and optic vesicle regions, respectively. A significant expansion of the pax2 expression region was observed in Sox4 deficient eyes at 18 hpf, indicating an increased midline Hh activity that altered the optic stalk and optic vesicle boundary. 2) Ptch2 is a Hh receptor and itself is a target of Hh activity, such that a high expression level of ptch2 indicates a high Hh activity. Sox4 deficiency resulted in a significantly increased expression of ptch2 at 12 hpf detected in a Tg(ptch2:EGFP) background. 3) Treatment of Sox4 deficient embryos with the Hh inhibitor cyclopamine at very early developmental stages from 5.5 hpf to 13 hpf (when midline Hh is the only source of Hh activity in the developing embryo) can significantly rescue the coloboma phenotype. 4) The expression of the Hh ligand ihhb was elevated in sox4 morphants from 8 hpf to 24 hpf. These observations provide a strong argument that Hh activity is upregulated in Sox4 deficient embryos at very early developmental stages and proper level of early Hh activity is required for normal choroid fissure closure (Figure 5.1).
Figure 5.1. Sox4 regulates choroid fissure closure through negatively regulating Hh signaling activity. A schematic drawing represents choroid fissure closure of the optic cup due to the presence of Sox4 during normal ocular development (A) and an aberration in this event in the condition of Sox4-deficiency (B). The expression of Sox4, partially through BMP signaling, limits the midline Hh signaling activity, which controls the expression level of Pax2 and governs the closure of the choroid fissure. Loss of Sox4 causes a reduction of BMP activity, hence an elevation in Hh and Pax2 expression, inhibiting the closure of the choroid fissure.
SOX4 is upregulated in many different types of human cancer cells and many cell signaling pathways that are commonly activated in cancer cells are able to stimulate SOX4 expression. SOX4 can either directly signal through some of its target proteins or transcriptionally regulate target gene expression. SOX4 is known to function upstream of Wnt signaling by directly interacting with β-catenin and activating several Frizzled Wnt receptors (Sinner et al., 2007). In addition, SOX4 can activate the Notch signaling pathway by transcriptionally activating HES2, ADAM10, and DLL1 (Moreno, 2010). A genome-wide promoter analysis of SOX4 in prostate cancer cell revealed 282 direct SOX4 target genes, which are involved in many cellular processes, including microRNA processing, tumor metastasis, transcriptional regulation, TGF-β, Wnt, Hh, and Notch pathways (Scharer et al., 2009).

Intriguingly, the Hh receptor gene PTCH1 was also detected as one of the SOX4 direct targets with high confidence (Scharer et al., 2009). Targeted deletion of Ptc1 in mice resulted in a large proportion of Ptc1+/− heterozygous individuals with medulloblastoma (MB) (Goodrich et al., 1997). SOX4 expression was upregulated in many cases of MB in human patients (Yokota et al., 2004). The expression of SOX4 and the Hh effector gene GLI1 was reported to be oppositely regulated in some cases of human MB cells, with SOX4 being upregulated and GLI1 being downregulated (Yokota et al., 2004). Moreover, Sox4 expression was repressed in rat kidney epithelial cells that were transfected with Gli1 (Yoon et al., 2002). Besides studies in cancer cells, there are very few reports for Hh signaling and Sox4 regulatory relationship during development. Previously, we have reported a similar ocular coloboma and an elevation in shh expression when another SoxC transcription factor sox11 was knocked down. Together with our finding in sox4 and ihhb activity, we postulate a negative regulatory relationship between SoxC proteins and the Hh signaling pathway during vertebrate eye development.
Further investigations are required to determine through what mechanisms SoxC proteins regulate Hh activity. The first question that remains to be examined is whether the negative regulation of Hh activity by Sox4 is direct or indirect. Sox4 is a transcription activator (Dy et al., 2008; van de Wetering et al., 1993). Although it may potentially inhibit target gene transcription through some unknown domains or partners, there are no such studies reported yet. We favor the indirect model that some unknown intermediate molecule(s) is(are) involved in the regulatory network acting as Hh inhibitors. We hypothesize that Sox4 negatively regulates Hh activity by activating the expression of Hh inhibitors.

We examined the expression levels of several potential intermediate molecules in Sox4 deficient embryos and found BMP signaling as a promising candidate for Hh repression. BMP signaling genes are strongly expressed in the dorsal retina and RPE, and surface ectoderm cells overlying the optic vesicle (Huang et al., 2015; Muller et al., 2007). BMP activity is required for the differentiation of the retina, RPE cells and lens vesicle (Muller et al., 2007). It also facilitates the epithelial flow of the optic cup that affects eye morphogenesis (Heermann et al., 2015). BMP receptor deficiency in the mouse lens placode resulted in ocular coloboma (Huang et al., 2015). Previously BMP7 has been reported to mediate choroid fissure closure upstream of Hh activity (Morcillo et al., 2006). We detected a significant decrease in the expression of BMP signaling ligands bmp4 and bmp7 in Sox4 deficient embryos. Overexpression of bmp7 mRNA in sox4 morphants can partially rescue the coloboma phenotype. Further experiments that can detect protein-DNA binding such as chromatin immunoprecipitation (ChIP) are required to provide direct evidence of Sox4 and BMP molecule interaction.

It is also likely that BMP signaling is not the only intermediate candidate repressing Hh. The Fgf signaling pathway also plays a critical role in regulating optic vesicle patterning. A mutual regulatory relationship between Fgf and Hh signaling pathways has been intensely studied during
ocular development and they work collaboratively to define the dorsoventral axis of the optic vesicle (Lupo et al., 2005). Fgf activity is also required for the initiation of shh expression within the retina for retinal neurogenesis in zebrafish (Vinothkumar et al., 2008). As mentioned in Chapter 2, we detected a significant reduction of the Fgf signaling receptor 2 (fgfr2). Fgfr1 and Fgfr2 conditional knockout in the mouse optic cup resulted in ocular coloboma and failure of RGC neurogenesis (Cai et al., 2013; Chen et al., 2013). Further examination is required to confirm the effect of Sox4 deficiency on Fgf activity. We would like also to conduct unbiased examinations such as RNA-seq or ChIP-seq to identify more Sox4 targets that are involved in ocular development.

We also reported a novel requirement of Sox4 in proper rod photoreceptor differentiation in Chapter 4. Sox4 morphants exhibit a specific delay in rod maturation during embryonic eye development. The sox4 mutant retina also has reduced numbers of mature rod photoreceptors during embryonic and juvenile stages. The normal expression of several rod progenitor and precursor genes crx, neuroD, and nr2e3 indicated that the rod defect was probably related to the terminal differentiation of rod photoreceptors. The delay in rod terminal differentiation is not due to the secondary effect of coloboma as it was observed in both sox4 morphants and in sox4aC2 MZ mutants without coloboma, indicating that this phenotype may be independent of ocular coloboma. We have two hypotheses to explain the delay in rod maturation phenotype: 1) Since we found the upregulation of midline Hh activity in Sox4 deficient embryos during early development, future experiment will address our hypothesis that the delay of rod maturation is also related to the upregulation of midline Hh activity. Early expression of extraretinal midline Hh signaling is known to initiate retinal neurogenesis prior to the intraretinal Hh activity by promoting the expression of the proneural gene ath5 in the retina (Kay et al., 2005; Stenkamp and Frey, 2003). Future experiments will include the analysis of mature rod numbers in Sox4 deficient embryos treated with either cyclopamine or with ihhb MO injection. 2) Our second
hypothesis is that sox4 expression within the retina plays a direct role in regulating rod photoreceptor maturation. As discussed in Chapter 1 and Chapter 4, studies in mice indicated the requirement of an optimal level of SoxC expression in the retina for proper rod photoreceptor development (Usui et al., 2013b). In addition, although we did not observe a change of transcript levels of rod progenitor or precursor genes, there might be an epigenetic modification of those genes facilitated by Sox4, as suggested in mouse studies (Benavente et al., 2014; Usui et al., 2013b). Future studies are required to decide whether the effect of Sox4 deficiency on rod maturation is through early extraretinal expression of sox4 on midline Hh expression or the late sox4 expression within the retina.

In summary, this dissertation includes a detailed examination for the requirement of Sox4 in regulating ocular morphogenesis and retinal neurogenesis (Figure 5.2). It provides a thorough expression profile of soxA and soxB in the brain, periocular tissues, and the retina during early ocular morphogenesis and retinal neurogenesis stages. Loss-of-function analyses conducted on sox4 knockdown and sox4 mutant zebrafish models demonstrated a specific role of Sox4 in regulating the choroid fissure closure and rod photoreceptor terminal differentiation. Mechanistic study of the ocular coloboma provided strong evidence that Sox4 negatively regulates midline Hh activity, especially the expression of ihhb. The elevated midline Hh activity through Sox4 deficiency in turn causes expanded optic stalk that inhibits the choroid fissure from closing. The increased cell death due to Sox4 deficiency also contributes to the severity of coloboma. An indirect regulatory relationship between Sox4 and Hh activity is proposed, with BMP signaling as a potential intermediate component.
Figure 5.2. Proposed models for the functions of Sox4 in regulating ocular morphogenesis and retinal neurogenesis. Proper Sox4 expression is required for an appropriate level of midline Hh activity (probably Ihhb) that governs the process of eye field segregation, optic vesicle patterning, and choroid fissure closure. In addition, Sox4 may also be required for choroid fissure closure by regulating cell survival independent of Hh signaling. During retinal neurogenesis, Sox4 regulates the state of proliferation of retinal progenitor cells and controls the timing of retinal precursor cell terminal differentiation.
SoxC transcription factors have their footprints in many developmental and pathogenic processes, regulating cell proliferation, differentiation, and survival (Penzo-Mendez, 2010; Vervoort et al., 2013). Different target genes and mechanisms are used in those processes in a context dependent manner. Our work on the regulation of SoxC in ocular development provides important evidence supporting the critical role of SoxC proteins in regulating cell fate specification and organogenesis. Although further analysis is required to continue identifying direct target genes of SoxC, the discovery of a regulatory relationship between SoxC proteins and the Hh signaling pathway during ocular development opens up a new avenue when we think about genetic regulation of embryonic development and pathogenic mechanisms of many diseases such as congenital ocular defects and cancer. Although there is limited data for the involvement of SoxC deficiency on human diseases, some of loss-of-SoxC ocular phenotypes are similar to many that are observed in human disorders. It is reasonable to speculate that SoxC deficiency may be involved in some of those diseases. Future studies will focus on the determination of the critical time period for the requirement of SoxC in ocular morphogenesis, the identification of the direct target(s) of SoxC, and how these targets affect Hh signaling activity and ocular development. We have also provided some evidence for a requirement of SoxC in rod photoreceptor neurogenesis, we are eager to elucidate additional functions of SoxC in regulating embryonic retinal neurogenesis and during adult retinal regeneration.

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