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ROLE OF SOX11 DURING VERTEBRATE OCULAR MORPHOGENESIS AND RETINAL NEUROGENESIS

Lakshmi Shashidharan Pillai
University of Kentucky, lakshmi.pillai@uky.edu

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Lakshmi Shashidharan Pillai, Student

Dr. Ann C. Morris, Major Professor

Dr. Dave Wetneat, Director of Graduate Studies
ROLE OF SOX11 DURING VERTEBRATE OCULAR MORPHOGENESIS 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

Lakshmi Pillai-Kastoori
Lexington, Kentucky

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

2015

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ABSTRACT OF DISSERTATION

ROLE OF SOX11 DURING VERTEBRATE OCULAR MORPHOGENESIS AND RETINAL NEUROGENESIS

Microphthalmia, anophthalmia, and coloboma (MAC) are distinct abnormalities demonstrating a continuum of developmental eye defects that contribute to 15-20% of blindness and severe vision deficiencies in children worldwide. The genetic etiology of MAC is large, complex and encompasses the whole developmental biology of the eye. Understanding how the eye develops will aid in identifying genes and developmental pathways involved in MAC. Although investigation of the genetic architecture of congenital anomalies is growing exponentially, much work remains to be accomplished to understand the complex, genetically heterogeneous congenital anomalies, which significantly impact childhood vision.

With an interest in elucidating the mechanisms that are involved in eye morphogenesis, I have characterized a SRY-Box transcription factor, Sox11, during zebrafish ocular development. The SRY (sex determining region Y)-box 11 (sox11) gene, codes for a transcription factor which functions as a regulator of cell fate, survival, and differentiation in the embryonic and adult nervous system. By titrating the levels of sox11 gene function in developing zebrafish embryos I have investigated the role of Sox11 during ocular morphogenesis and retinal neurogenesis. Chapter 1 of this dissertation provides a review of vertebrate eye development with a focus on emerging roles of SoxC proteins during vertebrate ocular morphogenesis. Chapter 2 presents data demonstrating that knockdown of both paralogs of sox11 in zebrafish results in microphthalmia, coloboma, as well as a specific deficit in mature rod photoreceptors. Additionally, we demonstrate for the first time that Sox11 regulates early ocular and photoreceptor development in part by maintaining proper levels of Hedgehog (Hh) signaling. Deficiency of Sox11 results in elevated Sonic Hedgehog a (Shha) transcript levels, which in turn leads to improper patterning of the optic vesicle into the proxio-distal territories. Furthermore, the data indicate that alterations in SOX11 gene dosage or mutation within the SOX11 coding region are potentially disease causing and contribute to human ocular defects like MAC and rod dysfunction. Chapter 3 presents data indicating that sox11 gene function is required during the critical period of neurulation (4-10 hours post fertilization) to guide choroid fissure closure and proper ocular morphogenesis to occur in the developing zebrafish. Chapter 4 is a technical report on the progress towards generating stable sox11a/b knockout zebrafish lines using the CRISPR/Cas9 genome editing approach. Transient F0 injected embryos and F0 adults carry mutations in the sox11a/b target site in addition to displaying ocular abnormalities similar to those previously reported in sox11 morphants. F1 juveniles are ready to be screened for establishment of mutagenesis efficiency and germ line transmission. Finally, in Chapter 5 I discuss how the results of each chapter demonstrate the functional requirement of Sox11 for ocular development. Furthermore, I discuss the implications of this work in the field of developmental biology and how the current data will shape future investigations.
My dissertation incorporates human genetics, biochemical analyses, and zebrafish reverse genetic analyses, and will help in better understanding the exact role of Sox11 during eye development at the cellular and molecular level. Moreover, by identifying Sox11 targets belonging to the Hh pathway, as well as novel targets of Sox11 regulation, these studies may also contribute to our understanding of the function of Sox11 in development and disease pathogenesis.

KEYWORDS: Eye development; Sox11; Coloboma; Hedgehog signaling; CRISPR/Cas9

Lakshmi Pillai-Kastoori
Student’s Signature

April XX, 2015
Date
ROLE OF SOX11 DURING VERTEBRATE OCULAR MORPHOGENESIS AND RETINAL NEUROGENESIS

By

Lakshmi Pillai-Kastoori

Ann C. Morris
Director of Dissertation

David F. Westneat
Director of Graduate Studies

April XX, 2015
Date
To Amma
ACKNOWLEDGEMENTS

“Success is the sum of small efforts, repeated day in and day out”- Robert Collier

I have this quote on top on my work bench strategically placed at my eye level. I have strived to live by it every single day that I have been in my graduate school. Although the cover of this dissertation displays my name alone, the work presented in this dissertation has been possible due to the earnest efforts of many.

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CHAPTER 1: KEEPING AN EYE ON SOXC PROTEINS: VERTEBRATE OCULAR MORPHOGENESIS AND CHOROID FISSURE DEVELOPMENT

Lakshmi Pillai-Kastoori¹, Ann C. Morris¹

¹Department of Biology, University of Kentucky, Lexington, Kentucky 40506-0225

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1.1 Introduction
Development of the vertebrate eye is a complex process consisting of a series of highly coordinated events that involve interactions between the neural ectoderm, surface ectoderm, and extraocular mesenchymal cells. Each phase of eye development depends both on inductive signaling pathways and the precise temporal expression of cell-intrinsic factors. Disruptions in these cellular signals or mutations in eye development genes result in a variety of sight-threatening pediatric ocular malformations. Therefore, to better understand anomalies in the structure and the function of the eye it is important to identify the key molecular players in each step of eye formation.

This review will provide an overview of the major stages of vertebrate eye development, highlighting some of the important molecular cues associated with each stage. In addition, we will focus on the emerging roles of SoxC proteins during vertebrate ocular morphogenesis and in human ocular malformations. Throughout the review, we will follow established nomenclature, designating human genes in all capital letters, rodent genes with the first letter capitalized, and zebrafish and Xenopus genes with all lower case letters; italic letters indicate genes, whereas non italicized names refer to proteins.
1.2 Vertebrate eye development and retinal neurogenesis

1.2.1 Development of the vertebrate eye field

During gastrulation the anterior neural plate is specified into domains that eventually form the eye field, telencephalon, diencephalon, and the hypothalamus. Critical inductive events prepare the presumptive eye field present within the anterior neural plate, which eventually migrates and integrates with nearby tissues. The eye field cells are surrounded rostrally and laterally by telencephalic precursors and medially by cells that will form the hypothalamus. Therefore, one of the first critical steps that eye precursor cells must take is to define their lineage and separate themselves from the surrounding brain tissues.

Formation and specification of naive uncommitted cells into an organized eye field domain requires the contribution of many signaling pathways and transcription factors (TFs). The fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and Wingless (Wnt) pathways guide movement of cells into the eye field and also help maintain the eye field territory (Esteve and Bovolenta, 2006). In addition, both canonical and non-canonical Wnt signaling pathways interact with each other to separate the eye field territory from the diencephalon and the telencephalon (Cavodeassi et al., 2005; Esteve and Bovolenta, 2006; Wilson and Houart, 2004). Balance between BMP and non-canonical Wnt (Wnt5, Wnt11) signaling patterns the anteroposterior axis of the neural plate, and initiates morphogenetic movements of cells in the anterior neural plate, contributing to the formation of the eye field (Cavodeassi et al., 2005; Wilson and Houart, 2004). Simultaneous inhibition of canonical Wnt signaling is also required for suppression of diencephalon fate markers, as misexpression of Wnt8b results in poor delineation of the border between the diencephalon and the eye field, and mutation in the Gsk3 binding domain of Axin1 results in conversion of telencephalon and eye field precursors into diencephalon due to constitutive activation of the Wnt/β-catenin pathway (Adler and Canto-Soler, 2007; Cavodeassi et al., 2005; Esteve and Bovolenta, 2006; Heisenberg et al., 2001; Kim et al.,
FGF modulation of ephrinB1 phosphorylation also plays a role in inducing the prospective progenitors to migrate, coalesce, and assemble themselves as an eye field (Chong et al., 2000; Moore et al., 2004).

In addition to receiving critical signals from the surrounding forebrain tissue, eye field progenitors themselves express the eye field transcription factors (EFTFs) \(\text{Rx1/Rax, Pax6, Lhx2, Six3, Otx2, ET, tll and Hes1}\) (Chow and Lang, 2001). Six3, Pax6, Otx2, and Rx1 specify progenitor cells to the retinal lineage, and also regulate morphogenetic cell movements that guide presumptive eye field cells the correct geographic location (Kenyon et al., 2001; Moore et al., 2004). Lhx2 is required to maintain optic identity and suppress alternative fates (Roy et al., 2013). Loss-of-function mutations in EFTFs result not only in the absence of an optic cup but also cause severe neuro-developmental anomalies in a variety of different animals such as mice, chicken, zebrafish, and humans (Chow and Lang, 2001; Lequeux et al., 2008; Porter et al., 1997; Stigloher et al., 2006; Tucker et al., 2001; Winkler et al., 2000). Notably, there are also TF’s (e.g. \(\text{Hes1}\) and \(\text{Otx2}\)) that are expressed outside the eye field domain which influence its formation. \(\text{Otx2}\) is not expressed within the \(\text{Rx}\) positive eye field region, however \(\text{Otx2}\) is needed to maintain the expression of \(\text{Six3}\) and \(\text{Hes1}\) in the anterior neural plate (Andreazzoli et al., 1999; Rhinn et al., 1998; Simeone et al., 1993). \(\text{Hes1}\) influences eye development by controlling the formation of the forebrain. \(\text{HESX1}\) mutations in humans result in variety of defects including optic nerve hypoplasia, and in mice \(\text{Hes1}\) null mutants display anophthalmia and microphthalmia (Chow and Lang, 2001; Dattani et al., 1998).

1.2.2 From one eye field to two optic vesicles
The eye field cells undergo cellular proliferation during gastrulation and eventually split into two bilateral domains in response to secreted factors originating from the ventral midline. High-resolution dynamic fate map studies have revealed the substantial structural changes that occur to
move the ventral diencephalon anlagen from a posterior to an anterior ventral position, resulting in bisection of the eye field (England et al., 2006; Varga et al., 1999). The process of eye field segregation requires axial Nodal/TGF-β and Hedgehog (Hh) signaling, which in turn establishes optic vesicle boundaries and patterns the proximodistal and ventronasal axes of the optic vesicles by modulating expression of TFs such as Pax2, Pax6, Vax1, and Vax2 (Barbieri et al., 1999; Barth and Wilson, 1995; Dressler and Woolf, 1999; Ekker et al., 1995; Hyatt et al., 1996; Muller et al., 2000; Nornes et al., 1990; Schulte and Cepko, 2000). Loss of Nodal-related proteins such as Squint, Cyclops, or One-eyed pinhead results in cyclopia and holoprosencephaly, underscoring the importance of TGFβ/Nodal signaling for eye field segregation (Pei and Feldman, 2009; Zhang et al., 1998). Likewise, mutations in the Hh signaling ligand SHH result in holoprosencephaly and cyclopia in humans and mice (Belloni et al., 1996; Chiang et al., 1996).

1.2.3 From a flat optic vesicle to a spherical optic cup
In the next phase of eye development, the symmetrical paired optic vesicles (OVs) evaginate from the ventral diencephalon and expand through the extraocular mesenchyme towards the surface ectoderm (Fuhrmann, 2010; Kessler and Melton, 1994; Li et al., 1997; Vogel-Höpker et al., 2000). This evagination step depends critically on paracrine retinoic acid (RA) signaling originating from the temporal mesenchyme (Adler and Canto-Soler, 2007; Cvekl and Wang, 2009). Upon physical contact with the overlying head surface ectoderm, a series of spatially and temporally complex structural changes ensues. The surface ectoderm at the point of contact thickens and forms a lens placode, which continues to invaginate, eventually forming the lens vesicle and detaching from the surface ectoderm. Concomitantly, the distal portion of the OV elongates laterally and undergoes invagination to form a bilayered optic cup (OC), which remains connected to the diencephalon via the optic stalk (for a detailed description see (Fuhrmann, 2010). Dynamic migratory behavior of cells present within the optic vesicle is crucial for eye morphogenesis (Ivanovitch et al., 2013; Kwan et al., 2012). Formation of the optic cup is an
important foundation on which depends the development of the iris, the ciliary body, the retina, and retina pigmented epithelium (RPE). The neural retina originates from the inner layer of the optic cup, whereas the outer layer of the optic cup differentiates into the RPE. A transient channel called the choroid fissure is present along the ventral side of the optic cup and optic stalk. This opening permits the entry of periocular mesenchymal cells (neural crest derived) that later form the blood vessels which provide nourishment to the growing eye; it also allows the axons of the ganglion cells to exit the eye and connect with the brain.

As optic cup morphogenesis and lens induction progresses, overlapping sets of TFs mark the lineage specification of distinct ocular tissues. The presumptive lens expresses Pax6, IGF-2, Prox-1, Six3, Sox1, and Sox2; the nascent neural retina expresses Pax6, Pax2, Rx, Lhx2, Chx10, and Optx2; the developing RPE expresses Mitf and Otx2; and the optic stalk expresses Pax2, Vax1, Vax2, and Six3 (Chow and Lang, 2001; Jean et al., 1999). In addition to establishing the competence of progenitor cells for specific lineages, TFs interact with each other to establish boundaries within and between individual ocular tissues. Within the developing optic vesicle, Pax6 and Tbx5 pattern the dorso-ventral axis, and BF-1/Foxg1 and BF-2/Foxd2 pattern the anterior-posterior axis. Along the medial-lateral axis, Pax2, Pax6, sox4, sox11, and Vax regulate the proximo-distal patterning of optic vesicle versus optic stalk territories (Figure 1) (Adler and Canto-Soler, 2007; Hatini et al., 1994; Horsford et al., 2005; Schwarz et al., 2000).
Figure 1.1. Vertebrate ocular morphogenesis. A schematic representation of the major stages of eye development is shown, with the presumptive telencephalon (T, red), eye field (EF, yellow), hypothalamus (H, green), and diencephalon (D, purple) indicated within the anterior neural plate. The transcription factors (TF’s) associated with each stage of eye development are indicated in blue boxes on the left of the figure. OV, optic vesicle; L, lens; NR, neural retina; RPE, retinal pigmented epithelium; CF, choroid fissure; A, anterior; P, posterior; N, nasal; T, temporal.
1.3 Optic cup morphogenesis

1.3.1 The anterior segment: lens, cornea, iris and ciliary body
All sensory placodes possess the capacity to form the lens before they acquire unique identities [reviewed extensively by (Streit, 2007)]. The preplacodal region gets patterned along the anterior-posterior axis, and precursors for the lens placode reside in the anterior region of the surface ectoderm. *Pax6* expression is critical for lens formation from the early preplacodal phase, and its expression is controlled at various stages of lens development by a variety of pathways. BMP and FGF signaling regulate *Pax6* expression during placode formation; *Six3* and *Meis* family members regulate *Pax6* activity in the presumptive lens ectoderm. Additionally, *Pax6* regulates the critical expression of *Sox2* in the presumptive lens epithelium, and as the placode is formed there is role reversal and *Pax6* controls the expression of *Six3* (Kamachi et al., 1998; Liu et al., 2006; Streit, 2007; Wawersik et al., 1999; Zhao et al., 2008). The last stages of lens formation lead to inductive events resulting in the formation of the cornea endoderm, iris, and the ciliary body stroma. Soon after the lens separates from the overlying surface ectoderm, periocular mesenchymal cells of neural crest origin invade the space between the lens and the surface ectoderm. The mesenchymal cells differentiate and contribute to the formation of the cornea, iris, and the ciliary body (Graw, 2010; Piatigorsky, 2001).

1.3.2 The RPE and retina
Concomitant with lens development, profound changes are occurring in the bi-layered optic cup. As mentioned above, the outer layer of the optic cup forms the RPE, and the inner layer forms the neural retina. The cells in the inner layer proliferate (accompanied by some pruning due to programmed cell death) to generate six classes of retinal neurons and one intrinsic glial cell type: retinal ganglion cells (RGCs), amacrine cells, horizontal cells, bipolar cells, cone photoreceptors, rod photoreceptors, and the Müller glia [for more extensive reviews on retinal development, see...
The RGCs are present in the ganglion cell layer (GCL), the interneurons (amacrine, horizontal and bipolar cells) and the cell bodies of the Müller glia are present in the inner nuclear layer (INL), and the photoreceptors are present in the outer nuclear layer (ONL). The genesis of the different retinal cell types occurs in a conserved temporal order but species-specific spatial order. In general, RGCs are the first cells born in the neural retina, followed by amacrine, horizontal cells, and cone photoreceptors. The last retinal cell types to differentiate are the bipolar cells, rod photoreceptors, and the Müller glia. Importantly, in mammals retinal neurogenesis occurs only during embryonic/perinatal development; however cold-blooded vertebrates such as teleost fish and some amphibians experience growth-associated retinal neurogenesis throughout their lifespan. Retinal progenitor cells (RPCs) attain competence to become RGCs by expressing a bHLH TF called \textit{ath5}. In addition, Hh and FGF signaling are required for the proper temporal expression of \textit{ath5} and the generation of RGCs (Martinez-Morales et al., 2005; Masai et al., 2005). Amacrine, horizontal, and bipolar cells are generated by an overlapping network of TFs such as \textit{Prox1}, \textit{Math5}, \textit{NeuroD}, \textit{Math3}, \textit{Ptf1}, \textit{Pax6}, \textit{Six3}, \textit{Mash1}, and \textit{Foxn4} for an extensive review see (DEMB and SINGER, 2012; Dyer et al., 2003; Ohsawa and Kageyama, 2008; Tomita et al., 2000). Photoreceptors are generated from a pool of \textit{Crx}- and \textit{Otx2}-positive cells, which later acquire competence to form either cone or rod photoreceptors by expressing the TFs \textit{TRβ2} and \textit{RXRγ} for cones, or \textit{Nrl}, \textit{Nr2e3}, and \textit{Ascl1} for rods (Swaroop et al., 2010).

The RPE is characterized by the presence of melanosomes, which produce and store melanin pigment. The RPE lies between the neural retina and the vascular choroid, and is sensitive to signals emanating from the adjacent periocular mesenchyme. The RPE is indispensable for retinal function. It forms part of the blood-retina barrier, facilitates adhesion between the neural retina and the surrounding choroid, captures free radicals, absorbs background light, is critical for
retinoid metabolism, and phagocytoses the spent outer segment tips of the photoreceptors (Boulton and Dayhaw-Barker, 2001; Martinez-Morales et al., 2005). Surprisingly, only a handful of TFs have been identified that are required for the development and differentiation of the RPE. These include Mitf, Otx1/Otx2, and Pax6 (Baumer et al., 2003; Bharti et al., 2012; Goding, 2000; Martinez-Morales et al., 2005; Raviv et al., 2014). TGFβ, FGF, BMP, and Hh signals from the surrounding mesenchyme also help induce the RPE fate during development (Chung et al., 2001; Dohrmann et al., 1993; Ertzer et al., 2007; Feijen et al., 1994; Fuhrmann et al., 2014).

1.3.3 Ocular vasculature and optic nerve
A mature eye has the highest oxygen demand per unit weight of any human tissue (Saint-Geniez and D'Amore, 2004). Early on, the developing eye is nourished by intraocular vasculature generated by the hyaloid system and the choroid vessels. The hyaloid artery enters the optic cup through the choroid fissure and traverses the primitive vitreous to land on the posterior portion of the developing lens. The hyaloid arteries rapidly spread out and form a dense capillary network and connect with a venous system at the anterior portion of the optic cup. The venous system is provided by the choroidal vasculature derived from the periocular mesenchyme. As soon as the hyaloid system and choroid vasculature merge, the hyaloid vasculature is formed and it provides all the nutrients and metabolites to the growing eye. Around the same time the more permanent retinal vasculature formation is initiated and in mammals hyaloid vasculature starts to regress (for excellent description see (Saint-Geniez and D'Amore, 2004).

The proper connection between the arterial-venous network is crucial for functional circulation of nutrients and oxygen within the ocular tissue (Bussmann et al., 2011; Haigh et al., 2003). Development and maturation of choroidal blood vessels depends on expression of the angiogenic factors VEGF, bFGF, PDGF and PEDF from the RPE, and neutralization of bFGF and VEGF within the RPE results in incomplete formation of choroid vessels (Lee et al., 2010; Rousseau et
al., 2003). Misexpression of FGFR1 results in immature choroidal vessels and mutation in VEGF causes severe vascular defects (Carmeliet et al., 1996; Ferrara et al., 1996). Notch signaling is important for capillary bed formation in mice, humans, and zebrafish. VEGF and Delta-like 4 (DII4) interact dynamically to regulate vascular patterning (Hellstrom et al., 2007; Shawber and Kitajewski, 2004). Finally, genetic studies in zebrafish suggest that Hh signaling specifies endothelial cell fate to directly regulate arterial differentiation (Williams et al., 2010).

In summary, organogenesis of the eye is a conserved process that is driven by the complex interaction between the surface ectoderm, neuroepithelia and the extraocular mesenchyme (Chow and Lang, 2001; Fuhrmann, 2010). While the events of ocular morphogenesis are broadly conserved across vertebrates, various stages exhibit species specific differences. For example, in zebrafish, lens development does not progress through a hollow lens vesicle stage as it does in mammals (Greiling et al., 2010). Additionally, unlike mammals, zebrafish retinal vasculature development does not involve regression of the hyaloid vessels (Alvarez et al., 2007). Despite these small differences, the information gathered from different vertebrate animal models has enriched our understanding of the key molecular players and signaling pathways in embryonic eye development.

1.4 Congenital ocular malformations

Estimates of the prevalence of congenital ocular defects vary by region and method of ascertainment, but in general range from 1-10 cases per 10,000 births (Royal National Institute of Blind People, RNIB; (Gregory-Evans et al., 2004). Improper execution of any stage of early eye development can result in sight-threatening ocular malformations. For example, abnormal signaling from the midline (caused by defects in Shh, Nodal, and RA signaling or mutations in Six3) can cause failure of the eye field to properly segregate into two bilateral domains, resulting in ocular hypotelorism or cyclopia in extreme cases, as well as holoprosencephaly (Belloni et al.,
Another group of ocular defects associated with abnormal morphogenesis includes microphthalmia (small eye), anophthalmia (absence of an eye), and coloboma (failure of choroid fissure closure), collectively called MAC. Mutations or altered gene dosage of several of the TFs and signaling molecules active during early eye development, such as VAX, SOX10, OTX2, RAX, SOX2, PAX2, PAX6, SIX3, and SHH can result in MAC phenotypes in humans (Bondurand et al., 1999; Dressler and Woolf, 1999; Lequeux et al., 2008). Although genetic factors contribute significantly to the aetiology of MAC, the causative mutations identified to date account for less than 20% of all cases (Gregory-Evans et al., 2004). Further investigations in animal models should help to identify novel MAC-causing genes.

Defects in ocular morphogenesis may also cause anterior segment dysgenesis, resulting in malformations of the lens, iris, cornea, or ciliary body. Mutations in PAX2 and PAX6 can cause aniridia (absence of the iris), isolated cataracts, and Peters anomaly (persistent adhesion between the cornea and lens) (Abouzeid et al., 2009; Bower et al., 2012; Glaser et al., 1992; Hill et al., 1992; Otteson et al., 1998). Mutations in PITX, FOX and MAF family members cause cataracts, anterior segment mesenchymal dysgenesis, and severe iris anomaly (Blixt et al., 2000; Cheong et al., 2007; Komatireddy et al., 2003; Semina et al., 2001; Shaham et al., 2009). Congenital cataracts are also highly prevalent in patients carrying mutations in lens intrinsic membrane proteins, gap-junction membrane channel proteins, and crystallins (Berry et al., 2000; Graw, 2003; Shiels and Bassnett, 1996).

Finally, ocular malformations are often associated with more extensive syndromes that involve defects in the development of the heart, nervous system, skeleton, or other tissues. Some examples are CHARGE syndrome (coloboma, heart anomaly, choanal atresia, retardation, genital and ear anomalies), Anophthalmia-Esohageal-Genital (AEG) syndrome, and Coffin-Siris syndrome (systemic abnormalities like skeletal defects, neurologic defects, cardiac
malformations, renal and genital defects) (Gregory-Evans et al., 2004; Hornby et al., 2003; Kelberman et al., 2006; Onwochei et al., 2000).

1.5 Hedgehog (Hh) signaling pathway and vertebrate eye development

Hedgehog signaling plays a fundamental role in embryonic patterning and organogenesis by acting as a morphogen and a mitogen (Amato et al., 2004; Choy and Cheng, 2012; Martí and Bovolenta, 2002). Originally, the Hh gene was identified in a saturation screen performed by Christiane Nüsslein-Volhard and Eric Wieschaus in Drosophila (Nusslein-Volhard and Wieschaus, 1980). Hh signaling is highly conserved from flies to mammals and is an excellent example of versatility in developmental biology (Amato et al., 2004). Although the fly genome encodes only a single hh gene, vertebrate genomes encode multiple hh genes differing in their function and tissue expression. Mammals and birds have three hh genes, namely Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert Hedgehog (Dhh); zebrafish have shha, shhb (previously called tiggywinkle Hedgehog), ihha, ihhb (previously known as Echidna hedgehog) and dhh (Ekker et al., 1995; Krauss et al., 1993). SHH is the one of the best studied morphogens (a molecule that establishes a concentration gradient and generates different biological outputs depending on the gradients)(McMahon et al., 2003). In order to achieve the optimum biological output, SHH can act as a short-range, long-range or a contact dependent inducing factor (Chuang and McMahon, 1999; Johnson and Tabin, 1995).

1.5.1 SHH signal secreting cell: Biosynthesis and release of SHH protein

Following translation, the SHH precursor peptide (~45 kDa) moves into the endoplasmic reticulum lumen (after cleavage of the signal sequence) and undergoes cholesterol dependent autocatalytic cleavage event to produce two secreted proteins. The N-terminal ~19 kDa fragment is the signaling ligand and the C-terminal ~25 kDa fragment has an autocatalytic intein-like domain with cholesterol-transferase activity (Porter, 1996). Two covalent modifications to the N-terminal SHH fragment, addition of a cholesterol group at the C-terminus mediated by the C-
terminal SHH fragment and addition of a palmitoyl moiety at the N-terminus catalyzed by HHAT (Hedgehog acyltransferase)/Skinny hedgehog, enables it to act as a morphogen (Buglino and Resh, 2012). The addition of the cholesterol moiety ensures proper trafficking and transport of the active N-terminal peptide (Lewis et al., 2001) and the palmitoyl group is required for long range signaling (50 μm in the imaginal wing disc of flies and up to 300 μm in the vertebrate limb bud) (Briscoe and Therond, 2013; Chen et al., 2004; Tabin and McMahon, 1997). Cholesterol- and palmitate- modified SHH fragment (hereafter referred to as SHH) gets transported to the plasma membrane, where the cholesterol moiety mediates multimerization of the SHH monomers in lipid rafts possibly by promoting interactions with heparin sulfate proteoglycans (Ryan and Chiang, 2012).

The release of mature SHH from the producing cell requires the synergistic activity of two proteins: Dispatched (DISP), a 12-pass transmembrane protein from the resistance-nodulation-division (RND) transporter family and vertebrate specific SCUBE family of secreted protein. DISP and SCUBE establish two distinct synergistic cholesterol dependent contacts with SHH, which allows hand-off of the hydrophobic SHH from DISP to SCUBE (Briscoe and Therond, 2013; Tukachinsky et al., 2012).

Full length SHH peptides that do not undergo processing are functional and act in juxtacrine signaling in vitro. However, mutations that cause improper or no cleavage of the SHH peptide result in developmental defects like holoprosencephaly where the cerebral hemispheres fail to divide into distinct halves (Belloni et al., 1996; Maity et al., 2005; Roessler et al., 1996; Traiffort et al., 2004).

1.5.2 SHH signal responding cells: Vertebrate Shh signaling transduction
Shh signaling pathway has been historically considered to have a core machinery conserved from insects to mammals (Ingham and McMahon, 2001; McMahon et al., 2003). However, recent
studies have reported evidence for divergence in the Shh transduction cascade between flies and mammals (Huangfu and Anderson, 2006; Marks and Kalderon, 2011; Varjosalo et al., 2006). SHH signal is propagated by the binding of SHH ligand to a receptor complex comprising the twelve-pass membrane proteins Patched1 and 2 (PTCH) (Marigo et al., 1996; Pepinsky et al., 1998) and a seven-pass transmembrane protein called Smoothened (SMO) (Ingham, 1998). In vertebrates, binding of SHH ligand to the PTCH receptor is facilitated by co-receptors like cell-surface proteins growth arrest specific (GAS), Hedgehog-interacting protein (Hhip), cell-adhesion molecule-related/downregulated by oncogenes (CDO) and the brother of CDO (BOC) (Allen et al., 2011; Beachy et al., 2010; Izzi et al., 2011). In the absence of SHH ligand, PTCH acts as a negative regulator of SMO, inhibiting its entry into the cilium and thereby preventing the activation of the pathway. Upon binding of SHH to PTCH receptors the non-stoichiometric inhibition of PTCH on SMO is relieved and SMO accumulates in the cilium in an inactive form (Aanstad et al., 2009; Goetz et al., 2009; Lin et al., 2014; Rohatgi et al., 2007). This release of SMO results in the activation of the only downstream effectors of Hh signaling, the zinc finger containing Glioma-associated oncogene family (GLI) proteins.

Vertebrates have three dual function GLI proteins, GLI1, GLI2, and GLI3, all containing a C-terminal activation domain. GLI2 and GLI3 also possess an N-terminal repressor domain (Dai et al., 1999; Sasaki et al., 1999). GLI proteins are evolutionarily conserved TF’s, and were among the first TF’s to have their DNA binding site determined and modeled into 3D structure (Jiang and Hui, 2008; Kinzler and Vogelstein, 1990; Pavletich and Pabo, 1993). Moreover, GLI proteins were the first developmental genes to be associated with human cancers (Kinzler et al., 1988; Vortkamp et al., 1991). Presence or absence of HH ligand influences the balance of activator (GLI\textsuperscript{A}) and repressor (GLI\textsuperscript{R}) forms of GLI. Absence of HH results in selective proteolytic cleavage of the C-terminus activator domain of GLI2/3 resulting in the synthesis of repressor forms of GLI2/3. Protein kinase A (PKA), casein Kinase 1 (CK1), Suppressor of fused (SUFU),
the kinesin Kif7, glycogen synthase Kinase 3 (GSK3), and the E3 ubiquitin ligase SCF$^{\text{Slimb}^{\beta}\beta\text{TrCP}}$
are required to convert GLI$^A$ to GLI$^R$. In the absence of HH ligand, SUFU binds and sequesters
GLI2/3 in the cytosol and blocks their translocation into the nucleus. SUFU simultaneously
promotes PKA-, CK1-, and GSK3$^\beta$-mediated phosphorylation of the C-terminal activation
domain residues of GLI2/3 and primes them for E3 ubiquitin ligase SCF$^{\text{Slimb}^{\beta}\beta\text{TrCP}}$ mediated
ubiquitination and degradation to generate GLI$^R$. In vertebrates, Hh signal transduction relies on
the primary cilium that bulges out of the surface of quiescent cells. Primary cilium and its
associated structures harbor many components of the Hh signaling pathway both under active and
inactive state, which results in control of GLI1 protein degradation and processing (Goetz and
Anderson, 2010; Goetz et al., 2009; Rohatgi et al., 2007).

Functional studies demonstrate that GLI2/3 mutant mice do not transduce Hh signaling and that
GLI2/3 are critical mediators of Hh signaling during embryogenesis. GLI1 appears to be
dispensable for embryonic growth in mammals (Buttitta et al., 2003; Motoyama et al., 2003). However, in contrast to what is observed in mammals, vertebrates like zebrafish and Xenopus
have apparent differences. For example, GLI1 and not GLI2 is the primary activator of Hh
signaling during embryogenesis (Karlstrom et al., 2003; Lee et al., 1997).

1.5.3 Hedgehog signaling pathway during vertebrate eye development
Hh signaling is a classical pathway used reiteratively during various stages of vertebrate
development. The Hh network of proteins has pleiotropic functions including control of cell
division, cell survival, cell growth, and cell fate specification and patterning. Hh signaling has
universal gene targets that are activated ubiquitously as well as specific targets that are activated
only under certain circumstances. The targets are varied as the targeting output depends on
several criteria: the type of signal receiving cell; the amount of signal received; and the spatio-
temporal period of exposure (Varjosalo and Taipale, 2008). The Hh gradient is fine-tuned and
made robust by the deployment of several categories of proteins that control the synthesis, secretion, processing, and transport of the mature HH peptide. Cellular responses to the gradient involve series of positive and negative feedback loops that maintain precise transcriptional control of the Hh signal and its targets.

The downstream Hh target genes make up a positive/negative feedback loop with the Hh pathway and include PTCH1 (Vissers et al., 2004), PTCH2, GLI1 (Dai et al., 1999), Hhip and BOC. PTCH1, PTCH2 and BOC repress Hh signal by blocking the expression of Cdo, Boc and Gas1 (Bakrania et al., 2008; Lee et al., 2010; Vokes et al., 2007).

The list of genes targeted by Hh signaling network includes secreted molecules like bone morphogenetic protein (BMP4), fibroblast growth factor (FGF4), vascular endothelial growth factor (VEGF)-A; genes involved in cell growth and division, N-myc, cyclinD, and many transcription factors critical for embryogenesis like Pax, Nkx, Dbx, Irx families. The number of genes targeted by Hh signals are growing, and new techniques like genome-wide in silico analyses are identifying new targets.

Many signaling pathways regulate key developmental stages of eye development. Amongst them Shh pathway is crucial during eye development. SHH is secreted from the ventral midline, which directs the bifurcation of the eye field into two bilateral OVs in both invertebrates and vertebrates (Chiang et al., 1996; Roessler et al., 1996). However, in zebrafish due to redundancy between HH proteins, reduction in levels of both Shha and Shhb result in partial cyclopia and other ventral midline anomalies (Nasevicius and Ekker, 2000; Schauerte et al., 1998). The second important role of Shh during early stage of eye development is OV patterning along the proximo-distal axis. Shh signals promote proximal fate of optic stalk and choroid fissure and repress distal cell fate of retina, RPE and lens. At the molecular level, pax2 is a direct target of Shh and Shh activates the
expression of *pax2* in the optic stalk and choroid fissure (Chiang et al., 1996; Perron et al., 2003; Zhang and Yang, 2001). Distinct proximo-distal domains are formed within the OV due to reciprocal transcriptional repression of *pax2* and *pax6* (Schwarz et al., 2000). Overexpression of *Shh* results in ectopic expression of *Pax2* in the distal domain of OV, thereby forming excess optic structure in the optic stalk region at the expense of *Pax6* expressing distal neural retina (Ekker et al., 1995; Lee et al., 2008; Macdonald et al., 1995; Perron et al., 2003; Pillai-Kastoori et al., 2014a). Loss of *Pax2* results in a variety of ocular defects like optic nerve hypoplasia, thinned retinal layers, and coloboma syndromes in mice (Favor et al., 1996), zebrafish (Lun and Brand, 1998) and humans (Sanyanusin et al., 1995a).

Hh signaling pathway is also important during retinal neurogenesis and has been well studied in a variety of model organisms. In vertebrates, neurogenesis starts in the ventral retina, adjacent to the choroid fissure and the first post-mitotic neurons are found adjacent to the optic stalk. *Shh* expression wave spreads through the differentiating retina in the GCL and the amacrine cells of the INL of zebrafish retina. Additionally, a basic helix loop helix TF *atonal homolog 5 (Ath5)* is essential for the generation of RGCs in frog, chickens, mouse, and zebrafish (Brown et al., 2001; Kay et al., 2005; Vetter and Brown, 2001). Mutations in *ath5* result in complete loss of RGCs in zebrafish and mice (Brown et al., 2001; Kay et al., 2005). *Ath5* wave sweeps across the differentiating retina and is maintained at the peripheral region of the retina. *Shh* expression is initiated in the RGC and then the wave extends as differentiation proceeds. Both *Shh* and *ath5* waves are essential for proper neurogenesis. Blockade of Hh signaling using cyclopamine causes severe retardation in retinal neurogenesis and a delay in *ath5* wave progression (Kay et al., 2005; Neumann and Nuesslein-Volhard, 2000), as is seen in *syu*<sup>Δ</sup> (Shh null) mutant retinas (Kay et al., 2005). In *syu* mutants Shh:GFP signal is initiated but the progression of *Shh* is inhibited suggesting that *Shh* is not required for initiating its own expression but is required for expansion of *Shh* wave (Neumann and Nuesslein-Volhard, 2000). *Lakrtiz* mutants that lack Ath5 protein do
not form RGCs, they do however have a thin GCL that contains misplaced amacrine cells (Kay et al., 2005; Shkumatava et al., 2004). Interestingly, as retinal differentiation proceeds, Shh exerts an opposite effect on RGCs, by inhibiting the terminal differentiation of retinal precursors into RGC fate, thereby controlling the number of mature RGCs produced within the retina (Chung et al., 2001). The dual role of Hh has been attributed to its morphogen characteristic, where low levels of Shh stimulate differentiation of precursors into mature RGCs, and high concentration of Hh behind the differentiation wave inhibit the remaining progenitors from adopting the RGC fate. Hh signaling from the amacrine cells is required for the survival of neurons in the INL as syu mutants display lot of dying neurons in the INL. Shha and Shhb have been identified in the RPE and in the subretinal space and their expression occur concurrent with the appearance of the first differentiated photoreceptors (PR). Therefore, it is proposed that secreted Hh signal from the RPE may play a role in propagation of PR differentiation across the developing zebrafish retina. However, one of the interpretations based on the mosaic embryo generated by Shkumatava et al., 2004, consisting of syu mutants and wild type Shh:GFP cells, was that amacrine-derived Hh signals, not RPE-derived Hh signal, promotes PR differentiation. Alternative explanations are that perhaps RPE-Hh is required for PR differentiation and amacrine-Hh is important for cell survival; that signals from both apical (RPE) and basal (amacrine) cell layers promote PR differentiation; or that the amount of Shh morphogen biologically available to the PR precursor must attain a threshold before the precursors can undergo terminal differentiation. There is precedence for the significance of threshold requirements and graded Hh signal during neuronal cell fate determination and specification in ventral spinal cord and hindbrain of mice (Briscoe and Ericson, 1999; Ericson et al., 1997).

In conclusion, during vertebrate embryogenesis, pattern formation, and cell fates are determined by variable concentrations of crucial inductive signals. A lot is known about the strength of ligand mediated receptor activation and the biochemical interaction between co-receptors; however, the
downstream translation of the graded signal into an all-or-none output that influences distinct cellular identity remains an open and exciting challenge. More importantly, how subtle deviations from the optimal concentrations tips the balance from a perfect/normal organogenesis to a diseased state.

1.6 The zebrafish as a model system to study eye development
Extensive study and characterization of the zebrafish eye has contributed to the basic understanding of the intricate events underlying vertebrate eye development. Embryonic development of the zebrafish retina is extremely fast and the birth date of almost all retinal cell types has been determined, aiding the studies of ocular development. In vivo time-lapse imaging and tracking of single cells have shed light on the complex rearrangements that occur during optic cup morphogenesis. In zebrafish, during neurulation the single sheet of neural plate is folded at the midline to form a neural tube resulting in the formation of a “neural keel.” Between 12-16 hours post fertilization (hpf) the specified eye field cells undergo orchestrated evagination and elongation steps as the hypothalamic anlagen migrates from posterior to anterior location and displaces the prospective eye and optic stalk cells laterally into the forming optic vesicles (OV) (England et al., 2006). In zebrafish, as the OV ends evagination around 14 hpf, the developing optic cup including the NR, RPE and the lens rotates anteriorly from a horizontal to a more vertical position. Lens formation is induced upon the contact of the evagination OV and the overlying surface ectoderm. OCM continues and as the RPE cells spread, lens cells converge medi ally and cells undergo rim movement between 18-24 hpf in order to join the optic cup layers both anteriorly and posteriorly. The optic stalk starts to lengthen around 24 hpf and allows for the eye to rotate once more and move away from the head. The choroid fissure is formed by 24 hpf and it starts closing around 36 hpf and is completely fused by 48 hpf due to the fusion of mesenchymal ligaments. Eye rotation stops at 36 hpf such that the choroid fissure is now repositioned from an anterior to a more ventral location.
In zebrafish retina, the first neurons to differentiate in the retina are the ganglion cells, which appear around 32 hours post fertilization (hpf). The first photoreceptor outer segments are formed by 55 hpf. Photoreceptor synaptic terminals are detectable at about 65 hpf, followed by the bipolar cell synaptic terminals at about 70 hpf. Synaptic transmission is initiated by the photoreceptors at about 80 hpf and the photoreceptors are completely mature by 5 days post fertilization (dpf). The zebrafish retina continues to add new neurons throughout life. New retinal neurons are produced from a small population of proliferative cells present at the ciliary margin zone (CMZ). In addition, there is a population of rod progenitor cells located at the base of the outer nuclear layer ONL that exclusively produces rod photoreceptors to maintain visual sensitivity as the retina expands. Similar to humans, zebrafish have a tri-layered retina separated by two synaptic cell free layers called plexiform layers, and zebrafish are also a diurnal species (unlike nocturnal rodents). However, in contrast to humans, the zebrafish retina possesses the remarkable ability to regenerate neurons in response to injury.

Zebrafish has one type of rod photoreceptor and four spectral subtypes of cone photoreceptors arranged in a precise mosaic pattern consisting of alternating rows of red/green double cones and blue/ultraviolet (UV) cones. The inner nuclear layer (INL) includes the cell bodies of at least 17 subtypes of bipolar cells, 4 subtypes of horizontal cells and at least 70 different types (28 subtypes) of amacrine cells (Jusuf and Harris, 2009). INL also contains the cells bodies of the only resident glia of the retina called the Müller glia. Müller glia are an important part of the retina as they help in a wide range of physiological functions. Müller glia help in recycling of the visual pigment all-trans retinal to 11-cis retinal in the phototransduction cascade, help in nutrient cycling, and in maintenance of constant metabolic rhythm for the retinal neurons. In zebrafish, Müller glia cells are also able to de-differentiate in response to injury to regenerate different types of retinal neurons.
1.7 SOXC and the eye
The morphological events, coordinated cell movements, and the sequential induction of eye specific genes regulating early ocular development have been well described. However, we do not yet fully understand how the structural and functional interactions between the cellular signals guide pattern formation in the anterior neural plate or the dynamic regulation of genes expressed in the optic progenitors at different stages of eye formation. With an interest in elucidating the mechanisms that are involved in retina pattern formation, we have characterized a SRY-Box transcription factor, Sox11, during zebrafish ocular development.

1.7.1. SOX C genes and proteins
SOX proteins are found extensively throughout the animal kingdom including, nematodes, arthropods, birds, rodents, reptiles, amphibians, mammals, and fish (Bowles et al., 2000; Nagai, 2001). Vertebrate genome encodes about 20 SOX genes that are grouped into 8 subgroups (A-H) on the basis of sequence similarity and genomic arrangements (Bowles et al., 2000; Kamachi and Kondoh, 2013; Koopman et al., 2004; Schepers et al.). SoxC family of Sox proteins represents three intron-less genes: SOX11 [MIM: 600898], SOX4 [MIM: 184430] and SOX12 [MIM: 601947] (Penzo-Méndez, 2010). These transcription factors contain a 78-amino-acid DNA binding high-mobility group (HMG) domain in the N-terminus and a transactivation domain (TAD) in the C-terminus. Like other Sox members, Sox4, Sox11 and Sox12 are >50% identical to the sex determining region on the Y chromosome (SRY) HMG box domain both at the nucleotide and amino acid level (Lefebvre et al., 2007; Nagai, 2001). Within the subgroup, Sox4, Sox11 and Sox12 share high degree of similarity within the HMG box domain as well as the sequences flanking the HMG box including the TAD domain (Bhattaram et al., 2010a). DNA site selections studies have shown that the HMG box domain consisting of three alpha helices and a beta strand prefers a core sequence of six bases 5’-(A/T)(A/T)CAA(A/T)G-3’, and it also binds to four-way DNA junctions without any sequence specificity (Grosschedl et al., 1994; King and
Weiss, 1993; Weiss, 2001). After the initial specific sequence recognition, the twisted L-shaped HMG domain induces a dramatic sharp architectural bend of 30-110° in the minor groove of the target DNA (Grosschedl et al., 1994; Lefebvre et al., 2007). The DNA bending causes the width of the minor groove to widen and also results in possible unstacking of base pairs. Thus, HMG box containing Sox proteins gain greater accessibility and plasticity in forming transcriptional enhanceosomes.

1.7.2 SOXC targets

In the past decade, several groups have started to unravel the role of SOXC proteins in diverse processes. SOXC family of proteins are extensively and abundantly expressed in cells of neural and mesenchymal lineage (Bhattaram et al., 2010a; Sock et al., 2004b). This family of proteins has a potent transactivation domain in the (TAD) in the C-terminus which is present among the SOXC members. Sox4/11/12 have remarkable conservation of residues (94-100%) within the HMG-box DNA binding domain and the C-terminal 33 residues which makeup the TAD domain (Bhattaram et al., 2010b; Dy et al., 2008b; Koopman et al., 1991). Although Sox4/11/12 have almost identical HMG box domain, they show different DNA binding efficiencies in vitro. Sox4 is more efficient than Sox11 and Sox12, suggesting that functional domains outside of HMG-box domain influence DNA binding capacities of SoxC proteins. Indeed, deletion of the TAD and acidic regions in Sox11/12 increases their efficiency to bind DNA targets in vitro (Dy et al., 2008b). Additionally, Sox4/11/12 have different transactivation capabilities as well, with Sox11 being more potent than Sox4 and Sox11 in vitro, most likely due to the different α-helical structure formed by their respective TADs (Dy et al., 2008b).

Thus, it is not far reaching to conclude that the co-expression of SoxC proteins does not always mean redundancy and that secondary and tertiary SoxC protein structures may provide unique target selection and biochemical advantage. Bergsland and his group also demonstrated that co-
expression of a wildtype and a truncated SoxC protein lacking the TAD causes a dominant negative repression of the wild type SoxC protein function (Bergsland et al., 2006).

Several Sox proteins bind DNA target sites that are only partially identical to the core Sox recognition site identified via in vitro studies (Lefebvre et al., 1997; Lefebvre et al., 1998). Vertebrate genome is riddled with such incomplete Sox recognition sequences and therefore, search of Sox target sites simply on the basis of DNA sequence can be a puzzling assignment. Although they are essential and play multiple roles in embryonic development and tissue differentiation, very few SOXC target genes have been identified. A single target gene has been identified for Sox4. Sox4 binds to the 3’ end enhancer region of CD2, a T-cell specific enhancer and transactivates the CD2 gene (Wotton et al., 1995). In response to genotoxic stress, Sox4 acts as DNA damage sensor and is required to activate and stabilize p53. Sox4 physically interacts with p53 protein, and stabilizes the protein by enhancing its transcriptional action via acetylation, thereby inhibiting Mdm2-mediated p53 ubiquitination (Pan et al., 2009).

In keeping with the role of SoxC during organogenesis, Sox4/11 is reported to target Tead2 (TEA domain family member 2). TEAD2 is a transcriptional activator of the Hippo signaling pathway that controls tissue and organ size (Barron and Kagey, 2014; Bhattaram et al., 2010a; Kamachi and Kondoh, 2013). As the embryo develops and neurogenesis ensues, Sox4/11 controls a critical step in transition from an immature neuron to a mature differentiated neuron (Bergsland et al., 2011). Sox4/11/12 expressed within post-mitotic immature neuron targets a neuron specific classIII β-tubulin known as Tubb3 or Tuj1(Romaniello et al., 2014). Tuj1 putative promoter region has three potential SoxC binding sites which can be bound by Sox4/11 in a gel shift assay (Bergsland et al., 2006). Sox12 also has the potential to also bind to Tubb3 but with a relatively lower transactivation activity (Hoser et al., 2008).
1.7.3 *SoxC expression and function in the developing eye*

The members of the SoxC family of transcription factors are expressed extensively in the developing vertebrate nervous system and are known to regulate neuronal cell fate determination and differentiation into specific lineage in a highly redundant manner (Bergsland et al., 2011; Bhattaram et al., 2010a; Dy et al., 2008a). Expression of SoxC genes is strong in the ventricular region of the brain, particularly in cells that have exited the cell cycle but have not yet differentiated into mature neurons (Bhattaram et al., 2010a). As development progresses, SoxC expression is limited to the forebrain and caudal part of the spinal cord suggesting that SoxC genes are downregulated as the nervous system matures (Jankowski et al., 2006).

With respect to the developing eye, in the mouse *Sox11* and *Sox4* expression are first detected in the central dorsal optic cup; however, they differ in the temporal aspect of their expression. *Sox11* appears first at E10.5 and then *Sox4* at E11.5 in a similar region of the central dorsal optic cup. As development proceeds, *Sox4* and *Sox11* expression fans out rapidly in a central to peripheral fashion spanning the whole of the retina. From E15.5-E18.5, *Sox4* and *Sox11* have an identical strong expression pattern in the GCL and an identical diffused expression pattern in the outer neuroblastic layer. While expression of *Sox11* fades by P3, *Sox4* persists in lower levels in the GCL and inner nuclear layer in the postnatal retina (Jiang et al., 2013; Usui et al., 2013a). Expression of *Sox4* and *Sox11* coincides with the expression of *Brn3b*, which is a ganglion cell marker, and *Islet1* which is an amacrine cell marker (Jiang et al., 2013; Usui et al., 2013a). Similar to what has been observed in mice, SoxC genes are also expressed in the developing eye of persistently neurogenic animals like zebrafish and *Xenopus* and become downregulated as development progresses (Cizelsky et al., 2013). However, both *Sox4/11* continue to be expressed in the retinal progenitor cell niche called the ciliary marginal zone (CMZ) throughout adulthood (Pillai-Kastoori et al., 2014b; Wen et al.).
Global inactivation of Sox11 and/or Sox4 in vertebrate embryos is lethal due to septation defects in the ventricular chamber of the heart. However, Sox12 knockouts are viable and fertile (Dy et al., 2008a; Hoser et al., 2008; Schilham et al., 1996). Compound Sox11<sup>+/−</sup>; Sox4<sup>+/−</sup> heterozygotes also die at birth, suggesting that cardiac development in mice requires an optimal gene dosage of both Sox4 and Sox11. Apart from cardiac defects, Sox11<sup>−/−</sup> homozygous mutant mice embryos exhibit a range of ocular defects, including Peter’s anomaly, open eyelids, microphthalmia, and coloboma (Sock et al., 2004a; Wurm et al., 2008a).

Variation in SoxC gene dosage also results in a persistent lens stalk and delayed lens maturation in zebrafish, mice, and Xenopus (Cizelsky et al., 2013; Wurm et al., 2008b). Cellular analysis of Sox11<sup>+/−</sup> embryos shows reduced mitotic profiles in the lens placode during lens invagination, suggesting that Sox11 is required for the separation of the lens vesicle from the surface ectoderm (Wurm et al., 2008a). Pax6 is known to be critical in lens development (Kondoh et al., 2004). Recent study in pax6 mutant mice, which has delayed lens development, and medaka embryos revealed that pax6 suppressed the expression of sox11 in the lens via miR-204 (Shaham et al., 2013).

In addition to ocular morphogenesis and lens defects, retinal neurogenesis is also disrupted in SoxC-deficient animals. Jiang and others created Sox4, Sox11, and Sox4/Sox11 conditional knockout mice using a Six3-Cre line, thereby removing Sox4 and/or Sox11 in the eye field and ventral forebrain from E9 onwards. They observed a modest reduction in RGCs in the single knockouts, and a complete loss of RGCs as well as significant reductions in other retinal neurons in the Sox4/Sox11-null retina (Jiang et al., 2013). Loss of Sox4 and Sox11 function in mice also resulted in reduction in the expression of histone H3 acetylation at proneural genes such as NeuroD, suggesting that Sox4 and Sox11 may influence retinal progenitor cell competence and differentiation by creating a specific epigenetic state. The expression of the RGC marker pou4f1
was absent in the retinas of both Sox4- and Sox11-deficient *Xenopus* embryos, indicating a defect in RGC differentiation (Cizelsky et al., 2013). Apoptosis was also significantly increased in Sox4- and Sox11-deficient *Xenopus* retinas, indicating a potential cause for the smaller eye phenotype and the retinal disorganization in these animals (Cizelsky et al., 2013).

*Sox4* and *sox11* expression are elevated in a zebrafish model of chronic rod photoreceptor degeneration and regeneration (Morris et al., 2011b), suggestive of their potential involvement in rod photoreceptor differentiation. Gain-of-function studies using mouse retinal explants demonstrated that overexpression of SoxC factors interferes with the maturation and terminal differentiation of rods and cones (as well as Müller glia), suggesting that SoxC factors inhibit photoreceptor differentiation (Usui et al., 2013b). It may not be far reaching to presume that SoxC factors may have species-specific functions in photoreceptor development, and/or that photoreceptor development pathway is critically sensitive to SoxC expression levels, such that deviations from the optimum meaningfully compromises terminal differentiation of photoreceptor progenitors.

### 1.8 SOXC and human ocular defects

In conjunction with the data from animal models, evidence implicating SOXC factors in human diseases that affect eye development is beginning to emerge. Tsurusaki et al. performed whole-exome sequencing on DNA samples from 92 patients with Coffin-Siris syndrome, a disorder characterized by developmental delay, abnormalities of the fingers and/or toes, and abnormal facial features. Two patients were identified with two novel *de novo* heterozygous mutations in the HMG domain of *SOX11*. One of the patients exhibited vision defects, along with several other clinical features of Coffin-Siris syndrome (Tsurusaki et al., 2014).

Patients suffering from coloboma often present with symptoms of a common syndrome called CHARGE syndrome also known as HALL-HITTNER SYNDROME (MIM #214800).
Pathogenic mutations in Chromatin remodeler chromodomain-helicase-DNA-binding protein 7 (CHD7) gene located on chromosome 8q12 account for more than 90% of patients with CHARGE syndrome (Aramaki et al., 2005; Bergman et al., 2011; Lee et al., 2015; Vissers et al., 2004). CHD7 is crucial for the formation of multipotent neural crest cells that migrate from the neural tube and contribute to a variety of tissue formation (Schulz et al., 2014). In addition, its ATP-dependent chromatin remodeling activity modulates transcription activity of target genes (Kim and Roberts, 2013; Sperry et al., 2014). Inactivation of CHD7 specifically in the mouse neural stem cells causes reduced neuronal differentiation and resulted in abnormal dendritic development of newborn neurons. Additionally, it also causes remodeling of SoxC promoter region into a closed chromatin state (Feng et al., 2013; Kim and Roberts, 2013). Gene expression profiling data from human glioblastoma patients (GMB) present in the Cancer Genome Atlas Project (TCGA) shows strong correlation of SOX4 and SOX11 with CHD7 expression (Feng et al., 2013). Extensive work done by Feng and colleagues strongly suggest that SOX4 and SOX11 are direct targets of CHD7 (Feng et al., 2013). Similarities between the phenotypes exhibited by Sox11/Sox4 null mice, sox4/sox11 zebrafish morphants and CHARGE patients, suggests that CHD7/SOX4/SOX11 may be related in a general pathway to regulate ocular development and disease progression in CHARGE syndrome patients.

1.9 Conclusions and Perspectives
Extensive work across several vertebrate models has begun to unravel the intricacies of ocular morphogenesis. One thing that we have learned from these studies is that a handful of signaling pathways control various aspects of oculogenesis and they are deployed re-iteratively throughout the course of embryonic eye development. These signaling pathways regulate the expression of several key TFs to pattern the developing eye into tissue-specific domains, and to control the precise and timely specification of progenitor cells for differing fates. SOX family members are critical regulators of embryonic development, and the SOXC family has been recently implicated
in eye development in a variety of animal models. Although it is clear that mutation or loss of SOXC proteins results in defects in ocular morphogenesis, lens development, and retinal neurogenesis, we do not know all of the transcriptional targets of SOXC proteins in the eye. The future lies in the investigation and identification of SOXC target genes, and in understanding their mechanism of action during ocular development.
Rationale, Overall Hypothesis and Specific Aims

Congenital ocular defects like microphthalmia, anophthalmia, and coloboma (MAC) arise due to faulty embryogenesis. Genetically, MAC phenotypes display heterogeneity, incomplete penetrance, and variable expressivity. Only ~27 genetic loci have been mapped in the coloboma gene network, however, these 27 loci explain less than 20% of the known cases of MAC (Gregory-Evans et al., 2013; Gregory-Evans et al., 2004; Maumenee and Mitchell, 1990). The apparent association of coloboma with a variety of genes across the genome and with different multi-system syndromes with varying inheritance patterns illustrates the genetic complexity of coloboma (Hornby et al., 2000; Onwochei et al., 2000). Further characterization of the signaling molecules and transcription factors and understanding the relationships between different regulators will help us understand both eye development and diseases affecting the developing eye.

The overall aim of this dissertation was to determine the role of Sox11 during vertebrate ocular morphogenesis and retinal neurogenesis. The working hypothesis was that Sox11 is required for proper eye formation and differentiation of rod photoreceptors. Presented in this body of work is evidence of requirement of Sox11 for proper ocular morphogenesis, specifically for choroid fissure closure, and rod photoreceptor differentiation. Furthermore, we present the first report of negative regulation of Sonic Hedgehog (Shh) signaling by Sox11 during ocular morphogenesis in zebrafish. Additionally, we took the findings from our animal model studies one step forward and performed human SOX11 sequence screen and provide evidence that alteration in SOX11 gene sequence or dosage levels contribute to pediatric human ocular abnormalities.
This work will be laid out in the following aims:

**Specific Aims**

**I.** The initial working hypothesis tested was that Sox11 is required for proper eye development and is required for rod photoreceptor differentiation. The specific aims were to:

- Identify the expression patterns of *sox11a* and *sox11b* during different stages of eye and retina development in zebrafish using in situ hybridization.
- Test the requirement of *sox11a/b* during ocular morphogenesis and retinal neurogenesis by using morpholino-mediated gene knockdown approach.

**II.** Based on results from the above experiments, the second hypothesis tested was that dysregulation of Hedgehog signaling (Hh) contributes to the abnormal ocular phenotypes displayed by *sox11* morphants. To test this hypothesis, the following experiments were performed:

- Evaluate the expression profiles of Hh target genes (*pax2.1; pax6a*) via fluorescent in situ hybridization at different stages of eye development.
- Evaluate the transcript levels of Hh pathway receptors, ligands, and effectors in control and *sox11*-deficient zebrafish embryos via quantitative real time PCR.
- Evaluate the expression of *patched2* gene expression using a transgenic *Tg(GBS-petch2.nlsEGFP)* zebrafish background in the presence of *sox11* morpholino at early stages of eye development.
- Test the contribution of Hh signaling to the abnormal ocular phenotypes displayed by *sox11* morphants by using pharmacological and antagonist of Hh signaling on *sox11* morphant embryos. Additionally, test the effect of *sox11* overexpression on Hh signaling during early eye development.
- Evaluate the contribution of *shha* ligand to the abnormal ocular phenotypes displayed by *sox11* morphants by co-knockdown of both *shha* and *sox11* in developing zebrafish embryos.
III. The data gathered helped the evolution of hypothesis III. The new hypothesis tested was that *sox11* acts as an activator of a negative regulator of *shha* transcription during eye development in *zebrafish*.

- Evaluate the transcript status of known negative regulators of *shha* transcription in *sox11* morphants via quantitative real time PCR, and perform proof-of-principle experiment (RNA rescue) once a likely candidate is identified.

IV. On the basis of studies in our lab and work done by others (Cizelsky et al., 2013; Jiang et al., 2013; Lo-Castro et al., 2009; Wurm et al., 2008b), we initiated a sequencing screen of patients belonging to MAC cluster in order to identify possible correlation between *SOX11* and human ocular abnormalities. This project was a fruitful collaboration between our lab, Dr. Ordan Lehmann at University of Alberta, Canada, and Dr. Adriana Lo-Castro at University of Rome, Italy. The hypothesis tested was that alteration in *SOX11* coding sequence or changes in gene dosage contributes to human ocular defects. The following experiments were performed:

- Carry out a *SOX11* sequence screen on a cohort of 79 MAC patient DNA samples and 400 control patient samples to identify sequence variations and establish statistical significance.
- Test the functional consequences of the sequence variations discovered in *SOX11* in zebrafish embryos and via an in vitro luciferase assay.
- Perform array comparative genomic hybridization (array CGH) on DNA from a patient with microphthalmia, unilateral optic nerve agenesis, and a *de novo* chromosome 2p25 deletion (Lo-Castro et al., 2009) to narrow the breakpoint region.

The four aims mentioned above constitute Chapter 2 of this dissertation.

V. In order to further understand the function of Sox11 during eye development, we tested the hypothesis that *sox11* gene function is required during early stages of embryonic development to guide both proper ocular morphogenesis and retinal neurogenesis. The following experiment was performed:
• Identify the critical temporal window of embryonic development that requires $sox11$ gene function for the proper closure of choroid fissure and rod photoreceptor development by employing Photo-morpholino approach.

VI. The final aim of the dissertation is to make stable $sox11a$ and $sox11b$ knockout lines using the CRISPR/Cas9 genome editing system, in order to study the requirement of $sox11$ gene function not only in the developing eye but also in the adult stage under regeneration/degeneration paradigm.
CHAPTER 2: SOX11 IS REQUIRED TO MAINTAIN PROPER LEVELS OF HEDGEHOG SIGNALING DURING VERTEBRATE OCULAR MORPHOGENESIS

Lakshmi Pillai-Kastoori¹, Wen Wen¹, Stephen G. Wilson¹, Erin Strachan², Adriana Lo-Castro³, Marco Fichera⁴, Sebastiano A. Musumeci⁵, Ordan J. Lehmann², and Ann C. Morris¹*

¹Department of Biology, University of Kentucky, Lexington, KY 40506-0225, USA
²Departments of Ophthalmology and Medical Genetics, University of Alberta, Edmonton, AB T6G 2H7, Canada
³Department of Neuroscience, Pediatric Neurology Unit, “Tor Vergata” University of Rome, 00133 Rome, Italy
⁴Laboratory of Medical Genetics, IRCCS Associazione Oasi Maria Santissima, 94018 Troina, Italy, and Medical Genetics, University of Catania, 95131 Catania, Italy
⁵Unit of Neurology, IRCCS Associazione Oasi Maria Santissima, 94018 Troina, Italy

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2.1 Abstract
Ocular coloboma is a sight-threatening malformation caused by failure of the choroid fissure to close during morphogenesis of the eye, and is frequently associated with additional anomalies, including microphthalmia and cataracts. Although Hedgehog signaling is known to play a critical role in choroid fissure closure, genetic regulation of this pathway remains poorly understood. Here, we show that the transcription factor Sox11 is required to maintain specific levels of Hedgehog signaling during ocular development. Sox11-deficient zebrafish embryos displayed delayed and abnormal lens formation, coloboma, and a specific reduction in rod photoreceptors, all of which could be rescued by treatment with the Hedgehog pathway inhibitor cyclopamine. We further demonstrate that the elevated Hedgehog signaling in Sox11-deficient zebrafish was caused by a large increase in shha transcription; indeed, suppressing Shha expression rescued the ocular phenotypes of sox11 morphants. Conversely, over-expression of sox11 induced cyclopia, a phenotype consistent with reduced levels of Sonic hedgehog. We screened DNA samples from 79
patients with microphthalmia, anophthalmia, or coloboma (MAC) and identified two novel heterozygous $SOX11$ variants in individuals with coloboma. In contrast to wild type human $SOX11$ mRNA, mRNA containing either variant failed to rescue the lens and coloboma phenotypes of Sox11-deficient zebrafish, and both exhibited significantly reduced transactivation ability in a luciferase reporter assay. Moreover, decreased gene dosage from a segmental deletion encompassing the $SOX11$ locus resulted in microphthalmia and related ocular phenotypes. Therefore, our study reveals a novel role for Sox11 in controlling Hedgehog signaling, and suggests that $SOX11$ variants contribute to pediatric eye disorders.
2.2 Author summary
Ocular coloboma is condition in which tissue is missing from a portion of the eye due to its abnormal development. Coloboma is also frequently associated with additional anomalies, including microphthalmia (abnormally small eye) and cataracts. Although some of the genes that cause coloboma have been identified, in the majority of cases the underlying genetic cause has not been determined. One pathway that has been implicated in coloboma is the Hedgehog (Hh) signaling pathway. In this study, we have taken advantage of the ability to titrate levels of gene expression in zebrafish to demonstrate for the first time that the transcription factor Sox11 is required to limit levels of Hedgehog (Hh) signaling during ocular development. We show that in the absence of Sox11, levels of the Sonic Hedgehog (Shh) ligand are greatly elevated, which disrupts the proper patterning of the optic stalk and optic vesicle. We also provide evidence that SOX11 dosage changes or mutations contribute to human coloboma, microphthalmia, and rod photoreceptor dysfunction. Thus, our work establishes a novel link between Sox11 and Hh signaling, and suggests that mutations in SOX11 contribute to pediatric eye disorders such as coloboma.
2.3 Introduction
Ocular coloboma arises when the embryonic choroid fissure in the ventral optic cup fails to close. It can cause significant pediatric visual impairment (Shah et al., 2012), and is often associated with other ocular abnormalities such as microphthalmia or anophthalmia (collectively referred to as MAC). Coloboma may also be observed in conjunction with dysgenesis of the anterior segment (front portion of the eye) or optic nerve, lenticular defects (such as cataract), or systemic congenital malformation syndromes (Chang et al., 2006). In addition to phenotypic heterogeneity, coloboma is genetically heterogeneous, exhibiting differing patterns of inheritance, variable expressivity, and reduced penetrance (Chang et al., 2006).

Among the signaling pathways that converge to regulate ocular morphogenesis, Hedgehog (Hh) signaling has a critical role and acts reiteratively during eye development (Amato et al., 2004). Hh signaling from the midline promotes the segregation of the single eye field into two optic primordia, and is required for the correct proximodistal and dorsoventral patterning of the optic vesicle (Amato et al., 2004; Ekker et al., 1995; Zhang and Yang, 2001). Once the optic cup has formed, intraretinal Hh signaling regulates the differentiation of retinal progenitor cells (Amato et al., 2004). Given its central role in eye development, it is unsurprising that mutations in genes encoding Hh pathway ligands (SHH) or targets (PAX2, VAX1) are associated with congenital ocular malformations in humans (Bakrania et al., 2010; Sanyanusin et al., 1995b; Schimmenti et al., 2003; Slavotinek et al., 2012). However, these mutations account for only a minority of patients; for the majority of MAC cases, the molecular defect has yet to be identified. Because of its potency, the spatiotemporal levels of Hh ligands must be tightly regulated throughout eye development; yet, very little is known about the factors that restrict their expression during oculogenesis. Such factors would represent excellent candidate genes for human coloboma and associated ocular defects, and potentially could be used to influence Hh signaling.
Here, we focus on the role of the SRY-box transcription factor Sox11 during eye development. Sox11 is a member of the group C family of SOX proteins, which also includes Sox4 and Sox12 (Penzo-Méndez, 2010). Sox11 is required for a variety of processes, including organogenesis and neurogenesis, craniofacial and skeletal development (Penzo-Méndez, 2010), as well as being implicated in carcinogenesis (including mantle cell lymphoma, medulloblastoma, and glioblastoma) (de Bont et al., 2008; Penzo-Méndez, 2010). Expression and functional studies support a role for Sox11 during several stages of eye development. In the mouse, Sox11 is expressed in the optic cup and periocular mesenchyme during early eye development, and in the developing lens and retina at later stages (Sock et al., 2004b; Wurm et al., 2008b). In the zebrafish retina, we previously found that Sox11 is upregulated in rod progenitor cells during rod photoreceptor regeneration (Morris et al., 2011a). Sox11+/− mice exhibit ocular abnormalities such as anterior segment dysgenesis, microphthalmia, a persistent lens stalk, delayed lens formation, and coloboma (Wurm et al., 2008b). Finally, some human chromosomal rearrangements resulting in ocular abnormalities have been mapped to the vicinity of the SOX11 locus at chromosome 2p25.2 (Aviram-Goldring et al., 2000; Heathcote et al., 1991; Lo-Castro et al., 2009; Tirado et al., 2009). These data together suggested intriguing roles for Sox11 in ocular morphogenesis and rod photoreceptor differentiation, however the underlying mechanisms were undefined.

In this study, we inhibited Sox11 activity in zebrafish embryos, and based on the resultant phenotypes demonstrate that the function of Sox11 in regulating lens development and choroid fissure closure is evolutionarily conserved, and that Sox11 is required for rod photoreceptor differentiation. We demonstrate that elevated Hh signaling causes the ocular phenotypes in Sox11-deficient zebrafish, and that Sox11 is required to repress expression of the Sonic hedgehog gene (shha). Finally, we identify SOX11 variants with reduced transactivation ability in MAC patients, and in parallel demonstrate that decreased SOX11 gene dosage results in congenital ocular abnormalities. In revealing a previously uncharacterized role for Sox11 upstream of Hh.
signaling, these studies may substantially extend our understanding of additional Sox11-dependent developmental and pathologic processes.
2.4 Results

2.4.1 Expression of sox11a/b during ocular development

Zebrafish possess two orthologs of mammalian Sox11, which are expressed in overlapping and distinct domains ((De Martino et al., 2000; Rimini et al., 1999), this study). Previous studies have shown that both sox11a and sox11b are maternally expressed prior to the midblastula transition, and are expressed in the region of the anterior neural plate that gives rise to the diencephalon at the onset of the segmentation period (De Martino et al., 2000). Using in situ hybridization with paralog-specific probes, we investigated the expression of sox11a and sox11b both within the forebrain during optic cup formation, and in the eye at later stages of retinal development. At 18 hours post fertilization (hpf), we detected expression of sox11a and sox11b in the telencephalon, and in the dorsal “corner” formed by the diencephalon and the evaginated optic stalk/optic vesicle (top panel arrows and second row closed asterisks, Figure 1A). We also detected faint expression of sox11a and sox11b at the ventral hinge of the optic stalk/optic vesicle axis (open asterisks, Figure 1A). However, we did not detect expression of sox11a or sox11b within the optic vesicle itself (Figure 1A). At 24 hpf, sox11a/b expression persisted in the diencephalon adjacent to the retina and in the telencephalon, and both paralogs were also expressed in the hypothalamus (Figures 1A, C). Within the developing retina at 24 hpf, sox11b was expressed diffusely across the lens and retinal neuroepithelium, and was distinctly visible in a small cluster of cells in the ventro-nasal retina (arrow, bottom right panel, Figure 1A), corresponding to the location at which retinal neurogenesis initiates (Hu and Easter Jr, 1999). As retinal development proceeded, sox11a expression was observed in the ganglion cell layer (GCL) at 48 hpf, whereas the expression of sox11b was detected in a few scattered cells across the central retina but was mostly restricted to the undifferentiated peripheral retina [Figure 1B; (Morris et al., 2011a)]. By 72 hpf, when retinal neurogenesis was mostly complete, both sox11a and sox11b were predominantly expressed in the persistently neurogenic ciliary marginal zone [Figure 1B; (Morris et al., 2011a)]; expression of sox11a also persisted in the GCL and in some cells in the inner half of the inner nuclear later.
(INL). Interestingly, the expression domains of both sox11 paralogs were adjacent to regions of shha expression in the ventral diencephalon at 18 and 24 hpf (Figure 1C), whereas at 48 hpf sox11a expression overlapped with the previously described location of shha in the GCL (Neumann and Nuesslein-Volhard, 2000; Vinothkumar et al., 2008).
Figure 2.1. Developmental expression of sox11. In situ hybridization with antisense probes for sox11a, sox11b, and shha was performed on whole embryos or on tissue sections at the indicated time points. (A) Sox11a and sox11b was expressed in the diencephalon adjacent to the optic vesicle (arrows in top row and asterisks in second row) at 18 hpf (top two rows) and 24 hpf (third and fourth rows). Sox11a expression was not detected in the lens or retina at 24 hpf (bottom left). Sox11b was expressed in a patch of cells in the ventronasal retina (arrow, bottom right) and more diffusely across the rest of the retina and lens. Top and third rows are dorsal views of flat-mounted embryos. Second and fourth rows are frontal sections through the head. Bottom panels are lateral views of dissected eyes; (n=20 embryos examined per time point, 3 independent repeats). (B) Transverse sections through the eye at 48 hpf (top) and 72 hpf (bottom). Sox11a expression was detected in the ganglion cell layer (GCL) and in few sporadic cells in the inner nuclear layer (INL); sox11b expression was observed in scattered cells across the central retina and in the peripheral retina. At 72 hpf, sox11a expression persisted in the GCL and in some cells in the INL; sox11a and sox11b were also expressed in the persistently neurogenic ciliary marginal zone (CMZ); n=20 embryos examined per time point, 3 independent repeats. (C) Expression patterns of sox11a (left), sox11b (center), and shha (right) in the developing brain at 18 hpf (top) and 24 hpf (bottom). The eye was removed to better image the brain. Expression of sox11a and sox11b, but not shha, was observed in the telencephalon. Expression of all three genes was detected in the hypothalamus and ventral diencephalon at 24 hpf (n=20 embryos examined per time point, 3 independent repeats). Scale bar=100µm; D, dorsal; V, ventral; A, anterior; P, posterior; hpf, hours post fertilization; OV, optic vesicle; L, lens; R, retina; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; CMZ, ciliary marginal zone; tel, telencephalon; hy, hypothalamus; di, diencephalon.
2.4.2 Knockdown of sox11a/b causes abnormal ocular morphogenesis

To investigate the function of Sox11 paralogs during eye development, translation of sox11a and sox11b was blocked with morpholino oligonucleotides (MOs), whose efficiency and specificity were confirmed using a second sox11 MO and a GFP reporter assay, respectively (Figures S1A, C). Zebrafish embryos were injected with sox11a and sox11b MOs simultaneously (hereafter referred to as sox11 morphants), as co-inhibition of both paralogs induced the highest prevalence of ocular phenotypes (Figure S1B). At 24 hpf, 72.9±7.4% of sox11 morphants displayed a misshapen, rudimentary, or absent lens (Figures 2A, S1B). Sox11 morphant lenses mostly recovered to a spherical shape by 2 days post fertilization (dpf), however at this stage a similar proportion of morphants (70.0±7.7%) displayed coloboma (Figures 2A, S1B). Sox11 morphant eyes were also hypopigmented ventrally, and microphthalmic compared to controls (Figures 2A, B). Histological sections revealed that the colobomatous retinas in sox11 morphants frequently extruded through the open choroid fissure into the brain (Figure 2C). Approximately 54% (15 of 28 individuals examined) of sox11 morphant retinas with coloboma also exhibited poor or reduced retinal lamination, suggesting a delay in retinal differentiation. In contrast, of the sox11 morphant retinas that did not display coloboma, only 14% were poorly laminated (4 of 29). This suggests that similar mechanisms may underlie the ocular morphogenesis and retinal developmental defects observed in sox11 morphants with coloboma. The presence of the coloboma prevented the retinal pigmented epithelium (RPE) from completely enclosing the posterior eye (Figure 2C), which likely accounts for the hypopigmented appearance of the ventral portion of the eye when viewed laterally (Figure 2A). This coloboma phenotype was reminiscent of the zebrafish blowout mutant, which has a mutation in patched2 (formerly named patched1), a negative regulator of Hh signaling (Lee et al., 2012; Lee et al., 2008). In addition to the ocular phenotypes, sox11 morphants also frequently displayed a downward kink of the tail, as well as brain abnormalities such as widened ventricles, likely reflecting Sox11’s expression and function in the posterior somites and developing brain, respectively (De Martino et al., 2000; Penzo-
Méndez, 2010). All of the morphant phenotypes were rescued by injection of wild type *sox11a* and *sox11b* mRNA, consistent with the morpholinos being specific for Sox11 (Figures 2A, 2D). Importantly, these phenotypes were also rescued by injection of human *SOX11* mRNA, indicating that the function of Sox11 in regulating early eye development is evolutionarily conserved (Figure 2D).

One mechanism that has been suggested to contribute to optic fissure closure defects is overproliferation of progenitor cells within the presumptive neural retina (Kim et al., 2007). To determine whether changes in mitotic activity underlie the lens and coloboma phenotypes in *sox11* morphants, we immunolabeled retinal sections from control and *sox11* morphants with an antibody to phosphohistone H3 (PH3). We observed a modest but significant increase in the number of PH3-positive cells in *sox11* morphant optic vesicle and retinas at 18 and 24 hpf, and a larger increase in proliferation relative to controls at 48 and 72 hpf (Figures S2C, D). However, the excess PH3-positive cells were not clustered in the ventral retina, optic stalk, or lens at 24 hpf (Figure S2D), by which time the abnormal ocular phenotypes are already apparent. Therefore, we conclude that overproliferation likely does not underlie the early ocular phenotypes of *sox11* morphants. We also performed TUNEL staining on sections from control and *sox11* morphant retinas (Figures S2A, B). This analysis revealed a variable but significant increase in TUNEL-positive cells in the optic vesicle of *sox11* morphants at 18 hpf. At 24 hpf, we did not detect elevated apoptosis in the retina or optic stalk of Sox11-deficient embryos. However, we did observe a significant increase in TUNEL-positive cells in the anterior lens of *sox11* morphants, which persisted through 72 hpf (Figures S2A, B). This increase in apoptotic cells in the lens may be related to the abnormal lens morphology we observed by light microscopy (Figure 2A).

Finally, we observed an increase in TUNEL-positive cells in the colobomatous tissue of *sox11* morphant retinas at 48 hpf (Figure S2B), indicating that this abnormal ocular structure negatively impacted the survival of the cells within it.
Figure 2.2. Sox11 knockdown disrupts ocular morphogenesis and causes coloboma in zebrafish. (A) Representative eye and body images of control and sox11 morphants (taken from the set of embryos analyzed in (D). At 24 hpf, approximately 70% of sox11 morphants displayed a malformed lens (arrowhead) and a posterior kink in the tail (arrow). At 2 dpf, a similar proportion of sox11 morphants displayed coloboma (bracket), and had a hypopigmented and underdeveloped ventral retina. Both the abnormal lens and coloboma phenotypes were rescued with co-injection of wild type zebrafish sox11 mRNA (bottom row). (B) Sox11 morphants were microphthalmic at 24 hpf. Eye area was normalized to body length (*p <0.0001, Student’s t-test; control MO: n=10 embryos examined; sox11 MO: n=12 embryos examined, 3 independent repeats). (C) Sections of 72 hpf control (left) and sox11 morphant eyes (right) stained with cresyl violet revealed the extrusion of the retina into the brain through the open choroid fissure of sox11 morphants (asterisk); n=6 individuals examined per group. The thickened appearance of the dorsal RPE in the sox11 morphant retina is a staining artifact and was not observed in fresh tissue sections. Scale bar = 50 μm. (D) Injection of zebrafish and human sox11 mRNA rescued the ocular phenotypes in sox11 morphants. Number of embryos analyzed: 24 hpf control MO, 4.18
Figure 2.2. Sox11 knockdown disrupts ocular morphogenesis and causes coloboma in zebrafish (contd.)

ng/embryo, n=1007; 2 dpf control MO, 4.18 ng/embryo, n=1001; 24 hpf sox11 MO, 4.18
ng/embryo, n= 309; 2 dpf sox11 MO, 4.18 ng/embryo, n=294; 24 hpf sox11 MO, 8.36 ng/embryo,
n=559; 2 dpf sox11 MO, 8.36 ng/embryo, n=392; 24 hpf sox11 MO 8.36 ng/embryo plus 2.0
ng/embryo zebrafish sox11 mRNA, n=185; 2 dpf sox11 MO, 8.36 ng/embryo plus 2.0 ng/embryo
zebrafish sox11 mRNA, n=167; 24 hpf sox11 MO, 8.36 ng/embryo plus 0.3 ng/embryo human
SOX11 mRNA, n=130; 2 dpf sox11 MO, 8.36 ng/embryo plus 0.3 ng/embryo human SOX11
mRNA, n=125. Three biological replicates were performed for all experiments. (*p<0.001,
Student’s t-test). D, dorsal; V, ventral; A, anterior; P, posterior; L, lens; R, retina; hpf, hours post
fertilization; dpf, days post fertilization; MO, morpholino; GCL, ganglion cell layer; INL, inner
nuclear layer; ONL, outer nuclear layer; RPE, retinal pigmented epithelium.
Figure S2.1. Efficiency and specificity of sox11 morpholinos. (A) Schematic representation of the pEF1α:GFP plasmid containing a portion of the sox11 5’ UTR placed upstream of the GFP reporter (top). The binding site for the sox11 morpholino is shown in red. Separate reporters were constructed for the sox11a and sox11b MOs. (Center) Lateral view (anterior at top) of 24 hpf embryos injected with EF1α- sox11a/b-GFP plasmids alone (left) or with both sox11 MOs. No GFP expression was detected in the embryo injected with sox11 MOs. (Bottom) Quantification of the proportion of GFP-positive embryos at 24 hpf. The sox11 MOs were highly effective at blocking GFP expression. Number of embryos analyzed: pEF1α- sox11-GFP plasmid alone, n=169; pEF1α- sox11-GFP + sox11 MOs, n=140, 3 independent repeats; *p=0.004, Student’s t-test. (B) Both sox11a and sox11b contribute to abnormal lens and coloboma phenotypes observed in sox11 morphants. The proportion of embryos displaying either phenotype was significantly higher when injected with sox11a and sox11b MOs simultaneously, compared to either MO alone. Number of embryos analyzed: 24 hpf control MO, n=463; 2 dpf control MO, n=441; 24 hpf sox11a MO, n=229; 2 dpf sox11a MO, n=214; 24 hpf sox11b MO, n=341; 2 dpf sox11b MO, n=316; 24 hpf sox11a + sox11b MO, n=271; 2 dpf sox11a + sox11b MO, n=262, 3 independent repeats. *p<0.001, Fisher’s exact test. (C) A second non-overlapping sox11 MO (that targeted both sox11a and sox11b simultaneously) produced the same coloboma phenotype in similar proportion to the first set. Number of embryos analyzed: control MO, n=186 embryos; sox11 MO, n=194, 3 independent repeats; *p<0.001, Fisher’s exact test. MO, morpholino; hpf, hours post fertilization; dpf, days post fertilization.
Figure S2.2. Cell proliferation and apoptosis in sox11 morphants. (A) Quantification of TUNEL+ cells in the optic vesicle, lens, and retina of control and sox11 and morphants from 18-72 hpf. Sox11 morphants had an elevated number of TUNEL+ cells in the optic vesicle at 18 hpf. Additionally, sox11 morphants consistently displayed more TUNEL+ cells in the anterior lens compared to controls from 24-72 hpf. Number of embryos analyzed: 18 hpf control MO, n=20; 18 hpf sox11 MO, n=22; 24 hpf control MO, n=15; 24 hpf sox11 MO, n=19; 48 hpf control MO, n=10; 48 hpf sox11 MO, n=13; 72 hpf control MO, n=12; 72 hpf sox11 MO, n=12; average of 3 independent biological replicates. **p<0.00001, *p<0.01, Student’s t-test. (B) Representative transverse sections of control (left column) and sox11 (right column) morphants at 18, 24, and 48 hpf, taken from the set of individuals analyzed in (A). At 48 hpf, TUNEL+ cells were detected within the colobomatous tissue and the region of the optic stalk in sox11 morphants (arrow, bottom right). (C) Sox11 morphant retinas had more PH3+ cells than controls from 18-72 hpf. Number of embryos analyzed: 18 hpf control MO, n=12; 18 hpf sox11 MO, n=15; 24 hpf control MO, n=20; 24 hpf sox11 MO, n=19; 48 hpf control MO, n=10; 48 hpf sox11 MO, n=13; 72 hpf control MO, n=14; 72 hpf sox11 MO, n=12; average of 3 independent biological replicates. **p<0.001, *p<0.01, Student’s t-test. (D) Representative transverse sections of control (left column) and sox11 (right column) morphants at 18, 24, and 48 hpf, taken from the set of individuals analyzed in (C). D, dorsal; V, ventral; MO, morpholino; hpf, hours post fertilization; ON, optic nerve; OV, optic vesicle; R, retina; L, lens.
2.4.3 Sox11 morphants possess fewer mature rod photoreceptors

Given that expression of sox11a/b is upregulated in adult zebrafish rod progenitor cells during rod photoreceptor regeneration (Morris et al., 2011a), we investigated whether Sox11-deficient embryos displayed altered rod development. Since we found that a significant proportion of sox11 morphant retinas with coloboma also displayed poor lamination, indicating a potential delay in retinal development, for analysis we divided the sox11 morphants into those with and without coloboma. This approach minimized the potential secondary effects on retinal development from the ocular morphogenetic defect masking any additional role for Sox11 in retinal neurogenesis. Using immunohistochemistry with cell-type specific antibodies, we found that sox11 morphants without coloboma (approximately 30% of morphant embryos) possessed well-laminated retinas with normal numbers of ganglion, amacrine, horizontal, and bipolar cells, Müller glia, and cone photoreceptors at 72 hpf (Figures S3A, B). In contrast, when control and sox11 MOs were injected into a rod photoreceptor-GFP transgenic reporter line (Fadool, 2003), we observed a significant reduction in mature rod photoreceptors in sox11 morphant retinas without coloboma at 3 dpf (control embryos, 34.9±7.4 rods/section; sox11 morphants, 8.7±8.9 rods/section; p<0.00001; Figures 3A, B). Furthermore, several retinal sections from sox11 morphants contained no detectable GFP-positive rods at 3 dpf. The reduction in mature rod photoreceptors in sox11 morphant retinas was confirmed by immunolabeling with the rod-specific antibody 4C12 (not shown), by fluorescent in situ hybridization (FISH) of retinal sections with a probe for rhodopsin (rho), and by quantitative RT-PCR (qPCR) for the rho transcript at 3 dpf (Figures 3C, D). Rod photoreceptor number could be rescued by injection of wild type sox11 mRNA (Figure 3B), demonstrating that the reduction in rods was due to Sox11 deficiency. To determine whether depletion of Sox11 blocks specification of the rod photoreceptor fate, we conducted FISH on 3 dpf retinal sections from control and sox11 morphants using probes for three genes associated with the rod photoreceptor lineage: neuroD, crx, and nr2e3 (Chen et al., 2005; Ochocinska and Hitchcock, 2009; Shen and Raymond, 2004). Interestingly, we found that expression of all three
rod lineage genes was qualitatively normal in \textit{sox11} morphant retinas, even those with coloboma and poor lamination (Figure 3C). We also verified by qPCR that \textit{nr2e3} transcript levels were not significantly different in \textit{sox11} morphants and controls (Figure 3D) Therefore, these data suggest that Sox11 is required for the terminal differentiation, but not the specification, of rod photoreceptor cells. Because the window of rod photoreceptor differentiation is longer than that of cones or other retinal neurons (Bruhn and Cepko, 1996; Stenkamp, 2007) we investigated whether rod photoreceptor number remained reduced in \textit{sox11} morphants later in development. The number of rods in \textit{sox11} morphant retinas was higher at 4 dpf than at 3 dpf, but remained significantly reduced relative to controls (\textit{sox11} morphants, 15.9±2.9 rods/section; controls, 57.9±5.4 rods/section; p<0.001; Figure S3C). Taken together, these data suggest that terminal differentiation of rods requires Sox11.
Figure S2.3. Retinal neurogenesis in sox11 morphants. (A) Retinal cell types were visualized by immunohistochemistry (ganglion, amacrine, horizontal, and bipolar cells) or with fluorescent reporter transgenic lines (Tg(gfap:GFP)mi2001 for Müller glia and Tg(3.2TαC-EGFP) for cones) in controls (left) and sox11 morphants (center, right) at 3 dpf. In sox11 morphants without coloboma (center), the retinas are well laminated and had normal numbers of ganglion, amacrine, horizontal, and bipolar cells, cone photoreceptors, and Müller glia. However, sox11 morphants with coloboma (asterisk; right) had poorly laminated retinas and reduced numbers of differentiated retinal cell types, indicating delayed retinal development. (B) Quantification of numbers of late-born retinal cell types in control and sox11 morphants without coloboma. Only rod photoreceptors displayed a significant reduction. Number of embryos analyzed: control MO, n=19; sox11 MO without coloboma, n=25, 3 independent repeats. **p<0.00001; ns=p>0.05, Student’s t-test. (C) At 4 dpf, sox11 morphants have more mature rod photoreceptors than at 3 dpf but the number remains significantly less than controls (*p<0.001, Student’s t-test); MO, morpholino; dpf; days post fertilization; L, lens; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; ON, optic nerve.
Figure 2.3. *Sox11* morphants lack mature rod photoreceptors. (A) Representative transverse retinal sections from XOPS-GFP zebrafish injected with control, *sox11* MO, or *sox11* MO plus zebrafish *sox11* mRNA at 3 dpf (from the set of individuals analyzed in (B). Even *sox11* morphants with well-laminated retinas and no evidence of coloboma (second panel) displayed greatly reduced numbers of mature rods compared to controls (left panel). Co-injection of wild type zebrafish *sox11* mRNA (right panel) rescued the rod deficiency at 3 dpf. (B) Quantification of the number of rod photoreceptors/section. Number of embryos analyzed: control MO, n=25; *sox11* MO without coloboma, n=25; *sox11* MO with coloboma, n=25; *sox11* MO plus zebrafish *sox11* mRNA, n=17. (**p < 0.00001; n.s., p >0.05, Student’s *t*-test). (C) Two-color fluorescent in situ hybridization (FISH) for *neuroD*, *crx*, *nr2e3*, and *rhodopsin* expression in control and *sox11* morphants with and without coloboma at 3 dpf. Expression of the rod lineage genes *neuroD* (top), *crx* (middle), and *nr2e3* (bottom) was qualitatively normal in *sox11* morphants with or without coloboma. However, *rhodopsin* expression (green) was greatly reduced compared to control morphants (left column). Number of embryos analyzed: n=14 per group, 3 independent biological replicates. (D) Quantitative RT-PCR (qPCR) performed on mRNA from control and *sox11* morphant heads at 3 dpf revealed a significant decrease in *rhodopsin* expression in *sox11* morphants compared to controls. However, *nr2e3* transcript levels were not significantly different between control and *sox11* morphants. Relative transcript abundance was normalized to *atp5h* levels and is presented as the mean fold-change in expression relative to controls (n=30 embryos per group, 3 independent biological replicates). *p <0.003; n.s, p>0.05, Student’s *t*-test. D, dorsal; V, ventral; L, lens; dpf, days post fertilization; MO, morpholino; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; ON, optic nerve.
2.4.4 Sox11 negatively regulates Hedgehog signaling

As mentioned above, the coloboma phenotype of sox11 morphants is similar to the zebrafish blowout mutant, in which increased Hedgehog signaling results in altered proximodistal patterning of the optic vesicle (Lee et al., 2008). To determine whether a similar defect was present in sox11 morphants, we performed FISH on retinal sections to examine the expression of pax2a and pax6a, which mark optic stalk and retinal territories, respectively. This revealed expansion of the pax2a domain in approximately 50% of sox11 morphant embryos at 18 and 36 hpf, while at later stages (48 hpf), expression persisted around the open choroid fissure, whereas it was barely detectable in controls (Figure 4A). These expression changes were verified by qPCR at 18 and 24 hpf. Although we did not observe a concomitant decrease in pax6a expression in the optic vesicle at 18 hpf, there was a significant reduction in transcript levels detected by qPCR at 24 hpf (Figure 4B). To further test whether Hh signaling was elevated in sox11 morphants, we made use of a recently described Hh signaling reporter line of zebrafish, which expresses GFP under the control of the patched2 (ptc2) promoter (Shen et al., 2013). Sections through the head of 24 hpf control and sox11 morphants on the ptc2:GFP background revealed both an increase in GFP expression and an expansion of the GFP-positive domains in the brain, retina, and RPE of sox11 morphants (Figure 5A). Taken together, these data strongly suggest that Hh signaling is indeed elevated in sox11 morphants.

To directly test this hypothesis, control and sox11 morphant embryos were treated from 5.5-13 hpf with the Hh inhibitor cyclopamine. This treatment window was chosen because it resulted in maximal rescue of colobomas in the blowout mutant (Lee et al., 2008). The proportion of embryos displaying a malformed lens at 24 hpf (21.3±8.8%) or coloboma at 2 dpf (10.1±3.8%) was significantly reduced after cyclopamine treatment, compared to vehicle-treated sox11 morphants (>70% for both phenotypes; p<0.0001; Figure 5B and Figure S4A). Moreover, cyclopamine treatment significantly increased the number of rods at 72 hpf (sox11 MO:...
20.3±11.1 rods/section; sox11 MO + cyclopamine: 43.8±10.5 rods/section; p=0.02; Figure 5C and Figure S4B), and corrected the lamination and differentiation defects that were associated with colobomatous retinas (Figure S4C). In a reciprocal experiment, embryos were injected with half the full dose of each sox11 MO, and treated with either a Hh agonist (purmorphamine) or vehicle control (DMSO) from 5.5-24 hpf. We used a sub-threshold dose of purmorphamine (75 μM), which did not cause coloboma when given alone (Figure 5B). In contrast, when the half dose of sox11 MO was combined with purmorphamine, the prevalence of lens malformations at 24 hpf and coloboma at 2 dpf significantly increased (sox11 MO half dose + purmorphamine: 57.9±10.2% malformed lens, 57.2±5.3% coloboma; sox11 MO half dose + DMSO: 24.2±3.5% malformed lens, 28.9±5.9% coloboma; p<0.0001; Figures 5B and S4A). Together, these data demonstrate that deficiency of Sox11 increases Hh signaling, resulting in defects in ocular morphogenesis and reduced rod photoreceptor number.

Finally, we injected sox11a and sox11b mRNA into wildtype zebrafish embryos and evaluated the prevalence of a cyclopic phenotype, which is classically associated with reduced Hh pathway activity (Amato et al., 2004), at 24 hpf. Injection of sox11a and sox11b mRNA caused a cyclopic phenotype in 33.8±2.9 % of the embryos, whereas only 4.2±0.7% of embryos had cyclopia when injected with a control td-tomato mRNA (p<0.001; Figure 5D). Taken together, these results demonstrate that Sox11 is required to limit Hh signaling during zebrafish ocular development.
Figure 2.4. Pax2.1 and pax6a expression is altered in sox11 morphants. (A) Fluorescent in situ hybridization on transverse sections from control and sox11 morphants with probes for pax2.1 and pax6a. The expression domain of f pax2.1 was expanded into the optic vesicle of sox11 morphants at 18 hpf (top right, arrows), and there was a modest retraction of pax6a expression compared to controls (top left; number of embryos analyzed: control MO, n=14; sox11 MO, n=13). At 36 and 48 hpf, in control retinas pax2.1 expression decreased and was only observed lining the optic nerve (asterisk; left middle and bottom rows); in contrast, pax2.1 expression was expanded and persisted around the open choroid fissure in sox11 morphant retinas (asterisks, right middle and bottom rows). Pax6a expression in the retina of sox11 morphants at 36 and 48 hpf appeared comparable to the control morphant retinas at this stage (number of embryos analyzed: 36 hpf control MO, n=7; 36 hpf sox11 MO, n=12; 48 hpf control MO, n=8; 48 hpf sox11 MO, n=14). (B) QPCR performed on mRNA from control and sox11 morphant heads at 18 and 24 hpf revealed a significant increase in pax2.1 expression at both 18 and 24 hpf, and a downregulation of pax6a expression at 24 hpf, in sox11 morphants compared to controls. Relative transcript abundance was normalized to atp5h (18 hpf) or gapdh (24 hpf) levels and is presented as the mean fold-change in expression relative to controls (n=50 embryos per group, 3 independent biological replicates. *p<0.05. D, dorsal; V, ventral; OV, optic vesicle; L, lens; hpf, hours post fertilization; MO, morpholino.)
Figure 2.5. *sox11* negatively regulates Hedgehog (Hh) signaling. (A) Transverse retinal sections from 24 hpf *ptc2:EGFP* zebrafish embryos injected with control or *sox11* MO. Sox11 morphants displayed elevated GFP expression in the brain as well as in the central and dorsal retina, and the dorsal RPE (number of embryos analyzed: control MO, n=8; *sox11* MO, n=10). (B) Treatment with the Hh inhibitor cyclopamine rescued the ocular phenotypes in *sox11* morphants. In contrast, treatment with the Hh agonist purmorphamine increased the prevalence of ocular phenotypes in embryos injected with a half dose of *sox11* MO. Number of embryos analyzed: 24 hpf *sox11*MO (plus 100% ethanol), n=393; 2 dpf *sox11* MO (plus 100% ethanol), n=319; 24 hpf *sox11* MO plus cyclopamine, n=276; 2 dpf *sox11* MO plus cyclopamine, n=263; 24 hpf half dose *sox11* MO (plus DMSO), n=258; 2 dpf *sox11* MO half dose (plus DMSO), n=241; 24 hpf uninjected plus purmorphamine, n=83; 2 dpf uninjected plus purmorphamine, n=81; 24 hpf half dose *sox11* MO plus purmorphamine, n=291; 2 dpf half dose *sox11* MO plus purmorphamine, n=270; 3 independent biological replicates. * and # p<0.0001, Fisher’s exact test. (C) Treatment with cyclopamine rescued rod photoreceptor number in *sox11* morphants. Rods were counted in retinal cryosections from 3 dpf embryos. Number of embryos analyzed: control MO, n=17; *sox11* MO, n=20; *sox11* MO plus cyclopamine, n=18; 3 independent replicates. *p=0.02, Student’s *t*-test. (D) Overexpression of zebrafish *sox11* increased the proportion of embryos with a cyclopic phenotype (right) compared to embryos injected with equimolar amounts of control *td-tomato* mRNA (left). Number of embryos analyzed: control mRNA, n=168; *sox11* mRNA, n=202, 3 independent biological replicates. *p<0.001, Fisher’s exact test. D, dorsal; V, ventral; A, anterior; P, posterior; hpf, hours post fertilization; dpf, days post fertilization R, retina; hy, hypothalamus; L, lens; MO, morpholino.
Figure S2.4. Elevated Hh signaling contributes to the abnormal ocular phenotypes displayed by sox11 morphants. (A) Representative brightfield images of sox11 morphants treated with cyclopamine, purmorphamine and their corresponding vehicle controls at 24 hpf and 2 dpf, taken from the set of embryos analyzed in Figure 5. Treatment with 75 uM purmorphamine alone did not cause any abnormalities (last column; 24 hpf control MO plus 75 uM purmorphamine alone, n=123; 2 dpf control MO plus 75 uM purmorphamine alone, n=114, 3 independent biological repeats). (B) Suppression of Hh pathway with cyclopamine rescued the rod photoreceptor defect in sox11 morphants at 3 dpf (right; number of embryos analyzed: sox11 MO, n=20; sox11 MO + cyclopamine, n= 18; 3 independent repeats). (C) Retinal cell types were visualized by immunohistochemistry (ganglion, cones, amacrine, horizontal, and bipolar cells) or with a transgenic fluorescent reporter lines (Tg(gfap: GFP)mi2001) for Müller glia in sox11 morphants (left) and sox11 morphants treated with cyclopamine (right) at 3 dpf. The retina of sox11 morphants treated with cyclopamine were well laminated and displayed normal distributions of all cell types (n=15 per group, 3 independent repeats). D, dorsal; V, ventral; A, anterior; P, posterior; MO, morpholino; hpf, hours post fertilization; L, lens.
2.4.5 Transcription of sonic hedgehog a (shha) is strongly upregulated in sox11 morphants

Zebrafish possess five Hedgehog ligands (Sonic hedgehog a and b, Indian hedgehog a and b, and Desert hedgehog), two Patched and one Smoothened receptor, and four Gli effectors. To determine whether expression of any of these pathway members was altered in sox11 morphants, we performed qPCR on mRNA prepared from 18 and 24 hpf control and sox11 morphant heads. At 18 hpf, no significant gene expression changes were observed, except for gli2a and gli3, which were both slightly elevated in sox11 morphants (Figure S5A). In contrast, at 24 hpf we observed a very strong increase (189-fold) in the expression of shha in sox11 morphants relative to controls, as well as a modest decrease in ihhb, ptc1, and gli2b expression, and a 2-fold increase in expression of ptc2 (Figure 6A). The increase in shha expression in sox11 morphants appeared to be dose-dependent, as injection of one-half the full dose of sox11 MOs resulted in only a 65-fold elevation in shha (Figure S5B). In situ hybridization revealed greatly increased shha signal intensity in regions of the sox11 morphant embryo that normally express shha, such as the ventral forebrain and the notochord (Figure 6B), with ectopic expression observed in the dorsal midbrain and telencephalon (Figure 6B). These results suggest that the ocular phenotypes in sox11 morphants are caused by elevated levels of Shha. However, we were puzzled that shha transcript levels were not significantly increased at 18 hpf (Figure S5A), and yet cyclopamine treatment from 5.5-13 hpf rescued the ocular defects of sox11 morphants. Therefore, we asked whether shha levels were elevated in sox11 morphants at earlier time points. We performed qPCR analysis on mRNA from control and sox11 morphants at 8, 10, and 12 hpf and found that shha levels are elevated approximately 2-fold in sox11 morphants at these time points (Figure 6C). Moreover, using the ptc2:GFP line, we detected increased GFP levels in the ventral midline of sox11 morphants at 12 hpf, confirming that Hh signaling was elevated at this stage (Figure 6D). Taken together, these results suggest that knockdown of sox11 results in elevated expression of shha, and an increase in Hh signaling, as early as 8-12 hpf when the optic vesicle is evaginating from the midline.
To test the hypothesis that elevated Shha levels cause the ocular phenotypes of sox11 morphants, we knocked down both shha and sox11 simultaneously (using our sox11 MOs and a previously described shha MO (Nasevicius and Ekker, 2000) ) and scored embryos at 24 hpf and 2 dpf for malformed lens and coloboma phenotypes, respectively. We used a low dose of the shha MO (3.14 ng/embryo), which by itself did not produce lens defects, coloboma, or rod photoreceptor defects (Figures 6E and S5C). The prevalence of ocular phenotypes was significantly reduced in the double morphants (sox11 MO: 70.3%±6.7% malformed lens, 75.4%±8.3% coloboma; sox11 + shha MOs: 28.9%± 9.2% malformed lens; 34.7% ± 2.7% coloboma; p< 0.0001; Figures 6E and S5C). Rod photoreceptor number was also significantly increased at 3 dpf in the double shha/sox11 morphants, however it did not reach the levels observed in controls (sox11 MO: 5.8±7.1 rods/section; sox11 + shha MOs: 14.6 ± 2.3 rods/section; p<0.05; Figure S5D). We performed qPCR analysis on sox11 morphants treated with cyclopamine and purmorphamine and confirmed that these treatments caused a decrease and an increase in shha transcript levels, respectively (Figures S5E and S5F). Cyclopamine treatment of sox11 morphants also restored expression of the Hh target gene ptc2 to control levels (data not shown). Moreover, qPCR analysis of embryos injected with control or sox11 mRNA confirmed that overexpression of sox11 resulted in a concomitant decrease in shha expression (Figure S5G). Finally, we determined that there was not a reciprocal regulation of sox11 by shha, because injection of the shha morpholino alone did not result in a change in expression of sox11a or sox11b (Figure S5H). Taken together, these results demonstrate that Sox11 controls levels of Hh signaling primarily through negative regulation of shha expression, and that limiting shha expression is essential for proper ocular morphogenesis.
Figure S2.5. Hh pathway gene expression changes in sox11 morphants. (A) QPCR performed on mRNA from sox11 morphant and control heads at 18 hpf reveal small increases in gli2a and gli3 expression in sox11 morphants compared to controls, but no significant change in shha expression. Relative transcript abundance was normalized to atp5h levels and is presented as the mean fold-change in expression relative to controls (B) At 24 hpf, sox11 morphants demonstrated a large increase in shha expression, which correlated with the dose of sox11 MO injected. Relative transcript abundance was normalized to gapdh levels and is presented as the mean fold-change in expression relative to controls (n=60 embryos per group, 3 independent biological repeats) *p<0.01, Student’s t-test. (C) Representative bright-field images of embryos injected with sox11 MO alone (left side), shha MO alone (middle), or both shha and sox11 MOs (right side), taken from the set of embryos analyzed in Figure 6E. (D) Co-knockdown of shha increased rod photoreceptor number in sox11 morphants at 3 dpf (number of embryos analyzed: control MO, n=11; shha MO, n=10; sox11 MO, n=16, sox11+ shha MO, n=15, 3 independent repeats) *p<0.05, Student’s t-test. (E) QPCR performed on mRNA from heads of sox11 morphants treated with vehicle (100% ethanol) or cyclopamine and compared to control morphants treated with vehicle revealed a significant reduction in shha expression in sox11 morphants treated with cyclopamine at 24 hpf. Relative transcript abundance was normalized to gapdh levels and is presented as the mean fold-change in expression relative to controls (n=40 embryos per group, 3 independent biological repeats) **p<0.0001, Student’s t-test. (F) QPCR for shha was performed
on mRNA from the 24 hpf heads of sox11 morphants injected with half the normal dose and treated with DMSO, sox11 morphants (half dose) treated with purmorphamine, and compared to control morphants treated with DMSO. An increase in shha expression was detected in sox11 morphants (1/2 dose) treated with purmorphamine compared to sox11 morphants (half dose) treated with DMSO, however the increase did not reach the threshold for statistical significance. Relative transcript abundance was normalized to gapdh levels and is presented as the mean fold-change in expression relative to controls (n=40 embryos per group, 3 independent biological repeats). (G) QPCR was performed on mRNA from the 24 hpf heads of zebrafish embryos injected with control (td-tomato) mRNA and embryos injected with zebrafish sox11 mRNA. This analysis revealed a significant decrease in shha expression in embryos overexpressing zebrafish sox11 mRNA compared to the controls. Relative transcript abundance was normalized to 18s rRNA levels and is presented as the mean fold-change in expression relative to controls (n=30 embryos per group, 3 independent biological repeats). **p<0.0001, Student’s t-test. (H) QPCR performed on mRNA from heads of 24 hpf embryos injected with shha MO or control MO revealed no significant change in expression of either sox11a or sox11b in shha morphants compared to controls. Relative transcript abundance was normalized to gapdh levels and is presented as the mean fold-change in expression relative to controls (n=45 embryos per group, 3 independent biological repeats). ns, p>0.05, Student’s t-test. D, dorsal; V, ventral; A, anterior; P, posterior; MO, morpholino; hpf, hours post fertilization; dpf, days post fertilization; L, lens.
Figure 2.6. *Shha* expression is upregulated in *sox11* morphants. (A) QPCR performed on mRNA from control and *sox11* morphant heads at 24 hpf revealed a dramatic upregulation of *shha* expression, and a small but significant increase in *ptc2* expression, in *sox11* morphants compared to controls (n=70 embryos per group, 3 independent biological replicates). Relative transcript abundance was normalized to *gapdh* levels. The Y-axis (log-scale) represents the mean ratio of *sox11* morphant to control expression for three biological and three technical replicates. *p*<0.01, Student’s *t*-test. (B) In situ hybridization with a *shha* probe on control (left) and *sox11* morphant (right) embryos at 24 hpf revealed expanded *shha* expression in *sox11* morphants throughout the brain and also in the notochord (inset) Numbers of embryos analyzed: n=15 embryos per group, 3 independent repeats. (C) QPCR performed on mRNA from control and *sox11* morphant heads at 8, 10 and 12 hpf demonstrated an upregulation of *shha* expression in *sox11* morphants compared to controls. Relative transcript abundance was normalized to *gapdh* levels and is presented as the mean fold-change in expression relative to controls (n=60 embryos per group, 3 independent biological repeats). **p<0.001, *p=0.01, Student’s *t*-test. (D) *Sox11* morphants (right) on the *ptc2:EGFP* background displayed elevated GFP expression in the midline at 12 hpf compared to control morphants (left). The bottom panels are an enlargement of the boxed area indicated in the top left panel. Number of embryos analyzed: control MO, n=34; *sox11* MO, n=41, 3 independent biological replicates. (E) Co-knockdown of *shha* and *sox11* reduced the proportion of embryos displaying abnormal lens and coloboma phenotypes at 24 hpf and 2 dpf, respectively. Number of embryos analyzed: 24 hpf control MO, n=186; 2 dpf control MO, n=165; 24 hpf *sox11* MO, n=199, 2 dpf *sox11* MO, n=182; 24 hpf *shha* MO, n=249; 2 dpf *shha* MO, n=231; 24 hpf *sox11*+ *shha* MO, n=207; 2 dpf *sox11*+ *shha* MO, n=190; 3 independent biological replicates. *p<0.0001, Student’s *t*-test. D, dorsal; V, ventral; A, anterior; P, posterior; hpf, hours post fertilization; dpf, days post fertilization; R, retina; di, diencephalon, tel, telencephalon; hy, hypothalamus; MO, morpholino.
2.4.6 Bmp7b can rescue the ocular phenotypes in sox11 morphants

Thus far, our data strongly suggest that Sox11 is required to limit levels of shha expression during ocular development. However, Sox11 and other members of the SoxC family have previously been shown to function as transcriptional activators rather than repressors (Bergsland et al., 2011; Chew and Gallo, 2009; Wiebe et al., 2003). Furthermore, a scan of the shha promoter revealed no perfect consensus binding sequences for Sox factors [not shown;(Wiebe et al., 2003)], and the expression domains of sox11 and shha only partially overlap in the ventral midline during ocular morphogenesis. Therefore, we hypothesized that Sox11 negatively regulates Shha indirectly and perhaps non-cell autonomously, by activating the expression of an upstream inhibitor of Shha. We searched the literature to identify candidate Shha repressors that are expressed in the forebrain during development, and then asked whether expression of any of these factors was reduced in sox11 morphant heads at 24 hpf (Figure 7A). We analyzed five candidate genes: bmp7b, fgfr2, tbx2a, tbx2b, and kras, which had been shown previously to negatively regulate shha expression during development. Of these five, only bmp7b showed significantly decreased expression in sox11 morphants compared to controls (Figure 7A). Bmp7b represents a good candidate intermediary between Sox11 and shha for several reasons. First, Bmp7b null mice display microphthalmia and optic fissure defects, similar to sox11 null mice (Morcillo et al., 2006). Second, bmp7 is expressed in the ventral midline and proximal optic vesicle in the mouse (Morcillo et al., 2006), and bmp7b is expressed in the forebrain adjacent to the optic vesicle in zebrafish at 18 hpf in a similar pattern to sox11 (Shawi and Serluca, 2008). Third, bmp7 expression was reported to be reduced in Sox11/− mice (Wurm et al., 2008b). And finally, a scan of the bmp7b promoter revealed two perfect Sox consensus binding sites (Wiebe et al., 2003) located approximately 950 bps upstream of the transcription start site (not shown).

Because we had detected elevated shha levels as early as 8 hpf in sox11 morphants, we asked whether bmp7b expression is also downregulated at that time. qPCR analysis revealed that bmp7b
transcript levels were significantly reduced at 8, 10, and 12 hpf in *sox11* morphants when compared to controls (Figure S6). Interestingly, *bmp7b* expression increased to just above control levels at 18 hpf, before declining significantly again at 24 hpf. This rebound in *bmp7b* expression at 18 hpf precisely mirrors the normal levels of *shha* expression in *sox11* morphants at this time (Figure S5A). Taken together, these data suggest that the initial decrease in *bmp7b* expression (and corresponding elevation of *shha*) caused by knockdown of *sox11* induces a compensatory pathway that works to bring transcriptional levels back to normal, but that the continued knockdown of *sox11* results in renewed dysregulation of *bmp7b* and *shha*.

We reasoned that if Bmp7b functions downstream of Sox11 and upstream of Shha, then expression of *bmp7b* in *sox11* morphants should rescue the ocular phenotypes caused by elevated Hh signaling. To test this hypothesis, we injected *bmp7b* mRNA into control and *sox11* morphant embryos, and determined the proportion of embryos that displayed lens defects and coloboma at 24 hpf and 2 dpf, respectively. We found that co-injection of *bmp7b* mRNA into *sox11* morphants significantly reduced the number of embryos displaying ocular phenotypes (*sox11* MO: 72.6±2.22% malformed lens, 74.5±1.8% coloboma; *sox11* MO + *bmp7b* mRNA: 35.6±6.9% malformed lens; 43.8±14.4% coloboma; p<0.001; Figure 7B and C), although the rescue was not as large as that observed with cyclopamine treatment. These data suggest that Sox11 negatively regulates *shha* at least in part through Bmp7b.
Figure 2.7. Bmp7b expression is reduced in sox11 morphants. (A) QPCR was performed on mRNA from control and sox11 morphant heads at 24 hpf for known repressors of shha transcription. A significant downregulation of bmp7b was observed in sox11 morphants compared to controls. Relative transcript abundance was normalized to gapdh levels and is presented as the mean fold-change in expression relative to controls (n= 50 embryos per group, 3 independent biological repeats). **p<0.01, Student’s t –test. (B) Injection of bmp7b mRNA significantly reduced the proportion of sox11 morphants displaying abnormal lens and coloboma phenotypes at 24 hpf and 2 dpf, respectively. Number of embryos analyzed: 24 hpf control MO, n=127; 2 dpf control MO, n=123; 24 hpf sox11 MO, n=282; 2 dpf sox11 MO, n=274; 24 hpf bmp7b mRNA, n=95; 2 dpf bmp7b mRNA, n=91; 24 hpf sox11 MO + bmp7b mRNA, n=140, 2 dpf sox11 MO + bmp7b mRNA, n=134; 3 independent biological replicates. *p<0.006. (C) Brightfield images of a representative sox11 morphant and a sox11 morphant rescued with bmp7b mRNA, taken from the set of embryos analyzed in (B). D, dorsal; V, ventral; A, anterior; P, posterior; hpf, hours post fertilization; dpf, days post fertilization; MO, morpholino.
2.4.7 Sox4 can compensate for the loss of Sox11
As functional redundancy between SoxC family members has been observed in mouse models (Bergsland et al., 2011; Dy et al., 2008b; Penzo-Méndez, 2010), we investigated whether another SoxC factor could compensate for the loss of Sox11 during zebrafish ocular morphogenesis. By in situ hybridization and qPCR, we observed elevated expression of the SoxC factor sox4a in sox11 morphants at 24 and 36 hpf, suggesting that sox11 deficiency induces a compensatory increase in sox4 expression (Figure S7A and B). We then injected sox4 mRNA into sox11 morphants and found that this significantly reduced the proportion of embryos with lens and coloboma phenotypes (Figure S7C). This result suggests that increased Sox4 expression may buffer the effects of Sox11 deficiency. Consistent with this hypothesis, we observed a significantly greater proportion of embryos with coloboma in sox4/sox11 double morphants than when either gene was knocked down alone (data not shown).
Figure S2.7. Sox4 compensates for the loss of Sox11. (A) Sox4a was diffusely expressed in the control retina at 36 hpf (left); however, sox4a expression was upregulated in the lens and retina of sox11 morphants (right; n=20 per group); scale bar=50 µm. (B) QPCR performed on mRNA from the heads of 24 hpf zebrafish embryos injected with sox11 MO or control MO reveal that sox4a expression is elevated in sox11 morphants compared to controls. Relative transcript abundance was normalized to gapdh levels and is presented as the mean fold-change in expression relative to controls (n=40 embryos per group, 3 independent biological repeats) *p<0.01, Student’s t-test. (C) Co-injection of sox4 mRNA rescued the lens and coloboma phenotypes of sox11 morphants at 24 hpf and 2dpf. Number of embryos analyzed: 24 hpf control MO, n=136; 2 dpf control MO, n=124; 24 hpf sox11 MO, n=179; 2 dpf sox11 MO, n=161, 24 hpf sox11 MO + sox4 mRNA, n=210, 2 dpf sox11 MO + sox4 mRNA, n=184, 3 independent biological replicates. *p<0.001, Fishers exact test; MO, morpholino.
2.4.8 Identification of SOX11 variants in patients with coloboma

To investigate whether SOX11 mutations contribute to patient phenotypes, the coding region was sequenced in DNA samples from 79 MAC patients (Ye et al., 2010). These DNA samples had been previously screened for mutations in two other coloboma-related genes, GDF3 and GDF6 (Asai-Coakwell et al., 2007; Asai-Coakwell et al., 2009; Ye et al., 2010). We identified heterozygous sequence changes in two probands (Figure 8A), both of whom are Canadians of white European ancestry. The first, a c.488G→T missense mutation in a coloboma patient, is predicted to result in a G145C amino acid alteration, considered damaging by SIFT analysis (http://sift.jcvi.org/). The second variant, a 12-nucleotide duplication (c.1106-1117) in a patient with bilateral iris and retino-choroidal coloboma (Figure 8B), is predicted to result in an in-frame, four amino acid duplication (S351-354dup). The affected amino acid residues are located outside previously defined functional domains and are conserved in chimp and macaque SOX11 (Figure 8A). These variants were absent from dbSNP and the 1000 Genomes databases, and from the NHLBI database comprising more than ten thousand exomes (Figure S8A). Sequencing of SOX11 from the probands’ family members revealed that the S351-354dup alteration was present in the proband’s mother, who did not exhibit a phenotype clinically (Figure 8C). In light of the rod photoreceptor phenotype in zebrafish sox11 morphants, an electretinogram (ERG) was performed on the mother carrying the S351-354dup alteration. This analysis demonstrated a reduction in scotopic b-wave amplitude, indicating reduced rod photoreceptor function (Figure S8B). In addition, her 10Hz dim white flicker response was appreciably reduced, and was associated with a change in latency. The mother was asymptomatic at the time the ERG was performed, which may reflect her young age (37 years). Her cone flicker response was normal.

Intrigued by the presence of phenotypic effects in a heterozygote only on targeted testing, 384 DNA samples derived from patients undergoing screening for hemochromatosis were sequenced,
which detected the S351-354dup variant in three individuals, whilst the G145C variant was absent. Unfortunately, these three carriers could not be recalled for clinical examination.

To determine whether the two SOX11 sequence variants had functional consequences, their ability to rescue the lens and coloboma phenotypes of zebrafish sox11 morphants was compared to wild type human SOX11 mRNA. Whereas wild type SOX11 mRNA significantly reduced the proportion of sox11 morphants displaying lens defects and coloboma, no significant rescue was observed with mRNA containing either SOX11 variant (Figures 8D and S8D), suggesting that both sequence changes compromise SOX11 function. Next, we utilized a luciferase reporter containing the promoter region of the SOX11 target gene GDF5 (Kan et al., 2013) to further analyze the functional consequences of the two mutations. Expression of increasing amounts of wild type SOX11 in COS-7 cells produced a dose-dependent increase in luciferase activity from the GDF5 reporter (Figure 8E). In contrast, transfection of equivalent amounts of either SOX11 variant did not enhance luciferase activity over the empty vector control (Figure 8E), although the variants showed comparable levels of protein expression by Western blot (Figure S8C). Equivalent results were obtained with the luciferase assay in two additional cell lines (HEK293 and HeLa; data not shown). To further confirm that the two SOX11 sequence variants are functionally compromised, we overexpressed them in zebrafish and quantified the proportion of embryos that exhibited a cyclopic phenotype at 24 hpf. Whereas injection of WT human SOX11 mRNA caused a significant increase in the proportion of cyclopic embryos compared to injection of control td-Tomato mRNA (35.3±11% in WT SOX11 injected vs. 61.16±10.7% in control injected; p<0.05), neither of the SOX11 sequence variants produced elevated levels of cyclopia (G145C, 10.0±4.8%, S351-354dup, 13.1±4.11%; Figures 8F, S8E). Taken together, these data suggest that the two variants compromise SOX11’s transactivation ability.
Finally, array comparative genomic hybridization (array CGH) was performed on DNA from a patient with microphthalmia, unilateral optic nerve agenesis, and a \textit{de novo} chromosome 2p25 deletion (Lo-Castro et al., 2009). This defined a 1.14 Mb segmental deletion (5,206,155-6,343,906; chromosome build GRCh37), encompassing an interval within which \textit{SOX11} is the only protein-coding gene (Figures 8G, S8F). Taken together, these data demonstrate that perturbed SOX11 function, either through mutation or decreased gene dosage, contributes to structural (microphthalmia/coloboma) or functional (rod photoreceptor) phenotypes.
**Figure 2.8. Association of SOX11 locus with MAC.** (A) Schematic representation of SOX11, indicating the positions of the two MAC sequence variants, and an alignment of the SOX11 protein sequence encompassing the two affected regions. (B) Photographs of the S351-354dup proband, indicating bi-lateral iris coloboma (top) and retino-choroidal coloboma (bottom). (C) Pedigree showing the S351-354dup proband and his parents. The proband’s mother also carries the S351-354dup mutation, but does not have coloboma. (D) SOX11 mRNA containing G145C (MI) or S351-354dup (MII) did not rescue the abnormal lens or coloboma phenotypes of sox11 morphants. Number of embryos analyzed: 24 hpf control MO, n=202; 2 dpf control MO, n=174; 24 hpf sox11 MO, n=148; 2 dpf sox11 MO, n=133; 24 hpf sox11 MO + wild type SOX11 mRNA, n=177; 2 dpf sox11 MO + wild type SOX11 mRNA, n=159; 24 hpf sox11 MO + MI SOX11 mRNA, n=203, 2 dpf sox11 MO + MI SOX11 mRNA, n=188; 24 hpf sox11 MO + MII SOX11 mRNA, n=219; 2 dpf sox11 MO + MII SOX11 mRNA, n=201; average of three independent biological replicates. *p < 0.0001, Fisher’s exact test. (E) GDF5-luciferase reporter activity. Transfection of either SOX11 G145C (MI) or S351-354dup (MII) did not significantly enhance luciferase levels. Firefly luciferase activity was normalized to Renilla luciferase and is represented as mean fold change over the empty vector (pGEM3Z) from three biological and six technical replicates (*p < 0.0001, Student’s t-test). (F) Whereas overexpression of wild type (WT) human SOX11 mRNA increased the proportion of embryos with a cyclopic phenotype compared to injection of control (td-Tomato) mRNA, human SOX11 mRNA containing G145C (MI) or S351-354dup (MII) did not cause cyclopia. Number of embryos analyzed: control mRNA, n=67; WT SOX11 mRNA, n=62; MI SOX11 mRNA, n=165; MII SOX11 mRNA, n=128, 3 independent biological repeats. *p<0.006 (G) Array CGH data for 2p25.2 demonstrating deletion breakpoints (red arrows) in a patient with agenesis of the optic nerve, microphthalmia, and developmental delay. The corresponding genomic region annotated by the UCSC genome browser is shown below; SOX11 (red box) is the only protein-coding gene within the deleted region. MAC, microphthalmia, anophthalimia, and coloboma; MO, morpholino.
Figure S2.8. Association of SOX11 locus with ocular abnormalities. (A) Amino acid sequence of human SOX11, with previously identified non-synonymous SNPs highlighted in green. The two variants identified in the MAC patients (positions indicated in red) are novel. (B) Scotopic ERG analysis of the proband’s mother carrying the S315-354dup variant, demonstrating a reduction in the b-wave amplitude. (C) Western blot for SOX11 and β-actin in COS-7 cells transfected with SOX11 expression constructs. Densitometric analysis was performed with ImageJ software. (D) Representative brightfield images of sox11 morphants co-injected with either WT, MI (G145C), or MII (S315-354dup) SOX11 mRNA at 24 hpf and 2 dpf, taken from the set of embryos analyzed in Figure 8D. (E) Representative brightfield images of embryos overexpressing human WT, MI, or MII SOX11 mRNA, taken from the set of embryos analyzed in Figure 8F. (F) Array CGH analysis of a proband with optic nerve agenesis and microphthalmia and her parents, confirming the presence of a de novo interstitial deletion at chromosome 2p25.2 (shaded gray). D, dorsal; V, ventral; A, anterior; P, posterior; MO, morpholino; hpf, hours post fertilization; dpf, days post fertilization; L, lens.
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<td>TGCCATCTCAGCAGAAAACCTTG</td>
<td>CACAGGCTCAGAAGACATGCA</td>
<td>Real Time-PCR</td>
</tr>
</tbody>
</table>
2.5 Discussion
This study reveals a novel role for Sox11 in maintaining the correct level of Hedgehog (Hh) signaling during ocular morphogenesis. We demonstrate that knockdown of Sox11 in zebrafish perturbs lens formation, induces coloboma, and reduces the number of differentiated rod photoreceptors – phenotypes that can be rescued by pharmacological inhibition of the Hh pathway (cyclopamine) or morpholino inhibition of shha. Comparable lenticular and coloboma phenotypes have also been observed in murine mutants (Wurm et al., 2008b), demonstrating that Sox11’s function in vertebrate ocular development is evolutionarily conserved. However, the perinatal lethality of Sox11 null mice has precluded a thorough in vivo assessment of rod photoreceptor differentiation, which mostly occurs postnatally. Expression of the rod photoreceptor genes Nrl, Nr2e3, and Sag (Rod arrestin) is significantly reduced in E16 retinas from Sox11/− mice (Usui et al., 2013a), suggesting that Sox11 does regulate aspects of rod photoreceptor differentiation in mammals. However, in retinal explants derived from Sox11 null mice and cultured for several days, reduced rod photoreceptor number was not observed (Usui et al., 2013a). Our data suggesting that early, midline-derived Shh influences rod photoreceptor differentiation (see below), indicates that retinal explants, being removed from the source of extra-retinal Shh, may not accurately reflect the in vivo response of retinal progenitor cells to their environment. In this context, the external embryogenesis, rapid pace of retinal development, and continual rod photoreceptor genesis in the zebrafish have benefitted our studies and permitted us to uncover for the first time both the mechanism of Sox11’s action during early ocular development, as well as a role for Sox11 in regulating rod photoreceptor differentiation.

A second key finding of our study is that Sox11 acts upstream of Hh signaling specifically by negatively regulating transcription of the ligand shha. In Sox11-deficient embryos, we observed a strong increase in shha expression in the ventral forebrain, as well as an expansion of the shha territory into the dorsal diencephalon and the telencephalon. Therefore, in addition to regulating
expression of *shha* expression in the ventral midline, our data suggest that Sox11 is also required to prevent activation of *shha* in the more dorsal regions of the brain. Within the retina, the expression of *sox11a* in the GCL at 48 hpf suggests that Sox11 continues to regulate Hh signaling during retinal neurogenesis.

The magnitude of the increase in *shha* expression in the absence of *sox11* (over 180-fold) at 24 hpf suggests that loss of *shha* transcriptional repression is accompanied by a significant positive transcriptional feedback loop. However, the Hh target gene *ptc2* demonstrated a much smaller increase in expression (2-fold) at this time, raising the question of why the dramatic upregulation in *shha* did not produce a correspondingly large transcriptional response. One possible explanation is that post-transcriptional mechanisms narrow the range of Shha protein expression in *sox11* morphants. Moreover, additional feedback mechanisms may work to attenuate the transcriptional response of Hh target genes such as *patched*. In any case, the elevated and expanded GFP expression in the Hh reporter line *ptc2:GFP*, as well as the rescue by cyclopamine and *shha* co-knockdown, strongly argue that the rise in *shha* transcription induced by *sox11* deficiency has functional consequences.

In the absence of Sox11, we observed an early expansion of the optic stalk marker *pax2.1* in the optic vesicle, and a later reduction in the retinal marker *pax6a*. Such altered proximodistal patterning of the optic vesicle has been observed in several models of elevated Hh signaling (Ekker et al., 1995; Lee et al., 2008; Macdonald et al., 1995; Perron et al., 2003; Zhang and Yang, 2001). The increased apoptosis in the lens and its abnormal development may be attributable to reduced *pax6a* expression in *sox11* morphants, since similar phenotypes were observed in a lens-specific *Pax6* conditional mutant mouse model (Shaham et al., 2009). In parallel, we suggest the expansion of *pax2.1* expression due to elevated levels of Shh enlarged the area of the optic vesicle that was specified as optic stalk, hindering closure of the choroid fissure and thus causing
coloboma. Elevated Hh signaling could also account for the increase in mitotic cells in the retina, as this pathway is known to be mitogenic (Martí and Bovolenta, 2002).

Previous studies in zebrafish have shown that blocking early Hh signaling, either with shha and shhb morpholinos or by cyclopamine treatment, caused a reduction in rhodopsin expression in the retina, suggesting that Hh signaling promotes rod photoreceptor differentiation (Stenkamp et al., 2000). However, murine studies have found that activation of the Hh pathway results in a non-cell autonomous inhibition of rhodopsin expression (Zhang et al., 2006), which is consistent with our results. Moreover, loss of Shh was shown to cause accelerated differentiation of rods and cones in a conditional mouse model (Liu et al., 2005). The seemingly paradoxical response to increased and decreased Shh levels is potentially explained by the requirement for precise Shh dosage, with either alteration resulting in reduced photoreceptor number. This accords with a comparable model for Shh’s effect on reactive astrocytes (Sirko et al., 2013), and is a well-recognized feature of transcription factors, as exemplified by the effects of altered Pax6 dosage in inducing microphthalmia (Schedl et al., 1996).

Interestingly, we observed a significant increase in shha expression at 8-12 hpf, when the optic vesicle is evaginating from the midline, and we confirmed that Hh signaling was increased at this time using a ptc2:GFP reporter line (Figure 6). Furthermore, treatment of sox11 morphants with cyclopamine during this developmental window was sufficient to restore rod photoreceptor number at 72 hpf. Thus, taken together, these data indicate that early, midline-derived Shh influences rod photoreceptor differentiation. This is not the first demonstration that early midline Hh signals influence later neurogenesis in the retina. It has been shown previously that the timely progression of ath5 expression in the retina, which coincides with the activation of neurogenesis, depends on axial Shh (Kay et al., 2005). As ath5-positive cells contribute significantly to the rod photoreceptor lineage (Brzezinski et al., 2012), it is plausible that elevated Shh coming from the...
midline in *sox11* morphants delays rod photoreceptor differentiation by influencing the cell-intrinsic neurogenic program of retinal progenitor cells.

Although the phenotypes of zebrafish *blowout* (*blw*) mutants and *sox11* morphants are similar with respect to coloboma, *blw* mutants do not appear to have a defect in the differentiation of rod photoreceptors or any other retinal cell types. This is surprising, given the well-described influence of Hh signaling on retinal neurogenesis (Amato et al., 2004; Ekker et al., 1995; Kay et al., 2001; Kay et al., 2005; Levine et al., 1997; Stenkamp, 2007; Stenkamp and Frey, 2003; Stenkamp et al., 2000; Wang et al., 2002). Moreover, patients with elevated Hh signaling due to heterozygous loss of function mutations in *PTCH* exhibit retinal abnormalities, and *PtchlacZ*/- mice display a delay in photoreceptor and horizontal cell maturation at P5, all of which is consistent with our data (Black et al., 2003). One possible explanation as to why *sox11* morphants and *blw* mutants differ in this aspect of their phenotype is that the mutation in *ptc2* may be a partial loss of function allele, which is supported by the observation that *ptc2* morphants display more severe phenotypes than *blw* mutants (Lee et al., 2008).

Since SoxC factors are generally considered to function as transcriptional activators rather than repressors (Bergsland et al., 2011; Chew and Gallo, 2009), we hypothesized that Sox11 regulates Shha indirectly, through the induction of a repressor. Indeed, we found that *bmp7b* expression was significantly reduced in *sox11* morphants, and that injection of *bmp7b* mRNA into *sox11* morphants could rescue the lens and coloboma phenotypes (Figure 7). As Bmp7 has previously been shown to antagonize Shh signaling (Bastida et al., 2009; Liem et al., 1995), our results are compatible with a model whereby Bmp7 functions downstream of Sox11 to limit Shh expression during ocular morphogenesis. However, since the magnitude of the *bmp7b* rescue was not as large as that observed with cyclopamine treatment, additional mechanisms linking Sox11 with the regulation of Hh signaling are likely.
So far, more than 27 genes are associated with coloboma in humans (Chang et al., 2006), however mutations in these account for less than 20% of cases. Consequently, it is important to define additional causative genes, both to extend understanding of pathogenesis and define pathways that may be amenable to therapeutic modulation. Our work, in combination with previous studies (Wurm et al., 2008b), strongly supports a contribution from SOX11 to coloboma phenotypes, however our data indicate that the relationship is complex. With a 50% reduction in gene dosage (2p25 segmental deletion; Figure 8G), a profound phenotype was observed. In contrast, milder coding changes (S351-354dup) with a low prevalence in the general population, resulted in incompletely penetrant phenotypes, with the unaffected carrier exhibiting a sub-clinical phenotype, only detectable on ERG testing. Since the variants had significantly reduced function on in vitro and in vivo assays, this suggests that such mild alleles contribute to MAC but may be insufficient to induce phenotypes alone in all cases. Coloboma, like many developmental defects, exhibits extensive phenotypic variability, suggesting complex relationships between disease genes and modifying alleles that complicate simple genotype-phenotype correlations. It is also possible that oligogenic inheritance is a factor in coloboma, in which individuals in non-penetrant families carry a combination of pathogenic alleles at two or more disease loci, as has been described for other genetically heterogeneous developmental disorders such as the ciliopathies (Davis and Katsanis, 2012). Furthermore, functional redundancy between Sox subgroup family members is also commonly observed (Bergsland et al., 2011; Bhattaram et al., 2010a; Dy et al., 2008a), suggesting that one SoxC family member may buffer the effects of mutation in a second. Consistent with this model, we observed elevated expression of the SoxC factor sox4 in sox11 morphants at 24 and 36 hpf, and found that the lens and coloboma phenotypes of sox11 morphants could be rescued by injection of sox4 mRNA (Figure S7). Finally, in light of the incompletely penetrant phenotypes evident with multiple other MAC-causing genes (Asai-Coakwell et al., 2009; Bakrania et al., 2008; Mihelec et al., 2009; Reis et al.,
2011; Ye et al., 2010), a similar additive contribution from other SOX gene variants is highly plausible.

In summary, we describe here a novel role for Sox11 in regulating levels of Shh during ocular morphogenesis. It will be interesting to determine whether dysregulated Hh signaling underlies any of the additional developmental defects observed in Sox11−/− mice, such as congenital cardiac malformations and craniofacial anomalies. Future studies will continue to explore the mechanisms of how Sox11 regulates Hh signaling and Shh transcription, as well as the identification of direct molecular targets of Sox11 transcriptional control.
2.6 Materials And Methods

2.6.1 Zebrafish

The Tg (XIRho:EGFP)\textsuperscript{fl1} transgenic line has been previously described (Fadool, 2003), and was generously provided by J.M. Fadool (Florida State University, Tallahassee, FL). The Tg (gfap:GFP)\textsuperscript{mi2001} line has been previously described (Bernardos and Raymond, 2006) and was obtained from the Zebrafish International Resource Center (Eugene, OR). The Tg (3.2T\alphaC-EGFP) line has been previously described (Kennedy et al., 2007), and was generously provided by S.E. Brockerhoff (University of Washington, Seattle, WA). Tg(\textit{GBS-ptch2:nlsEGFP}) has been previously described (Shen et al., 2013) and was kindly provided by R. Karlstrom (University of Massachusetts, Amherst, MA). Zebrafish (\textit{Danio rerio}) were reared, bred, and staged according to standard protocols (Kimmel et al., 1995; Westerfield, M., 1995). All animal procedures were carried out in accordance with the policies established by the University of Kentucky Institutional Animal Care and Use Committee (IUCAC).

2.6.2 Morpholino (MO) injection and analysis

Morpholinos (MOs) were obtained from Gene Tools, LLC (Philomath, OR) and were prepared and injected as previously described (Forbes-Osborne et al., 2013). The following MOs were used in this study: standard control MO, 5’-CCTCTTACCTCAGTTACAATTTATA-3’; \textit{sox11a} MO1, 5’ –GTGCGTTGTCAGTCCAAAATATCAA-3’; \textit{sox11b} MO1, 5’ –CATGTTCAAACACACTTTTCCCTCT; \textit{shha}-MO: 5’CAGCACTCTCGTCAAAGCCGCATT (Nasevicius and Ekker, 2000). The specificity of the \textit{sox11} morphant phenotype was confirmed using a second \textit{sox11} morpholino placed further downstream of the first set (completely non-overlapping with \textit{sox11a} MO1, and overlapping by only 4 nucleotides with \textit{sox11b} MO1). Because the target site for this morpholino extended into the coding region (which is highly similar in sequence for both genes) it simultaneously targets both \textit{sox11a} and \textit{sox11b} (\textit{sox11} MO2, 5’ –TCCGTTTGCPGCACCATG-3’; the “P” indicates a
photo-cleavable moiety that was not used in this study). The *sox11* MO2 produced the same coloboma phenotype as the first set of MOs (Figure S1C). All data presented in this study are from embryos injected with *sox11a* MO1 and *sox11b* MO1. Unless stated otherwise, embryos were injected with 4.18 ng each of *sox11a* MO1 and *sox11b* MO1, 4.18 ng of the standard control MO, or with 3.14 ng of *shha* MO. We also confirmed that no abnormal phenotypes were observed when embryos were injected with 8 ng of standard control MO. To determine the efficiency of the *sox11* MOs, PCR fragments corresponding to the 5’UTRs of *sox11a* and *sox11b* encompassing the morpholino target sequences were amplified (using primers listed in Table 1) and cloned upstream and in frame with the EGFP gene in the pEF1α:GFP plasmid (Addgene plasmid 11154). One-cell stage zebrafish embryos were injected with 100 pg/embryo of pEF1α:GFP plasmid containing the MO binding site in the presence or absence of the *sox11* MOs. GFP expression in injected embryos was analyzed by fluorescence microscopy at 24 hpf.

2.6.3 mRNA synthesis and injection
Zebrafish *sox11a* and *sox11b* or human wild type and variant *SOX11* coding sequences were PCR amplified (using primers listed in Table 1) and cloned into the pGEMT-easy vector (Promega). The pCRII-*bmp7b* plasmid has been previously described (Shawi and Serluca, 2008) and was a kind gift from Dr. S. Fabrizio (The Novartis Institutes for Biomedical Research, Cambridge, MA). The constructs were linearized and mRNA was prepared using the mMESSAGE mMACHINE kit (Ambion) according to manufacturer’s instructions. Zebrafish *sox11a* and *sox11b* mRNAs (1.0 ng each), human *SOX11* mRNA (0.3 ng), zebrafish *bmp7b* mRNA (1.0 ng) or zebrafish *sox4a* and *sox4b* (0.5 ng each) were injected into zebrafish embryos at the one-cell stage. For mRNA rescue experiments, the mRNAs were either co-injected with *sox11* MOs, or were injected sequentially after injection of the MOs. As both methods produced similar results, the data presented here are for co-injection of mRNA and morpholino. Injections were always
performed in triplicate, and a minimum of 55 injected embryos were analyzed in each experiment. For mRNA overexpression experiments, embryos were injected with either a control (tdTomato) mRNA, zebrafish sox11a/b mRNA, or human WT, G145C (MI), or S351-354dup (MII) SOX11 mRNA, all at equimolar concentrations. The control mRNA was synthesized from pRSET-B-td-Tomato (kindly provided by Dr. D.A. Harrison, University of Kentucky, Lexington, KY). To compare control versus sox11a/b mRNA, 0.003 pmol of each mRNA was injected. To compare control versus human WT and variant SOX11 mRNA, 0.0133 pmol of each mRNA was injected. Zebrafish embryos were injected at the one-cell stage, and embryos were scored for cyclopic phenotypes (one single eye in the center of the head, two eyes that were almost fused at the midline, or one normal eye and one vestigial eye) at 24 hpf.

2.6.4 Patient analysis
To screen for mutations in human SOX11, PCR was performed using three sets of overlapping primers that spanned the entire coding region of the single-exon SOX11 gene. The amplicons were sequenced on an ABI Prism 3100 capillary sequencer (Applied Biosystems), analyzed using DNABaser v.3.1.5 and sequence alignments were performed using ClustalW. Mutations were confirmed by bi-directional Sanger sequencing and RFLP analysis of the SOX11 amplicons. Half of the 384 control DNA samples were screened by RFLP analysis, using TseI (NEB) for the G145C variant and SfcI (NEB) for the S351-S354dup variant, and the other half were screened by direct Sanger sequencing of the SOX11 coding region. Array CGH analysis was performed using a custom designed Nimblegen 4x72 whole human genome array. Oligonucleotide probes were spaced approximately every 75 bp across a 2.65 Mb region at 2p25.2, and backbone probes covered the rest of the genome. Four technical replicates were performed on the proband’s DNA, and two replicate hybridizations were performed for each parental DNA sample. Array hybridization and scanning were performed by the Roy Carver Center for Genomics at the University of Iowa (Iowa City, IA). Array data were analyzed using the segMNT analysis.
program (Nimblegen). Informed consent was obtained from all participants. Study approval was provided by the University of Alberta Hospital Health Research Ethics Board and the Ethics Committee of the IRCCS Oasi Maria SS Onlus, Troina, Italy.

2.6.5 Pharmacological manipulations
Cyclopamine (Sigma) was resuspended at 1 mM concentration in 100% ethanol and diluted in fish water for exposure. A dose response curve was generated by exposing wild type embryos to 0.5, 1.0, and 2.0 µM of cyclopamine from 5.5-13 hpf, and the dose (2.0 µM) at which no abnormal phenotype and negligible toxicity was observed was used for control and sox11 morphants. Purmorphamine (Calbiochem) was resuspended at 50 mM concentration in DMSO and diluted in fish water for exposures. Wild type embryos were exposed to 10-100 µM of purmorphamine from 5.5-24 hpf, and the dose (75 µM) at which no ocular phenotypes were observed was used to treat control and sox11 morphants.

2.6.6 Whole mount in situ hybridization, two-color fluorescent in situ hybridization (FISH) and immunohistochemistry
Whole mount in situ hybridization (WISH) and immunohistochemistry were performed essentially as previously described (Forbes-Osborne et al., 2013). For FISH embryos were manually dechorionated and fixed in 4% paraformaldehyde (PFA) made with diethyl pyrocarbonate (DEPC)-treated PBS at 4°C overnight. The fixed embryos were sequentially cryoprotected in 10% sucrose-DEPC and 30% sucrose-DEPC at 4°C overnight. Embryos were then embedded in OCT (Ted Pella, Redding, CA) and frozen at -80°C. Ten-micron sections were collected using a cryostat (Leica CM1900, Leica Biosystems, Buffalo Grove, IL), placed on Superfrost plus glass slides (Fisher Scientific, Waltham, MA) and air dried at room temperature overnight. The sections were post-fixed in 1% PFA-DEPC and rehydrated in PBST-DEPC. The sections were permeabilized for 10 minutes with 1µg/ml proteinase K. Sections were acetylated in triethanolamine buffer plus 0.25% acetic anhydride (Sigma-Aldrich, Saint Louis, MO), and
then rinsed in DEPC treated water. Sections were hybridized with digoxigenin (DIG) and fluorescein (FITC) labeled probes (2.5 ng/µl) in hybridization buffer (0.25% SDS, 10% dextran sulfate, 1X Denhardt’s solution, 200µg/ml torula yeast tRNA, 50% de-ionized formamide, 1mM EDTA, 600mM NaCl, and 10mM Tris pH 7.5 in DEPC-treated water) at 65°C in a sealed humidified chamber for a minimum of 16 hours. Following hybridization, the slides were rinsed in 5X SSC and then with pre-warmed 1X SSC/50% formamide. Endogenous peroxidase activity was quenched with 1% H₂O₂ for 30 minutes. Sections were blocked using 0.5% PE blocking solution (Perkin Elmer Inc, Waltham, MA) for at least 1 hour. For two-color FISH, sections were incubated first with anti-DIG-POD Fab fragment (Roche, Indianapolis, IN) at 4°C overnight. Subsequently, probe signal was detected using the TSA plus Cy3 kit (Perkin Elmer Inc, Waltham, MA) following the manufacturer’s instructions. For the second color detection, the sections were treated with 1% H₂O₂ for 30 minutes and then incubated with anti-FITC-POD Fab fragment (Roche, Indianapolis, IN) at 4°C overnight. Subsequently, the FITC-labeled probe signal was revealed using TSA plus Fluorescein (Perkin Elmer Inc, Waltham, MA). Finally, sections were counterstained with 4’, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Saint Louis, MO), mounted in 40% glycerol, and imaged on an inverted fluorescent microscope (Nikon Eclipse Ti-U; Nikon Instruments, Melville, NY) using a 40x objective.

The sox11a, sox11b and shha cDNAs were amplified (using primers listed in Table 1) and cloned from 48 hpf whole embryo cDNA. The sox11b and NeuroD antisense probes have been previously described (Morris et al., 2011a). The pax6a, crx, and nr2e3 probes have been previously described, and were kindly provided by Y.F. Leung (Purdue University, Indiana). The pax2.1 probe has been described previously (Lee et al., 2008) and was a gift from J.M. Gross (University of Texas, Austin, TX). The following primary antibodies and dilutions were used: Zpr-1 (1:20; ZIRC), which labels red-green cones; Zn-8 (1:10; ZIRC), which labels ganglion cells; anti-Prox-1 (1:2000; Millipore), which recognizes horizontal cells; anti-PH3 (1:500;
Millipore), which marks cells in G2/M phase; 5E11 (1:10; J.M. Fadool, Florida State University), which labels amacrine cells; and anti-PKCα (1:300; Santa Cruz Biotechnology), which labels bipolar cells. Alexa Fluor secondary antibodies (Molecular Probes, Invitrogen) and Cy-conjugated secondary antibodies (Jackson ImmunoResearch) were all used at 1:200 dilution. Sections from the same region of the eye were analyzed for quantification purposes. One section was quantified per individual embryo (for both control and sox11 morphants).

2.6.7 TUNEL Assay
Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed on retinal cryosections using the ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer’s instructions. Sections from the same region of the eye were analyzed for quantification purposes. One section was quantified per individual embryo (for both control and sox11 morphants).

2.6.8 Real-Time Quantitative RT-PCR
RNA extracted from the heads of control, sox11, and shha morphant embryos at various time points was used to perform first-strand cDNA synthesis (GoScript Reverse Transcriptase System; Promega). Real time PCR was performed using either Maxima SYBR Green qPCR master mix (Thermo Scientific) or FastStart SYBR Green Master (Roche) on an iCycler iQ Real Time PCR Detection system (Bio-Rad) or LightCycler 96 (Roche) with primers listed in Table 1. Three biological replicates were performed for each experiment. The gene expression change was determined using a relative standard curve quantification method with gapdh, atp5h, or 18s rRNA (McCurley and Callard, 2008) expression as the normalization control.
2.6.9 Statistics
Statistical analysis was performed on all data using the GraphPad Prism 6.02 software. Continuous data were analyzed using Student’s-t-test and Fisher’s exact test. For all graphs, data are represented as the mean ± the standard deviation (s.d.).

2.6.8 Dual Luciferase Assays
COS-7 cells were transfected with the pcDNA3 expression vector (Invitrogen) containing the coding region of wild type, G145C, or S351-354dup SOX11; the pGL3 Firefly Luciferase reporter vector (Promega) containing the GDF5 core promoter was a kind gift from Akinori Kan (Harvard Medical School, Boston, MA) (Kan et al., 2013); and the pRL-TK vector (Promega) containing Renilla luciferase driven by a ubiquitous tyrosine kinase promoter to control for transfection efficiency. Transfections were performed using Fugene 6 (Promega), following manufacturer’s instructions. The total mass of DNA and molar ratios of pGL3 and pRL-TK were held constant across transfections, which were repeated a minimum of 6 times. Dose response curves were generated using wild type SOX11 at 0:100, 1:20, 1:10, and 1:5 molar ratios to the GDF5 reporter. The mutant SOX11 variants were transfected at a 1:5 molar ratio to the GDF5 reporter. Firefly and Renilla luciferase activity were measured 24-36 hours post transfection using the DualGlo Luciferase Assay System (Promega). Data was analyzed as follows: Firefly luciferase (FFLuc) was baselined against untransfected control (UTC) samples (=FFLuc – UTC) and normalized using the Renilla luciferase (RLuc). The Relative Luciferase Activity (RLA) was calculated as (FFLuc-UTC)/RLuc and compared between experimental and control transfections.
2.7 Acknowledgements
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CHAPTER 3: CRITICAL PERIOD OF SOX11 FUNCTION FOR PROPER OCULAR MORPHOGENESIS AND ROD PHOTORECEPTOR DEVELOPMENT IN ZEBRAFISH

Lakshmi Pillai-Kastoori¹, Ann C. Morris¹
¹Department of Biology, University of Kentucky, Lexington, KY 40506-0225, USA

Keywords: Photo morpholino; Sox11; ocular morphogenesis; coloboma; zebrafish eye

3.1 Introduction
Eye formation is a very delicate biological process that results from the sculpting and molding of sheets of cells to form a perfect tissue. Disruptions in this process can cause congenital ocular malformations leading to pediatric visual impairment. With the motivation to study eye development as a way to understand ocular pathologies, I have characterized a SRY-Box transcription factor, Sox11, during zebrafish ocular development.

The sox11 zebrafish morphants display a variety of abnormal ocular phenotypes including abnormal lens shape associated with delayed lens development, coloboma due to failure of choroid fissure closure, and a specific reduction in the number of mature rod photoreceptors. Importantly, inhibition of Hedgehog (Hh) pathway activity as early as 5-13 hpf is sufficient to rescue abnormal eye defects resulting from the loss of sox11 function. Further analysis revealed that shh transcript levels are robustly upregulated in sox11 deficient embryos as compared to controls. Knockdown of shha significantly rescues the abnormal ocular phenotypes present in the sox11 morphants.
Although we know that the *sox11* morphants display delayed lens development, coloboma, and reduced rod photoreceptors, we do not know when *sox11* gene function is required to regulate proper ocular morphogenesis and rod neurogenesis. To begin to address this question, we want to manipulate *sox11* gene function at different stages of eye development to distinguish between early and late functions of *sox11*.

The goal of the project described below is to determine the critical period of requirement for *sox11* during eye development and rod neurogenesis in zebrafish.

The *sox11* morphants have increased *shha* mRNA levels as early as 8 hpf and have abnormally shaped optic vesicles at 12 hpf in addition to delayed lens, coloboma and rod defects at 1, 2.5 and 3 dpf respectively. I therefore hypothesized that *sox11* gene function is required during optic vesicle patterning stages (6-18 hpf) and this early function regulates both ocular morphogenesis and rod neurogenesis. To test this hypothesis, we designed a study to control temporal expression of *sox11* using photo-morpholinos.

In this study, in order to gain temporal control over the translation-blocking capacity of *sox11* morpholino, we used photo-morpholino technology to inhibit *sox11* function(s) at various stages of ocular morphogenesis and retinal neurogenesis. Photo-morpholino technology employs the use of a photo-cleavable moiety placed strategically in the middle of the morpholino sequence. Upon radiation of a 365 nm UV light, the morpholino sequence containing the moiety breaks into two pieces.

In order to stop *sox11* MO activity (GENE-ON), we designed an antisense *sox11* morpholino that can be inactivated by exposure to UV light. Embryos injected with Antisense-Photo- *sox11* MO (AS-P-*sox11*) as well as uninjected control individuals were placed under 365 nm UV light for 5-10 minutes to break the AS-P-*sox11* MO sequence. The embryos were allowed to develop
to various stages and were scored for delayed lens development, coloboma, and rod photoreceptor
defects at 24 hours post fertilization (hpf), 2.5 dpf and 3 dpf respectively. The control morpholino
to compliment the AS-P-sox11 MO is a Sense-Photo-sox11 MO (S-P-sox11) sequence which
does not bind to the target site and contains a Photo moiety in the center of the sequence.

3.2 Results
3.2.1 Knockdown of sox11 using antisense-Photo-sox11 morpholino (AS-P-sox11) results in
abnormal ocular morphogenesis and causes coloboma in zebrafish.
To first determine the efficiency of AS-P-sox11 MO, zebrafish embryos were injected with AS-P-
sox11 MO (hereafter referred to as sox11-P morphants). I have previously shown that co-
inhibition of both sox11 paralogs (Figure S1B), resulted in lens defects in 72.9±7.4% of sox11
morphants at 24 hpf (Figures 2A, S1B), and caused coloboma in a similar proportion (70.0±7.7%)
of injected embryos at 2 days post fertilization (Figures 2A, S1B). At 24 hpf, unlike the
conventional sox11 morphants, none of the sox11-P morphants displayed an abnormal lens
phenotype. Instead, sox11-P morphants displayed a non-circular eye structure where the ventral
lips of the eye do not meet ventrally, rather one of the two lips appears misshapen and contorted
(Figures 3.1B, 4.5). At 24 hpf, 82.3 ±6.7% of sox11-P morphants displayed this abnormal eye
shape. At 2 dpf, 65.6 ±5.8 % sox11-P morphants displayed an open choroid fissure resulting in
coloboma (Figures 3.2, 3.5).
Figure 3.1. Knockdown of sox11 using AS-P-sox11 MO results in abnormal eye structure. (A) Representative eye and body images of control and sox11 morphants. At 24 hpf, approximately 82% of sox11 morphants displayed a malformed eye (red outline) and a heart edema. Three biological replicates were performed for all experiments. D, dorsal; V, ventral; A, anterior; P, posterior; L, lens; R, retina; hpf, hours post fertilization; MO, morpholino.
3.2.2 *sox11-P* morphants display poor retinal lamination and fewer mature rod photoreceptors

I investigated whether, like *sox11* morphants, *sox11-P* morphants displayed altered rod development. Since we found that 65.6 ±5.8% of *sox11-P* morphant retinas with coloboma also displayed poor lamination (31/31 *sox11*-P morphants), indicating a potential delay in retinal development, for analysis we divided the *sox11*-P morphants into those with and without coloboma. This approach minimized the potential secondary effects on retinal development from the earlier ocular morphogenetic defect. Using immunohistochemistry, I found that *sox11*-P morphants without coloboma (approximately 35% of morphant embryos) also lacked well-laminated retinas at 72 hpf (15/17 =88.2% *sox11*-P morphants). Furthermore, when *sox11*-P MO were injected into a rod photoreceptor-GFP transgenic reporter line (Fadool, 2003), I observed a significant reduction in GFP-positive rod photoreceptors in *sox11*-P morphant retinas irrespective of the presence of coloboma at 3 dpf (uninjected embryos, 36.5±7.7 rods/section; *sox11*-P morphants, 5.7±8.9 rods/section; p<0.0001; Figures 3.2B’-B’, C-C’, 3.6). Moreover, several retinal sections from *sox11*-P morphants contained no detectable GFP-positive rods at 3 dpf.

Taken together, it appears that AS-P-*sox11* MO even at the optimum dosage significantly affects overall retinal neurogenesis and retinal lamination. *Sox11*-P morphants lack the presence of mature cell bodies and three retinal layers. It is tricky to delineate the observed primary and secondary effects of the AS-P-*sox11* MO. Does AS-P-*sox11* MO cause reduction in mature rod photoreceptors or the reduction in mature rods occurs as a consequence of delay in retinal neurogenesis?
Figure 3.2. Knockdown of sox11 using AS-P-sox11 MO results in coloboma. At 2 dpf, approximately 62% sox11-P morphants displayed coloboma (red lines), and had a hypopigmented and underdeveloped ventral retina. Three biological replicates were performed for all experiments. D, dorsal; V, ventral; A, anterior; P, posterior; L, lens; R, retina; dpf, days post fertilization; MO, morpholino.
Figure 3.3. *sox11*-P morphants display immature retinas. Representative transverse retinal sections from XOPS-GFP zebrafish injected with control, AS-P-*sox11* MO 3 dpf (from the set of individuals analyzed in Figure 3.6). Even *sox11*-P morphants with no evidence of coloboma (red asterix) (B-B’) displayed greatly reduced numbers of mature rods and thick RPE (red arrow) compared to controls (A-A’). D, dorsal; V, ventral; L, lens; R, retina; hpf, hours post fertilization; MO, morpholino; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigmented epithelium; AS, antisense; rho, rhodopsin.
3.2.3 Sox11 gene function is required between 4-10 hpf for proper ocular morphogenesis in zebrafish embryos.

Embryos injected with AS-P-sox11 MO as well as uninjected control individuals were placed under 365 nm UV light at various times after injection for 5-10 minutes to break the AS-P-sox11 MO sequence. The embryos were then scored for delayed lens development, coloboma, and rod photoreceptor defects at 24 hpf, 2.5 dpf and 3 dpf, respectively. The schema (Figure 3.4) details the treatment period(s) and predictions.

I injected 0-1 cell stage zebrafish embryos with 5.0 ng/embryo of AS-P-sox11 MO + 80 pg/embryo GFP reporter plasmid (containing sox11 MO binding site upstream of the GFP). A subset of the injected and uninjected embryos were exposed to 365 nm UV light at 1 hpf and 4 hpf under 4X aperture, ND#2, for 5 minutes; these exposure conditions were chosen because they were the least toxic to the embryos and the most efficient at inducing cleavage of the morpholino. Cleavage of the photo-morpholino was tracked using the GFP reporter plasmid. The uncleaved AS-P-sox11 MO will bind to the sox11 MO binding sequence upstream of the GFP sequence and inhibit GFP expression from the reporter plasmid, in addition to inhibiting translation of endogenous sox11. Following UV exposure, efficient breakage of the AS-P-sox11 MO should relieve the translational inhibition and allow for the production of both Sox11 protein and the GFP. Embryos were tracked every hour for the presence of GFP signal and sampled at 24 hpf, 2 dpf and 3 dpf for abnormal ocular phenotypes.

GFP signal (> 3 GFP+ cells/embryo) was first confirmed in majority of injected embryos 6-7 hours post UV treatment irrespective of the stage (1 hpf vs. 4 hpf) of UV treatment. Majority of the embryos had abundant GFP expression (>12 GFP+ cells) around 10 hpf irrespective of the stage of UV treatment. After UV treatment at 1 hpf, a subset of embryos injected with AS-P-sox11 MO were sampled at 24 hpf, 2 dpf and 3 dpf. At 24 hpf, only 4.04±3.05 % displayed irregular eye structure and RPE defects, and at 2 dpf only 4.04±3.05 % displayed coloboma.
These results suggest that UV inactivation of the sox11-P MO was successful and the presence of sox11 gene product is crucial for allowing ocular morphogenesis to proceed normally in the majority of embryos. However, at 3 dpf sox11-P morphants that were exposed to UV light at 1 hpf still continued to possess reduced number of rod 4.3±7.13 photoreceptors and very poorly laminated retinas (Figure 3.6).

After UV treatment at 4 hpf, a subset of embryos injected with control MO, S-P-sox11 MO, AS-P-sox11 MO were sampled at 24 hpf, 2 dpf and 3 dpf. After breakage of the AS-P-sox11 MO sequence at 4 hpf the proportion of embryos with non-circular eye at 24 hpf (11.67±8.12 %) and coloboma (7.08±7.53%) was significantly reduced compared to non-UV treated control sox11 P-morphants (>65% for both phenotypes; p<0.0001; Figure 3.5). However, at 3 dpf sox11-P morphants still continued to possess reduced number of rod photoreceptors and poorly laminated retinas (sox11-P MO-UV, 0.9±1.4; sox11-P MO+UV, 3.3±2.7). This was a surprising observation since sox11-P morphants displayed normal gross eye morphology with circular eye shape and fused choroid fissure.

Nevertheless, presence of sox11 gene function acquired by inactivating AS-P-sox11 MO soon after injection, suggests that (a) AS-P-sox11 MO can be inactivated with the UV treatment of 5 minutes, (b) presence of sox11 gene function is necessary for proper eye structure and closure of choroid fissure and lastly, (c) presence of AS-P-sox11 MO reagent or UV treatment is acting as a confounder in the present experiment and impeding retinal neurogenesis

3.2.3 The presence of photo moiety within the AS-P-sox11 MO sequence appears to be toxic to retinal neurogenesis

In order to identify the cause of the defects in retinal lamination and neurogenesis in sox11-P morphants, I injected 0-1 cell stage zebrafish embryos with either 5.0 ng/embryo of AS-P-sox11 MO, 5.0 ng/embryo of sense-P-sox11 MO, 5.0 ng/embryo of conventional sox11a/b MO, or 5.0
ng/embryo of control MO. Subsets of all the MO injected and uninjected embryos were exposed to 365 nm UV light at 1 hpf under 4X aperture, ND#2, for 5 minutes. Histological examination of the sosx11-P morphants at 3 dpf revealed a significant reduction in the number of mature rod photoreceptors in AS-P-sosx11 MO (4.3±7.13 rods/section), sense-P-sosx11MO (9±9.77 rods/section), sosx11a/b MO (1.92±3.5 rods/section), compared to control retinas (38.95±5.6) (Figure 3.6). Furthermore, to rule out the contribution of the UV treatment itself to delayed retinal neurogenesis I compared non-UV treated sense-P-sosx11 morphants with control morphants. At 3 dpf S-P-sosx11 morphants without UV treatment (which should not possess active morpholino) still have reduced rod photoreceptors compared to controls (12.44±7.7 vs 36.5±7.7 rods/section) (Figure 3.6). The presence of reduced numbers of mature rod photoreceptors in embryos injected with sense-P-sosx11MO ± UV treatment suggests that it is the photo moiety itself, independent of sosx11 knockdown, that is causing immature looking, poorly laminated retinas.

In the light of this observation, we cannot proceed with using photo morpholino technology to address the two key questions of (a) the critical time period of sosx11 gene function for proper ocular morphogenesis and (b) whether sosx11 has two independent and temporally separable roles to guide early ocular morphogenesis and later retinal neurogenesis.
Figure 3.4. Schematic representation of Photo morpholino strategy to test temporal requirement of Sox11 during ocular morphogenesis and retinal development. Embryos will be injected with Antisense-Photo- sox11 MO (AS-P-sox11 MO) and injected individuals will be placed under 365 nm UV light for 5-10 minutes to break the AS-P-sox11 MO sequence. The embryos will allowed to develop to various stages and will be scored for delayed lens development, coloboma, and rod photoreceptor defects at 24 hours post fertilization (hpf), 2.5 dpf and 3 dpf respectively.
Figure 3.5. Inactivation of *sox11* Photo morpholino significantly reduces the proportion of abnormal ocular phenotypes in zebrafish. AS-P *sox11* MO injection results in abnormal eye and coloboma phenotypes. The proportion of embryos displaying either phenotype was significantly higher in the minus UV treatment category compared to the plus UV category. Number of embryos analyzed: 24 hpf control MO-UV, n=192; 2 dpf control MO-UV, n=192; 24 hpf AS-P-*sox11* MO-UV, n=195; 2 dpf AS-P-*sox11* MO-UV, n=193; 24 hpf S-P-*sox11* MO-UV, n=184; 2 dpf S-P-*sox11* MO-UV, n=184; 24 hpf control MO+UV, n=127; 2 dpf control MO +UV, n=127; 24 hpf AS-P-*sox11* MO+UV, n=48; 2 dpf AS-P-*sox11* MO+UV, n=48; 24 hpf S-P-*sox11* MO+UV, n=151; 2 dpf S-P-*sox11* MO+UV, n=151, 3 independent repeats. *p<0.001, Fisher’s exact test.
Figure 3.6. *Sox11*-P morphants display delayed retinal neurogenesis. Representative images of 3 dpf embryos injected with control and sox11 MOs with UV (at 1 hpf) and without UV treatment. Embryos injected with S-P-sox11 MO display retarded, poorly laminated retina with or without UV treatment. Inactivation of AS-P-sox11 MO at 1 hpf does not rescue the rod defect in sox11 morphants. D, dorsal; V, ventral; L, lens; dpf, days post fertilization; MO, morpholino; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; ON, optic nerve. Red asterix indicates colobomatous region of the retina.
Figure 3.7. Presence of the Photo moiety within sox11 MO sequence contributes to delayed retinal neurogenesis in sox11-P morphants. Quantification of the number of rod photoreceptors/section sampled from embryos presented in Figure 3.6. Number of embryos analyzed: UIC-UV, n=75, control MO-UV, n=19; AS-P-sox11 MO-UV, n=9; S-P-sox11 MO-UV, n=7; UIC+UV, n=24; control MO+UV, n=12; AS-P-sox11 MO+UV, n=14; S-P-sox11 MO+UV, n=15; sox11a/b MO+UV, n=15, 3 independent repeats. n.s., p >0.05, Student’s t- test. UIC, uninjected control; MO, morpholino; AS, antisense; UV, ultra violet light.
3.3 Discussion
Morpholinos have been useful to study and examine the molecular mechanisms of a variety of developmental systems in a range of animal models such as zebrafish, medaka, carp, chicken, *Xenopus*, sea lamprey, sea urchin, mouse and *Drosophila* (Agaisse et al., 2005; Eisen and Smith, 2008; Friedman and Perrimon, 2006; Heasman et al., 2000; Sauka-Spengler and Barembaum, 2008). They have proven to be a powerful antisense reagent for studying gene function(s) in both *in vitro* and *in vivo* paradigms. Using antisense MO we have shown that zebrafish embryos with reduced levels of *sox11a/b* display abnormal ocular morphogenesis and coloboma. In addition, *sox11* morphant retinas possess reduced number of mature rod photoreceptors. To better understand the temporal requirements of *sox11* gene function during zebrafish ocular development, we proposed to use photo-morpholinos to determine the following: (a) what is the critical period of development during which *sox11a/b* gene function is required for proper ocular morphogenesis, and (b) does *sox11* have two independent roles in ocular morphogenesis and later retinal neurogenesis or is early expression of *sox11* sufficient to regulate both processes?.

Given that *sox11* morphants display ocular defects as early as 12 hpf with abnormally shaped optic vesicle and additionally, display increased *shha* mRNA levels as early as 6 hpf, we tested the hypothesis that *sox11* gene function is required during the optic vesicle patterning stages (6-18 hpf) and that this early function simultaneously regulates both ocular morphogenesis and rod neurogenesis. To test this hypothesis, we designed a study to control temporal expression of *sox11* using photo-morpholinos.

We used GFP reporter plasmid with *sox11* MO binding sequence cloned upstream of the GFP tag as a proxy for Sox11 protein due to unavailability of a working antibody against zebrafish Sox11. Therefore, when the antisense *sox11*-P MO is in an active form, GFP signal will be absent and
upon UV exposure (which cleaves the antisense sox11-P MO), GFP signal is present, acting as a proxy for Sox11 protein. It is established that maturation of the GFP chromophore and spectral signal is directly coupled with GFP protein folding. Therefore, there should be correlation between the target protein and the fate of the folded GFP protein (Reid and Flynn, 1997; Wachter, 2007). However, in our study we did not use a fusion protein construct, and therefore it is not easy to make a correlation between GFP and Sox11 protein maturation and folding. After UV inactivation, GFP signal was first visualized after 6-8 hours post treatment in 2-3 cells throughout the embryo. This marked lag between the UV treatment and the first signs of GFP signal, plus the small amount of signal makes inferences of return of sox11 gene function difficult.

Inactivation of the sox11 antisense photo morpholino at 4 hpf significantly reduced the proportion of embryos with abnormal lens shape and coloboma at 24 hpf and 2 dpf respectively (Figure 3.5) compared to embryos injected with conventional sox11 MO. These data suggest that sox11 is required between 4-10 hpf for the proper development and morphogenesis of the optic cup and the closure of the optic fissure. Although this window is quite broad, it is nonetheless a better estimation of the requirement period of sox11 gene function than we started with. We can narrow this window even further by injecting a hsp70:sox11-polyA construct, which upon heat shock at varying time points within the 4-10 hpf window will make full length sox11 mRNA and rescue the abnormal phenotypes. The time point at which no more significant rescue is detectable will be the last time point at which sox11 should be necessary for proper ocular morphogenesis.

Our study provides a decent estimate for the requirement for sox11 gene function for proper ocular morphogenesis and choroid fissure closure. However, the sense/Antisense-Photo-sox11 MO used itself does harm to the normal retinal neurogenesis in zebrafish embryo (Figure 3.6). It is possible that the two pieces of morpholino sequence generated as a result of UV inactivation might act as toxic byproducts hindering normal retinal neurogenesis. Embryos injected with both
sense and antisense-Photo-sox11 MO ± UV treatment displayed very poor retinal lamination, RPE defects, and lack of mature retinal neurons. Therefore, using the sox11-photo morpholino tool to address the question of sox11 gene functions requirement for retinal neurogenesis is not feasible.
3.4 Material and Methods

3.4.1 Zebrafish
Zebrafish (Danio rerio) were reared, bred, raised, and maintained at 28°C under a 14 hour light and 10 hour dark cycle according to standard protocols (Kimmel et al., 1995; Westerfield, 1995). All animal and procedures were carried in accordance with the policies established at the University Of Kentucky Institutional Animal Care and Use Committee (IUCAC). The Tg (XIRho:EGFP)f1 transgenic line (hereafter referred to as XOPS:GFP) has been previously described (Fadool, 2003), and was generously provided by J.M. Fadool (Florida State University, Tallahassee, FL).

3.4.2 Morpholino (MO), photo MOs and light exposure
Morpholinos (MOs) were obtained from Gene Tools, LLC (Philomath, OR) and were prepared and injected as previously described (Pillai-Kastoori et al., 2014b). The following MOs were used in this study: standard control MO, 5’-CCTCTTACCTCAGTTACAATTTATA-3’; sox11a MO1, 5’ –GTGCGTTGTCAGTCCAAAATATCAA-3’; sox11b MO1, 5’ –CATGTTCAAACACACTTTTCCCTCT. Photo sox11 morpholino sequence was placed further downstream of the first set (completely non-overlapping with sox11a MO1, and overlapping by only 4 nucleotides with sox11b MO1). Because the target site for this morpholino extended into the coding region (which is highly similar in sequence for both genes) it simultaneously targets both sox11a and sox11b (AS-P-sox11 MO, 5’ –TCCGTTTGCPGACCATG-3’; the “P” indicates a photo-cleavable moiety). Unless stated otherwise, embryos were injected with 5 ng each of sox11a MO1and sox11b MO1, AS-P-sox11 MO, S-P-sox11 MO, and 5 ng of the standard control MO. For UV exposure, embryos were placed in a petri dish and illuminated with a broad spectrum UV light using the 365 nm DAPI filter cube on an inverted fluorescent microscope (Nikon Eclipse Ti-U; Nikon Instruments, Melville, NY) using a 4x objective at ND#2 setting for 5 minutes.
3.4.3 Reporter assay to confirm sox11 gene “ON” function
To determine that AS-P-sox11 MO sequence is cleaved and Sox11 function is turned “ON” following UV exposure, PCR fragments encompassing the morpholino target sequence were amplified (using primers listed in Table1) and cloned upstream and in frame with the EGFP gene in the pEF1α:GFP plasmid (Addgene plasmid 11154). One-cell stage zebrafish embryos were injected with 80 pg/embryo of pEF1α:GFP plasmid containing the MO binding site in the presence or absence of the AS-P-sox11 MO. GFP expression in injected embryos was analyzed by fluorescence microscopy from 6-24 hpf.

3.4.4 Immunohistochemistry
Immunohistochemistry were performed essentially as previously described (Forbes-Osborne et al., 2013). Briefly, 10 µm transverse retinal section generated from injected XOPS:GFP embryos were fixed, hydrated and were counterstained with 4’, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Saint Louis, MO), mounted in 40% glycerol, and imaged on an inverted fluorescent microscope (Nikon Eclipse Ti-U; Nikon Instruments, Melville, NY) using a 40x objective.

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CHAPTER 4: GENERATION OF ZEBRAFISH SOX11A AND SOX11B MUTANTS USING CLUSTERED REGULARLY INTERSPACED SHORT PLAINDROMIC REPEATS (CRISPR) TECHNOLOGY

Lakshmi Pillai-Kastoori¹, Ann C. Morris¹

¹Department of Biology, University of Kentucky, Lexington, KY 40506-0225, USA

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4.1 Introduction
Targeted gene inactivation via classical homologous recombination approach or newer genome editing approaches using engineered nucleases enable investigators to determine gene function and genotype-phenotype correlations. Classical approach is a laborious process with low-efficiency of disruption of the chromosomal target site, requiring extensive selection/screening steps, and possibility for antagonistic background mutagenic effects. Importantly, mouse-style knockout approach cannot be used on zebrafish due to the current lack of established zebrafish embryonic stem cell lines. Morpholino mediated gene knockdown approach in zebrafish has provided researchers with quick, inexpensive, and high-throughput alternative. However, morpholino approach provides only transient inhibition of gene function which can be insufficient, and presents variability between hands and laboratories, along with the possibility of unpredictable off-target site effects (Kok et al., 2015). These important limitations obstruct researchers’ ability to make inferences about the genotype-phenotype relationship and limits practical application of morpholino technology.

Recently, a new approach that enables researchers to manipulate any gene of interest in diverse types of cells or organisms has revolutionized the field of genetic engineering. This genome
editing technology uses the type II clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas9) system. CRISPR is an adaptive immune system of bacteria that protects against invading foreign DNA via RNA guided DNA cleavage (Gaj et al.; Makarova et al., 2011; Wiedenheft et al., 2012); it has become a powerful tool for genome editing in various model organisms (Li et al., 2013; Mali et al., 2013; Ota et al., 2014) including zebrafish (Hwang et al., 2013).

4.1.1 CRISPR/Cas9 system

CRISPR/Cas systems have been classified into three major categories on the basis of information gathered from phylogenetic and comparative genomic analyses. Cas9 protein belongs to the type II CRISPR/Cas system and is reported to be sufficient for RNA mediated silencing of foreign DNA (Makarova et al., 2011).

CRISPR/Cas systems confer immunity by employing small RNAs for sequence-specific detection and silencing of foreign nucleic acids. In type II CRISPR/Cas9-mediated immunity discovered in Streptococcus pyogenes, silencing occurs in three steps: adaptation, expression, and interference. In the adaptive phase, upon viral challenge short fragments of foreign sequence (protospacers) are integrated into the host (bacteria and archaea) chromosome at the proximal end of the CRISPR array (Bhya et al., 2011; Jinek et al., 2012; Terns and Terns, 2011; Wiedenheft et al., 2012).

The second stage in type II CRISPR/Cas9-mediated immunity is expression, in which the CRISPR locus is transcribed to generate a long primary transcript (pre-crRNA) which then is processed into short CRISPR RNAs (crRNAs). In type II systems, pre-crRNAs are processed by a different mechanism where a trans-activating crRNA (tracrRNA) complementary to the pre-crRNA repeat sequence (belonging to the foreign DNA) forms a duplex with pre-crRNA. The
duplex formation triggers pre-crRNA processing by the housekeeping double-stranded RNA-specific ribonuclease RNase III (Deltcheva et al., 2011; Wiedenheft et al., 2011). In the third step of DNA interference, tracrRNA:crRNA guided Cas9 protein cleaves invading target DNA at the NGG-trinucleotide protospacer adjacent motif (PAM). Cas9 nuclease has homology to HNH-like and RuvC-like endonuclease domain and they cleave complementary and noncomplementary DNA strands respectively (Gesner et al., 2011; Hale et al., 2009; Jinek et al., 2012; Makarova et al., 2011).

In conclusion, CRISPR/Cas9-genome editing method involves dual-RNA structure that guides Cas9 endonuclease to introduce double stranded site-specific break(s) in the target DNA. Target recognition by Cas9 endonuclease requires both a seed sequence in the crRNA and a NGG-trinucleotide protospacer adjacent motif (PAM) adjacent to the crRNA-binding region in the target DNA (Jinek et al., 2012).

4.1.2 RNA guided endonuclease system (RGEN)
tracrRNA:crRNA guided Cas9 protein targets and cleaves target DNA efficiently in nature. The alternative of a single RNA guided Cas9 is robust and enables programmed DNA cleavage and genome editing (Jinek et al., 2012). In such a system, a single custom guide RNA containing complementary sequence to the target site recognizes target DNA sequence located adjacent to the PAM motif NGG, and the DNA target sites is then cleaved by a complex consisting of gRNA and Cas9 endonuclease. Subsequently, the DNA breaks are repaired by error-prone, non-homologous end joining (NHEJ), which in turn results in insertion and/or deletion (indel) mutations within the target site. Therefore, indel mutations within the coding sequence results in gene disruption/inactivation (knockout).

CRISPR/Cas9 gene editing tool box contains two components, small gRNA and the nuclease Cas9 (Jinek et al., 2012). The gRNA oligonucleotide has a 20 nucleotide sequence to target the
protospacer sequence of the target with a PAM motif present at the 3’ end of the protospacer, which makes it useful for high throughput targeted mutagenesis. Compared to other techniques available, the Cas9/gRNA system is far easier for engineering and practical application. The only synthesis involved is of a specific gRNA oligo of ~100 nucleotides for any new target sequence, and the Cas9 endonuclease protein (in a plasmid form or mRNA encoding this protein), which is universal for all target sites. Several groups have reported single gene disruptions (Chang et al., 2013; Hruscha et al., 2013; Hwang et al., 2013; Jao et al., 2013; Sung et al., 2014) as well as multi-locus targeting in zebrafish (Jao et al., 2013; Xiao et al., 2013).

In this study, I designed two gRNAs targeting distinct locations within the coding region of both sox11a and sox11b in order to generate stable sox11 knockout lines. By generating stable knockout lines we will be able to characterize the phenotype due to permanent loss of sox11 gene function in the eye not only in the developing embryo but also in the juvenile and adult stages. Additionally, these sox11 mutant lines can be used to study the function of sox11 under retinal injury and regeneration.

4.2 Results
4.2.1 RGEN design and synthesis
To generate stable knockout lines for sox11a and sox11b, I designed four customized single-stranded gRNAs composed of nucleotide sequences complementary to the target sequences that guide the Cas9 endonuclease to the target site of interest (Figure 4.1A, 4.1B). Two gRNAs were designed to target two separate regions: (a) 5’ to the HMG box and (b) 3’ end of the coding region. A nuclear localized zebrafish codon-optimized cas9 (nls-zCas9-nls) mRNA was used for RGEN mutagenesis (Jao et al., 2013). The gRNAs were synthesized by direct in vitro transcription from synthetic oligonucleotides as templates to generate optimum RNA quality and quantity. To demonstrate the efficiency of the CRISPR/Cas9 system in zebrafish we chose to disrupt the tyrosinase (tyr) genomic locus, which has been previously shown to be suitable and
accessible for CRIPSR/Cas9 studies (Jao et al., 2013). The Tyrosinase gene encodes a copper-containing enzyme that acts as catalyst for melanin pigment production by oxidizing tyrosinase (Page-McCaw et al., 2004). Therefore, disruption of tyr gene results in an obvious abnormal hypopigmentation of skin melanophores and the retinal pigment epithelium (RPE) in zebrafish.
Figure 4.1. Schematic representation of sox11 genes and CRISPR target site. (A) sox11a gene is a single exon gene with a short 3’UTR and long 5’UTR. The first target site namely sox11a-C1gRNA is 5’ to the HMG domain and the second sox11a-C1gRNA is present towards the end of the coding region. The distance between the two targets is 942 bps. (B) sox11b gene is a single exon gene with a short 3’UTR and long 5’UTR. The first target site namely sox11b-C1gRNA is within the HMG domain and the second sox11b-C1gRNA is present towards the end of the coding region. The distance between the two targets is 1195 bps. Bps, base pairs; gRNA, guide RNA; C,CRISPR.
Figure 4.2. Test of mutagenesis in F0 embryos via HRMA analysis and Sanger sequencing. (A) PCR products from a subset of *sox11a* gRNA+ nls-zCas9-nls injected embryos (mutli-color lines) are subjected to a high resolution melt curve and normalized to uninjected curves (blue lines), revealing the melting difference caused by injection. (A’) Difference curve allows easy visualization of melt deflections between *sox11a* gRNA+ nls-zCas9-nls injected and uninjected controls. (B) PCR products from a subset of *sox11b* gRNA+ nls-zCas9-nls injected embryos (mutli-color lines) are subjected to a high resolution melt curve and normalized to uninjected curves (blue lines), revealing the melting difference caused by injection. (B’) Difference curve allows easy visualization of melt deflections between *sox11b* gRNA+ nls-zCas9-nls injected and uninjected controls. (C) Sequences of *sox11a* mutations in embryos injected with *sox11a* gRNA+ nls-zCas9-nls. The wild-type reference CRISPR sequence with the target site and PAM highlighted in grey. The net change of each indel mutation is noted at the Right of each sequence (+, insertion; −, deletion). The number of times a mutant allele was identified is indicated as nX. (D) Sequences of *sox11b* mutations in embryos injected with *sox11b* gRNA+ nls-zCas9-nls. The wild-type reference CRISPR sequence with the target site and PAM highlighted in grey. The net change of each indel mutation is noted at the Right of each sequence (+, insertion; −, deletion). The number of times a mutant allele was identified is indicated as nX.
4.2.2 Sox11 gene disruption by CRISPR/Cas9-sgRNA complex in F0 zebrafish embryos

I injected Cas9 mRNA (400-1000 pg/embryo) with various sgRNAs (100 pg/embryo) into the yolk sac of zebrafish embryos at the 0-1 cell stage. Injection of up to 450 pg of Cas9 mRNA did not perturb normal development in >80% of the injected population. However, injection of less than 400 pg of Cas9 mRNA did not result in detectable mutations.

Successful RGEN mediated mutagenesis results in a mixture of wildtype and mutant alleles in F0 injected founders. PCR products generated from such F0 embryos will contain both wildtype and mutant sequences, resulting in heteroduplex DNA which will melt at a lower temperature than PCR amplicons from wildtype (homoduplex) controls (Figure 4.2A-B). This can be detected using high resolution melt curve analysis (HRMA). HRMA was performed on genomic DNA extracted from single control and CRISPR-injected embryos and was a successful tool in identifying potential mutants. High resolution melt curve analysis (HRMA) on PCR products encompassing the CRIPSR target site and Sanger sequencing analyses displayed distinct characteristics of RGEN-induced mutations in F0 embryos (Figure 4.2C-C’).

4.2.3 Cas9:sox11a/b-specific sgRNA injected F0 embryos display defective phenotypes

A subset of F0 zebrafish embryos coinjected with Cas9 mRNA: tyr sgRNA showed defects in melanin synthesis, demonstrating that some of their somatic tissues underwent bi-allelic conversion for the tyrosinase gene. These results indicate that Cas9 mRNA:sgRNA coinjection can exert sufficient gene-disrupting events to induce null mutations at endogenous loci at early stages of development in zebrafish embryos (Figure 4.3A-F).
A subset of Cas9:sox11a/b–sgRNA F0 embryos displayed both abnormal lens defects and coloboma at 24 hpf and 2 dpf, respectively (Figures 4.4A-B, 4.5), as well as a slight reduction in the number of mature rod photoreceptors at 5 dpf compared to uninjected wild type controls (Figures 4.4C, 4.5). The abnormal ocular phenotypes displayed by F0 embryos were identical to the ocular phenotypes of sox11 morphants (Pillai-Kastoori et al., 2014a), although not surprisingly the frequency of abnormal phenotypes in sox11 morphants is much higher (>70%). Nevertheless, the abnormal lens, coloboma, and rod photoreceptor phenotypes suggest functional loss of sox11a/b in mosaic F0 Cas9 mRNA:sox11a/b sgRNA embryos.
Figure 4.3. Biallelic disruption of tyrosinase (tyr) by Cas9 generates mosaic pigmentation phenotypes. tyr gRNA and nls-zCas9-nls RNA were injected into wild-type embryos and scored for phenotypes at 2 dpf. (A-D) Lateral views of wildtype (A,C) and tyr gRNA + nls-zCas9-nls RNA injected embryos at 2dpf (B, B’, D). (B-B’) tyr gRNA + nls-zCas9-nls RNA injected embryos displayed varying degree of hypopigmentation throughout the body. (C-D) Mosaic pigmentation was also observed in the eye of the embryos injected with tyr gRNA + nls-zCas9-nls RNA. (E) PCR products from a subset of tyr gRNA + nls-zCas9-nls injected embryos (multi-color lines) are subjected to a high resolution melt curve and normalized to uninjected curves (blue lines), revealing the melting difference caused by injection.(F) Difference curve allows easy visualization of melt deflections between tyr gRNA + nls-zCas9-nls injected and uninjected controls.
Figure 4.4. Efficient disruption of sox11a and sox11b by Cas9 results in ocular abnormalities. sox11 gRNA, tyr gRNA and nls-zCas9-nls RNA were injected into wild-type embryos and scored for phenotypes at 24hpf, 2 dpf and 5 dpf. (A) Lateral views of wildtype and sox11 gRNA + nls-zCas9-nls RNA injected embryos at 24 hpf. Compared to wildtype, sox11g RNA_nls-zCas9-nls injected embryos display abnormal shape of the lens, eye structure (red arrowhead) and thick RPE phenotype. (B) Lateral views of wildtype and sox11 gRNA + nls-zCas9-nls RNA injected embryos at 24 dpf. Compared to wildtype, sox11gRNA_nls-zCas9-nls injected embryos display variable degree of coloboma. (C) Representative transverse retinal sections from XOPS-GFP zebrafish uninjected and injected with sox11 gRNA, tyr gRNA and nls-zCas9-nls RNA at 5 dpf (from the set of individuals analyzed in (A-B)). Number of mature rod photoreceptors in tyr gRNA + nls-zCas9-nls RNA injected embryos were comparable to wildtype retina, however, embryos injected with sox11a/b gRNA + nls-zCas9-nls RNA showed reduced number of rods at 5 dpf. Red arrow head in C (right panel) indicates improper lamination od INL and ONL in the central retina. Three biological replicates were performed for all experiments. D, dorsal; V, ventral; A, anterior; P, posterior; L, lens; hpf, hours post fertilization; dpf, days post fertilization; MO, morpholino; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.
4.2.4 CRISPR/Cas9 system induced sox11a/b gene modifications are heritable

To test whether the sox11a/b gene modifications induced by the CRISPR/Cas9 system were heritable, I outcrossed the adult F0 animals derived from sox11a-C1+C2 gRNA injected-embryos with wild type animals to generate F1 progeny. Using HRMA analysis and BamHI-mediated restriction fragment length polymorphism (RFLP) analysis on samples from F1 progeny, I identified 2 out of 3 potential founders for a mutation in the sox11a-C2 target site. Sequencing of the sox11a-C1+C2 target site amplicon confirmed the presence of either a single base pair deletion or a 42 base pair insertion at the sox11a-C2 site but not in the sox11a-C1 site for 2 out of 4 F1 embryos tested (Figure 4.7A, A’, C). Unfortunately, I was also unable to detect embryos with mutations in both C1 and C2 sites of sox11a. Furthermore, no embryo with complete deletion/drop out of the region between C1 and C2-sox11a region was discovered. However, further screening of larger sample size is currently ongoing.

I identified 1 founder carrying a mutation in the sox11b-C1 target site. Sequencing of the sox11b-C1 target site confirmed the presence of either a single base pair deletion or a 35 base pair insertion in 2 out of 3 F1 embryos tested (Figure 4.7 B, B’, D). The single base pair deletion result in a frame shift mutation without the presence of a premature stop codon.
Figure 4.5. Embryos injected with sox11a/b gRNA + nls-zCas9-nls display abnormal ocular morphogenesis and coloboma. Embryos with injected with a mixture of single gRNA or double gRNAs along with Cas9 mRNA. Coinjection of 100 pg of sox11a and sox11b gRNA along with 450 pg nls-zCas9-nls per embryos results in abnormal lens and coloboma in zebrafish. Coinjection of 42 pg/embryo sox11a (C1+C2) + 168 pg/embryo nls-zCas9-nls. At 24 hpf 30% of embryos injected with single or double gRNA targeting sox11a and display abnormal shape of the lens, eye structure (red arrowhead) and thick RPE phenotype and about 40-50% display coloboma at 2 dpf. At 24 hpf 25-60% of embryos injected with single or double gRNA targeting sox11b and display abnormal shape of the lens, eye structure (red arrowhead) and thick RPE phenotype and 30-60% display coloboma. Three biological replicates were performed for all experiments.
Figure 4.6. Embryos injected with sox11a/b gRNA + nls-zCas9-nls lack mature rod photoreceptors. Quantification of the number of rod photoreceptors/section from transverse retinal sections from XOPS-GFP zebrafish injected with tyr gRNA, sox11a gRNA and sox11b gRNAs + nls-zCas9-nls. Number of embryos analyzed: WT, n=7; tyr gRNA + nls-zCas9-nls, n=9; sox11a gRNA (C1) + nls-zCas9-nls, n=5; sox11a gRNA (C2) + nls-zCas9-nls, n=4; sox11b gRNA (C1) + nls-zCas9-nls, n=5; sox11b gRNA (C2) + nls-zCas9-nls, n=5. Three biological replicates were performed for all experiments.
Figure 4.7. Test of mutagenesis in F1 embryos via HRMA analysis and Sanger sequencing.

(A) PCR products from a subset of embryos *sox11a* gRNA+ nls-zCas9-nls derived from adult founder animals (multi-color lines) are subjected to a high resolution melt curve and normalized to wildtype curves (blue lines), revealing the melting difference caused by injection. (B) Difference curve allows easy visualization of melt deflections between *sox11a* gRNA+ nls-zCas9-nls derived from adult founder animals wildtyped controls. (A’) PCR products from a subset of *sox11b* gRNA+ nls-zCas9-nls derived from adult founder animals s (multi-color lines) are subjected to a high resolution melt curve and normalized to wildtype curves (blue lines), revealing the melting difference caused by injection. (B’) Difference curve allows easy visualization of melt deflections between *sox11b* gRNA+ nls-zCas9-nls derived from adult founder animals and wildtype controls. (C) Sequences of *sox11a* mutations in embryos derived from adult founder animals that were injected with *sox11a* gRNA+ nls-zCas9-nls. The wild-type reference CRISPR sequence with the target site and PAM highlighted in grey. The net change of each indel mutation is noted at the Right of each sequence (+, insertion; –, deletion). (D) Sequences of *sox11b* mutations in embryos derived from adult founder animals that were injected with *sox11b* gRNA+ nls-zCas9-nls. The wild-type reference CRISPR sequence with the target site and PAM highlighted in grey. The net change of each indel mutation is noted at the Right of each sequence (+, insertion; –, deletion).
| ACM576  | HRMA_sox11a_CRISPR#1_Forward   | TCGCAGAGAGCAGACGTACA | 165 bps |
| ACM577  | HRMA_sox11a_CRISPR#1_Reverse  | GCACCAGTCTGGGTTTATCG |
| ACM578  | HRMA_sox11a_CRISPR#2_Forward  | TCTAGGTCCTGCCACGTC   | 217 bps |
| ACM579  | HRMA_sox11a_CRISPR#2_Reverse  | GCTCAGGCGTGCAATAGTCT |
| ACM580  | HRMA_sox11b_CRISPR#1_Forward  | AACCGGACTGGTGCAAGA   | 140 bps |
| ACM581  | HRMA_sox11b_CRISPR#1_Reverse  | CCCAGCCTTTTGAGGAT    |
| ACM582  | HRMA_sox11b_CRISPR#2_Forward  | AGTGCAGCAACTCAAGC    | 205 bps |
| ACM583  | HRMA_sox11b_CRISPR#2_Reverse  | CGTCTCCTGTCGTCAGTA   |
4.3 Discussion
In the present study, we demonstrate successful application of CRISPR/Cas9 genome editing tool to introduce modifications at sox11a and sox11b loci in zebrafish. Preliminary findings of this work are (a) the Cas9-sox11a/b specific-sgRNA complex can generate site-specific disruptions (~50-66.6%) that includes a variety of indels/substitutions in the sox11a and sox11b loci in zebrafish; (b) we were able to reproduce Cas9/tyr-gRNA mediated bi-allelic conversion of the tyrosinase locus in our laboratory (Jao et al., 2013), suggesting that the reagents and the screening techniques are appropriate.

We simultaneously observed distinct abnormal ocular phenotypes in the Cas9-sox11a/b specific-sgRNA injected embryos (which were not observed in tyr-gRNA injected embryos) similar to the abnormal ocular phenotypes presented by sox11 morphants and Sox11 null mice (Pillai-Kastoori et al., 2014a; Wurm et al., 2008b), indicating that the CRISPR/Cas9 system is successful in faithfully generating mutations to modify sox11a/b loci. Importantly, by testing the F1 progeny derived from confirmed F0 founders, we confirm that mutations including large deletions are heritable and are present in the F1 progenies. Therefore, we expect it to be reasonable and feasible to intercross F1 heterozygous adults to generate homozygous F2 mutants. The F1 embryos are being raised to adulthood (currently ~1 month old) in our laboratory for immediate investigation of mutagenesis efficiency.

CRISPR/Cas9 system with its inherent simplicity has revolutionized reverse genetic toolbox in zebrafish. Zebrafish offers tremendous appeal in the field of developmental and regenerative biology; now with the addition of an amenable knockout technology we are in a better position to study cellular, molecular and genetic mechanisms of early ocular morphogenesis and retinal development.
Reduced level of Sox11 results in abnormal ocular morphogenesis in mice and zebrafish; and mutations in SOX11 contributes to ocular abnormalities in humans. Successful creation of sox11a/b knockout lines will offer a stable and long lasting environment to test the outcomes as a result of Sox11 gene inactivation on early ocular morphogenesis as well as in adult stage. In addition, Sox11 knockout lines can be used in a regeneration paradigm to test the role/requirement of Sox11 in the regeneration of retinal neurons.
4.4 Materials and Methods

4.4.1 Zebrafish
Zebrafish (*Danio rerio*) were reared, bred, raised, and maintained at 28°C under a 14 hour light and 10 hour dark cycle according to standard protocols (Kimmel et al., 1995; Westerfield, M., 1995). All animal and procedures were carried in accordance with the policies established at the University Of Kentucky Institutional Animal Care and Use Committee (IUCAC). The Tg (XIRho:EGFP)f11 transgenic line (hereafter referred to as XOPS:GFP) has been previously described (Fadool, 2003), and was generously provided by J.M. Fadool (Florida State University, Tallahassee, FL).

4.4.2 Guide Oligonucleotides
The target sites for *sox11a* and *sox11b* were selected to be within the coding region. Two unique sites were selected each flanking the HMG box domain. Single strand guide RNA (gRNA) oligonucleotides for each 20 base pair (bp) target site were designed using ZiFit (http://zifit.partners.org/ZiFiT/), selecting T7 promoter to generate a 100 ~bp amplicon. The oligos for each gRNA (100uM) were annealed (5 minutes at 95-100°C, then cooled at room temperature for 3-5 hours) and ligated to BsaI (NEB: R0535S) digested pDR247 vector (Addgene: 42250). Recombinant vectors containing the sgRNA insert were digested with DraI (NEB: R0129S) and the released insert was PCR amplified using DraI primer. The PCR product was purified (QIAquick PCR purification kit) and in vitro transcribed using Ambion-MEGAscript T7 Transcription Kit (Life Technologies#AM1334). gRNA targeting *tyrosinase* gene was used as a positive control for the study. pT7tyrgRNA (Addgene: 46761) plasmid was linearized using BamHI (NEB: R3136T) and in vitro transcribed using Ambion-MEGAscript T7 Transcription Kit (Life Technologies: AM1334).
4.4.3 Cas9 mRNA
pCS2-nCas9n (Addgene:47929) plasmid was linearized with NotI (NEB: R3189L) and capped mRNA was generated using Ambion mMESSAGE mMACHINE SP6 Transcription Kit (Life Technologies:AM1304).

4.4.4 Microinjections
The optimum dose was finalized after a dose curve analysis and following are the doses injected into one cell stage embryos: 100 pg/embryo of sox11a or sox11b sgRNA + 450 pg/embryo of Cas9 mRNA; 75 pg/embryo of tyr sgRNA + 250 pg/embryo of Cas9 mRNA.

4.4.5 High resolution melt curve analysis (HRMA)
Genomic DNA was isolated from individual 24 hpf embryos using 20ul Thermopol buffer (95°C for 10 minutes) and 10 mg/ml proteinase K (5ul; 55°C for 1 hour; 95°C for 10 minutes). HRMA was performed using LightCycler 480 High Resolution Melting Master (Roche) according to manufacturer’s instructions on a LightCycler 96 Real-Time PCR System (Roche). Primer sequences used for HRMA study are listed in Table 2.

4.4.6 Sanger Sequencing
Purified PCR products were cloned using pGEM®-T Easy Vector Systems (Promega: A1360). Samples were sequenced bi-directionally using Sanger sequencing and the results were analyzed using BLAST (http://blast.be-md.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

4.4.7 Restriction Fragment Length Polymorphism (RFLP)
Possible disruptions in sox11 C2 region were identified using BamHI mediated RFLP analysis. PCR products encompassing the whole sox11a coding region were digested with BamHI (NEB) to reveal polymorphic fragments.
4.5 Acknowledgements
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CHAPTER 5: SUMMARY AND DISCUSSION

Congenital colobomas are a major cause of pediatric visual dysfunction and blindness. Ocular coloboma can occur in isolation or is often observed in conjunction with other multisystem defects in the central nervous system and/or other systemic functions. Ocular coloboma results from developmental abnormalities during embryogenesis, however the aetiology of coloboma is still not well understood, and in addition to genetic inheritance, environmental factors may also be causal.

Our current knowledge of the molecular and cellular process governing optic fissure closure is poorly understood, however, this is rapidly changing with a great deal of genetic information being generated from pedigree analysis and corresponding investigation in animal models. An impressive range of animal models (dog, chicken, rat, mouse, and zebrafish) have been reported to have ocular phenotypes due to disruption of genes that are orthologous to human coloboma-causing genes. Therefore, it is beneficial to utilize both human and animal model data in our effort to dissect the molecular mechanisms underlying choroid fissure closure and development of coloboma.

Earliest developmental processes are guided by a complex network of transcription factors, diffusible signaling molecules, and cell cycle regulators. Together these signals guide ocular morphogenesis, cell migration, cellular proliferation, and cell death, which ultimately specifies cellular identities that occupy different ocular compartments. As discussed in Chapters 1 and 2, till date about 27 genes have been associated with colobomatous syndromes and few transcription factors; have been shown by mutational screening to cause coloboma. In addition, these transcription factors also play critical roles during development of the CNS, including the eye. Only a small percentage of coloboma cases can be explained by the mutations in the above
mentioned genes. In order to understand the pathogenesis underlying inherited ocular coloboma, we first need to understand the developmental aspect of choroid fissure closure and ocular morphogenesis.

The goal of this dissertation was to determine the requirement of Sox11 in the developing vertebrate eye and retinal neurogenesis. This was accomplished within the framework of three major hypotheses. The first hypothesis tested was that Sox11 is required for proper ocular development and rod photoreceptor development, which is supported by the data presented in Chapters 2 and 3. Reduced levels of either sox11a and or sox11b resulted in ocular abnormalities like abnormal lens shape, coloboma, and reduced numbers of mature rod photoreceptors. In chapter 2, we also showed a novel finding that Sox11 negatively regulates Sonic Hedgehog signaling (Shh) to guide proper ocular morphogenesis. Sox11-deficient embryos have an abundance of Sonic Hedgehog ligand (Shha) and suppression of both Shha and Shh pathway rescues all the abnormal ocular phenotypes. The second underlying hypothesis tested was that mutations in SOX11 are associated with human ocular abnormalities like microphthalmia, anophthalmia, and coloboma. The data presented in Chapter 2 demonstrates that variation(s) within the SOX11 coding region and altered SOX11 gene dosage contributes to human ocular colobomas. The third major hypothesis tested was that Sox11 is required during a critical window during ocular morphogenesis and it is this early signal that guides both ocular morphogenesis and retinal neurogenesis. We attempted to test the hypothesis using a photo morpholino approach to control temporal gene expression of sox11a/b. In Chapter 3, the data presented partially supports the hypothesis that early Sox11 signal is essential for choroid fissure closure. However, the data also implicates the drawback of the photo morpholino tool itself in being inadequate to test the hypothesis in its entirety. Finally, in Chapter 4, I present a technical report detailing the protocol to develop stable germline sox11a/b mutants using CRISPR/Cas9 genome editing tool. Data in Chapter 4 shows that transient sox11a/b gRNA+ Cas9 mRNA injected embryos display abnormal
eye phenotypes that are similar to sox11 morphants. Furthermore, mutations identified in F0 adults and F1 embryos encourage us to keep the breeding program going in order to confirm the presence of germline mutation in the F1-F3 generations. Presented in this final chapter is a discussion of the data presented in the previous chapters in support of the role of Sox11 during vertebrate ocular development, the consequences of the findings and the future directions this work takes us. I postulate that Sox11 is required for proper ocular morphogenesis in part by limiting midline derived Sonic Hedgehog signaling and that this early Sox11>>Shh relationship controls the stage for downstream retinal neurogenesis as well. I also postulate that variations in SOX11 gene sequence or alterations in SOX11 gene dosage contribute to human ocular abnormalities.

Data presented in this dissertation support the important role of sox11a/b during ocular morphogenesis and retinal neurogenesis in zebrafish. Sox11a/b transcripts are maternally inherited by the zebrafish oocytes, and as gastrulation proceeds the expression is restricted to the epiblast. During early embryonic development, sox11 is expressed in the anterior neural plate and in the neuroectoderm overlying the axial mesoderm. As discussed in Chapters 1 and 2, expression patterns of sox11a/b can be either overlapping or distinct depending on the time and or stage of the embryonic development. Sox11a/b are expressed in the developing CNS, in the forebrain, midbrain and in the hindbrain. Interestingly, sox11a/b is not expressed in the mese-metecephalic border, where pax2.1 is present. Therefore, expression of sox11a/b is interspersed with that of ligands of Hedgehog signal (Shh) as well as targets of Hh signal (Pax2.1). Sox11 is expressed within the retina and the lens early on and as the retina matures the expression of sox11a/b is restricted to the CMZ, while being absent from the central differentiated retina.
Figure 5.1. Sox11 is required for proper ocular morphogenesis in vertebrates. Sox11 controls the proper formation of the lens, closure of the choroid fissure (yellow arrows) as well as terminal differentiation of rod photoreceptors (red columns) in zebrafish by maintaining proper levels of midline derived Hedgehog (Hh) signaling. Zebrafish sox11 morphants display expansion of pax2 expression domain during ocular morphogenesis suggesting that there is abnormal patterning of the optic vesicle in sox11 morphants. Abnormal phenotypes displayed by sox11 morphants such as abnormal lens shape, open choroid fissure, reduced number of mature rod photoreceptors can be rescued by treatment with Hedgehog antagonist called cyclopamine.
By reducing the amount of Sox11a/b protein made within the developing zebrafish embryo, we expected a negative effect on ocular morphogenesis and retinal development. Indeed, we observed smaller eye size, lenticular defects, and open choroid fissure in sox11 morphants which was incompletely penetrant (~70% of the injected embryos displayed abnormal ocular defects). This was very similar to what has been reported for Sox11 null mice, where only 82% of Sox11 null mice displayed coloboma. Additionally, systematic mutation screening of SOX11 coding region in 79 patients belonging to the MAC cluster resulted in the identification of two patients carrying heterozygous mutations. We already know that coloboma is a genetically heterogeneous disorder and as discussed in Chapter 1, it is not an exception, rather a rule that the phenotype of coloboma is incompletely penetrant in the affected population (Onwochei et al., 2000).

We must consider that patients may have genotype-phenotype discrepancy; which only points to the possibility that they may be carrying a specific set of mutation burden and this gets even more complicated with the presence of modifying alleles. We identified the presence of one of the variations (12nt. duplication) in ~1% of the control patient population and the mother of the proband carrying this variation does not display coloboma. How do we explain the disparity between genotype-phenotype which is quite common for developmental defects? Affected control individuals can carry an extra rare “carrier” variant in recessive coloboma causing genes which perhaps in the presence of a unique genetic or environmental trigger can contribute to the presentation of complex ocular disease. Since we only performed single candidate gene sequencing approach by sequencing the coding region of SOX11, we do not have any information on the status of the patient’s full genome profile. One important question is how many MAC patients reveal monogenic/oligogenic inheritance pattern in MAC genes or related coloboma genes? In this respect we should adopt a high-throughput sequencing project on MAC and/or coloboma defects. Exon capture combined with high-throughput sequencing will provide
identification of unknown coloboma causing genes and may even be used in the future for
diagnostic purposes.

In Chapter 2, we also present data supporting the role of Sox11 in rod photoreceptor
development. Sox11 morphants display reduced numbers of mature rod photoreceptors. Sox11
morphants retina have rod precursors as marked by the presence of crx and Nr2e3, however, the
retinas showed marked reduction in rhodopsin which marks mature differentiated rods. These
data suggest that although rod precursors are formed in sox11 morphants they do not undergo
terminal differentiation in a timely manner. Sox11 null mice display microphthalmia and
coloboma; however, they die immediately after birth due to septation defects. Therefore, it is
difficult to determine the effect of Sox11 deletion on murine rod photoreceptor differentiation,
much of which occurs postnatally. However, expression of the rod photoreceptor genes as
discussed in Chapter 2 is significantly reduced in embryonic retinas from Sox11−/− mice (Usui et
al., 2013b), additionally, rod photoreceptor defect displayed by the mother of the proband
carrying the 12 nt. duplication suggests that SOX11 may contribute to rod dysfunction in
mammals.

One of the novel findings of my dissertation is the negative regulation of Shh signaling pathway
by Sox11 during early eye development. Our results suggest that Sox11 acts upstream of Hh
signaling specifically by negatively regulating transcription of shha ligand. As shown in Chapter
2, the expression domains of sox11a, sox11b, and shha do overlap in the ventral midline,
Interestingly, in sox11 morphants we observed ectopic expression of shha within the
telencephalon, which is where sox11a/b are normally present. Although the exact mechanism of
this ectopic expression is not known, it is possible that sox11 prevents the expression of shh in the
telencephalon. Finally, the magnitude of the increase in shha expression in the absence of sox11
(over 180-fold) suggests that loss of repression of shha is accompanied by a positive feedback
loop with respect to *shha* transcription. This idea is supported by Neumann and Vollhord’s work (Neumann and Nuesslein-Volhard, 2000), where they showed that Shh is required for its own expression and can activate its own expression, this feature in the background of *sox11* deficiency, perhaps sends *shha* transcription into an overdrive.

What then does this mean in terms of ocular development and retinal neurogenesis? Does reduced level of *sox11a/b*, cause increase in Shh signaling pathway, which then causes coloboma and rod defects? Treatment of *sox11*-deficient embryos with Shh pathway inhibitor (cyclopamine) recues all the abnormal ocular phenotypes as well as rod photoreceptor defects. A converse experiment of synthetically stimulating Shh pathway in *sox11* morphant background increases the proportion of embryos with abnormal ocular phenotypes. Furthermore, co-inhibition of *sox11a/b* and *shha* significantly reduces the proportion of embryos with abnormal ocular phenotypes. All these findings collectively, support the idea that it is indeed the over activation of Shh pathway due to reduced levels of Sox11 that causes lenticular defects, induces coloboma and reduces the number of mature rod photoreceptors. Moreover, all this is mediated by the upregulation of *shha* transcription.

It is important to point out that, inhibition of Shh pathway in the above mentioned experiments was done at a very early stage of ocular morphogenesis (5.5-13 hpf). Yet, how do we see a remarkable rescue on rod photoreceptor defect at 3 dpf? As discussed in Chapter 2, interestingly, we observed a significant increase in *shha* expression at 8-12 hpf, when the optic vesicle is evaginating from the midline, and we confirmed that Hh signaling was increased at this time using a *ptc2:GFP* reporter line and transcript analysis via QPCR. It has been postulated that there exists a neurogenic timer early during eye development that sets the stage for future retinal neurogenesis. Indeed, Kay et al. showed that the timely progression of *ath5* expression in the retina, which coincides with the activation of neurogenesis, depends on axial Shh (Kay et al.,
Atonal-homologue 5 (ath5) is a proneural gene expressed as retinoblasts exit the cell cycle and start the process of differentiation. Shh derived from the ventral-midline region regulates the timing of ath5 expression in the zebrafish retina. In mice, ath5-positive cells contribute significantly to the rod photoreceptor lineage (Brzezinski et al., 2012). Therefore, it is plausible that elevated Shh coming from the midline in sox11 morphants delays rod photoreceptor differentiation by influencing the cell-intrinsic neurogenic program of retinal progenitor cells.

Previous studies have demonstrated a link between the Hh pathway and ocular defects (coloboma and rod photoreceptor development), both in vitro and in vivo (Dakubo et al., 2008; Levine et al., 1997; Osakada et al., 2008; Stenkamp et al., 2000). Work by Shkumakava et al and Stenkamp et al., have shown that Shh signal is required for both optic stalk formation and retinal neurogenesis. Furthermore, it has been shown that reduction in midline Shh signal negatively impacts rhodopsin expression in the retina, suggesting that Hh signaling promotes rod photoreceptor differentiation (Shkumatava et al., 2004; Stenkamp et al., 2000). On the contrary, it was shown that conditional loss of Shh results in accelerated differentiation of rods and cones in mice (Wang et al., 2005). On the other hand, an unexpected finding by Yu et al., (Yu et al., 2006) showed that constitutively active Shh pathway can non-cell autonomously delay expression of rhodopsin in rod precursors in mice, much like what we see in sox11 morphants. The seemingly puzzling reaction to altered Shh levels can be potentially explained by the requirement for optimal Shh dosage, and sensitivity of rod photoreceptor development to such alterations.
Figure 5.2. Sox11 maintains optimal levels of midline derived Hedgehog signaling during vertebrate ocular morphogenesis. Deviation from the optimal levels of midline derived Hedgehog signaling results in a variety of ocular abnormalities. Increased levels of Hh signaling causes optic cup, choroid fissure, lens, and neurogenesis defects in mice, zebrafish, and Xenopus. Reduced levels of Hh signaling causes defects such as cyclopia or hypotelorism (reduced distance between the eye globes). Sox11 maintains proper levels of Hh signaling primarily by negatively regulating the transcription of sonic Hedgehog a (shha) by acting as an activator of an upstream repressor of shha called Bmp7b.
Till date several roles of Shh pathway have been discovered and it is reported to be used reiteratively through embryogenesis. We report here a novel regulation of Shh pathway via Sox11. We show that increasing amounts of Sox11 reduced midline derived Shh signal, resulting in cyclopic phenotypes in zebrafish embryos. SoxC factors are generally considered to behave as transcriptional activators rather than repressors (Bergsland et al., 2006; Chew and Gallo, 2009); therefore, we tested the hypothesis that Sox11 indirectly inhibits expression of shha. Using a candidate gene approach, we tested several known repressors of Shh transcription and found Bmp7b to be the most attractive candidate. Previous reports from (Bastida et al., 2009; Liem et al., 1995; Morcillo et al., 2006) have shown that Bmp7 is known to repress SHH and is required before SHH for choroid fissure formation in mice.

It is most certainly possible that Bmp7b is only one of the regulators of Shha pathway downstream of sox11 and there are several that are still unidentified. miR-210 is a microRNA best known for regulating cellular responses to hypoxia and is reported to repress Shh transcription in vitro. miR-210 was found to be upregulated 45-fold in sox11 morphants at 24 hpf. It was surprising to observe elevated miR-210 levels in sox11-deficient embryos, which have elevated shh transcript levels. While this is data is in conflict with our prediction, it is certainly interesting given the discoveries of microRNA as negative modulators of eye development. Although its role in vertebrate eye development has not been previously investigated, it cannot be ruled out that mir-210 has Shh pathway independent roles in eye development and contributes to abnormal phenotypes observed in sox11 morphants. We do know that miR-210 overexpression can activate Notch signaling pathway and that increased Notch signaling expands the expression domain of Sox11. It is known that both Shh and Notch pathways are critical for regulating stem cell niche and cortical layer formation. They both act in tandem during the development of the dorsal forebrain. Recent work (Dave et al., 2011) has shown that Notch and Shh signaling cooperate to maintain and expand the pool of neocortical stem cells.
Figure 5.3. Midline derived Hedgehog signaling is critical for proper retinal neurogenesis. Midline derived sonic Hedgehog (Shh) signaling is crucial for proper initiation of retinal neurogenesis. Midline derived Shh along with intra-retinal Shh then progressively controls retinal neurogenesis by controlling the expression of fibroblast growth factor’s (Fgf) 8, 19 to initiate retinal ganglion cell differentiation.
One future goal will be to elucidate whether the upregulation of miR-210 expression in sox11-deficient embryos results in elevated Notch signaling and whether Notch and Shh intersect to guide choroid fissure closure and retinal neurogenesis.

Since development is all about the right signals at the right time and the right place, I wanted to investigate when exactly during eye development is Sox11 required and is of consequence. My approach was to perform a temporal control of sox11a/b gene expression during eye development. Due to the absence of sox11a/b mutant, I addressed the question using photo morpholinos, which have been shown to work robustly in zebrafish (Tallafuss et al., 2012). It is clear from the data gathered that sox11 is required at the time of neurulation (4-10 hpf) for proper eye development and choroid fissure closure in zebrafish. However, there are a couple of drawbacks to the tool itself that complicate our interpretation of the data. First, I used GFP reporter as proxy for Sox11 protein, the GFP signal itself was visible only after 6-7 hours post UV inactivation of the photo MO (Gene “ON”). This did not help in narrowing of the critical window for Sox11 function. Secondly, the presence of the Photo moiety itself appeared to be toxic to retinal neurogenesis as injection with a sense control MO with Photo moiety also generated a severely retarded retina lacking any signs of lamination and differentiated neurons. Nevertheless, the data suggest that sox11 gene function is critical between 4-10 hpf for proper eye development and embryos with sox11 gene “ON” have reduced chances of having abnormal eye defects and coloboma. Second future goal will be to address this question at hand with a better approach/tool like (a) creating inducible transgenic sox11 zebrafish line where sox11 is under the control of a inducible promoter like hsp70 in an attempt to control the timing of sox11 inactivation; and (b) once the critical window is narrowed, we can perform a control experiment where exogenous sox11 wildtype mRNA is provided to the embryo for possible rescue of the abnormal ocular phenotypes.
The field of developmental biology in zebrafish is moving away from investigations where morpholino are the sole tool used for reverse genetics (Kok et al., 2015). The era of customizable nucleases to perform genome editing is here for zebrafish and currently the best practice is to verify morpholino data with a comparable mutant. We realize the potential of knockout lines and hence have started the project to create sox11a/b mutants using CRISPR/Cas9 genome editing tool. The preliminary data gathered from the F0 and F1 stages are encouraging and I plan to continue breeding the animals and identify sox11a and sox11b germ line mutants. The use of the said mutants will not only help us understanding eye development but also help in investigation the role of sox11a/b during regeneration of the retina under injury conditions. The third future goal will be to confirm the sox11 MO data and study the role of sox11 during ocular morphogenesis and retinal neurogenesis in sox11 mutants.

I have postulated thus far that elevated midline derived Shh signaling due to deficiency of Sox11 disrupts ocular morphogenesis and reduces the number of mature rod photoreceptors. Disruption of Shh paracrine activity in sox11 morphants can alter the responsiveness of progenitors to growth factors (such as CNTF and LIF) which can further aggravate the developmental stages. The manifestation of abnormal ocular phenotypes correlates with mutations or deletions of SOX11 loci and that identified mutations render SOX11 protein weak its transactivation capabilities and suppression of SHH pathway. It is certainly possible that modifier alleles, environmental factors, and/or the genotype of the other SoxC family members further confound the observations. Sox11 is required during neurulation (4-10 hpf) to guide proper ocular morphogenesis, however, whether this early signal guides retinal neurogenesis as well as is yet to be determined. We know that Shh is required for retinal neurogenesis, and the signal emanating from the ventral retina initiates neurogenesis and later Shh from the retinal ganglion cells propagates Shh wave sweeping the entire developing retina. We also know from the elegant work done by Uwe Strähle’s group (Vinothkumar et al., 2008), that without Fgf3, 8 and Shh itself, this
Shh wave cannot be initiated at the ventral retina and without Fgf19 and Shh the wave does not propagate within the retina; therefore Fgf’s essentially acts as a gateway to retinal neurogenesis. This complex relationship between Shh and Fgf and their interdependence, is tempting and leads us to speculate that sox11 morphants may experience changes in several more pathways.

SOX11 and SHH appear together in yet another abnormal developmental situation; both SOX11 and SHH are associated with several types of cancer, including pediatric brain tumors such as medulloblastoma (de Bont et al., 2008; Northcott et al., 2012). Intriguingly, SOX11 is strongly expressed in classical medulloblastoma, but only weakly expressed in desmoplastic medulloblastoma (Lee et al., 2002). Recent technological advances have led to the division of medulloblastoma into different subgroups based on molecular phenotype (Northcott et al., 2012). Medulloblastomas with desmoplastic histology are almost exclusively restricted to the SHH-driven subgroup, which accounts for approximately 30% of medulloblastoma cases (Kool et al., 2012). This suggests a potential negative relationship between SOX11 and SHH signaling in medulloblastoma, similar to what we have demonstrated during ocular morphogenesis. Given that Shh antagonists currently used to treat medulloblastoma often fail due to acquisition of drug resistance (Northcott et al., 2012), it would be interesting to determine whether forced expression of SOX11 in the SHH medulloblastoma subgroup could augment current therapies to inhibit tumor progression.

In this dissertation, I have analyzed the molecular roles of Sox11 during vertebrate ocular morphogenesis and retinal neurogenesis, reaching three main conclusions:

1. Sox11a/b are expressed in the telencephalon, and in the dorsal hinge formed by the diencephalon and the evaginated optic stalk/optic vesicle. Expression of sox11 at neurulation is
important for proper eye development. Reduction in Sox11 results in lenticular defects, coloboma, and reduced number of mature rod photoreceptors.

(2) Sox11 negatively regulates midline derived Shh signaling pathway, and loss of this negative regulation results in abnormal ocular phenotypes in zebrafish embryos. Sox11 appears to regulate Shh via Sox11>bmp7b>Shh signaling.

(3) Altered SOX11 gene dosage or mutations in SOX11 correlates with human ocular abnormalities and retinal dysfunction.

A major question remains: What are the direct molecular targets of Sox11 during different stages of eye and retinal development, and how is this targeting accomplished? Future directions will attempt to elucidate what exact molecular mechanisms underlie the ocular abnormalities in the absence of proper Sox11 gene function, as well as spatio/temporal ocular tissue specific requirement of Sox11, and how this can be casual for the manifestation of ocular deformities.
BIBLIOGRAPHY


Barth, K.A., Wilson, S.W., 1995. Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. Development 121, 1755-1768.


410.1097/1001.icu.0000243020.0000282380.f0000243026.


Kamachi, Y., Uchikawa, M., Collignon, J., Lovell-Badge, R., Kondoh, H., 1998. Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction. Development 125, 2521-2532.


Lefebvre, V., Dumitriu, B., Penzo-Méndez, A., Han, Y., Pallavi, B., 2007. Control of Cell Fate and Differentiation by Sry-related High-mobility-group Box (Sox) Transcription Factors. The international journal of biochemistry & cell biology 39, 2195-2214.


Lefebvre, V., Li, P., de Crombrugghe, B., 1998. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene.


Liu, W., Lagutin, O.V., Mende, M., Streit, A., Oliver, G., 2006. Six3 activation of Pax6 expression is essential for mammalian lens induction and specification.


VITA

Lakshmi Pillai-Kastoori

Born: Mumbai, India

Education

2005    BSc. Biotechnology, Ramnarain Ruia College, University of Mumbai
2007    MSc. Life Sciences, Ramnarain Ruia College, University of Mumbai
2009    Began graduate school MCDB Program, Dept. of Biology, University of Kentucky (UKY)

Scholastic Honors

2012-2015   The Lyman T. Johnson Graduate Student Fellowship (Competitive Renewal), UKY
2014        Gertrude Flora Ribble Graduate Student Fellowship, UKY
2013        Treasurer of the Biology Graduate Student Association
2011         The Company of Biologists, International Travel Fellowship, Cambridge, UK

Publications


**Paper Presentations**