SOX2 IS CRITICAL FOR THE MAINTENANCE OF QUIESCENCE AND HOMEOSTASIS IN NASCENT MÜLLER GLIAL CELLS.

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Genetics and Molecular Biology in the School of Medicine.

Chapel Hill
2014

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**ABSTRACT**

Tessa Crowl: SOX2 is critical for the maintenance of quiescence and homeostasis in nascent Müller glial cells.  
(Under the direction of Larysa Pevny and Ellen Weiss)

SOX2 is a HMG-box transcription factor that defines neural stem cell populations from the earliest stages of embryogenesis to adulthood. Previous studies have shown that SOX2 is critical for specifying neural competence in retinal progenitor cells in early mouse optic cup development, but its function in postnatal retinal progenitor cells is largely unexplored. Moreover, SOX2 expression is maintained in the adult retina in a population of Müller glia, which retain progenitor characteristics and have the capacity to generate neurons following injury, although our understanding of the role of SOX2 in Müller glia of the developing retina is limited. In this study, via conditional and specific ablation, we address the functions of SOX2 in both postnatal retinal progenitors and in Müller glia in the developing retina. Our hypothesis is that SOX2 is essential for cell cycle dynamics, progenitor cell identity, and homeostasis in both postnatal retinal progenitor cells and in differentiated Müller glia. We find that loss of SOX2 in postnatal progenitors results in the degradation of Müller glia morphology and the entrance of Müller glia into the cell cycle. Additionally, we find that hypomorphic levels of SOX2 during early retinal development induced Müller glia proliferation in late development. Lastly, we report that Müller glia proliferation is enhanced during their gliotic stress response.

In neural stem/progenitor cells, there are networks of basic helix-loop-helix (bHLH) transcription factors that regulate proliferation and differentiation. In this study, we address whether the misexpression of proneural bHLH factors, specifically Neurogenin-2 (NGN2), affects cell fate choices in postnatal retinal progenitors. Our hypothesis is that bHLH factor misexpression will influence retinal
progenitors to undergo neurogenesis at the expense of gliogenesis. We find that NGN2-misexpressing cells preferentially become amacrine neurons and rod photoreceptors and avoid the Müller glial cell fate. This result indicates that NGN2 is capable of influencing retinal progenitors to generate neurons at the expense of glial cells and encourages the production of amacrine cells over other retinal neurons.
ACKNOWLEDGEMENTS

I would like to thank the following individuals for their support, both academically and personally, that made this project a success:

Larysa Pevny, PhD
Ellen Weiss, PhD

Natalia Surzenko, PhD
Lee Langer, PhD

Amelia Bachleda; Scott Hutton, PhD; Whitney Heavner, PhD; Danielle Matsushima;
Rex Williams, PhD; Janet Berrios, Matt Fagan

Eva Anton, PhD; Terete Borras, PhD; Steve Crews, PhD; Bob Duronio, PhD; Jeremy Kay, PhD

Jared Chrispell, PhD; Shoji Osawa, PhD; Yubin Xiong

Allison Deal, MS; Anna Snavely, MS; Bill Snider, MD; Raluca Dumitru, MD, PhD;
Vladimir Ghukasyan, PhD; Manzoor Bhat, PhD; Cynthia Andoniadou, PhD; Franck Polleux, PhD; Randal
Hand, PhD; Robert Peterson, PhD; Megumi Aita, PhD
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<tbody>
<tr>
<td>B6/CD1</td>
<td>mixed mouse strain (C57BL6/J and CD-1)</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BK</td>
<td>big conductance</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRALBP</td>
<td>cellular retinaldehyde binding protein</td>
</tr>
<tr>
<td>Crb1</td>
<td>Crumbs homologue-1</td>
</tr>
<tr>
<td>DIV</td>
<td>days <em>in vitro</em></td>
</tr>
<tr>
<td>E8.5</td>
<td>embryonic day 8.5</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GCL</td>
<td>ganglion cell layer</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GS</td>
<td>glutamine synthetase</td>
</tr>
<tr>
<td>HC</td>
<td>horizontal cell</td>
</tr>
<tr>
<td>HMG</td>
<td>high mobility group</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IPL</td>
<td>inner plexiform layer</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced pluripotent stem cell</td>
</tr>
<tr>
<td>ISH</td>
<td><em>in situ</em> hybridization</td>
</tr>
<tr>
<td>NBL</td>
<td>neuroblast layer</td>
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<tr>
<td>NF</td>
<td>Neurofilament</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NGN1</td>
<td>Neurogenin-1</td>
</tr>
<tr>
<td>NGN2</td>
<td>Neurogenin-2</td>
</tr>
<tr>
<td>NICD</td>
<td>NOTCH1 intracellular domain</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NPCs</td>
<td>neural progenitor cells</td>
</tr>
<tr>
<td>NSCs</td>
<td>neural stem cells</td>
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<tr>
<td>OLM</td>
<td>outer limiting membrane</td>
</tr>
<tr>
<td>ONL</td>
<td>outer nuclear layer</td>
</tr>
<tr>
<td>OPL</td>
<td>outer plexiform layer</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>P12</td>
<td>postnatal day 12</td>
</tr>
<tr>
<td>PH3</td>
<td>phosphohistone H3</td>
</tr>
<tr>
<td>RPCs</td>
<td>retinal progenitor cells</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigmented epithelium</td>
</tr>
<tr>
<td>RGC</td>
<td>retinal ganglion cell</td>
</tr>
<tr>
<td>SC</td>
<td>supporting cells (of the cochlea)</td>
</tr>
<tr>
<td>SGZ</td>
<td>subgranular zone</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SOX2</td>
<td><em>Sry-related box 2</em></td>
</tr>
<tr>
<td>SRY</td>
<td><em>Sex-determining region Y</em></td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>TASK</td>
<td>tandem pore</td>
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Chapter 1: Introduction

During embryonic development, the nervous system is derived from the neuroepithelial layer of the neural plate, the sides of which eventually fold inwardly to form the neural tube. The neural tube gives rise to a diverse array of neural structures, such as the brain and the eye, as a result of a complex coordination of intracellular and extracellular factors that control patterning and specification events. At a cellular level, neural stem cells (NSCs) divide in order to self-renew and are capable of giving rise to both neurons and glia (Turner and Cepko, 1987). The proper formation of mature neural structures requires that NSCs proliferate and differentiate at regulated time points. This requires a balance of transcription factors that either promote or inhibit neurogenesis, and in neural tissues, is partially regulated by a network of basic helix-loop-helix (bHLH) factors (Hatakeyama and Kageyama, 2004). Another transcription factor that controls the ability of NSCs to retain their proliferative capacity throughout embryogenesis is the HMG-box transcription factor SOX2 (Sry-related box 2) (Pevny and Nicolis, 2010). SOX2 is required for the neural potential of NSCs from the earliest stages of pre-implantation to the final cell division, as well as throughout neural differentiation (Avilion et al., 2003; Cavallaro et al., 2008; Favaro et al., 2009). Humans with Sox2 mutations have developmental malformations of the eye and brain (Fantes et al., 2003; Kelberman et al., 2006). In addition to regulating NSCs during embryogenesis, SOX2 is required for the maintenance of NSCs and neurogenesis in the adult mammalian brain (Ferri et al., 2004; Favaro et al., 2009). Moreover, SOX2 continues to be expressed in a population of cells in the retina that retain the capacity for neurogenesis—the Müller glia (Taranova et al., 2006a; Karl and Reh, 2010; Gallina et al., 2013). In this study, we investigate the consequences following the loss of SOX2 in late retinal progenitor cells or specifically in Müller glia, and
analyze this factor’s effects on cell cycle dynamics. Furthermore, we determine whether late retinal progenitor cells are competent to change their fate to give rise to a different set of neurons and glia when they are influenced by ectopic expression of proneural bHLH factors.

To introduce the topics discussed in this study, this chapter will address the following subjects: (1) the anatomy, function, and development of the eye, (2) Müller glia development, basic functions, involvement during disease as it transitions to a state of gliosis, and their ability to function as an adult stem cell population, (3) introduction to SOX2 and its molecular dynamics, the role of SOX2 during early development, neural specification, maintenance of NSCs, differentiation, retinal development, and in Müller glia.

1.1 Eye Structure and Development

1.1.1 Eye Anatomy and Function

The eye is arguably the most fascinating organ in the body as it consists of a complex machinery of neurons, muscles, and connective tissue that coordinate the process of capturing light, translating it into a chemical signal, and transferring it to the brain for interpretation. The retina is a sensory tissue that absorbs the light and is made up of one primary glial cell type and six neuronal cell types, which together comprise 55 subtypes (See Fig. 1.1A for a description of eye anatomy) (Masland, 2001b). As light enters the eye, it is focused on the retina by the lens. The light travels through the thickness of the retina to reach the rod or cone photoreceptor outer segments that absorb the light and hyperpolarize, resulting in a reduction in glutamate release relative to dark conditions (Fig. 1.1B) (Masland, 2001a). In bright light conditions, cone photoreceptors absorb certain wavelengths of light depending on the single type of opsin they express. Mice have two cone photoreceptor cell types that express opsins that either absorb light of a short wavelength (blue, S-opsin) or a medium wavelength (green, M-opsin); humans, meanwhile, have a third type of cone that absorbs at a long wavelength (red, L-opsin). These cones
synapse onto one or a few bipolar interneurons in the outer plexiform layer (OPL) (Fig. 1.1B). Different subtypes of bipolar cells have specific responses to glutamate signals from the cones and may be depolarized (ON bipolar) or hyperpolarized (OFF bipolar) as a result. The activated bipolar cells then relay to one of the three specific synaptic layers within the inner plexiform layer (IPL), where they contact with a specific retinal ganglion cell (RGC) (Masland, 2001a). The axons of the RGCs make up the optic nerve, which transmits the information to the brain for processing. An alternate visual pathway is active in dim light, where rod photoreceptors have a predominant role. Rods express a rhodopsin pigment that absorbs light and causes the rod cell to hyperpolarize. The synapses of several rods converge onto a single type of bipolar cell, the rod bipolar, giving them increased sensitivity compared to cones. The rod bipolar cells then synapse onto AII amacrine cells before transmitting the output to RGCs. Horizontal and amacrine cells are interneurons that regulate the intermediate processing of information. The horizontal cells provide inhibitory feedback to the photoreceptors, and amacrine cells provide feedback to the stimulating bipolar cells, and also synapse with each other and RGCs (Masland, 2012). Müller glial cells maintain retinal architecture as well as provide trophic support to neurons. All of these retinal cell types form the complex networks that are required for phototransduction, and by deciphering their roles in this network, we can gain insight into the process that gives us vision.

1.1.2 Eye Formation and Retinal Development

Eye development begins during gastrulation as a specification of the eye field, which is located in the central portion of the developing forebrain (Adelmann, 1929). During neurulation, two lateral optic pits are formed from the bulging lateral walls of the diencephalon at mouse embryonic day 8.5 (E8.5) (Graw, 2010). By E9.0, the optic pits enlarge to form the optic vesicles that lie near the surface ectoderm. The optic vesicle is connected to the primitive forebrain lumen by the optic stalk, which will give rise to the glia that support the optic nerve (Kuwabara, 1975). The overlying surface ectoderm thickens, forming the lens placode. The close proximity of the lens placode with the optic vesicle induces
the neuroectoderm to invaginate to form the optic cup at E10.0 (Spemann, 1924). The inner layer of the optic cup will become the retina, while the outer layer will become the retinal pigmented epithelium (RPE). The lens placode invaginates in a similar manner to form the lens, while the remaining surface ectoderm becomes the cornea (Graw, 2010).

Retinal differentiation begins in the central optic cup around E11 near the optic nerve head, spreading towards the periphery in a wave-like fashion (Prada et al., 1991; Hu and Easter, 1999). The differentiation of cells occurs in an order that is highly conserved among most vertebrate species, and the generation of cell types occurs in overlapping phases (Livesey and Cepko, 2001b). Retinal differentiation occurs primarily in two major waves: during embryogenesis, the RGCs are generated first, followed by the horizontal cells, cone photoreceptors, and amacrine cells. The second wave occurs during postnatal stages, and includes rod photoreceptors, bipolar cells, and finally, Müller glia (Young, 1985a; Cepko et al., 1996; Hatakeyama and Kageyama, 2004). Within the developing retina, progenitor cell proliferation occurs throughout the neuroblast layer, and following terminal cell divisions, postmitotic cells migrate to their positions in the neuronal layers and differentiate. Retroviral tracing has revealed that retinal progenitor cells are capable of giving rise to multiple cell types following the final cell division, indicating the presence of a common multipotent progenitor, rather than a progenitor cell that undergoes various intermediate stages prior to differentiation (Turner and Cepko, 1987). Although all retinal cell types arise from a common progenitor, progenitor cells are competent to form various cell types at specific time points during development, and this competency state is partly controlled by external secreted factors, such as Wnt, Sonic hedgehog (Shh), and fibroblast growth factor (FGF) (Esteve and Bovolenta, 2006; Wallace, 2008). Additionally, internal regulators of progenitor competency include a network of transcription factors, specifically the bHLH family, that promote progenitor cell proliferation (Hes1/5) or neurogenesis (Neurogenin1/2, NeuroD1/2, Math3/5) (Reh and Levine, 1998; Cepko, 1999). Therefore, an extensive network of transcription factors and secreted factors are required
to control the proper timing of differentiation and specific cell type generation to develop a fully functional, mature retina.

1.2 Müller Glia

1.2.1 Müller glia Development

Müller glia are the principal glial cell in the retina and function both to maintain retinal homeostasis and provide trophic support to neurons. Müller glia are one of seven retinal cell types produced from a single multipotent retinal progenitor cell, and fate tracing studies in the postnatal rat show that retinal progenitor cells undergo a terminal cell division to give rise to a single Müller glia and a rod photoreceptor cell (Turner and Cepko, 1987). Thus, Müller glia lack a “glial precursor” intermediate stage, and instead are generated directly from a progenitor capable of producing neurons and glial cells. Müller glia are produced in the second wave of retinogenesis, and are the final cell type to be generated (Young, 1985a).

The generation of Müller glia at a specific developmental time point and appropriate cell numbers depends on a coordinated balance of transcription factors and signaling pathways. One of the most critical determinants for Müller glia production is the Notch signaling pathway. Misexpression of the downstream effectors, the negative basic helix-loop-helix (bHLH) factors Hes1, Hes5, and Hey2, in the P0 retina induces glial production at the expense of neurons (Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001). Retinas in Hes1-null mice can no longer produce Müller glia, yet can still generate all of the retinal neurons (Tomita et al., 1996). Overexpression of Notch through the constitutively active form NICD (Notch intracellular domain) promotes an increase in cells with Müller glial features (Bao and Cepko, 1997; Furukawa et al., 2000; Scheer et al., 2001). The Notch pathway is required for the maintenance and proliferation of retinal progenitor cells (Jadhav et al., 2006a), including at developmental time points when Müller glia are not produced (i.e., embryonic stages).
Therefore, Notch is not the master regulator of glial cell genesis, but rather may act to preserve a population of cells to become Müller glia by preventing neurogenesis throughout development. In summary, the production of Müller glia requires the coordination of many transcription factors and signaling pathways, especially the components of the Notch signaling pathway.

Other factors that have been shown to be determinants of Müller glial cell fate are the sex determining box (SRY)-related SOX factors. Retroviral overexpression of SOX2 in E17 mouse retinal explants induces Müller glia and amacrine cell production, possibly through the promotion of cell cycle exit (Lin et al., 2009). In addition, SOX9, whose expression pattern overlaps with SOX2 in proliferating progenitor cells in the retina as well as in mature Müller glia, is required for Müller glial cell production (Poche et al., 2008). This group demonstrated that ablation of SOX9 in progenitors results in the absence of Müller cells in the adult retina, despite the presence of relatively normal neuronal cell numbers.

In another study, Lillien (1995) characterized the expression of the epidermal growth factor receptor (EGFR) as increasing during the late stages of retinal development, when Müller glial cell production is prominent. The viral misexpression of EGF receptor increased glial cell production in late retinal progenitor cells but was unable to affect the fate of early progenitors, which do not normally produce glia (Lillien, 1995; Lillien and Wancio, 1998). This result suggests that rather than directly specifying a glial fate, EGFR helps to distinguish the competency states of late retinal progenitors from early retinal progenitors in order to promote the differentiation of Müller glia at the appropriate time.

In addition to the role of SOX factors and EGFR in the production of Müller glial cells, cell cycle regulatory factors also influence the Müller glial cell fate. The CDK inhibitor p27^{kip1} is responsible for proper cell cycle exit in cycling progenitors, and its expression is maintained in mature, quiescent Müller glial cells (Zhang et al., 1998). The expression of the amphibian homolog p27^{Xic1} gradually increases throughout the retinogenesis period, with its highest expression levels observed during the final stages of cell production during gliogenesis (Ohnuma et al., 1999). Overexpression of p27^{Xic1} in the *Xenopus*
retinal progenitors causes cell cycle arrest and favors Müller cell production, while blocking its expression causes a decrease in Müller cells. Thus, a cell cycle factor that controls the timing of cell cycle exit plays a role in Müller glia fate determination. Interestingly, overexpression of p27Kip1 in the mouse retina is insufficient to induce Müller glia production, suggesting a requirement for other factors during glial fate specification in mammals (Dyer and Cepko, 2001b). However, since p27Kip1 expression is maintained in mature Müller glia in mammals, it may play a role in the maintenance of their quiescence in the adult.

Following the generation of Müller glia during late retinogenesis, their maturation process continues for the first few postnatal weeks (Bringmann et al., 2006). Their bipolar structure extends throughout the retinal thickness, and their endfeet form the inner and outer limiting membrane. This morphology provides a scaffold for migration of nascent postmitotic neurons, as shown by in vitro studies (Willbold et al., 1997). The structure of Müller glia allows neurons to grow neurites and establish mature synaptic wiring during the first postnatal week. Early Müller glia retain the flexibility to undergo mitosis readily and lack many features of mature glial cells, being characterized by a depolarized resting membrane potential and a lack of inward currents. However, after maturation, Müller cells exhibit an increase in inward K+ currents and hyperpolarize to adult levels of -80 mV (Bringmann et al., 1999a). This K+ current is due to the gradual increase in the number of inward rectifying Kir4.1 K+ channels in the membrane during the second postnatal week, at which point Müller glia provide neuronal support but have a greatly reduced proliferative capacity (Bringmann et al., 2000; Kofuji et al., 2002). In addition, Müller glia begin to function in neurotransmitter recycling by expressing the glutamate transporter (GLAST) and glutamine synthetase (GS), the enzyme that converts glutamate to glutamine (Germer et al., 1997). These changes in Müller glia morphological and electrophysiological properties distinguish their transition from glial-like progenitor cells to mature glial cells with neuronal support functions.
1.2.2 Müller Glia Function

Müller glial cells have an important function in maintaining retinal homeostasis, both structurally and physiologically. Müller glia span the thickness of the retina; their soma reside in the inner nuclear layer (INL), and their endfeet at the termini of their processes make up the inner and outer limiting membranes that maintain the structural integrity of the retina. As evidence of their requirement for normal retinal architecture, selective ablation of Müller cells results in dysplasia, neuronal apoptosis, and eventual retinal degeneration (Dubois-Dauphin et al., 2000). In addition to their main apicobasal processes, Müller glia have numerous smaller side processes that ensheath neighboring neurons, including axons and synapses (Reichenbach et al., 1993). Müller glia also act as molecule exchange mediators between neurons and the external environment through contact with blood vessels and the vitreous. A primary function of Müller glia is to maintain retinal K⁺ homeostasis by absorbing excess K⁺ released by active neurons. This process is aided by their expression of specialized channels, such as Kir4.1 and Kir2.1, tandem-pore (TASK), and Ca²⁺-dependent big conductance (BK) channels (Bringmann et al., 1997; Kofuji et al., 2002; Skatchkov et al., 2006). Additionally, Müller glia provide neurons with lactate and pyruvate for glucose oxidative metabolism, (Poitry-Yamate et al., 1995), take up metabolic waste (Tsacopoulos and Magistretti, 1996), develop and maintain the blood-retinal barrier (Tout et al., 1993), recycle neurotransmitters and create precursors of neurotransmitters for neurons (Matsui et al., 1999), maintain water and ion homeostasis (Newman and Reichenbach, 1996), and control the excitability of neurons through release of neuroactive substances including ATP, glutamate, and D-serine (Newman and Zahs, 1998; Stevens et al., 2003; Newman, 2004). Müller glia also play a role in visual metabolism through their expression of cellular retinaldehyde binding protein (CRALBP), which recycles photo pigments by converting 11-trans-retinol to 11-cis-retinol (Das et al., 1992). Interestingly, in addition to these physiological functions, it has been suggested that the cylindrical shape and parallel orientation of Müller glia allows for enhanced transmittance of light through the retinal tissue to the
photoreceptor outer segment layer (Franze et al., 2007). These extensive and specific roles for Müller glia are evidence for their necessity to the proper functioning and homeostasis of the retinal tissue.

1.2.3 Gliosis and Disease

In addition to maintaining retinal homeostasis and aiding in proper visual function, Müller glia mediate the retinal stress response—gliosis. During gliosis, glial cells react to stressful stimuli (such as retinal damage or degeneration) in an attempt to minimize tissue damage and preserve neuronal function. However, some downstream effects of gliosis perpetuate damage and contribute to neuronal cell death (Bringmann et al., 2009). Gliosis is induced following exposure to most stressful stimuli, but the characteristics of these responses can be nonspecific or specific to the type of stimulus. Nonspecific gliotic responses include cellular hypertrophy, proliferation, and upregulation of intermediate filaments. A hallmark of gliosis is the upregulation of the intermediate filament glial fibrillary acidic protein (GFAP) (Bignami and Dahl, 1979). Other gliotic responses are specific to the type and severity of insult, such as the downregulation of GS following the loss of glutamate-releasing neurons during photoreceptor degeneration, presumably because of a decreased need for converting glutamate to glutamine (Grosche et al., 1995). Alternatively, increased GS expression is observed in gliosis during hepatic retinopathy in order to detoxify the increased concentrations of ammonia (Reichenbach et al., 1995). In addition to different specificities in gliotic responses, significant variability is observed among the population of Müller glia with respect to the degree of gliosis they exhibit. Fischer and Reh (2003) demonstrated that when the chick retina is exposed to N-methyl-D-aspartate (NMDA) to induce excitotoxic cell damage, there is a variable response in Müller glia; 65% of the population lacked GFAP and proliferated, while the remaining 35% of the cells expressed GFAP and remained quiescent. In addition, the manifestation of the gliotic response is determined by the specific disease or injury. Acute insults (e.g. retinal detachment) tend to induce Müller cell proliferation, while slow photoreceptor degeneration usually induces gliosis but is generally not associated with Müller glia proliferation (Bringmann et al., 2009).
Gliosis can be classified into two types: conservative gliosis and massive gliosis (Bringmann et al., 2000). Conservative gliosis involves a more mild response and can be beneficial to neurons, but it can progress to a more severe, harmful form of massive gliosis. Conservative gliosis is characterized by an upregulation of GFAP, cellular hypertrophy, and an absence of proliferation (Bringmann et al., 2009). In this condition, Müller glia display little to no decrease in potassium currents and only a slight membrane depolarization. There is also a decreased expression of glial genes that are active in a physiologically normal state, such as GS and CRALBP (Lewis et al., 1994; Lieth et al., 1998; Joly et al., 2008). The human retinopathies that are associated with conservative gliosis include retinal detachment, diabetic retinopathy, and retinal degeneration (Bringmann et al., 2006). In mouse models, the experimental retinopathies typically associated with conservative gliosis include Borna disease virus-induced retinitis, rds mice which exhibit photoreceptor dystrophy, optic tract damage, and white light-induced retinal degeneration (Felmy et al., 2001; Pannicke et al., 2001; Iandiev et al., 2006). On the other hand, massive gliosis is characterized by proliferation, glial scar formation, and complete loss of gene expression of most normal physiological support genes (Bringmann et al., 2009). Massive gliosis is associated with human proliferative vitreoretinopathies (Bringmann et al., 2006). The potassium conductance of Müller glia drops down to 5% of normal, leading to a significant membrane depolarization (Bringmann et al., 1999b) Glial scars consist of Müller glia that proliferate to form cell masses and then transdifferentiate into contractile myofibrocytes (Guidry, 2005). The transition from conservative to massive gliosis is typically triggered by a breakdown in the blood-retinal barrier, an event that is accompanied by an influx of immune and growth factors. Both conservative and massive gliosis are influenced by the condition and severity of the stress stimuli, and the course of gliosis determines whether the retinal tissue is likely to recover or degenerate.

The critical role of Müller glia in the maintenance of homeostasis and during states of gliosis indicate that their function or dysfunction determines the state of the retinal tissue. As such, it is
important that Müller glia are able to withstand and respond to stressful stimuli to preserve homeostasis in the retinal environment. To maintain this balance, Müller glia have the following characteristics: energy reserves of glycogen, high antioxidant content, regenerative potential, and receptors for prosurvival neurotropic factors (Bringmann et al., 2009). However, conditions that result in the dysfunction or loss of Müller glia have drastic effects on the retinal tissue and oftentimes lead to blindness. For example, mutations in the human Crumbs homologue-1 (Crb1) leads to blindness in patients with retinitis pigmentosa, a retinal degeneration characterized by a loss of photoreceptors. Crb1 is a transmembrane protein that regulates the number and size of the Müller glia apical microvilli (Richard et al., 2006) and is located near the adherens junctions, which form the outer limiting membrane that borders the photoreceptors. Loss of Crb1 leads to a disruption of the adherens junctions and microvilli, leading to degeneration of photoreceptors and the underlying retinal pigmented epithelium (van de Pavert et al., 2007). Therefore perturbation of Müller glial structure or function can have devastating effects on the function of the retina.

In addition to upregulating GFAP during gliosis, Müller glia also increase the expression of the intermediate filaments Vimentin and Nestin in their processes (Bignami and Dahl, 1979; Bringmann and Reichenbach, 2001; Lewis and Fisher, 2003). GFAP upregulation is typically the first response to injury and occurs very quickly after a damaging stimulus (Kaur et al., 2007); for example, GFAP is upregulated 1-3 hours after ischemia-hypoxia and within 2 hours following increased intraocular pressure in glaucoma (Kim et al., 1998). One of the primary functions of intermediate filaments during gliosis is the prevention of further structural damage by stabilizing the Müller glia, preventing the loss of the inner and outer limiting membranes in conditions such as retinal detachment (Lundkvist et al., 2004; Verardo et al., 2008). Intermediate filaments can also function as a pathway to transfer information from the cell periphery to the nucleus in order to facilitate repair following injury. For example, Vimentin transports phosphorylated ERK1/2 to the perinucleus along mitochondrial tracks, an effect that is essential to
efficient neurite growth and regeneration (Perlson et al., 2004). In addition, since Nestin is a neural progenitor marker, its upregulation during gliosis is thought to be an indicator of the transition of Müller glia to progenitor cells (Fischer and Omar, 2005). The upregulation of intermediate filaments is essential to the progression of gliosis. Mice lacking GFAP and Vimentin have fewer structural changes and glial scarring after injury than do wild type mice (Pekny et al., 1999). During retinal detachment, glial scarring is reduced without GFAP and Vimentin, and as a result, there is aberrant neurite sprouting (Verardo et al., 2008).

Gliosis often leads to the formation of glial scars, which in most cases are barriers to regeneration. The purpose of glial scar is to create a physical barrier to block further damage by pathogenic factors in the surrounding tissue. A glial scar forms when Müller glial processes grow into spaces in the neural retina left behind following neuronal degeneration or injury. During photoreceptor degeneration or aging, Müller glial processes grow into the subretinal space below the outer nuclear layer (Fan et al., 1996). During retinal reattachment, which occurs following detachment, Müller glial processes grow into the vitreous to form epiretinal membranes, resulting in the eye condition proliferative retinopathy (Fisher and Lewis, 2003). In addition, Müller glial processes grow into spaces such as the degenerating RPE layer, blood vessel lumens in diabetic retinopathy, or the vitreous in age-related macular degeneration (Bek, 1997; Wu et al., 2003).

The formation of glial scars contributes to the limited capacity for retinal regeneration in warm blooded vertebrates. Inhibitory extracellular matrix and cell adhesion molecules are expressed on Müller glia membranes and are chemical inhibitors of axonal growth and new neurons (Ponta et al., 2003). The structural matrix components chondroitin sulfate proteoglycans are normally present during development and help to create boundaries that guide ganglion cell axons; however, these components prevent regeneration when expressed by Müller glia during gliosis (Silver, 1994). The focus of many ophthalmic studies has been to increase retinal regeneration by eliminating glial scars and preventing
Müller glia hypertrophy through the degradation of extracellular matrix with matrix metalloproteinases or chondroitinases (Silver, 1994; Zhang et al., 2007). In addition, in stem cell transplantation studies in the eye, the addition of chondroitinases facilitates exogenous cell integration into host tissue (Bull et al., 2008). These studies demonstrate that the downstream effect of glial scars can present significant barriers to regeneration.

1.2.4 Müller glia as adult stem cells

In response to injury or disease, Müller glia maintain the capacity to dedifferentiate, undergo cell division, and give rise to neurons in order to regenerate damaged tissue (Karl and Reh, 2010). The ability to revert to a proliferating progenitor cell and give rise to neurons varies greatly among vertebrates, with a high efficiency in lower vertebrates, such as fish and reptiles, but very limited capacity in higher vertebrates, such as birds and mammals.

Müller glia in all vertebrates resemble neural progenitor cells with respect to their morphology, cell cycle migration patterns, and transcriptional profile. With respect to their morphology, Müller glia maintain the bipolar form of retinal progenitors, with processes that span the apicobasal axis of the retina. Additionally, when retinal progenitors or Müller glia undergo cell divisions, they follow a distinct pattern of interkinetic nuclear migration that corresponds with various stages of the cell cycle (Baye and Link, 2007). During S phase, the soma migrates towards the basal (vitreal) side of the retina, proceeding towards the apical (scleral) side during G1 phase, and then undergoing M phase at the apical surface. During G2, the soma travels back to the neuroblast layer (progenitors) or INL (Müller glia) in the basal direction. With regards to gene expression, Müller glia and retinal progenitors have a significant overlap in their transcriptional profile (Jensen and Wallace, 1997; Blackshaw et al., 2004; Roesch et al., 2008), including several components of the Notch signaling pathway (Notch1 and Hes1/5), inhibitors of the Wnt pathway (Dkk3), and members of the Sonic Hedgehog pathway (Shh, Ptc, Smo, Gli). In addition, Müller glia maintain expression of the multiple neural progenitor genes: Sox2, Pax6, Chx10, Sox9, Lhx2, Rx and...
Myc (Gallina et al., 2013). However, despite a strong resemblance to retinal progenitors, Müller glia are quiescent and rarely proliferate in the adult mammalian retina, even in response to injury (Bringmann et al., 2009). Regardless, these numerous similarities with retinal progenitors are indicative of the regenerative potential of Müller glia, and further studies into Müller glial cell cycle dynamics and neurogenic potential will facilitate the determination of their potential therapeutic value.

### 1.2.4.1 Müller glia progenitor cell potential: Lower vertebrates

Retinal regenerative capabilities in lower vertebrates, such as fish and amphibians, are particularly robust. Remarkably, the urodelian amphibians (i.e., salamanders) are capable of regenerating their retina following its complete surgical removal (Araki, 2007). Rather than Müller glia, the source of the new retinal tissue is from RPE that has transdifferentiated into neurons. Very little is known about the ability of Müller glia to serve as a source of stem cells in amphibians, and they are thought to have limited regenerative abilities compared to the robust regenerative capacity of the RPE (Reh, 1987). Teleost fish, on the other hand, are incapable of regenerating a retina from their RPE cells but have a remarkable capacity for retinal regeneration from their population of Müller glial cells (Raymond and Hitchcock, 2000; Bernardos et al., 2007). In a non-injured homeostatic retina, the Müller glia of the fish slowly proliferate to generate exclusively rod photoreceptors. Upon injury to the retina, the Müller glia can act as multipotent progenitor cells to give rise to all retinal neurons and regenerate the retinal tissue. Since the discovery of the progenitor capabilities of Müller glia, the zebrafish has served as a model of retinal regeneration; therefore, the molecular pathways regulating this process have been extensively studied in the last decade (Lenkowski and Raymond, 2014). One gene that is critical for zebrafish Müller glia dedifferentiation and retinal regeneration is the proneural transcription factor Ascl1a, which is the homolog of the mammalian Mash1 that specifies progenitors to differentiate into neurons (Ramachandran et al., 2010). Ascl1a is upregulated in Müller glia following injury in order to promote their dedifferentiation and conversion to progenitors through the positive regulation of
Lin28, a known inhibitor of *let-7* miRNAs. Ramachandran et al. (2010) also showed that the expression of certain pluripotency factors, namely, *klf4, oct4*, and *c-myc*, were maintained in the uninjured retina and were increased in the injured retina. Conversely, the pluripotency factors *sox2, lin28, nanog*, and *c-myc* were not expressed in the uninjured retina but were induced upon injury. The maintenance and induction of these factors following injury supports a model in which zebrafish Müller glia resemble an endogenous population of induced pluripotent stem cells (iPSCs) that are capable of dedifferentiating, proliferating, and gaining a new cell identity. Other pathways that contribute to proliferation and neurogenic capacity of Müller glia in fish include those that are critical to retinal development: Notch, EGFR/MAPK, and Wnt/β-catenin (Wan et al., 2012). Despite the ability of fish to regenerate functional retinal tissue, the efficiency of this process is relatively low due to considerable cell death among the progeny of proliferating Müller glia, with only a 30% survival rate of these cells after 2 weeks (Fausett and Goldman, 2006). This high death rate is possibly due to the inability of the new cells to make proper connections in the pre-existing neural wiring. These studies on amphibian and fish retinal regeneration shed light on mechanisms that could potentially be utilized for human retinal regeneration.

1.2.4.2 Müller glia progenitor cell potential: Higher vertebrates

In contrast to amphibians and fish, birds, and especially mammals, have a more limited ability to regenerate neurons. In contrast to fish Müller glia, which continuously divide during homeostasis, the Müller glia in birds and mammals are quiescent but can be stimulated upon injury. Interestingly, the neurogenic capacity of Müller glia was first discovered in chicks, rather than in fish (Fischer and Reh, 2001). Following excitotoxic retinal injury induced by intraocular injections of NMDA, the resulting proliferating cells expressed GS, Chx10, Pax6, and CASH1 (chick homolog of mammalian Mash1), identifying them as Müller glia with enhanced neural progenitor cell characteristics. Fate tracing of the proliferating cells using BrdU confirmed that a very small subset became neurons, others generated Müller glia, but most remained undifferentiated progenitors. As opposed to fish Müller glia, while a
majority of chick Müller glia enter the cell cycle following injury (Fischer and Reh, 2003), they were only capable of undergoing a single round of cell division. One pathway shown to be involved in chick Müller glia proliferation is Notch (Hayes et al., 2007). Specifically, the Notch components Notch1, Dll1, and Hes5 are upregulated upon Müller glia proliferation. When Notch signaling is inhibited using DAPT prior to injury, the Müller glial cells are unable to proliferate. However, when Notch is inhibited after injury-induced Müller glia proliferation, there is an increase in the generation of new neurons, which is consistent with the requirement for Notch downregulation prior to neurogenesis (Nelson et al., 2006). These results indicate that Notch has a dual role during regeneration to both promote Müller glia dedifferentiation/proliferation and to prevent aberrant or premature neuronal differentiation.

In the mammalian retina, the most common response to injury is gliosis, a process that presents significant barriers to regeneration (see section 1.2.3). It was long believed that the mammalian retina was incapable of regeneration, until Ooto et al. (2004) showed that rat Müller glia are capable of dividing in response to injury by NMDA injection. All of the proliferating cells in the INL identified as Müller glia due to GS expression, yet these cells made up a very rare minority among the total population of Müller glia. After 2 weeks, 70% of the original proliferating cells (tracked by BrdU) retained GS, but a small minority became rod photoreceptors and bipolar cells, indicating that mammalian Müller glia were not only capable of proliferation but also have neurogenic abilities. Retinal injury induced by a number of excitatory amino acids other than NMDA, such as kainic acid and domoic acid, were able to coerce Müller glia into the cell cycle (Sahel et al., 1991). Various other stimuli were able to activate Müller glia proliferation, such as activation of the Shh pathway (Wan et al., 2007), neurotoxic injury through ERK activation (Karl et al., 2008), treatment with the l-glutamate analogue dl-α-amino adipic acid (Takeda et al., 2008), and through the Notch and Wnt pathways (Del Debbio et al., 2010).

Following retinal injury, mammalian Müller glia increase the expression of progenitor-associated genes, such as Pax6 (Osakada et al., 2007), Notch components (Karl et al., 2008), and Nestin (Close et al.,
2006). However, mammalian Müller glia do not regain a complete progenitor cell identity, as they lack expression of essential proneural genes following injury, such as Mash1 (the mammalian correlate of Ascl1) and Neurogenin-2 (Ngn2), which presumably limits their ability to generate neurons. In order to directly ascertain their neurogenic ability, Das et al. (2006) isolated Müller glia from P10-P21 rat retinas; 0.5-1% of the cells were able to proliferate and generate Sox2-expressing neurospheres upon exposure to fibroblast growth factor-2 (FGF2). Under differentiation protocols, these cells were capable of generating functional neurons and losing their progenitor and glial character. This study also showed that when injury-activated Müller glia were injected into the retina, they were able to generate retinal neurons. Therefore, mammalian Müller glia do not have a strong inclination to generate neurons, yet they have neurogenic capacity under specific conditions.

Even though Müller glia can be induced to proliferate in mammals, they make up a very small subset of the total Müller glia population. Close et al. (2006) found that they could increase the number of proliferating Müller glia by treating retinas with growth factors, such as EGF, following light damage. Additionally, when using explant culture as a stress model, the number of proliferating Müller glia can be increased by 20-fold following the addition of the Wnt3a ligand (Osakada et al., 2007). Furthermore, the number of proliferating Müller glia is age-dependent. Injection of EGF in a postnatal day 14 (P14) rat induces only a tenth of the number of proliferating cells as an EGF injection at P10, and that number decreases to nearly zero when the EGF injection is administered at P21 (Close et al., 2006). Presumably, the loss of responsiveness to EGF with age is correlated with the downregulation of the receptor EGFR on Müller glia during a similar time period (Close et al., 2006). These results indicate that the mammalian Müller glia have limited potential to proliferate, yet can be stimulated to proliferate more readily when induced by growth factors or activation of endogenous pathways.

Despite the similarity in gene expression profiles between lower vertebrates and mammals, the latter have a diminished ability for regeneration. Extrinsic and intrinsic factors contribute to the
nonpermissive nature of mammalian Müller glia cell cycle entry and neurogenic ability. For example, the retinal environment encourages proliferation during retinogenesis but is unfavorable in the adult retina. When mammalian Müller glia-derived neurospheres are cultured with E3 chick retinal cells, representing an early retinogenesis environment, the generation of the early-born retinal cell type, retinal ganglion cells, increased by ~5-fold compared to neurospheres cultured alone (Das et al., 2006). When these neurospheres are cultured with P1 rat retinas from the late retinogenesis period, the production of the two late-born cell types, rod photoreceptors and bipolar neurons, increased by ~4-fold and ~3.5-fold, respectively. Therefore, the retinal environment promotes or inhibits the generation of neurons in a temporal-dependent manner, and similar to retinal progenitors, Müller glia are susceptible to these signals. One intrinsic factor responsible for controlling cell cycle entry of Müller glia is the CDK inhibitor and tumor suppressor p27\(^{kip1}\), which is maintained at high expression levels in the adult retina (Dyer and Cepko, 2000). Following neurotoxic retinal injury, p27\(^{kip1}\) is downregulated in Müller glia, coincident with their cell cycle entry. It is likely that p27\(^{kip1}\) keeps Müller glia proliferation in check, a hypothesis that is strongly supported by the fact that p27\(^{kip1}\) is negatively regulated by Notch and Wnt pathways (Castelo-Branco et al., 2003; Murata et al., 2005; Sarmento et al., 2005), both of which are increased in neurotoxin-damaged retinas (Das et al., 2006). Although several extrinsic and intrinsic factors regulate Müller glia proliferation, further studies are necessary to understand the discrepancy in regenerative abilities between lower and higher vertebrates, as well as the full capacity of mammalian Müller glia as a source of neurons.
1.3 SOX2

1.3.1 SOX2 Molecular Dynamics

**1.3.1.1 SOX2 Discovery and Overview**

The transcription factor SOX2 is a decisive player in the regulation of numerous aspects of development from the earliest stages of embryogenesis to tissue homeostasis in adulthood. Sox2 was first identified in a gene screen for factors related to the *sex-determining gene Y* (*Sry*) that contain high mobility group (HMG)-box DNA binding domains (Gubbay et al., 1990). It was later independently discovered as a regulator of the *Fgf4* gene, which is critical for the survival of the early mouse embryo (Dailey et al., 1994; Yuan et al., 1995). One characteristic of SOX2 that drew the attention of the embryonic stem cell field was its conserved expression throughout the developing central nervous system (CNS) in vertebrates, including zebrafish, chick, mouse, and human (Collignon et al., 1996; Pevny and Placzek, 2005). Sox even has homologues in the developing CNS of *Drosophila*, namely, *Fish-hook* (Nambu and Nambu, 1996) and *Dichaete* (Russell et al., 1996). Since its early discovery and characterization, SOX2 has been implicated in the maintenance of stem cell identity, specification of the CNS, neuronal differentiation, and adult stem cell maintenance (Pevny and Nicolis, 2010). SOX2 is also involved in the development of many tissues, including the CNS (Kishi et al., 2000; Zappone et al., 2000), trachea (Que et al., 2009), taste bud sensory cells (Okubo et al., 2006), and inner ear sensory cells (Dabdoub et al., 2008), and the palate (Langer et al., 2014). Additionally, SOX2 expression has been observed in a number of brain tumors, and it has been shown that cancer stem cells have a requirement for SOX2, similar to NSCs (Lee et al., 2006; Nicolis, 2007; Schmitz et al., 2007; Gangemi et al., 2009). The findings that have given SOX2 the most attention in recent years is its ability, in cooperation with the transcription factors Oct3/4, c-Myc, and Klf4, to reprogram adult cells into induced pluripotent stem cells (iPSCs), which can be utilized for the generation of new tissue (Takahashi and Yamanaka, 2006).
These studies highlight the importance for SOX2 in numerous aspects of development and disease, and further understanding of SOX2 and its regulation of stem cell potential will hopefully lead to advances in human therapeutics.

1.3.1.2 SOX2 Structure

There are currently 20 different Sox genes that have been identified in mouse and human; Sox genes are defined as containing more than 50% amino acid similarity to the HMG domain of Sry (Schepers et al., 2002). The HMG domain is made up of 3 alpha helices that are organized in an L-shape as it binds to target DNA in the minor groove. As a result of binding, the DNA is bent to various degrees depending on the SOX protein and binding sequence (Remenyi et al., 2003). The HMG domain of all SOX factors bind to a similar DNA motif: $^A/\gamma^A/\gamma^C^A/\gamma^G$. Binding of the SOX HMG domain to the regulatory region of DNA does not activate gene transcription; rather, it is the interaction with a binding partner that allows transcriptional activity (Kamachi et al., 2000). Furthermore, in addition to the role of the HMG domain in DNA binding, it also serves as the primary interface for protein partner interactions (Wilson and Koopman, 2002; Kondoh and Kamachi, 2010). For example, SOX2 interacts with its partner OCT3/4 through its HMG domain (Yuan et al., 1995). Another role for the HMG domain of SOX is nuclear importation, due to the nuclear localization sequence in the amino and carboxy terminus of the SOX HMG domain (Sudbeck and Scherer, 1997). This sequence allows SOX proteins to enter the nucleus to regulate transcriptional activity. This HMG box is highly conserved among the SOX proteins and can maintain transcriptional activity even in chimeric proteins in which the native SOX or SRY HMG sequence is replaced with another HMG domain (Kamachi et al., 1999; Bergstrom et al., 2000).

1.3.1.3 SOX2 Binding Partners and Target Genes

Since SOX2 is expressed in multiple cell types and is involved in several functions throughout development, the regulation of specific target genes at certain times depends on its interaction with
different partner proteins (Kamachi et al., 2000). These partner proteins bind DNA at a neighboring site to the binding site of the HMG domain and create functionally active transcriptional complexes. The presence or absence of a specific partner protein with a SOX protein allows the transcriptional complex to be active only under certain circumstances, allowing for temporal and tissue specificity in its various regulatory roles. Additionally, the requirement for a partner protein limits the number of potential binding sites due to the requirement for sites for both SOX and the partner protein, which further contributes to specificity in target genes.

Among the partners for SOX2 are OCT3/4, BRN2, OTX2, and PAX6, and these cofactors interact with SOX2 at various developmental stages. SOX2 interacts with OCT3/4 in the pregastrulation mammalian embryo to regulate the gene expression of Fgf4 (involved in postimplantation development), the pluripotency factor UTF1, and osteopontin (involved with cell adhesion/migration in embryogenesis) (Yuan et al., 1995; Botquin et al., 1998; Nishimoto et al., 1999; Kamachi et al., 2000). In addition, the SOX2/OCT3/4 complex binds to the Hoxb1 enhancer, which is responsible for hindbrain development (Di Rocco et al., 2001). Another SOX2 binding partner is the class III POU factor BRN2, and this complex activates the neural stem cell gene Nestin in the embryonic mouse neural primordium of the spinal cord (Tanaka et al., 2004). With the binding partners OTX2 and PAX6, SOX2 is involved with early optic cup development. In Xenopus, SOX2 complexes with OTX2, a transcription factor essential for proper eye formation in humans (Ragge et al., 2005a), to activate the Rax gene, a transcription factor that is critical for specification of the eye field and optic vesicle formation (Mathers et al., 1997; Zhang et al., 2000; Danno et al., 2008). PAX6 is another eye-related transcription factor that interacts with SOX2. PAX6 is the “master regulator” of eye development, and homozygous loss of Pax6 in mice and humans results in anophthalmia (absence of an eye) (Hill et al., 1991; Glaser et al., 1994). The SOX2/PAX6 complex activates the expression of both its genes in vitro, binding at the N3 enhancer of SOX2 and the PAX6 head ectoderm-specific enhancer (Aota et al., 2003; Inoue et al., 2007). The SOX2/PAX6 pair is
present in the embryonic head ectoderm at the lens placode, acting to specify lens tissue (Kamachi et al., 2001; Smith et al., 2009). Additionally, the SOX2/PAX6 pair binds to the DC5 enhancer of the delta-crystallin gene to induce lens cell differentiation, and the presence of both transcription factors is required to bind the enhancer in vivo (Kamachi et al., 1995). In conclusion, the SOX2 partner code helps determine the specificity of binding sites in target genes of certain tissues at distinct developmental time periods.

1.3.2 Role of SOX2 during Embryogenesis and the Development of the Central Nervous System

1.3.2.1 SOX2 in Early Embryogenesis

SOX2 activity begins in the pre-implantation embryo, at the very earliest stages of life. The mammalian blastocyst is made up of the trophectoderm, which gives rise to the extraembryonic ectoderm and eventually the placenta, as well as the inner cellular mass, which gives rise to the epiblast that is responsible for forming the 3 germ layers of the embryo: ectoderm, mesoderm, and endoderm. SOX2 is expressed in the morula early in development and eventually is confined to the inner cellular mass of the blastocyst (Avilion et al., 2003). SOX2 expression is maintained throughout the epiblast, and by mid-primitive streak stages it is found in the anterior portion of the presumptive neuroectoderm. By embryonic day 9.5 (E9.5), SOX2 is found throughout the brain, neural tube, sensory placodes, branchial arches, and gut endoderm. Targeted disruption of SOX2 results in embryonic lethality due to a failure to form the epiblast (Avilion et al., 2003). However, in these pre-implantation embryos, maternal SOX2 expression persists in the zygote, and subsequent studies showed that when RNAi targeting removes both zygotic and maternal SOX2 mRNA, embryos become arrested at the morula stage and are unable to form the blastocyst (Keramari et al., 2010). These results point to an early requirement for SOX2 in mammalian development.
From the earliest stages of development to terminal differentiation, SOX2 is expressed in undifferentiated, proliferating stem/progenitor cells, and this pattern is highly conserved in fish, amphibians, birds, mammals, as well as flies (in the *Drosophila* SOX2 homolog, *Dichaete*) (Pevny and Placzek, 2005). Together with the transcription factors OCT4 and NANOG, SOX2 acts to maintain pluripotency of embryonic stem (ES) cells by regulating pathways in self-renewal and inhibiting differentiation programs (Boyer et al., 2005; Kim et al., 2008). This pluripotency is also highly dosage-dependent in ES cells. Small decreases in SOX2 promote the differentiation of ES cells into trophectodermal cells (Chew et al., 2005), whereas small increases in SOX2 can drive ES cells to differentiate into a range of differentiated cell types (Kopp et al., 2008). Thus, SOX2 is a critical component to self-renewal properties and maintenance of ES cells.

1.3.2.2 SOX2 and Neural Fate Specification

In addition to its roles in ES cells and early development, SOX2 is involved in the specification of neural fate during embryogenesis. Expression of the SOXB1 factors, SOX1-3, defines ectodermal cells that are competent to form neural primordium. These factors are later expressed in cells that are committed to a neural cell fate, and this pattern is conserved in many species, including chick, *Xenopus*, and *Drosophila* (Rex et al., 1997; Mizuseki et al., 1998; Cremazy et al., 2000). In *Xenopus* embryos, SOX2 defines the prospective neuroectoderm at the beginning of gastrulation, and its expression is upregulated following overexpression of the neural inducing signal Chordin, indicating that it is involved in neural fate specification (Mizuseki et al., 1998). Additionally, when SOX2 is overexpressed by mRNA injection into *Xenopus* embryos, neural differentiation of ectoderm is initiated when co-injected with FGF, but not from SOX2 alone. This result indicates that SOX2 and FGF together are responsible for making the ectoderm amenable to receiving extracellular signals for neural induction (Mizuseki et al., 1998). In mouse ES cells, forced expression of either SOX1 or SOX2 promotes the generation of neuroectodermal cells at the expense of mesoderm and endoderm (Zhao et al., 2004). Taken together,
these findings demonstrate that while SOX2 may not be the master regulator of neural induction, it acts in conjunction with other neural inducing signals to coordinate neural potential. Additionally, due to the overlapping expression of SOXB1 factors in neuroectoderm and evidence that these factors participate in neural determination, it is likely that there is a functional redundancy in neural fate specification (Pevny and Placzek, 2005).

1.3.2.3 SOX2 and Neural Stem Cell Identity

Consistent with its role in neural fate specification, SOX2 is critical to the maintenance of neural stem cell (NSC) identity. In Sox2EGFP knock-in mice, Sox2 expression (as shown by EGFP) is present throughout the anterior-posterior axis in the developing CNS from neural plate stages into adulthood (Ellis et al., 2004). Isolated SOX2-EGFP-expressing cells from the embryonic or adult CNS have multipotent potential and can form neurospheres in vitro that differentiate into neurons, astrocytes, and oligodendrocytes. In the chick embryonic spinal cord, Sox2 is expressed in proliferating NSCs until the final cell division and is downregulated upon neuronal differentiation (Graham et al., 2003). Constitutive expression of SOX2 by in ovo electroporation into neural progenitors of the chick spinal cord is sufficient to maintain progenitor characteristics and inhibit neuronal differentiation. Inhibition of SOX2 signaling in these cells results in cell cycle exit and delamination from the ventricular zone, similar to the migration of neural progenitors following terminal cell division (Altman and Bayer, 1984). Additionally, cells in which SOX2 is inhibited lose neural progenitor markers and gain the expression of differentiated neurons. In a similar study, overexpression of SOXB1 factors (SOX1-3) in chick spinal cord prevented the generation of neurons, and the authors found that the ability of proneural bHLH factors to induce neuronal differentiation depended on their repression of SOX1-3 (Bylund et al., 2003). Following an experimental inhibition of SOX2 (independent of bHLH factors), neural progenitors underwent premature neuronal differentiation, yet a fully differentiated neuronal phenotype required the presence of bHLH factors. In order to elucidate the function of SOX2 in the developing mouse brain,
Sox2 was deleted in neural progenitors using Nestin-CRE mice (Miyagi et al., 2008). Among the isolated and cultured forebrain progenitors, there was a decrease in the number of cells capable of forming neurospheres. However, these cells retained self-renewal and neurogenic properties as a result of the maintenance of SOX3 expression, which compensated for SOX2 loss. In an independent study, when Sox2 was deleted from mouse CNS progenitors at E12.5, the mice showed minor brain defects by birth (Favaro et al., 2009). However, the development of the hippocampus was impaired, resulting in an almost complete loss of NSCs and neurogenesis in the dentate gyrus (the site of neurogenesis in the hippocampus) during the first week following birth. The loss of neurogenesis in the dentate gyrus resembled the phenotype following loss of Shh, which was also lost in these Sox2-mutants. Ectopic expression of Shh in vivo resulted in the partial rescue of neurogenesis in the dentate gyrus, and chromatin immunoprecipitation identified Shh as a SOX2 target gene (Krieg and Schipper, 1996). Together, these results suggest that SOX2 plays a significant role in the maintenance of neurogenesis and NSC identity.

1.3.2.4 Requirement of SOX2 during Differentiation

In addition to its roles in NSCs, SOX2 is important for proper cell cycle exit and neuronal differentiation. Cavallaro et al. (2008) studied the role of SOX2 during neuronal differentiation by isolating and culturing NSCs from embryonic brains of mice that were hypomorphic for SOX2. These cells had relatively normal self-renewal properties but displayed defects in neuronal differentiation, characterized by abnormal neuronal morphology and decreased expression of mature neuronal genes. SOX2 has also been demonstrated to be important for the early stages of differentiation; overexpression of SOX2 using a lentivirus in Sox2-hypomorphic cells rescued the differentiation defect when expressed during early stages of differentiation, but not during later stages (Cavallaro et al., 2008). Additionally, the abnormal neurons formed from Sox2-hypomorphic NSCs aberrantly expressed glial genes, and the authors found that SOX2 directly binds to the GFAP promoter to inhibit astroglial differentiation in
neuronal-committed cells, indicating that SOX2 promotes a neural fate by suppressing glial differentiation.

1.3.2.5 SOX2 and Retinal Development

Previous studies of the function of SOX2 in the developing brain and spinal cord have highlighted a role in the maintenance of ES cells, NSCs, and neurogenesis (Pevny and Nicolis, 2010). However, studies of the specific function of SOX2 have been difficult due to its overlapping expression with SOX1 and SOX3 throughout the developing CNS, especially given that these SOXB1 factors have been shown to compensate for each other when one is downregulated or ablated (Collignon et al., 1996; Pevny et al., 1998; Bylund et al., 2003; Tanaka et al., 2004; Miyagi et al., 2008). However, despite their overlapping expression patterns in the developing CNS, only SOX2 is present in the developing optic cup following the downregulation of SOX1 and SOX3 after optic vesicle invagination (Kamachi et al., 1998; Le et al., 2002; Taranova et al., 2006a). Therefore, the optic cup is a good in vivo model for studying SOX2 in the developing CNS.

SOX2 is expressed in proliferating retinal progenitor cells throughout the developing retina and is downregulated upon cell cycle exit and differentiation into neurons (Taranova et al., 2006a). Taranova et al. (2006a) created an allelic series of mice that have reduced levels of SOX2 expression, and when crossed with mice that are heterozygous for a null allele of Sox2 (Sox2^{EGFP}) (Ellis et al., 2004), the progeny express 20-40% of wild type SOX2 protein levels. These mice display a range of eye phenotypes, from mild bilateral microphthalmia (small eyes) to severe anophthalmia (absence of eyes), which is consistent with the phenotypes found in humans with SOX2 mutations (Fantes et al., 2003; Hagstrom et al., 2005; Ragge et al., 2005b; Zenteno et al., 2005; Schneider et al., 2009). Additionally, Sox2-hypomorphic retinas are thinner than wild type retinas and display rosette formations (Taranova et al., 2006a). Although most of the retinal cell types are formed by adulthood, there is a reduction in the number of retinal ganglion cells (RGCs), as well as their axons (which make up the optic nerve), and as a result, the size of
the optic nerve is greatly reduced. Interestingly, an upregulation of GFAP is observed in the processes of the adult Müller glia, which could be a direct or indirect result of SOX2 reduction, depending on whether a) SOX2 regulates GFAP directly or b) gliosis is a secondary response to the tissue disruption and rosettes. When Sox2 is conditionally ablated in the retina at E10.5 using a Pax6 enhancer-driven Cre (αP0CREiresGFP), retinal progenitors lose their ability to proliferate and are unable to differentiate into neurons. Additionally, components of the Notch signaling pathway were downregulated in these Sox2-ablated retinas, indicating that the effects of SOX2 were likely mediated through regulation of the Notch pathway. In support of this, ChIP and transfection assays identified Notch1 as a direct target of SOX2 (Taranova et al., 2006a).

Subsequent studies of the Sox2-ablated retinas in the αP0CREiresGFP mice showed that SOX2 is responsible for regulating the neural competence of retinal progenitor cells during development (Matsushima et al., 2011). In the normal embryonic retina, SOX2 expression defines the neurogenic boundary of the retina that will give rise to neurons and glia, while PAX6 is expressed in a gradient that is highest in the non-neurogenic retina that will give rise to the ciliary epithelium and RPE. Ablation of SOX2 in retinal progenitors results in the loss of neural progenitor cell markers at E16.5, such as Notch1 and Hes5, as well as a loss in differentiated neurons, and coincident upregulation in PAX6 expression (Matsushima et al., 2011). By P0, the retinal tissue in Sox2-ablated retinas undergoes a cell fate conversion from neural retina to a single layered morphology characteristic of wild type ciliary epithelium. When the Sox2-ablated retinas were also haploinsufficient for Pax6, the cell fate conversion phenotype was significantly rescued and the neural retina restored, indicating that a proper antagonistic relationship between SOX2 and PAX6 is required for proper eye formation (Matsushima et al., 2011). These studies point to an important role for SOX2 in the establishment of neural competency and maintenance of retinal progenitors, as well as the critical dosage requirements of SOX2 to proper eye development.
1.3.3 Human SOX2 haploinsufficiency

Human SOX2 monoallelic mutations have been found to be associated with a number of developmental abnormalities, both ocular and otherwise (Kelberman et al., 2006; Williamson et al., 2006; Kelberman et al., 2008; Schneider et al., 2009). SOX2 mutations account for a majority of the cases of human anophthalmia and microphthalmia (Schneider et al., 2009), and the frequency of these developmental eye defects is 1-3/10,000 births. Other ocular abnormalities found in patients with SOX2 mutations include cataracts, optic nerve hypoplasia, coloboma, retinal and choroidal dystrophy, high myopia, and anterior segment dysgenesis (Kelberman et al., 2006; Bakrania et al., 2007). In addition to eye abnormalities, these patients display other phenotypes, such as male genital anomalies, pituitary gland hypoplasia, and brain abnormalities in the hypothalamus, hippocampus, septum, corpus callosum, and neurohypophysis (or posterior pituitary) (Kelberman et al., 2006; Schneider et al., 2009). Consistent with these neurological defects, some SOX2 haploinsufficient patients display learning disabilities, motor delay, and seizures (Kelberman et al., 2006; Sisodiya et al., 2006). A majority of SOX2 mutations occur de novo, although there are a few cases of familial inheritance (Schneider et al., 2009). Patients with missense mutations display milder phenotypes compared those with nonsense or frameshift mutations. Among the 40 patients analyzed with nonsense or frameshift mutations, 89% were anophthalmic or microphthalmic, 5% had other ocular phenotypes, and 6% had normal eyes (Schneider et al., 2009).

Mouse models of hypomorphic Sox2 exhibit many of the same eye and brain developmental abnormalities seen in humans with SOX2 haploinsufficiency (Ferri et al., 2004; Taranova et al., 2006a; Cavallaro et al., 2008; Favaro et al., 2009). However, in contrast to humans, heterozygous mice appear to be relatively normal, except for reduced pituitary size and testicular atrophy with age (Kelberman et al., 2006), indicating that mice seem to be less sensitive to SOX2 deficiency than humans. In the present study, we show that sensitivity to SOX2 deficiency also seems to be mouse strain-dependent (Chapter 4). Another interesting aspect of the analyses of phenotypes resulting from SOX2 mutations in both
humans and mice is the variability with respect to the range, penetrance, and severity of phenotypes, even among genetically identical (monozygotic) human twins or mouse litter mates (Taranova et al., 2006a; Zenteno et al., 2006). This observation indicates that even very small differences in SOX2 dosage, potentially due to stochastic events, may result in dramatic phenotypic differences between individuals.

1.3.4 SOX2 in Adult Stem Cell Populations

1.3.4.1 SOX2 in Adult Neural Precursor Cells

In addition to its role in preserving stem cell identity during development, SOX2 is also essential to the maintenance of adult progenitor cells in several tissues, including the brain, dermal papilla of the hair follicle, trachea, stomach, and testes (Ellis et al., 2004; Brazel et al., 2005; Driskell et al., 2009; Que et al., 2009; Arnold et al., 2011). In the subventricular zone (SVZ) of the adult brain, there are populations of glial cells (B-cell astrocytes) that function as neural stem cells and generate neurons and oligodendrocytes (Kriegstein and Alvarez-Buylla, 2009). The dentate gyrus of the hippocampus contains a population of astrocyte-like cells that are capable of neurogenesis (Alvarez-Buylla and Garcia-Verdugo, 2002). Using SOX2<sup>EGFP</sup> mice, Ellis et al. (2004) showed that SOX2 is expressed in both the glial-like cells and the neural precursors in both the SVZ and the dentate gyrus; EGFP-positive cells were also observed in ependymal cells of the spinal cord, another site of CNS neurogenesis (Johansson et al., 1999). When the neural progenitor cells from SOX2<sup>EGFP</sup> adult mice were isolated and cultured, they revealed self-renewal properties and were capable of differentiating into neurons, oligodendrocytes, and astrocytes (Ellis et al., 2004). As a complement to the <i>in vitro</i> work, lentiviral/retroviral fate tracing studies <i>in vivo</i> revealed that SOX2-expressing neural progenitor cells in the adult hippocampus gave rise to neurons and astrocytes, revealing their multipotentiality (Suh et al., 2007). In adult mice with hypomorphic levels of SOX2, a 65% reduction in the number of proliferating cells was observed in the dentate gyrus of the hippocampus, and a 55% reduction in proliferation in the SVZ (Ferri et al., 2004). Additionally, there was
a significant loss in neurogenesis in these areas, as shown by a decrease in the number of newly generated granule neurons in the hippocampus and early neurons in the SVZ. These results indicate that SOX2 is required for self-renewal and multipotency in adult neural progenitor cells.

**1.3.4.2 SOX2 in Müller Glia**

SOX2 expression is maintained in the adult retina in the cells that retain regenerative potential—Müller glia, as well as in the cholinergic amacrine cells (Taranova et al., 2006a). Consistent with its expression in Müller glia, there is evidence that SOX2 plays a role in determining Müller glial cell fate. Retroviral introduction of Sox2 into E17 mouse retinal explants induce Müller glia and amacrine cell production at the expense of rod photoreceptors (Lin et al., 2009). Furthermore, in the SOX2-overexpressing retinal explants, a significant decrease in progenitor cell proliferation is observed after 3 days and 5 days *in vitro* compared to the control-transfected retinas. Suppressing Sox2 by RNAi in E17 mouse explants resulted in a significant decrease in the number of cells in the INL, an effect that was specifically due to loss of amacrine cells; however, the authors did not address whether there was an effect on Müller glia or other retinal cells (Lin et al., 2009). Another study, in which SOX2 was inhibited by morpholino injections into *Xenopus* embryos, resulted in a 3.5-fold increase in the number of Müller glial cells (Van Raay et al., 2005). Therefore, in mouse retinas SOX2-overexpression induced Müller glia production, while in *Xenopus*, a similar result was found when SOX2 was inhibited. The discrepancy between the overexpression and knock-down results could reflect species-specific differences or the different experimental setups; the retroviral SOX2 overexpression was specific to mouse retinal tissue, whereas Sox2 morpholinos were injected into *Xenopus* 8-cell blastomeres.

The role of SOX2 has also been studied in immortalized cell lines derived from human retinas. These cultured cells have characteristics of Müller glia/progenitor cells based on their transcriptional profile, which includes expression of CRALBP, GS, PAX6, NOTCH1, and SOX2 (Limb et al., 2002; Lawrence et al., 2007). SOX2 downregulation by transfection of shRNA constructs in the Müller glial cell line
induced cells to rapidly adopt a neuronal-like morphology which was associated with the upregulation of several neuronal genes and a coincident loss of glial-specific identity (Bhatia et al., 2011). Moreover, the number of Ki67-positive cycling cells was reduced from 15% to 1%, and SOX2 silencing eventually resulted in apoptosis, indicating a role for SOX2 in granting proliferative abilities to Müller glia and preventing cell death.

Yet another role for SOX2 in Müller glia is its involvement in gliosis and proliferation following retinal injury. Following neurotoxic injury to the P1 chick retina with NMDA, Sox2 is upregulated by 4-fold one day post-injury, a result that was based on qPCR analysis of central retinas (Hayes et al., 2007). This upregulation occurs during the stages of Müller glia proliferation, and SOX2 is subsequently downregulated as the proliferating cells begin differentiating into neurons. Based on RT-PCR analysis in injured zebrafish retinas, SOX2 is upregulated by nearly 10-fold at 2 days post-injury, along with other progenitor and neurogenesis factors (Ramachandran et al., 2010). These studies reveal a role for SOX2 in the proliferative responses of Müller glia following retinal injury.

1.4 Summary and Hypothesis

The importance of SOX2 in progenitor/stem cell identity has been demonstrated across a range of neural progenitor cell populations throughout embryogenesis and adulthood, including in early retinal development. However, the role of SOX2 has not been investigated in late retinal progenitor cells or specifically in Müller glia in the context of a developing retina. Our hypothesis is that SOX2 is critical in postnatal retinal progenitors to maintain cell cycle quiescence of nascent Müller glia. Secondly, we hypothesize that postnatal progenitors are capable of altering their cell fate by switching from a gliogenesis to a neurogenesis program when influenced by bHLH factors.

To test these hypotheses, we address the following questions, providing analysis and discussion of the results:
1) What are the consequences of SOX2 loss in postnatal retinal progenitor cells? In Chapter 3, we show that loss of SOX2 in late progenitor cells results in the degradation of Müller glia morphology and induces cell cycle entry in these cells. Additionally, through rescue experiments, we show that SOX2 mediates the maintenance of Müller glia morphology through the regulation of the Notch signaling pathway.

2) Does SOX2 regulate the progenitor cell qualities of Müller glia, with respect to their cell cycle dynamics, neurogenic abilities, gliotic stress response, and survival? In Chapter 4, we demonstrate that SOX2 hypomorphism during early development induces Müller glia proliferation during late development. We also show that SOX2-deficient Müller glia are more likely to proliferate when they are in a state of gliosis, and that a cell cycle event leads to cell death.

3) Are late retinal progenitors capable of altering their cell fate following misexpression of proneural bHLH factors? In Chapter 5, we show that postnatal progenitor cells avoid the Müller glial cell fate following misexpression of Neurogenin-2 (NGN2), and instead generate amacrine neurons.
Figure 1.1. Eye anatomy and retinal cell types

(A) Eye anatomy. Light enters the eye through the cornea, and is focused on the retina by the lens. The retina absorbs the light, and converts it into electrical signals, and then sends the information to the brain via the optic nerve. The interior of the eye is made up of a transparent vitreous, and the retina is surrounded by the choroid, which is the connective tissue that contains a vascular network that supports the eye. The exterior layer is the sclera. The ciliary body is a pigmented circumferential tissue inside the eye made of muscle and ciliary processes. (B) Retinal cell types. The ONL is made up of rod and cone photoreceptors, which synapse in the OPL with the bipolar and horizontal cells. The INL is made up of bipolar, horizontal, amacrine, and Müller glial cells. The bipolar and amacrine cells synapse with retinal ganglion cells (RGCs) in the IPL. The ganglion cells and a subset of amacrine cells reside in the GCL, and the axons of the ganglion cells make up the optic nerve. The retinal pigmented epithelium (RPE) is the outer epithelial layer of cells that nourish the photoreceptor outer segments. Abbreviations: RPE- retinal pigmented epithelium, GCL- ganglion cell layer, IPL- inner plexiform layer, INL- inner nuclear layer, OPL- outer plexiform layer, ONL- outer nuclear layer.

Retina Schematic in (B) is modified with permission from (Surzenko et al., 2010).
Chapter 2: Materials and Methods

2.1 Mouse breeding and characterization of alleles

All animal work was carried out in accordance with the University of North Carolina IACUC and DLAM approval. The mouse lines described in this study were maintained either on a C57BL/6J background or a mixed C57BL/6J:CD-1 background. Studies were done on the C57BL/6J background unless otherwise indicated. The generation of Sox2C (Sox2COND) mouse line was described previously (Taranova et al., 2006a). Briefly, the Sox2C allele was generated by inserting loxP sites flanking the Sox2 open reading frame into the endogenous locus (Fig 4.1A). CAGG-CreERTM transgenic mice were a gift from Dr. A.P. McMahon (Hayashi and McMahon, 2002). Rosa26Reporter (Soriano, 1999) and CALSL-NICD (Tg(ACTB-Notch1)1Shn) (Yang et al., 2004) transgenic mice were obtained from Jackson Laboratories (Bar Harbor, ME). The Glast-CreERT2 allele consists of a tamoxifen-inducible Cre driven by the Glast promoter (gift from Dr. Jeremy Nathans, Fig. 4.1A). The Ai9 transgene expresses tdTomato following a CRE-mediated excision of a STOP codon (Fig. 4.1A)(Madisen et al., 2010). By utilizing mice containing either the CAGG-CreERTM or the Glast-CreERT2 allele combined with the Sox2C allele, Sox2 was ablated in a ubiquitous or Müller glia-specific manner, respectively, upon 4-OH-tamoxifen administration. The Rosa26Reporter and the Ai9 allele are used as fate tracers to track cells that have activated CRE. Genotyping primers are listed in Table S2.1.

2.2 Retinal explant culture

Retinal explants were prepared as described in Hatakeyama and Kageyama, with minor modifications (Hatakeyama and Kageyama). Briefly, P0-P1 retinas (Chapters 3 and 5) or P5 retinas (Chapter 4) were cultured on 30-mm filter inserts (Millicell), 2 retinas per filter, with the retinal ganglion
cell layer facing upward. The culture medium consisted of 50% MEM with Heps and L-glutamine (Gibco), 25% Hanks Balanced Salt Solution (HBSS; Gibco), 25% heat-inactivated horse serum (Gibco), 5.75 mg/mL glucose and penicillin/streptomycin (Gibco). Each well contained 1 mL medium; 0.5 mL of medium was replaced daily. 4-hydroxytamoxifen (Sigma) was dissolved in 100% ethanol and added to the culture medium at 1 μM for the first 24 hours. Retinas were cultured at 34°C in 5% CO₂ for 5-7 days.

Thymidine analogs (BrdU, EdU) were used for specific experiments, as follows. Chapter 3: BrdU (Sigma) was added to the culture medium at 1 μM for 2 hours prior to fixation. Chapter 4: EdU (Life Technologies) was added to the medium each day after the first 24 hours (Figs. 4.1B, 4.4, 4.5, 4.6). For fate tracing analyses, EdU was added only on the 5th day in vitro for 2 hours, followed by immediate fixation or fixation after 2 additional culture days (Figs. 4.1C, 4.7).

2.3 DNA constructs

Chapter 3: The following DNA constructs were used: pCIG2 (Addgene), pCAG-H2BEGFP (Konno et al., 2008), GLASTp-dsRED2 (a gift from Dr. N. Gaiano, Baltimore, MD; (Mizutani, 2007 #2635)), pCRALBP-dsRED (Addgene; (Matsuda and Cepko, 2007)), pCALNL-dsRED and pCALNL-EGFP (a gift from Dr. Cepko, Boston, MA), GLASTp-dsRED2 (a gift from Dr. N. Gaiano, Baltimore, MD). To generate pCRALBP-CreEGFP-Nuc, the CreEGFP sequence was excised from pCAG-CreEGFP-Nuc (a gift from Dr. Jaime Rivera, University of Massachusetts, Worcester, MA) using EcoRI, blunt-ended, and digested with NotI. The CreEGFP-Nuc fragment was inserted into pCRALBPdsRED2 following the removal of the DsRed2 cassette using the Smal and NotI restriction sites.

Chapter 5: Constructs containing the cDNA of the bHLH factors Neurogenin-1 (NGN1), Neurogenin-2 (NGN2), NeuroD2, and Mash1 were used in this analysis. In each construct, the bHLH cDNA was followed by a IRES-EGFP expression cassette and was under the control of a ubiquitous CMV-enhancer and chicken β-actin promoter (CIG2)(Hand et al., 2005) and (Polleux, unpublished). The control plasmid is the CIG2 promoter was followed by an IRES-EGFP sequence (CIG2-EGFP)(Addgene).
2.4 In vitro retina electroporation and time-lapse imaging

In the transfection experiments, retinas were dissected at P0, and DNA was electroporated into the apical side using an in vitro electroporation chamber (Nepagene) containing 80 μL of 2-5 μg/mL DNA in HBSS, essentially as described in Donovan and Dyer (Donovan and Dyer). Five 30-V pulses of 50 ms with 950 ms interpulse intervals were delivered to the chamber. Following electroporation, retinas were either prepared for time-lapse imaging (Chapter 3) or cultured as described above for 5-7 days for immunohistochemistry (Chapter 5). For time-lapse imaging, retinas were subsequently embedded in 5% low melting point agarose/MEM blocks and sectioned vertically into 200-μm slices using a microtome (Leica) and cultured as described above. Retinal slices were imaged on days 3-4 of culture using an inverted Olympus FV1000 laser scanning microscope equipped with an enclosure for environmental control (Precision Plastics); temperature and CO₂ were set to 34°C and 5%, respectively. Z-stack images (1.5-2.5 μm apart) were collected at 800 x 800 resolution using a 40X dry objective every 30-50 minutes over a course of 12-24 hours. The collected image series were processed using ImageJ and Adobe Photoshop software.

2.5 Immunohistochemistry, in situ hybridization, X-gal staining and EdU labeling

In preparation for immunohistochemistry, retinas were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Tissue was immersed progressively in 10%, 20%, and 30% sucrose in PBS before embedding in agar-sucrose gel and OCT medium (Tissue-Tek) and frozen at -80°C. 14μm tissue sections were collected on slides that were were blocked in 5% goat serum, 0.1% Triton-X for 3 hours, and incubated overnight at 4°C with primary antibodies in 3% goat serum. Secondary antibodies were incubated at 25°C for 1 hour. The antibodies and dyes used in this study are listed in Table S2.2. Antigen retrieval method was used for antibodies against PH3, PCNA and BrdU.
Slides were heated to 60-80°C in a buffer containing 10 mM Sodium Citrate/0.001% Tween-20, pH 6.0. X-gal staining was performed as described in Matsushima et al (2011). In situ hybridization was performed by the University of North Carolina In Situ Hybridization Core Facility. Specifically, 14-μm frozen retinal tissue sections were labeled using DIG-labeled antisense RNA probes followed by enzymatic detection according to manufacturer protocols (Roche). The following probes were used: Chx10 (Horsford et al., 2005), Hes5 (Chenn and Walsh, 2002) and Notch1 (Lardelli and Lendahl, 1993). EdU was fluorescently labeled through a copper catalyzed reaction using the Click-iT EdU Alexa Fluor 488 Imaging Kit from Life Technologies (C10337).

Images were obtained using a Leica inverted microscope (Leica DMIRB) equipped with either SPOT (RT Color Diagnostics) or Retiga (SRV-1394) cameras, a laser scanning upright microscope (Zeiss LSM 510), or an inverted Olympus FV1000 laser scanning microscope, and processed using Adobe Photoshop software.

2.6 Western blots and analysis

Western blots were performed as described in (Osawa et al., 2011). Individual retinas isolated from P5 mouse eyes were solubilized in Lee’s buffer containing 1mM DTT and SDS (Lee et al., 2004), and tissue was heated at 95°C, homogenized by a motorized pestle, and then the supernatant was isolated. Protein levels were determined using BioRad DC Protein Assay Kit according to the manufacturer’s directions. Individual eyes were loaded into separate lanes on the 10% SDS-PAGE gel, with 35 μg of protein loaded per lane. Following the transfer, nitrocellulose membranes were incubated in the Odyssey blocking buffer (Licor Biosciences), and incubated in primary antibody overnight at 4°C. The primary antibodies used were SOX2 (1:1000) (R&D, MAB2018) and β-actin (1:5000) (Sigma A1978), and the secondary antibody used was anti-mouse IRDye 800CW (Licor Biosciences). Images were obtained using the Odyssey Infrared Imaging System (Licor Biosciences). The pixel intensity of each band on the immunoblot was measured using the Odyssey 2.0 software. For comparison across different samples,
SOX2 levels were normalized to the pixel intensity values for β-actin. Then, for each strain background, the values were normalized to the control genotype Sox2+/++; the graphs in Fig. 4.2 show the averages of these values. For this analysis, we analyzed n=4 animals (8 separate eyes) for each genotype.

### 2.7 Statistical analysis

**Chapter 3**: Retinas from at least 3 pups of each genotype from independent litters were analyzed for the conditions and molecular markers described. Cells were counted on random, non-consecutive 14-μm sections through the central retinas of individual pups from separate litters. Unpaired two-tailed Student t-test, or one-way ANOVA and Tukey’s Multiple Comparison Test (Prism software, GraphPad) were used for analysis. Dividing GLASTdsRED2-labeled cells were counted on Z-stacks collected from retinal slices imaged for 12 hours. The number of counted cells ranged from 230 to 691 per genotype.

**Chapter 4**: (Fig. 4.5) In order to quantify the number of proliferating cells in central retinas, retinas were exposed to EdU on days 1-5 of the culture period, processed for immunohistochemistry, and stained for EdU, GFAP, and Hoescht. The central retinal slice was identified by GFAP, which marks the optic nerve head. The central slice as well as the preceding and following 2 sections were included in the analysis (5 slices used for each retina). Consecutive photos of the retinal slices were taken on a Leica inverted microscope and assembled using Adobe Photoshop. The central retina was defined as the area excluded from the 2 aligned brackets (bracket size: 700 μm) at the periphery of both ends of each slice (Fig. 4.5A). EdU cells were counted within these central regions for each slice. For the analysis, n=2 animals were counted for each genotype (4 retinas for each strain background). The data were analyzed using a mixed modeling analysis in Statistical Analysis Software (SAS) (performed by the UNC Biostatistics Core). To account for the repeated measures within mice, a mixed modeling method known as random coefficient modeling was used. This method correctly accounts for the correlation among...
Chapter 4: (Fig. 4.6) For proliferation and stress analysis, retinal areas were determined to be “stressed” on the basis of areas of high GFAP staining and retinal tissue disorganization or “non-stressed” in areas lacking GFAP staining and tissue disorganization. The number of Ki67-positive cells was counted within the “stressed” or “non-stressed” areas of a given size (735 µm x 735 µm). For each animal (both retinas), 5 “stressed” and 5 “non-stressed” areas were counted, and the graph displays the average number of Ki67-positive cells per area within each category. For this analysis, n=5 animals were analyzed for each genotype. The data were analyzed using a mixed modeling analysis (random coefficient modeling) in SAS (performed by the UNC Biostatistics Core). The models for Ki67 accounted for clustering within animal and estimated the main effects of genotype and stress level.

Chapter 5: (Fig. 5.4) For the quantification of Müller glia in CIG2-EGFP- and CIG2-NGN2-EGFP-electroporated retinas, cells were counted that were double-labeled for SOX9 and EGFP and then divided by the total number of EGFP-positive cells. These cells were counted from a single slice of a z-stack from central retina areas of a given size: 350 µm x 270 µm. For this analysis, n=2 animal were analyzed for each genotype, 6 areas per animal.
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### Primary antibodies

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### Fluorescent dyes

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Chapter 3: SOX2 maintains the quiescent progenitor cell state of postnatal retinal Müller glia.¹

3.1 Introduction

During central nervous system (CNS) development, both neurons and glia derive from a population of radial neural progenitors. SOX2, a member of the HMG-box transcriptional activators (of the SOXB1 subfamily), plays an essential role in progenitor cell maintenance in the developing and adult CNS (Pevny and Lovell-Badge, 1997; Ferri et al., 2004; Wegner and Stolt, 2005; Favaro et al., 2009). Disruption of SOX2 function in the embryonic and adult CNS results in both a loss of neural progenitor cells (NPCs) and a decrease in neurogenesis. To date, the key biological function of SOX2 that constitutes the requirement for NPC maintenance has not yet been elucidated. In this study, we address this question by genetically dissecting the role of SOX2 in the postnatal mouse retina.

In the developing retina, six neuronal and one glial cell types are produced by retinal progenitor cells (RPCs) in a distinct temporal order that is conserved among vertebrates (Livesey and Cepko, 2001b). To ensure the correct final composition of the retina with respect to the ratios of neuronal and glial cell types, a delicate balance between RPC maintenance and differentiation must be preserved throughout the course of retinogenesis. This balance is achieved through the coordinated actions of transcription factors regulating neuronal cell fate specification, such as those of the basic helix-loop-helix (bHLH) and homeobox families, and the molecular mechanisms acting to maintain the undifferentiated state of RPCs (Marquardt, 2003; Ohsawa and Kageyama, 2008). We previously demonstrated that SOX2 is essential for the maintenance of both RPC undifferentiated state and their

¹ Surzenko, N.†, Crowl, T.†, Bachleda, A., Langer, L. and Pevny, L. (2013) 'SOX2 maintains the quiescent progenitor cell state of postnatal retinal Muller glia', Development 140(7): 1445-56.

† These authors contributed equally to this work.
neurogenic potential (Taranova et al., 2006a; Matsushima et al., 2011). Ablation of Sox2 in embryonic RPCs abrogates neurogenesis through the loss of the key RPC characteristics – their molecular identity, morphology, and proliferative and differentiation capacity. In this study we dissect the mechanism of SOX2 function in the maintenance of NPC identity.

During the postnatal period, RPCs primarily give rise to rod photoreceptors, bipolar cells, and Müller glia (Morgan et al.) (Marquardt and Gruss, 2002; Ohsawa and Kageyama, 2008). Among these late-born cell types, Müller glia are the only cells to maintain both an RPC-like radial morphology and gene expression profile, including expression of SOX2 (Taranova et al., 2006a; Roesch et al., 2008; Nelson and Hyde, 2012). Under homeostatic conditions, Müller glia support neuronal function by maintaining retinal architecture and providing trophic support. However, recent evidence suggests that Müller glia do not irreversibly exit the progenitor state, as they can re-enter the cell cycle in response to retinal injury and maintain the potential to give rise to neurons (Fischer and Reh, 2001; Hitchcock and Raymond, 2004; Ooto et al., 2004; Karl et al., 2008; Del Debbio et al., 2010). Thus, Müller glia are considered a presumptive neural stem cell population in the retina and a key target of potential retinal regeneration therapies.

In this study, we utilize the transition of postnatal RPCs towards Müller glia cell fate as an experimental premise to address the role of SOX2 in NPC maintenance. We combine mouse genetics and real-time imaging approaches to demonstrate that SOX2 is required to maintain progenitor characteristic of Müller glia in the early postnatal period. Ablation of Sox2 causes nascent Müller glia to lose their radial morphology, resulting in the disruption of retinal cellular organization. Real-time imaging of early postnatal Müller glia reveals that in contrast to Sox2-expressing quiescent Müller glia, Sox2 mutant Müller glia undergo ectopic cell division.

The re-entry of Müller glia into the cell cycle in response to retinal injury is tightly associated with their neurogenic capacity (Fischer and Reh, 2001; Karl et al., 2008; Jadhav et al., 2009). Several
pathways that regulate embryonic NPC identity have been implicated in this process (Roesch et al., 2008; Jadhav et al., 2009). One of the key regulators of both neural progenitor identity and gliogenesis is the Notch signaling pathway (Jadhav et al., 2006a; Jadhav et al., 2009). Components of the Notch pathway, including its transcriptional downstream regulators HES1/5, not only serve as markers of Müller glia during postnatal development but also play a crucial role in establishing their cell identity (Tomita et al., 1996; Hojo et al., 2000; Takatsuka et al., 2004). Furthermore, HES1 regulates the quiescent state of neural stem cells (NSCs) through its sustained non-oscillatory expression, and can act as a safeguard against irreversible cell cycle exit during quiescence (Sang et al., 2008; Sang and Coller, 2009; Shimojo et al., 2011). We previously demonstrated that Notch1 is a direct transcriptional target of SOX2 in the developing CNS (Taranova et al., 2006a; Matsushima et al., 2011). Here we show that expression of both Notch1 and Hes5 is lost following ablation of Sox2 in the postnatal retina. Moreover, we demonstrate that genetic induction of Notch signaling restores Müller glia identity to Sox2 mutant cells. However, Notch is insufficient to re-establish the quiescent state of nascent Sox2 mutant Müller glia.

Collectively, these results reveal a new role for SOX2 in the maintenance of progenitor characteristics of postnatal Müller glia, and demonstrate that sustained SOX2 expression in the postnatal retina is required to maintain Müller glia cell quiescence.

3.2 Results

3.2.1 Sustained expression of SOX2 and Notch1 define Müller glia in the postnatal and adult retina.

During the first postnatal week, the pool of RPCs rapidly decreases as they undergo terminal cell divisions (Young, 1985b). We analyzed the dynamics of SOX2 expression in the developing retina between postnatal days 0 and 10 (P0-P10) and compared it with that of Notch1 - a known regulator of Müller glia specification (Fig. 3.1) (Jadhav et al., 2006a; Jadhav et al., 2006b; Jadhav et al., 2009; Nelson
and Hyde, 2012). At P0, SOX2 and Notch1 are expressed in retinal progenitors within the neuroblast layer (NBL) of the retina (Fig. 3.1A, G). SOX2 expression is also maintained in a subset of amacrine (starburst) cells, occupying the future inner nuclear (INL) and ganglion cell layers (GCL) (Fig. 3.1A, arrowhead) (Taranova et al., 2006a). By P4, SOX2 and Notch1 expression in the outer nuclear layer (ONL) is significantly reduced, concomitant with a decrease in the numbers of progenitor cells (Fig. 3.1B, H) (Young, 1985a; Young, 1985b). By P7, SOX2 and Notch1 are no longer detected in the ONL, and their expression is restricted predominantly to the INL (Fig. 3.1C, I). This corresponding pattern of SOX2 and Notch1 expression is observed at P10 and is maintained into adulthood (Fig. 3.1D-F, J-L).

To determine the identity of SOX2-expressing cells in the INL, we compared the expression of SOX2 at P10 with the following markers of postmitotic neurons and glia: NEUROFILAMENT (NF, horizontal cells), RHODOPSIN (rod photoreceptors), ISLET-1 (subsets of amacrine, ganglion and bipolar cells), GLUTAMINE SYNTHETASE (GS; Müller glia) and CRALBP (Morgan et al.) (Fig. 3.1M-R). We find that SOX2 is mutually exclusive of NF (Fig. 3.1M) and RHODOPSIN (Fig. 3.1N). SOX2-positive amacrine cells express ISLET-1 (Fig. 3.1O, arrowhead), whereas ISLET-1-expressing bipolar cells in the INL do not co-express SOX2 (Fig. 3.1O, arrow) (Elshatory et al., 2007). Cells that maintain SOX2 in the INL are Müller glia, as defined by the elongated cell body morphology and expression of both GS and CRALBP (Fig. 3.1P-R). In summary, SOX2 and Notch1 display overlapping patterns of expression in the postnatal mouse retina and define retinal progenitor cells and differentiating Müller glia.

3.2.2 Temporally-regulated ablation of Sox2 and genetic induction of Notch signaling in the early postnatal retina.

The Notch signaling pathway plays an important role in regulating NPC fate decisions; sustained activity of Notch1 is essential for the establishment and maintenance of Müller glia cell identity (Furukawa et al., 2000; Jadhav et al., 2009). The role of SOX2 in Müller glia cells is less well understood. We therefore sought to examine and compare the respective roles of SOX2 and Notch1 in the postnatal
retina during development of Müller glia. To establish an experimental system allowing for analysis of SOX2 in differentiating Müller glia cells, we first generated \( \text{Sox}2^{\text{COND/COND}; \text{CAGGCre-ER}^\text{TM}} \) (referred to as \( \text{Sox}2^\text{MUTANT} \)) animals, in which CRE expression is regulated by the ubiquitously active chicken β-actin promoter, and CRE activation is mediated by 4-hydroxytamoxifen (TM) (Fig. S3.1A) (Hayashi and McMahon, 2002). To ablate Sox2 during the period of Müller glia cell development, P0-P1

\( \text{Sox}2^\text{MUTANT} \) retinas were treated with TM \emph{in vitro} and cultured for 5-7 days (Fig. S3.1B). We find that expression of SOX2 in \( \text{Sox}2^\text{MUTANT} \) retinas is reduced within 2 days, and is no longer detected in the INL at 4-5 days of culture (Fig. 3.2C; Fig. S3.1S, T), while in \( \text{Sox}2^{+/+}; \text{CAGGCre-ER}^\text{TM} \) (referred to as \( \text{Sox}2^\text{CONTROL} \)) retinas cultured for 5 days, SOX2 is maintained in nascent Müller glia in the INL and in a subset of amacrine cells (Fig. 3.1A; Fig. S3.1S, T). Both retinal structural organization and expression of Müller glia and rod photoreceptor markers are preserved in \( \text{Sox}2^\text{CONTROL} \) retinas treated with TM at P0-P1 and cultured for 5 days (Fig. S3.1C-R). When the fates of mosaically-labeled RPCs are examined in TM-treated \( \text{Sox}2^\text{CONTROL} \) and \( \text{Sox}2^\text{MUTANT} \) retinas using electroporation of the CRE reporter construct, pCALNL-EGFP, no significant changes in the ratios of late-born Müller glia, rod photoreceptors and bipolar cells are detected (Fig. S3.2), suggesting that gradual TM-induced loss of Sox2 in the postnatal retina does not dramatically affect cell fate choices of postnatal RPCs, thus making newly-specified Müller glia amenable to further analysis of SOX2 function in these cells.

We previously showed that \emph{Notch1} is a downstream transcriptional target of SOX2 in the embryonic RPCs (Taranova et al., 2006a; Matsushima et al., 2011). To determine whether ablation of Sox2 in the postnatal retina leads to changes in the components of the Notch signaling pathway we examined expression of \emph{Notch1} and \emph{Hes5} in \( \text{Sox}2^\text{MUTANT} \) compared to \( \text{Sox}2^\text{CONTROL} \) retinas treated with TM and cultured for 5 days. We find that in contrast to \( \text{Sox}2^\text{CONTROL} \) retinas, in which both \emph{Notch1} and \emph{Hes5} are maintained in the INL, expression of \emph{Notch1} and \emph{Hes5} is downregulated in \( \text{Sox}2^\text{MUTANT} \) retinas (Fig. 3.1D; Fig. S3.1S, T).
3.2G and K).

We next used a transgenic approach to genetically induce Notch signaling in Sox2\textsuperscript{MUTANT} retinas via CRE-dependent activation of the \textit{CALSL-NICD} transgene, encoding the NOTCH1 intracellular domain (NICD) (Yang et al., 2004). We find that NICD activity restores the expression of \textit{Notch1} and \textit{Hes5} in Sox2\textsuperscript{MUTANT};\textit{CALSL-NICD} retinas (Fig. 3.2H and L). These results demonstrate that expression of \textit{Notch1} and its downstream effector, \textit{Hes5}, in the postnatal retina is downregulated in the absence of SOX2, and can be ectopically maintained in SOX2-deficient retinas through activation of the \textit{CALSL-NICD} transgene, thereby establishing an experimental premise to probe the functional relationship between SOX2 and Notch1 in the development of postnatal Müller glia.

3.2.3 Reduced density and disorganization of Müller glia in Sox2\textsuperscript{MUTANT} retinas.

The vast majority of Müller glia in the postnatal retina arise from RPCs that produce both rod photoreceptors and Müller glia during their final divisions (Turner and Cepko, 1987; Gomes et al., 2011). We therefore examined whether Müller glia and rod photoreceptors in Sox2\textsuperscript{MUTANT} retinas are correctly specified. We find that in contrast to SOX9-expressing Müller glia in Sox2\textsuperscript{CONTROL} retinas, which are confined to the INL, cell bodies of Müller glia in Sox2\textsuperscript{MUTANT} retinas treated with TM at P0-P1 and cultured for 5 days are found in both the INL and ONL, and are significantly disorganized (Fig. 3.2O, arrowheads). Furthermore, compared to Sox2\textsuperscript{CONTROL} retinas, the total density of SOX9-positive nascent Müller glia in Sox2\textsuperscript{MUTANT} retinas is reduced (mean ± S.E.M.: 134.8 ± 6.6 vs. 87.0 ± 3.7; p < 0.0001) (Fig. 3.2M vs. O; U). Conversely, the density of NR2E3-expressing rod photoreceptor precursors in Sox2\textsuperscript{MUTANT} retinas is moderately increased compared to Sox2\textsuperscript{CONTROL} retinas (mean ± S.E.M.: 468.5 ± 17.1 vs. 520.6 ± 26.4; p > 0.05) (Fig. 3.2V), while the ONL in Sox2\textsuperscript{MUTANT} retinas is disorganized.

Consistent with the role of NOTCH1 in regulating Müller glia and rod photoreceptor cell fates in the developing retina, genetic activation of NICD in Sox2\textsuperscript{CONTROL};\textit{CALSL-NICD} TM-treated retinas promotes Müller glia and attenuates photoreceptor cell genesis (Fig. 3.2M vs. N; Q vs. R) (Bao and Cepko, 1997;
Jadhav et al., 2006a; Jadhav et al., 2006b; Yaron et al., 2006; Jadhav et al., 2009; Nelson and Hyde, 2012). Specifically, the density of SOX9-positive Müller glia in Sox2<sup>CONTROL</sup>;CALSL-NICD retinas is increased compared to Sox2<sup>CONTROL</sup> retinas (mean ± S.E.M.: 167.7 ± 3.7 vs. 134.8 ± 6.6; p < 0.001) (Fig. 3.2M vs. N; U), while the density of NR2E3-positive rod photoreceptor precursors is decreased (mean ± S.E.M.: 416.5 ± 15.2 vs. 468.5 ± 17.1; p > 0.05) (Fig. 3.2O vs. P, N; U). However, the vast majority of Müller glia in Sox2<sup>MUTANT</sup>;CALSL-NICD retinas remain displaced throughout the INL and ONL (Fig. 3.2P, arrowheads).

Changes in density and disorganization of nascent Müller glia in Sox2<sup>MUTANT</sup> and Sox2<sup>MUTANT</sup>;CALSL-NICD retinas are also observed by immunostaining against PAX6, VIMENTIN and NESTIN (Fig. S3.3, S4). These data demonstrate that Müller glia in Sox2<sup>MUTANT</sup> retinas are formed, but are reduced in number and are significantly disorganized. Furthermore, activation of the Notch signaling pathway in Sox2 deficient background restores the density of Sox2-mutant Müller glia. However, NICD activity does not re-establish the correct laminar position of Müller glia in Sox2<sup>MUTANT</sup>;CALSL-NICD retinas, suggesting a role for SOX2 in postnatal Müller glia cells specifically.

### 3.2.4 Aberrant Müller glia morphology and laminar disorganization in Sox2<sup>MUTANT</sup> retinas.

Müller glia are essential for the establishment and maintenance of the laminar organization in the postnatal retina (Willbold et al., 1997; Bringmann et al., 2006). Mutations leading to reduced density of Müller glia, or altering their ability to function as a structural scaffold, are associated with the disruption of the inner and outer retinal membranes and of neuronal stratification, eventually resulting in retinal degeneration (Dubois-Dauphin et al., 2000; van Rossum et al., 2006). Reduced density and aberrant localization of Müller glia throughout the INL and ONL in SOX2-deficient retinas, combined with
the observed disorganization of the ONL, led us to examine the laminar architecture in Sox2\textsuperscript{MUTANT} retinas. Gross morphological analyses (Fig. 3.3A-D), coupled with β-catenin (Fig. 3.3E-H), Phalloidin (Fig. 3.3I-L) and Neurofilament (Fig. 3.3S, T) staining, marking adherens junctions, F-actin and horizontal cells, respectively, reveal the disruption of the outer limiting membrane (OLM), and disorganization of the inner and outer plexiform layers (IPL and OPL) in Sox2\textsuperscript{MUTANT} retinas when compared to Sox2\textsuperscript{CONTROL} retinas.

We next assessed the morphology of postnatal Müller glia in Sox2\textsuperscript{CONTROL} and Sox2\textsuperscript{MUTANT} retinas using immunostaining against CRALBP and GLAST (Fig. 3.3M-R). In Sox2\textsuperscript{CONTROL} retinas, Müller glia display a characteristic radial morphology - cell bodies located within the INL and processes that extend towards the apical and basal retinal boundaries (Fig. 3.3M, O, Q). In contrast, the radial morphology of Sox2\textsuperscript{MUTANT} Müller glia is disrupted, and the cell bodies are irregularly shaped and mislocalized to the ONL (Fig. 3.3N, P, T; Fig. S3.1T). Reduced density and aberrant morphology of Müller glia in Sox2\textsuperscript{MUTANT} retinas, and the disruption of the OLM, are accompanied by the disorganization of the ONL, such that RHODOPSIN-positive photoreceptors are no longer contained within a defined cellular layer, but protrude through the breaks in the OLM and form rosette-like structures (Fig. 3.3U vs. V). The severity of retinal disorganization in Sox2\textsuperscript{MUTANT} retinas correlates with the kinetics of TM-induced Sox2 ablation (Fig. S3.1T), while a subset of Sox2\textsuperscript{MUTANT} retinas display severe retinal degeneration at day 7 of culture (Fig. S3.1U) (Table S3.1). Together, these data reveal the aberrant morphology of postnatal Sox2\textsuperscript{MUTANT} Müller glia and the defects in retinal laminar architecture caused by ablation of SOX2 in the postnatal retina.

3.2.5 Disruption of cell cycle quiescence in nascent Sox2\textsuperscript{MUTANT} Müller glia.

Cell cycle quiescence, along with the confinement of RPC marker expression strictly to the INL, serve to distinguish newly-specified Müller glia from proliferating RPCs (Fig. S3.3). However, postnatal Müller glia that are forced to re-enter the cell cycle either by the administration of mitogenic factors or
in response to retinal injury, are capable of undergoing interkinetic nuclear migration (Mehdorn et al.) and cell division (Close et al., 2006; Karl et al., 2008; Joly et al., 2011; Ueki et al., 2012). SOX2 is critical to cell cycle dynamics in the retina, cortical subventricular zone, and glial cells in the hippocampus (Pevny and Nicolis, 2010 2004). Therefore, we asked whether displacement of Müller glia cell bodies to the ONL in Sox2\textsuperscript{MUTANT} retinas is associated with dysregulation of their cell cycle state. We first assessed incorporation of BrdU (2 hour pulse) and expression of PH3, marking cells in the S and M phases of the cell cycle, respectively, in Sox2\textsuperscript{CONTROL} and Sox2\textsuperscript{MUTANT} retinas (Fig. 3.4A-D). We find that by day 5 in culture, cells that incorporate BrdU and express PH3 are no longer present in the central regions of Sox2\textsuperscript{CONTROL} retinas (Fig. 3.4A, C), while they persist in the peripheral retinal regions (Fig. 3.4A, inset; Fig. S3.1M, inset). In contrast, BrdU- and PH3-positive cells are observed in both central and peripheral retinal regions in Sox2\textsuperscript{MUTANT} retinas (Fig. 3.4B, D). The total number of PH3-positive cells is significantly increased in Sox2\textsuperscript{MUTANT} relative to Sox2\textsuperscript{CONTROL} retinas (mean ± S.E.M: 32.70 ± 3.88 vs. 16.20 ± 4.05; p= 0.0055) (Fig. 3.4O). Consistent with both the decrease in Müller glia cell density and the increase in proliferating cells, expression of the cell cycle inhibitor, p27\textsuperscript{Kip1}, is reduced in Sox2\textsuperscript{MUTANT} retinas (Fig. 3.4E vs. F).

We next examined expression of PAX6, marking Müller glia cells, as well as amacrine and horizontal cells (HC) in the INL (Fig. 3.4G-H’’). In accordance with the depletion of dividing RPCs in the central retinal regions at day 5 of culture, PAX6-positive cells are rarely detected in the ONL of Sox2\textsuperscript{CONTROL} retinas, while postnatal Müller glia in the INL maintain PAX6 expression (Fig. 3.4G, arrow, G’; Fig. S3.4A, A’, arrowheads). On the contrary, PAX6-positive cells that undergo INM and co-express PCNA (Fig. 3.4H, H’, arrows; Fig. S3.5B, D, F, F’, arrows), and PAX6-positive/PCNA-negative cells with round morphology that have exited the cell cycle (Fig. 3.4H, H’, H’’, arrowheads; Fig. S3.5B, D, F, F’’, arrowheads), are found in the ONL of Sox2\textsuperscript{MUTANT} retinas. Furthermore, consistent with the INM of Sox2\textsuperscript{MUTANT} Müller glia, the number of SOX9-expressing Müller glia that are displaced to the ONL is
increased in $\text{Sox2}^{\text{MUTANT}}$ relative to $\text{Sox2}^{\text{CONTROL}}$ retinas (Fig. 3.4P). Importantly, cells that incorporate BrdU in $\text{Sox2}^{\text{MUTANT}}$ retinas are located in both the INL and ONL and co-express CRALBP (Fig. 3.4I-N, arrowheads; inset in N), confirming their identity as Müller glia. Thus, ablation of Sox2 in the postnatal retina leads to re-entry of Müller glia into the cell cycle.

### 3.2.6 $\text{Sox2}^{\text{MUTANT}}$ postnatal Müller glia undergo cell division.

To determine whether postnatal Müller glia that undergo INM and upregulate mitotic markers in $\text{Sox2}^{\text{MUTANT}}$ retinas undergo cell division, we electroporated P0-P1 retinas with GLASTp-dsRED2 plasmid and monitored the behavior of labeled cells in TM-treated retinal slices at days 3-4 of culture using time lapse microscopy (Fig. 3.5; Fig. S3.6) (Mizutani et al., 2007). GLAST-dsRED2 marks a small subset of electroporated cells, the vast majority of which exhibit characteristic radial morphology of Müller glia at day 4 in culture (Fig. S3.6E, F). Consistent with their identity as Müller glia, GLAST-dsRED2-positive cells in $\text{Sox2}^{\text{CONTROL}}$ retinas display long processes extending towards apical and basal retinal boundaries, do not undergo INM, and maintain cell body position within the INL (Fig. 3.5A and A', Fig. S3.6B; Movie 1). In contrast to Müller glia in $\text{Sox2}^{\text{CONTROL}}$ retinas, and in accordance with the expression of proliferative markers, a subset of $\text{Sox2}^{\text{MUTANT}}$ Müller glia captured during 24-hour imaging period at days 3-4 of culture migrate towards the apical retinal surface and undergo cell division (Fig. 3.5B and B'; Movies 2-4). Importantly, daughter cells resulting from $\text{Sox2}^{\text{MUTANT}}$ Müller glia cell divisions do not re-establish radial morphology, losing both RPC and Müller glia identity (Movies 2-4). Together, these results demonstrate that in the absence of SOX2, postnatal Müller glia cells divide at the apical retinal boundary, suggesting a role for SOX2 in the maintenance of nascent Müller glia cells by preventing their depletion through cell division.

### 3.2.7 Mosaic ablation of Sox2 in the postnatal retina leads to cell division of nascent Müller glia.

The ability of postnatal and adult Müller glia cells to re-enter the cell cycle is tightly associated
with their response to retinal injury (Dyer and Cepko, 2000; Ooto et al., 2004; Jadhav et al., 2009; Joly et al., 2011). To confirm that cell divisions of postnatal Müller glia are caused by the loss of SOX2 in these cells specifically, as opposed to being triggered by non-cell autonomous factors, we ablated Sox2 in a mosaic manner. pCRALBP-CreEGFP-Nuc construct, encoding CreEGFP fusion protein driven by the CRALBP regulatory element, was used to electroporate retinas at P1 (Fig. 3.6A) (Matsuda and Cepko, 2004; Matsuda and Cepko, 2007). Fate-mapping analysis of pCRALBP-CreEGFP with the Cre reporter, pCALNL-dsRed, in retinas cultured for 5 days confirms its restricted activity predominantly in postnatal Müller glia (Fig. S3.7A-D). At day 5 of culture, CreEGFP co-localizes with SOX2 and CRALBP strictly in Müller glia in the INL (Fig. 3.6C; Fig. S3.7E-G). In contrast to Müller glia in Sox2<sup>+/+</sup> retinas, which maintain their quiescent state, a subset of CreEGFP-expressing Müller glia in Sox2<sup>COND/COND</sup> retinas express PH3 (Fig. 3.6E, arrowhead), migrate to the apical retinal boundary (Fig. 3.6F, arrowhead) and undergo cell division (Fig. 3.6G; Movie 5). These results, combined with the observed Müller glia cell divisions in TM-treated Sox2<sup>MUTANT</sup> retinas, strongly suggest that SOX2 is required in nascent Müller glia cells specifically to prevent their depletion through cell division.

### 3.2.8 Induction of ectopic NOTCH1 activity restores Müller glia cell identity to Sox2<sup>MUTANT</sup> cells, but does not secure their quiescent state.

Radial morphology and cell cycle quiescence are hallmark features of postnatal Müller glia cells, and are disrupted due to loss of SOX2 in the postnatal retina. To examine, whether besides Müller glia cell density, genetic activation of the Notch signaling pathway restores these characteristic features of Müller glia, we examined Müller glia cell morphology and retinal laminar architecture in Sox2<sup>MUTANT;CALSL-NICD</sup> retinas (Fig. 3.7). In contrast to the aberrant morphology of Müller glia in Sox2<sup>MUTANT</sup> retinas, the morphology of Müller glia in Sox2<sup>MUTANT;CALSL-NICD</sup> retinas is restored by the activity of NICD. Despite the displacement of Müller glia cell bodies to the ONL in Sox2<sup>MUTANT;CALSL-NICD</sup> retinas, their processes are maintained at the apical and basal retinal boundaries (Fig. 3.7C vs. D).
Furthermore, restoration of Müller glia cell density and morphology in Sox2\textsuperscript{MUTANT};CALSL-NICD retinas is accompanied by the restoration of the OLM, marked by phalloidin staining (Fig. 3.7G vs. H). These data demonstrate that Notch signaling pathway functions downstream of SOX2 to maintain Müller glia cell molecular identity and radial morphology.

We next assessed whether the quiescent state of Sox2-mutant nascent Müller glia is restored by the NICD activity in Sox2\textsuperscript{MUTANT};CALSL-NICD retinas. We find that expression patterns of PCNA, marking predominantly Müller glia in the INL, are indistinguishable in Sox2\textsuperscript{CONTROL} and Sox2\textsuperscript{CONTROL};CALSL-NICD retinas (Fig. 3.8A, B; Fig. S3.3Q-T), while mitotic marker PH3 is undetectable (Fig. 3.8E, F). In sharp contrast, expression of PCNA and PH3 is observed throughout the INL and ONL of Sox2\textsuperscript{MUTANT};CALSL-NICD retinas (Fig. 3.8D, H).

To further examine the effect of ectopic NICD activity on the mitotic behavior of postnatal Müller glia, we followed the fates of GLAST-DsRED2-labeled cells in slices of both Sox2\textsuperscript{CONTROL};CALSL-NICD and Sox2\textsuperscript{MUTANT};CALSL-NICD retinas using time lapse imaging at days 3-4 of culture (Fig. 3.8I, J; Movies 6-9). In accordance with the lack of PH3 expression, GLAST-DsRED2-labeled Müller glia cells in Sox2\textsuperscript{CONTROL};CALSL-NICD retinas do not undergo INM or cell division (Fig. 3.8J, Movie 6). In contrast, GLAST-DsRED2-expressing Müller glia in Sox2\textsuperscript{MUTANT};CALSL-NICD retinas undergo INM and divide at the apical retinal boundary (Fig. 3.8I, Movies 7-9). The proportions of GLAST-DsRED2-labeled cells undergoing cell division during 12-hour imaging periods in both Sox2\textsuperscript{MUTANT} (mean ± S.E.M.: 16.14 ± 1.86; n=19) and Sox2\textsuperscript{MUTANT};CALSL-NICD retinas (mean ± S.E.M.: 16.68 ± 1.90; n=11) are significantly higher than in Sox2\textsuperscript{CONTROL} (mean ± S.E.M.: 1.73 ± 0.58; n=24) and Sox2\textsuperscript{CONTROL};CALSL-NICD retinas (mean ± S.E.M.: 0.37 ± 0.37) (p < 0.0001) (Fig. 3.8J). These findings, coupled with the restoration of Müller glia cell density and morphology in Sox2\textsuperscript{MUTANT};CALSL-NICD retinas, provide evidence that SOX2 maintains the identity of postnatal Müller glia via the Notch signaling pathway, but functions in a Notch-independent manner to prevent the aberrant cell division of nascent Müller glia.
3.3 Discussion

In this study, we show that SOX2 plays an essential role in maintaining the retinal structural organization and the quiescence of nascent Müller glia. Utilizing real-time imaging we find that the loss of SOX2 forces Müller glia to aberrantly divide into a pair of postmitotic daughter cells, leading to Müller glia cell depletion and retinal degeneration. We further establish that activation of Notch signaling restores Müller glia characteristics to Sox2 mutant cells, but is insufficient to secure their progenitor state quiescence. We propose a model by which SOX2 maintains a quiescent progenitor cell state of nascent Müller glia by preventing their progression through the cell cycle to terminal division.

Müller glia represent a dormant progenitor cell population. They maintain characteristics of postnatal RPCs, including radial morphology and the expression of NPC markers. Although they retain a neurogenic capacity, Müller glia proliferate and give rise to new neurons only when stimulated by signals that arise in response to retinal injury (Roesch et al., 2008; Jadhav et al., 2009). Here, we show that the ablation of Sox2 in the postnatal retina results in a gradual depletion of Müller glia, as evidenced by a decrease in the expression of the molecular markers of postnatal Müller glia cells, including SOX9, PAX6, p27Kip1, and PCNA. Moreover, this ablation is accompanied by the disruption of Müller glia radial morphology.

Unlike the subsets of specialized adult glial stem cells in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, Müller glia constitute the principal glial cell population in the retina, and thus their primary role lies in maintaining retinal homeostasis (Kriegstein and Alvarez-Buylla, 2009). Spanning the entire retinal width, Müller glia define the retinal boundaries through formation of intercellular junctions to function as a neuronal scaffold, and play the decisive role in the establishment of retinal laminar pattern and polarity (Willbold et al., 1997; Bringmann et al., 2006). Consistent with this role, the loss of Müller glia in the postnatal retina results in retinal degeneration (Rich et al., 1995; Dubois-Dauphin et al., 2000). In agreement with
these observations, we find that the gradual loss of Müller glia cells in Sox2\textsuperscript{MUTANT} retinas leads to severe retinal disorganization followed by retinal degeneration, suggesting an essential role for SOX2 in the maintenance of retinal homeostasis through its function in nascent Müller glia cells.

In the developing and adult CNS, SOX2 expression defines multipotent NPCs (Ellis et al., 2004). Consistent with its expression pattern, SOX2 controls the maintenance of NPC identity; disruption of SOX2 function is associated with the loss of proliferative and differentiation capacity in embryonic and adult NPCs (Ferri et al., 2004; Favaro et al., 2009; Pevny and Nicolis, 2010). However, to date, the precise mechanism underlying the requirement for SOX2 in the maintenance of NPCs has not been characterized. In this study, we demonstrate that SOX2 prevents the depletion of Müller glia through aberrant cell division. Cell cycle quiescence is an essential feature of Müller glia that distinguishes them from postnatal RPCs and enables them to function in maintaining retinal homeostasis. Using real-time imaging, we show that, unlike quiescent Müller glia, Sox2\textsuperscript{MUTANT} Müller glia undergo interkinetic nuclear migration and cell division at the apical retinal boundary. Divisions of Müller glia in Sox2\textsuperscript{MUTANT} retinas produce daughter cells that do not display morphological and molecular characteristics of Müller glia, losing both Müller glia and progenitor cell identity. These observations are supported by studies demonstrating that both embryonic and adult neural progenitor cells are incapable of neuronal or glial differentiation when the function of SOX2 is perturbed (Bylund et al., 2003; Graham et al., 2003; Ferri et al., 2004; Holmberg et al., 2008; Favaro et al., 2009; Ehm et al., 2010; Matsushima et al., 2011).

Therefore, we conclude that daughter cells resulting from Sox2\textsuperscript{MUTANT} Müller glia divisions exit the cell cycle and transiently acquire a rather complex identity.

Similar to SOX2, Notch signaling serves to maintain NPC identity in the developing and adult CNS (Austin et al., 1995; Tomita et al., 1996; Henrique et al., 1997; Furukawa et al., 2000; Takatsuka et al., 2004; Yaron et al., 2006; Holmberg et al., 2008; Ehm et al., 2010). Studies in multiple systems, including
the developing mouse retina, provide evidence for a possible interaction between SOX2 and Notch (Takatsuka et al., 2004; Taranova et al., 2006a; Yaron et al., 2006; Holmberg et al., 2008; Ehm et al., 2010; Matsushima et al., 2011). Together, these studies establish that the ability of Notch signaling to maintain NPC identity depends on functional SOXB1 proteins.

Examination of the relationship between Notch signaling and SOX2 in nascent Müller glia reveals that Sox2MUTANT Müller glia downregulate both Notch1 and its downstream target, Hes5, confirming that the expression of components of the Notch signaling pathway in Müller glia depends on SOX2. Importantly, the depletion of Müller glia in Sox2MUTANT retinas is prevented by the genetic induction of Notch signaling, as evidenced by the restoration of Müller glia radial morphology, leading to an improvement of retinal architecture. Misexpression of NICD itself has little effect on cell proliferation in the postnatal retina (Bao and Cepko, 1997; Furukawa et al., 2000). Secondly, through real-time visualization of Müller glia in Sox2MUTANT;CALSL-NICD retinas, we hypothesize that the daughter cells resulting from Sox2MUTANT Müller glia cell divisions may be capable of re-establishing radial morphology and cell body position in the INL. Together, the increase in Müller glia cell density and restoration of their radial morphology indicate that NICD activity specifically rescues the fates of Sox2MUTANT Müller glia cells.

Collectively, these data strongly suggest that, in addition to maintaining Müller glia cell identity via the Notch signaling pathway, SOX2 functions in a Notch-independent manner to prevent the depletion of nascent Müller glia through cell cycle progression and terminal cell division. Thus, we uncoupled the roles of SOX2 and Notch signaling in Müller glia cell development. We established that while Notch signaling functions as a Müller glia cell fate determinant, SOX2 is essential to prevent their terminal cell cycle exit. We further show that there is an obligatory requirement for at least one round of cell division in the absence of SOX2 as a prelude to terminal progenitor cell differentiation.
Together, our data highlight the essential roles of SOX2 and Notch signaling pathway in regulation of the quiescent state of Müller glia in the postnatal mouse retina. The mechanisms by which quiescent cells preserve their ability to resume proliferation after weeks or even years of cell cycle arrest are not known. Our study provides a general paradigm of how SOX2 functions in NPCs and will have implications for our understanding of the pathways that balance stem cell quiescence and proliferation in the developing and adult CNS.
Figure 3.1. **SOX2 and Notch1 are expressed in postnatal retinal progenitor cells and in Müller glia.**

(Garcia-Feijoo et al.) Expression of SOX2 in the mouse retina was assessed by immunohistochemistry at the indicated ages (arrowhead in A – amacrine cell; arrows in E, F – Müller glia). (G-L) Notch1 expression was examined by *in situ* hybridization (ISH). (M-R) SOX2 does not co-localize with NF (M) or RHODOPSIN (N). SOX2 is co-expressed with ISLET-1 in amacrine cells (O, arrowhead) but not in bipolar cells (O, arrow). SOX2 co-localizes with GS (P, Q – higher magnification image of P) and CRALBP (R) in Müller glia at P10 and in the adult retina. AM – amacrine cells; GS – Glutamine Synthetase; IPL – inner plexiform layer; NBL – neuroblast layer; NF – NEUROFILAMENT. Scale bars: (P) 65 µm; (R) 30 µm.
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Density of SOX9\textsuperscript{+} cells

V

Density of Nr2E3\textsuperscript{+} cells

# of cells per 4x10\textsuperscript{4}μm\textsuperscript{2}

Genotype
Figure 3.2. Loss of Notch1 activity and disorganization of Müller glia in Sox2\textsuperscript{MUTANT} retinas. 

(A-D) SOX2 expression is not detected in Sox2\textsuperscript{MUTANT} (C) and Sox2\textsuperscript{MUTANT};CALSL-NICD (D) TM-treated retinas cultured for 5 days. (E-L) Expression of Notch1 (E-H) and Hes5 (I-L) is lost in Sox2\textsuperscript{MUTANT} (G, K) retinas, and is restored by NICD activity in Sox2\textsuperscript{MUTANT};CALSL-NICD retinas (H, L) (ISH). (M-P) SOX9-expressing Müller glia are displaced to the ONL in both Sox2\textsuperscript{MUTANT} (O, arrowheads) and Sox2\textsuperscript{MUTANT};CALSL-NICD retinas (P, arrowheads). (Q-T) Nr2E3-expressing rod photoreceptor precursors are reduced in number in Sox2\textsuperscript{CONTROL};CALSL-NICD (R) and Sox2\textsuperscript{MUTANT};CALSL-NICD (T) retinas, and are disorganized in Sox2\textsuperscript{MUTANT} retinas (S). (U-V) Total densities of SOX9-positive (U) and Nr2E3-positive (V) cells were quantified on sections of TM-treated retinas of indicated genotypes. ***- p < 0.0001; ns – not significant. Scale bar: (T) 45 µm.
Figure 3.3. Defects in retinal lamination and Müller glia morphology in Sox2<sup>MUTANT</sup> retinas.

(A-L) Immunohistochemical analysis of sectioned retinas reveal disorganization of lamination in Sox2<sup>MUTANT</sup> retinas treated with TM and cultured for 5 days (B, D, F, H, J, L are high magnification images of A, C, E, G, I, K). (A-D) In Sox2<sup>MUTANT</sup> retinas, the ONL, INL and GCL are disorganized and cells are displaced beyond the OLM (C, arrowhead) and IPL (C, arrow), shown by Nissl staining (A, B vs. C, D). (E-H) While β-catenin is enriched in adherens junctions of the IPL (E, arrow) and OLM (E, F, arrowheads) of Sox2<sup>CONTROL</sup> retinas, β-catenin staining in the IPL (G, arrow) and OLM (G, H, arrowheads) of Sox2<sup>MUTANT</sup> retinas is discontinuous. (I-L) Breaks in the IPL (arrows) and OLM (arrowheads) of Sox2<sup>MUTANT</sup> retinas are visualized by the uneven distribution of F-actin (Phalloidin), normally enriched in the IPL (I, K, arrows) and OLM (I-L, arrowheads). (M-R) Immunostaining against CRALBP (M, N) and GLAST (O, P) reveals radial morphology of Müller glia in Sox2<sup>CONTROL</sup> retinas (M, O, Q), while Müller glia in Sox2<sup>MUTANT</sup> retinas are disorganized and displaced to the ONL (N, P, R, arrowheads). (S, T) The OPL, marked by NEUROFILAMENT, is disorganized in Sox2<sup>MUTANT</sup> retinas (T) compared to Sox2<sup>CONTROL</sup> retinas (S) cultured for 7 days. (U, V) Rod photoreceptors form rosette structures in Sox2<sup>CONTROL</sup> (V) compared with Sox2<sup>MUTANT</sup> retinas (U) cultured for 7 days. GCL – ganglion cell layer; IPL – inner plexiform layer; OLM – outer limiting membrane. Scale bars: (R) 40 µm; (L) 15 µm.
Figure 3.4. Müller glia in Sox2\textsuperscript{MUTANT} retinas express mitotic cell cycle markers.

(A-D) The number of cells incorporating BrdU (A, B) and expressing PH3 (C, D) in the central and peripheral regions of Sox2\textsuperscript{MUTANT} retinas is increased (insets in A, B). (E, F) Expression of p27\textsuperscript{Kip1} in the INL of Sox2\textsuperscript{MUTANT} retinas (F) is decreased compared to Sox2\textsuperscript{CONTROL} retinas. (G-H") PAX6 is not detected in the ONL, but marks amacrine cells, Müller glia and horizontal cells (HC) (G', arrowhead) in the INL (G, G') in Sox2\textsuperscript{CONTROL} retinas. In Sox2\textsuperscript{MUTANT} retinas PAX6 is found in elongated cells (H, H', arrows) and in cells with round morphology in the ONL (H, H', H", arrowheads). (I-N) CRALBP-positive Müller glia do not incorporate BrdU in Sox2\textsuperscript{CONTROL} retinas (I, K, M), but are BrdU-positive in Sox2\textsuperscript{MUTANT} retinas (J, L, N, arrowheads, inset). (O) The percentage of SOX9-expressing Müller glia displaced to the ONL is increased in Sox2\textsuperscript{MUTANT} (mean ± S.E.M: 12.16 ± 2.23) compared to Sox2\textsuperscript{CONTROL} retinas (mean ± S.E.M: 3.31 ± 0.84) (Two-tailed t-test; p=0.0005). Scale bars: (H) 45 µm; (H") 25 µm.
Figure 3.5. Cell division of Müller glia in Sox2\textsuperscript{MUTANT} retinas.

(A) Time-lapse imaging of GLASTp-DsRED2-expressing cells in slices of TM-treated Sox2\textsuperscript{CONTROL} retinas at day 3-4 of culture illustrates Müller glia radial morphology, maintenance of apical and basal cellular processes (arrowheads), and limited cell body movement (diagram in A’). (B) A GLASTp-DsRED2-labeled Müller glia imaged in the Sox2\textsuperscript{MUTANT} retina migrates to the ONL and undergoes cell division, followed by separation of the two daughter cells (arrows), and splitting of the basal cellular process (arrowheads) (diagram in B’). Image series were collected on 200 µm retinal sections every 40 minutes over 18-20 hours. Scale bars: (A) 30 µm; (B) 20 µm.
Figure 3.6. Mosaic ablation of Sox2 leads to ectopic cell division of nascent Müller glia.

(A) Schematic illustration of pCRALBP-DsRed2 and pCRALBP-CreEGFP-Nuc DNA constructs. (B) CRALBP-DsRED2 co-localizes with CRALBP-CreEGFP in Müller glia in retinas co-electroporated at P1 and cultured for 5 days. (C) In Sox2\textsuperscript{+/+} retinas CRALBP-CreEGFP is co-expressed with SOX2 in the INL (C, arrowheads).

(D) SOX2 is not detected in EGFP-positive cells in Sox2\textsuperscript{COND/COND} retinas. (E, F) A subset of CRALBP-CreEGFP-expressing Müller glia in Sox2\textsuperscript{COND/COND} retinas express PH3 (E, arrowhead, inset) and are displaced to the ONL (F, arrowheads). (G) CRALBP-CreEGFP/CRALBP-DsRED2 double-labeled Müller glia in Sox2\textsuperscript{COND/COND} retinas undergo interkinetic nuclear migration and cell division (arrows) (Movie 5).

Confocal images in (G) were collected from 200 µm thick retinal sections, every 40 minutes over 18 hour period. Hrs – hours. Scale bars: (D): 45 µm; (G): 35 µm.
Figure 3.7. Activation of Notch signaling in Sox2\textsuperscript{MUTANT};CALSL-NICD retinas restores Müller glia cell morphology and retinal architecture.

(A-D) CRALBP reveals restoration of Müller glia morphology and retinal architecture in Sox2\textsuperscript{MUTANT};CALSL-NICD retinas, compared to Sox2\textsuperscript{MUTANT} retinas (C vs. D). (E-H) The integrity of the OLM is restored in Sox2\textsuperscript{MUTANT};CALSL-NICD retinas (H, arrowhead) compared to Sox2\textsuperscript{MUTANT} retinas (G, arrowhead). (I) Schematic comparison of Müller glia morphology and retinal organization between Sox2\textsuperscript{CONTROL}, Sox2\textsuperscript{MUTANT}, and Sox2\textsuperscript{MUTANT};CALSL-NICD retinas. Müller glia in Sox2\textsuperscript{MUTANT} retinas are reduced in number and are displaced to the ONL. NICD activity restores the number of Müller glia, but not their INL position. Müller glia – green; rod photoreceptors – red; amacrine cells – gray, pink. INL – inner nuclear layer. Scale bars: (H) 45 µm.
Figure 3.8. NICD activity does not restore the quiescent state of Müller glia in Sox2\textsuperscript{MUTANT};CALSL-NICD retinas.

(A-D) PCNA-expressing cells are present in both the INL and ONL of Sox2\textsuperscript{MUTANT};CALSL-NICD retinas (D). (E-H) PH3-expressing cells are found in Sox2\textsuperscript{MUTANT} (G) and Sox2\textsuperscript{MUTANT};CALSL-NICD retinas (H). (I) Daughter cell resulting from a dividing GLAST-dsRED2-labeled Müller glia (arrowhead) re-establishes radial morphology in Sox2\textsuperscript{MUTANT};CALSL-NICD retina. (J) The percent of dividing GLAST-dsRED2-positive Müller glia is significantly increased in Sox2\textsuperscript{MUTANT} and Sox2\textsuperscript{MUTANT};CALSL-NICD retinas (p < 0.0001) compared with Sox2\textsuperscript{CONTROL} and Sox2\textsuperscript{CONTROL};CALSL-NICD retinas. Image series in (I) were collected on 200 µm sections every 40 minutes over a 12 hour period. OLM – outer limiting membrane. *** - p < 0.0001. Scale bars: (H) 45 µm; (I) 30 µm.
SUPPLEMENTARY FIGURES

A. Sox2<sup>COND</sup> allele

B. Sox2<sup>COND/COND</sup>; CAGGCre-ER<sup>TM</sup>

4-OH-tamoxifen

Filter insert

Culture medium

C. PH3 Hoechst

D. PCNA Hoechst

E. SOX9

F. CRALBP

G. RHODOPSIN

H. INL

I. OPL

J. ONL

K. INL

L. OPL

M. INL

N. OPL

O. ONL

P. INL

Q. OPL

R. P0 + 2 DIV

P0 + 4 DIV

NF

NF CRALBP

S. Sox2<sup>CONTROL</sup>; Rosa26R

Sox2<sup>MUTANT</sup>; Rosa26R

Zap73

ZXO5

T. P0 + 5 DIV

Sox2<sup>CONTROL</sup>

Sox2<sup>MUTANT</sup>

CRALBP

U. P0 + 7 DIV

Sox2<sup>CONTROL</sup>

Sox2<sup>MUTANT</sup>
Figure S3.1. Temporal and spatial development of neuronal and glial cell types and CRE-mediated ablation of Sox2 in cultured retinas.

(A) Schematic diagrams of the Sox2-conditional allele and CAGGC4-Er$^{TM}$ transgene. (B) Schematic illustration of retinal explant culture technique and ablation of Sox2 using TM. (C-Q) Comparison of the proliferative status and differentiation of postnatal neural progenitor cells into temporally appropriate retinal cell types between P0 (C-G) and P5 (H-L) retinas in vivo versus Sox2$^{CONTROL}$ retinas isolated at P0, treated with TM and cultured for 5 days in vitro (M-Q). (C-G) At P0, neural progenitor cells, marked by PCNA (D) and SOX9 (E), are present throughout the NBL and express PH3 during cell division (C). (F and G) Müller glial cells and rod photoreceptors are not yet formed by P0, as evidenced by low levels of CRALBP (F) and RHODOPSIN (G) expression, respectively. (H-L) At P5, mitotic progenitor cells are significantly reduced in number in the central retina (H), but still persist in the peripheral retina (inset in H), while PCNA (I) and SOX9 (J) become restricted to the INL. By P5, Müller glial cells and rod photoreceptors have differentiated (K, L). (M-Q) Correct morphology and laminar localization of Müller glial cells and rod photoreceptors in cultured retinas. (R) Formation of the outer plexiform layer (OPL), marked by Neurofilament, during culture period and correct localization of MG marked by CRALBP. (S) Expression of b-galactosidase (LacZ) in Sox2$^{MUTANT};R26R$ retinas treated with TM and cultured for 2 days. Sox2 expression is downregulated in Sox2$^{MUTANT}$ retinas at day 2 of culture. (T, U) In control retinas, SOX2 is expressed in the INL and amacrine cells after 5 (T) or 7 (U) days in culture. Expression of SOX2 in Sox2$^{MUTANT}$ retinas cultured for 5 days (T) is either absent or detected in a small number of amacrine cells, indicating temporal variation in the efficiency of TM-mediated DNA recombination. Following a 7-day culture period, SOX2 expression is not detected in Sox2$^{MUTANT}$ retinas (U). In control retinas, CRALBP expression pattern reflects typical morphology of Müller glia at both 5 (T) and 7 (U) days in culture. Following a 5 day culture period, Müller glia in Sox2 mutant retinas are moderately or significantly disorganized (T), and after 7 days in culture, a subset of retinas display significant loss of Müller glial cells and retinal degeneration (U). DIV – days in vitro; INL – inner nuclear layer; NBL – neuroblast layer; NR – neural retina; ONL – outer nuclear layer; OPL – outer plexiform layer; RPE – retinal pigmented epithelium; TM – 4-hydroxytamoxifen; NF – NEUROFILAMENT. Scale bars: (Q, S) 65 µm; (U): 45 µm.
Figure S3.2. Fate-mapping analysis of RPCs in Sox2\textsuperscript{CONTROL} and Sox2\textsuperscript{MUTANT} retinas. (Fernandez-Bueno et al.) P1 Sox2\textsuperscript{CONTROL} and Sox2\textsuperscript{MUTANT} retinas electroporated with pCALNL-EGFP, cultured for 5 days and immunostained for markers of late-born cell types. No significant differences are detected in the numbers of labeled bipolar cells (CHX10, A-B), Müller glia (SOX9, C-D), or photoreceptors (CRX, E-F) (G).
Figure S3.3. Expression of markers of differentiating Müller glia in control retinas cultured for 5 days.
Expression of Müller glial cell markers was analyzed using immunohistochemistry on sections of TM-treated Sox2<sup>CONTROL</sup> retinas dissected at P0 and cultured for 5 days. (A-D) Expression of SOX2 in the INL (A, arrowheads) coincides with CRALBP (arrowheads in B, C and D – larger magnification image of C). (E-H) SOX2-expressing cells within INL (E, arrowheads) express PAX6 at lower levels (F and G, arrowheads). SOX2/PAX6 double-positive cells exhibit elongated cell body morphology of Müller glia (arrowheads in H – higher magnification image of G). (I-L) The subset of INL cells that express PAX6 (I, arrowheads) co-express Müller glial cell marker CRALBP (arrowheads in J, K and L – higher magnification image of K). (M-P) VIMENTIN (Histofine) (M) co-localizes with CRALBP (N) in cell bodies of Müller glia (O, P – higher magnification image of O). (Q-T) Expression of PCNA (Q) is detected in the majority of CRALBP-positive cells (R) within the INL (S, T – higher magnification image of S, arrowheads). (U-X) NESTIN (U) is enriched at the basal retinal boundary and localizes to Müller glial cell processes (W and X – higher magnification image of W, arrowheads), marked by CRALBP (V, W and X). Scale bars: (W) 45 µm; (X): 15 µm.
Figure S3.4. Expression of Müller glial cell markers is downregulated in Sox2\textsuperscript{MUTANT} retinas, but can be restored by activation of NOTCH1 signaling.

(A-L) Expression of PAX6 (A–D’), VIMENTIN (E–H) and NESTIN (I–L), marking cell bodies (A–H) and radial processes (I–L) of Müller glial cells in the INL, respectively, are analyzed in sections of Sox2\textsuperscript{CONTROL} (A, A’, E and I), Sox2\textsuperscript{CONTROL};CALSL-NICD (B, B’, F and J), Sox2\textsuperscript{MUTANT} (C, C’, G and K), and Sox2\textsuperscript{MUTANT};CALSL-NICD (D, D’, H and L) P0 retinas treated with TM and cultured for 5 days. (A–D’) Fewer PAX6-positive cells with elongated cell body morphology are detected in the INL of Sox2\textsuperscript{MUTANT} retinas (C and C’ – higher magnification image of C, arrowhead) compared to Sox2\textsuperscript{CONTROL} retinas (A and A’ – higher magnification image of A, arrowheads). Genetic activation of NICD causes a moderate expansion of PAX6 expression domain in Sox2\textsuperscript{CONTROL};CALSL-NICD retinas (B and B’ – higher magnification image of B). In Sox2\textsuperscript{MUTANT};CALSL-NICD retinas, PAX6-positive cells are increased in number (D and D’ – higher magnification image of D) and displaced towards the ONL (arrowhead in D). (E–H) Expression of VIMENTIN is reduced in Sox2\textsuperscript{MUTANT} retinas (G), compared to Sox2\textsuperscript{CONTROL} retinas (E), and is increased in Sox2\textsuperscript{CONTROL};CALSL-NICD (F) and Sox2\textsuperscript{MUTANT};CALSL-NICD retinas (H). (I–L) Expression of NESTIN, localized to Müller glial cell processes in control Sox2\textsuperscript{CONTROL} retinas (I), is reduced in Sox2\textsuperscript{MUTANT} retinas (K), and is increased in Sox2\textsuperscript{CONTROL};CALSL-NICD (J) and Sox2\textsuperscript{MUTANT};CALSL-NICD retinas (L). Scale bars: (D’) 15 µm; (L) 45 µm.
Figure S3.5. Co-expression of PAX6 and PCNA identifies dividing Müller glial cells in Sox2\textsuperscript{MUTANT} retinas. (Garcia-Feijoó et al.) Expression of PAX6 (A, B) and PCNA (C, D) was assessed in sections of TM-treated control (A, C, E and E') and Sox2\textsuperscript{MUTANT} retinas (B, D, F and F') at day 5 of culture. In Sox2\textsuperscript{CONTROL} retinas, co-expression of PAX6 and PCNA marks predominantly cell bodies of Müller glial cells in the INL (arrows in A, C and E), and occasional cells in the ONL (arrow in E' – higher magnification image of E). Many PAX6/PCNA double-positive elongated cells are found in the ONL of Sox2\textsuperscript{MUTANT} retinas (arrows in B, D, F and F' – higher magnification image of F). Cells with round cell body morphology and high PAX6 expression, located in the ONL (arrowheads in B, D, F and F' – higher magnification image of F), do not express PCNA and are found only in Sox2\textsuperscript{MUTANT} retinas. Scale bar: (F): 45\,\mu m; (F') 20\,\mu m.
**Figure S3.6. Visualization of Müller glial cells in cultured retinal slices.**

(A) Schematic illustration of the *in vitro* electroporation of postnatal retina. (B) Müller glial cell expresses GLASTp-DsRED2 3 days following electroporation at P0. (C) Slices of electroporated retinas are cultured *in vitro* for 4 days. (D-F) - larger magnification images of boxed area in C) Retinas were co-electroporated with pCIG2, encoding EGFP driven by CAG promoter, and GLASTp-dsRED2, marking Müller glial cells. Confocal image stacks in (D-F) were collected from 200 µm retinal slices cultured for 4 days (average intensity Z projections are shown). Scale bars: (F) 30 µm.
Figure S3.7. Fate-mapping analysis of pCRALBP-CreEGFP confirms its expression predominantly in postnatal MG.

(A-B) P1 retinas were co-electroporated with pCAG-Cre (A) or pCRALBP-CreEGFP (B) and Cre reporter construct, pCALNL-dsRED, and cultured for 5 days. In contrast to expression of the dsRED reporter predominantly in rod photoreceptors (PR) in the ONL of retinas electroporated with pCAG-Cre (arrow in A), the majority of dsRED-positive cells in retinas electroporated with pCRALBP-CreEGFP are MG (upper arrow in B). (C) The average number of dsRED-positive cells is significantly reduced in retinas electroporated with pCRALBP-CreEGFP (mean ± S.E.M.: 54.70 ± 4.733; n=10) compared to retinas electroporated with pCAG-Cre (mean ± S.E.M.: 125.1 ± 13.07; n=10), as determined by unpaired student t-test (p<0.0001), suggesting that CRALBP regulatory element is active in a subset of postnatal retinal progenitor cells. Each construct was used at a concentration of 1 µg/µl. (D) The proportion of dsRED-positive MG among all labeled cells is significantly increased in retinas electroporated with pCRALBP-CreEGFP (mean ± S.E.M.: 57.12 ± 2.02; n=9) compared to retinas electroporated with pCAG-Cre (mean ± S.E.M.: 19.65 ± 1.45; n=9) (p<0.0001), while the proportion of labeled photoreceptors (PR) is significantly reduced in retinas electroporated with pCRALBP-CreEGFP (mean ± S.E.M.: 28.88 ± 2.40 vs 68.48 ± 1.94; n=9) (p<0.0001). No significant difference was observed in the proportions of labeled bipolar and amacrine cells (BP/AC). (E-F) CRALBP-CreEGFP co-localizes with CRALBP in the INL. INL – inner nuclear layer; ONL – outer nuclear layer. Scale bar: (B) 65 µm.
Table S3.1. Representative frequencies of observed severe retinal lamination defects.

<table>
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<th>Days in culture starting at P0</th>
<th>Genotype</th>
<th>Proportion of pups with severe retinal phenotype</th>
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<td>1/9</td>
</tr>
<tr>
<td></td>
<td>$Sox2^{+/+}$;CAGG-CreER&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>1/10</td>
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<tr>
<td></td>
<td>$Sox2^{COND/COND}$;CAGG-CreER&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>13/16</td>
</tr>
<tr>
<td>7</td>
<td>$Sox2^{+/+}$, $Sox2^{+/COND}$, $Sox2^{COND/COND}$</td>
<td>1/5</td>
</tr>
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<td></td>
<td>$Sox2^{+/+}$;CAGG-CreER&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>$Sox2^{COND/COND}$;CAGG-CreER&lt;sup&gt;TM&lt;/sup&gt;</td>
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Chapter 4: SOX2 maintains the quiescence and survival of Müller glia in a dosage-dependent manner.

4.1 Introduction

The primary role for Müller glia in the retina is to maintain retinal homeostasis by providing trophic support to neurons as well as providing a structural scaffold via their radial morphology (Bringmann et al., 2006; Kriegstein and Alvarez-Buylla, 2009). Müller glia are also responsible for regulating the extracellular retinal environment and protecting neurons during stress through the release of neurotrophic factors and antioxidants. In contrast to the beneficial roles of Müller glia, in the case of most retinal diseases, they enter a state of reactive gliosis, which is characterized by an upregulation of the intermediate filament, glial fibrillary acidic protein (GFAP). While early stages of gliosis are generally neuroprotective, advanced gliosis results in changes in the retinal environment and release of proinflammatory cytokines, leading to neuronal cell death (Bringmann et al., 2006). In addition, during advanced gliosis, the Müller glia become proliferative, leading to glial scarring and perpetuates retinal disease and neurodegeneration (Bringmann et al., 2009). Because Müller glia contact all seven types of retinal neurons and have a crucial role in nearly every retinal disease (Bringmann et al., 2009), they are promising candidates for therapeutic targets, and understanding their function will be critical for implementing potential therapies.

In addition to their homeostatic functions as glial cells, Müller glia retain several properties of progenitor cells. First, they resemble neural progenitors in their radial morphology. Secondly, they maintain expression of a number of progenitor genes throughout adulthood, including Sox2, Sox9 and Notch signaling components (Notch1, Hes1/5) (Roesch et al., 2008; Jadhav et al., 2009). Furthermore, a subset of Müller cells retain expression of the progenitor markers Pax6 and Chx10. A cDNA microarray revealed that retinal progenitors share a greater similarity in their gene expression profile with Müller
glia (43%) than with retinal neurons (Livesey et al., 2004). The strong overlap in gene expression between Müller glia and progenitors suggests that they are a type of late progenitor. Unlike neurons, Müller glia do not irreversibly exit the cell cycle. Under certain circumstances (e.g., injury), Müller glia may become reactive and undergo cell division. As Müller glia transition from quiescence to active proliferation, their expression profile of cell cycle components is altered (Bringmann et al., 2009). The ability of Müller glia to re-enter the cell cycle is very limited in mammalian retinas but is particularly robust in non-mammalian vertebrates. However, mammalian Müller glia can be stimulated to proliferate and regenerate neurons under certain circumstances. For example, Müller glia can be induced to proliferate and form bipolar or rod cells following NMDA and growth factor administration in rats (Ooto et al., 2004) or to form amacrine cells under the same conditions in mice (Karl et al., 2008). Due to their potential for regenerating damaged neurons, it is important to study the molecular mechanisms governing Müller glia cell cycle entry and neurogenic potential.

SOX2 is important for early eye development and is thought to regulate the expression of Rax, a gene critical for eye morphogenesis in *Xenopus* (Danno et al., 2008). In the mouse eye, SOX2 is expressed in proliferating neural progenitors and is downregulated in differentiating neurons (Ellis et al., 2004; Taranova et al., 2006a; Matsushima et al., 2011). When Sox2 is ablated in retinal progenitors by a retina-specific *Pax6* enhancer-driven *Cre*, there is a loss of progenitor cell competence and a cell fate conversion to non-neural ciliary epithelium (Taranova et al., 2006a; Matsushima et al., 2011). Humans with SOX2 mutations display a range of abnormalities, including microphthalmia (small eyes), anophthalmia (absence of eyes), pituitary defects, and hypothalamic hamartomas (Fantes et al., 2003; Sisodiya et al., 2006). Due to the requirement of SOX2 in neural progenitors of the eye and other parts of the CNS, future studies of the role of SOX2 using the retina as a model will potentially lead to a better understanding of human developmental disorders and neurological diseases.
SOX2 is essential for maintaining populations of adult progenitor cells in several adult tissues (Ellis et al., 2004; Brazel et al., 2005; Driskell et al., 2009; Que et al., 2009; Arnold et al., 2011). Moreover, SOX2 has also been shown to be important in a human Müller glial cell line (Bhatia et al., 2011). Specifically, the downregulation of SOX2 by transfection of shRNA constructs into the Müller glial cell line induced the cells to rapidly adopt a neuronal-like morphology due to the upregulation of several neuronal genes and a coincident loss of glial-specific identity. Moreover, the number of proliferating cells was reduced from 15% to 1%, and SOX2 silencing eventually resulted in apoptosis, indicating a role for SOX2 in Müller glial survival. In addition to SOX2’s importance to adult stem cell maintenance, it may also play a role in the Müller glial cell fate. Retroviral introduction of SOX2 into E17 mouse retinal explants induced Müller glial and amacrine cell production at the expense of rod photoreceptors (Lin et al., 2009). Therefore, SOX2 is critical for the generation and maintenance of adult stem cell populations.

Müller glia display qualities of quiescent cells in the sense that they are in a non-dividing state, but are capable of re-entering the active phases of the cell cycle when induced by proliferative signals. This quiescent state is important for preventing stem cell exhaustion and senescence (Mira et al., 2010). In this study, we examined whether the requirement for SOX2 in the maintenance of Müller glial cell quiescence is progenitor cell-dependent or a Müller glia-dependent requirement. We addressed this question by analyzing proliferation following a postnatal Müller glia-specific ablation of SOX2, and comparing it to proliferation in retinas that have hypomorphic and wild type levels of SOX2 in the retina. We found that retinas with hypomorphic levels of SOX2 prior to P5 exhibited increased Müller glia proliferation later in postnatal retinal cultures, and this effect was specific to the mouse strain background. Also, we report that when P5 retinas were exposed to cellular stress in vitro, the Müller glia were more likely to proliferate if SOX2 was ablated, indicating that SOX2 is important for regulating the switch from non-proliferative to proliferative gliosis. Lastly, we show that the fate of proliferating Müller
GLIA is most likely cell death. These results reveal the importance of SOX2 dosage levels in the maintenance of Müller glial cell quiescence, gliosis, and survival in the postnatal retina.

4.2 Results

4.2.1 Hypomorphic SOX2 levels result in phenotypic abnormalities in postnatal mice.

In order to study the role of SOX2 in postnatal Müller glia, we used mice carrying the Sox2<sup>C</sup> and Glast-CreER<sup>T2</sup> alleles, allowing for the excision of the Sox2 gene in a temporally controlled manner (Fig. 4.1A). Previous evidence shows that SOX2 is critical for retinal progenitor cells in a dosage-specific manner (Taranova et al., 2006a; Langer et al., 2012). Specifically, mice with hypomorphic levels of SOX2 (15-40% of wild type) display abnormalities, such as retinal thinning, rosettes in the photoreceptor layer, and a range of eye size defects, ranging from mild microphthalmia to severe anophthalmia. Since the Sox2<sup>C</sup> allele is flanked by LoxP sites and contains a Neo sequence, we wanted to assess whether these insertions affected the expression of Sox2 and resulted in variable SOX2 levels in pre-experimental conditions. In order to analyze SOX2 protein levels in animals carrying the Sox2<sup>C</sup> allele for postnatal retina experiments, we performed Western analyses on isolated P5 retinas in different mouse strains of the following genotypes: Sox2<sup>+/+</sup>, Sox2<sup>+/C</sup>, and Sox2<sup>C/C</sup> (Fig. 4.2). The protein levels in each eye were measured independently to account for a potential left-right asymmetry in SOX2 expression, similar to asymmetric eye abnormalities found in humans with hypomorphic levels of SOX2 (Schneider et al., 2009; Gerth-Kahlert et al., 2013). In the C57BL/6J strain, the Sox2<sup>+/C</sup> and Sox2<sup>C/C</sup> eyes had 94.1% (p=0.56) and 49.0% (p<0.005) of normalized Sox2<sup>+/+</sup> levels, respectively (Fig. 4.2A, B, n=4). Therefore, the conditional allele results in hypomorphic Sox2 expression, perhaps as a result of the presence of the exogenous 3’ and 5’ flanking sequences in this allele.

Because the Sox2<sup>C/C</sup> retinas had significantly lower SOX2 levels, we sought to determine if these animals displayed any previously characterized or novel SOX2 hypomorphic phenotypes. The Sox2<sup>C/C</sup>
animals were visibly smaller compared to their littermates (10 of 16 Sox2\(^{C/C}\) animals compared to 1 of 11 in Sox2\(^{+/+}\) animals, Table 4.1). The mean weight was 2.3 g for Sox2\(^{C/C}\) animals at P5, compared to 3.2 g for both Sox2\(^{+/+}\) and Sox2\(^{+/C}\) animals (Graph 4.1, p<0.05 for Sox2\(^{C/C}\) vs. Sox2\(^{+/+}\) and Sox2\(^{+/C}\)). The actual number of Sox2\(^{C/C}\) animals born was also far less than the expected Mendelian ratio, and even fewer animals survived to the experimental age, P5 (data not shown). These size defects have not yet been described in Sox2 hypomorphic animals. In addition, eye defects, such as microphthalmia, anophthalmia, and coloboma (failure of the ventral optic fissure to fuse) have been previously characterized and associated with SOX2 mutations in humans and mice (Taranova et al., 2006a; Schneider et al., 2009). In this analysis, 1 of 13 Sox2\(^{C/C}\) animals and 1 of 16 Sox2\(^{+/C}\) animals displayed unilateral microphthalmia, compared to 0 of 9 Sox2\(^{+/+}\) animals. Moreover, 1 of 13 Sox2\(^{C/C}\) and 1 of 16 Sox2\(^{+/C}\) animals displayed coloboma, compared to 0 of 9 in Sox2\(^{+/+}\) animals (Table 4.1). These results suggest that the Sox2\(^{C/C}\) animals have lower SOX2 levels compared to Sox2\(^{+/+}\) animals, and a subset display previously characterized and novel SOX2 hypomorphic phenotypes.

While microphthalmia and anophthalmia have been associated with SOX2 mutations in mice (Taranova et al., 2006b), the C57BL/6J strain of mice has been known to exhibit a high incidence of microphthalmia (Smith et al., 1994). Because of this, we wanted to determine if the observed defects and reduced SOX2 levels in the Sox2\(^{C/C}\) animals were specific to the C57BL/6J strain. If the SOX2 levels in Sox2\(^{C/C}\) animals of a mixed strain background were found to be comparable to Sox2\(^{+/+}\) animals, the results of a Müller glia-specific ablation of SOX2 would be directly related to the genetic manipulation and therefore more meaningful. To perform this analysis, we generated Sox2\(^{+/C}\); Glast-Cre\(^{ER}\)\(^{T2}\) breeder mice that were 50% C57BL/6J and 50% CD-1. These mice were mated to generate Sox2\(^{+/+}\), Sox2\(^{+/C}\), and Sox2\(^{C/C}\) animals of a mixed background (B6/CD1). Westerns were performed on isolated P5 retinas in order to determine the relative levels of SOX2 in each genotype (Fig. 4.2C, D). In these mixed-strain mice, the levels of SOX2 in Sox2\(^{+/C}\) and Sox2\(^{C/C}\) retinas were not significantly lower than levels in Sox2\(^{+/+}\)
retinas (92.3% (p=0.30) and 80.1% (p=0.06), respectively). Thus, the relative SOX2 levels in Sox2C/C retinas were greatly increased in the mixed B6/CD1 background compared to the C57BL/6J background, making it a better model for studying a temporally controlled ablation of SOX2. To assess if the mixed background animals displayed SOX2 hypomorphic phenotypes, we examined them at P5. Of the 15 B6/CD1 Sox2C/C animals, none displayed small body size (3.3 g, vs. 3.5 g in Sox2+/+) or unilateral microphthalmia, but 3 of 12 displayed mild to severe coloboma (Table 4.1). None of the B6/CD1 Sox2+/+ or Sox2+/C animals displayed any of these abnormalities. These results suggest that the degree of SOX2 hypomorphism is strain-dependent. However, while B6/CD1 Sox2C/C animals had SOX2 levels that were comparable to Sox2+/+ with increased body size and symmetrical eyes, a subset still displayed coloboma.

The Sox2C/C animals in both strain backgrounds were highly variable with respect to the exhibited range of gross abnormalities. To determine if there is a correlation between phenotype and SOX2 levels, we compared the SOX2 protein levels of Sox2C/C animals with and without abnormalities relative to the levels in Sox2+/+ control mice (Table 4.2). In the C57BL/6J animals, 2 of 4 Sox2C/C animals were drastically smaller than their littermates; yet, the SOX2 protein levels relative to Sox2+/+ were around 50%, approximately the same as the other 2 Sox2C/C animals, which were regularly sized. Of the B6/CD1 animals, the Sox2C/C animal with severe coloboma had approximately the same relative SOX2 level as Sox2C/C animals with normal eyes (=85% of Sox2+/+ levels). Despite the variability of phenotypic abnormalities exhibited by Sox2C/C animals, the SOX2 levels remained relatively constant in Sox2C/C mice within each strain, indicating that there must be variability between individuals of the same genotype within a given mouse strain.

Together, these results indicate that the SOX2 phenotypic abnormalities are mouse strain-specific. The C57BL/6J Sox2C/C mice exhibit roughly half of the SOX2 levels as the Sox2+/+ mice, and most are significantly smaller. The B6/CD1 Sox2C/C mice exhibit SOX2 levels comparable to the Sox2+/+ mice,
and they do not have size defects, but some have eye abnormalities. These data helped interpret the results in the subsequent analyses of the role of SOX2 in postnatal proliferation.

4.2.2 Glast-CreERT2 is localized to Müller glia and efficiently ablates SOX2

Our previous work has shown that when SOX2 is ablated ubiquitously from early postnatal progenitor cells, a subset of nascent Müller glial cells undergo a cell division (Surzenko et al., 2013). To determine if this effect is specific to the loss of SOX2 in Müller glial cells or to embryonic hypomorphic levels of SOX2, we utilized C57BL/6J mice carrying the following alleles: Sox2C, the Müller glia-specific tamoxifen-inducible Glast-CreERT2 allele, and the reporter Ai9 allele, to give Sox2C/C; Glast-CreERT2; Ai9 animals (Fig. 4.1A). In addition to the control Sox2+/C; Glast-CreERT2, we used the Sox2C/C; Ai9 animals to determine possible effects of hypomorphism on proliferation. Isolated P5 retinas were exposed to 4-OH-tamoxifen in vitro for 24 hours to activate CRE and then cultured for 5 days (Fig. 4.1B). After fixation, retinas were analyzed by immunofluorescence for expression of CRALBP, a Müller glia cell marker. In the Sox2+/C; Glast-CreERT2; Ai9 retinas, the Ai9-tdTomato-expressing cells co-expressed CRALBP (Fig. 4.3A-C); thus, the CRE activity is specific to Müller glia. Furthermore, in control Sox2+/C; Glast-CreERT2; Ai9 retinas, Ai9-tdTomato marks the SOX2-expressing Müller glia in the inner nuclear layer (INL, purple arrowheads in Fig. 4.3D-F). Importantly, SOX2 is also expressed in starburst amacrine cells in the INL and ganglion cell layer (GCL) (yellow arrowhead), but Ai9-tdTomato is absent from these cells and from the other neuronal cell layers in the retina, further indicating the specificity of Glast-CreERT2 activity. In Sox2C/C; Ai9 retinas, Ai9-tdTomato is absent from the retina due to a lack of CRE activity, and SOX2 expression is maintained in Müller glia (Fig. 4.3G-I). In the Sox2C/C; Glast-CreERT2; Ai9 retinas, Ai9-tdTomato is localized to the Müller glial cell bodies, where SOX2 has been mosaically eliminated (purple arrowheads in Fig. 4.3J-L). SOX2 expression is maintained in the starburst amacrine cells, where Glast-CreERT2 is not expressed (yellow arrowheads in Fig. 4.3J-L). These results indicate that the experimental arrangement permit the comparison of the dosage effects of control (Sox2+/C; Glast-CreERT2; Ai9 or Sox2+/C; Glast-
CreER$^{T2}$; Ai9), SOX2 hypomorphic (Sox2$^{C/C}$; Ai9), and SOX2-ablated (Sox2$^{C/C}$; Glast-CreER$^{T2}$; Ai9) Müller glia in the postnatal retina.

4.2.3 Increased proliferation in retinas with hypomorphic SOX2 levels

To assess whether SOX2 dosage level affects proliferation in the postnatal retina, we compared the incorporation of the S-phase marker and thymidine analogue EdU in Sox2$^{+/C}$; Glast-CreER$^{T2}$, Sox2$^{C/C}$, and Sox2$^{C/C}$; Glast-CreER$^{T2}$ retinas. Retinas from C57BL6/J mice were exposed to EdU during the 5 day experimental period (Fig. 4.1B) before fixation and immunohistochemistry for CRALBP (Fig. 4.4). In the central areas of Sox2$^{+/C}$; Glast-CreER$^{T2}$ retinas, there were very few EdU cells, and the Müller glia appeared morphologically normal, with processes extending from the apical to basal sides of the retina (Fig. 4.4A-C). In the Sox2$^{C/C}$ retinas, there was an increase in EdU-incorporated cells that were identified as Müller glia based on colocalization with CRALBP (Fig. 4.4D-F, D’-F’). In addition, the Sox2$^{C/C}$; Glast-CreER$^{T2}$ retinas had an increased number of proliferating Müller glial cells (Fig. 4.4G-I, G’-I’). To further support the identity of these cells, Müller glia, as marked by expression of GS, SOX9, and CRALBP, also coexpressed proliferation markers PH3 (phosphohistone H3), Ki67, PAX6, and PCNA (proliferating cell nuclear antigen) (data not shown). Thus, both of the genotypes with hypomorphic levels of SOX2 (Sox2$^{C/C}$ and Sox2$^{C/C}$; Glast-CreER$^{T2}$) had increased Müller cell proliferation.

To quantify the number of proliferating cells within the retinas of Sox2$^{+/C}$; Glast-CreER$^{T2}$, Sox2$^{C/C}$, and Sox2$^{C/C}$; Glast-CreER$^{T2}$ animals, consecutive photos were taken of retinas that were exposed to EdU during the entire experimental period (i.e., from P5 to P10) (Fig. 4.5). Since retinal differentiation occurs in a central to peripheral gradient, progenitors continue to proliferate in the periphery during the experimental period. By defining the boundaries for the peripheral and semi-peripheral areas (marked by the blue brackets in Fig. 4.5), the remaining retina was defined as central retina (green bracket in Fig. 4.5). EdU-positive cells were counted in the central retina areas, with each data point representing the number of EdU-positive cells per retina microtome slice (Fig. 4.5B, C). Five consecutive slices in this
central area were evaluated for each retina. Retinas from both C57BL/6J and B6/CD1 backgrounds were counted for comparison. In the central retinas of C57BL/6J Sox2\(^{+/c}\); Glast-Cre\(^{ERT2}\) animals, the number of EdU-positive cells per slice was always less than 10 (mean, 3.2). In contrast, in Sox2\(^{c/c}\) and Sox2\(^{c/c}\); Glast-Cre\(^{ERT2}\) central retinas, the number of EdU-positive cells per slice was extremely variable, with a range of 0-61 (mean, 23.0) and 0-40 (mean, 13.5), respectively. The Sox2\(^{c/c}\) retinas had significantly more proliferation than the Sox2\(^{+/c}\); Glast-Cre\(^{ERT2}\) retinas (p<0.05). Despite the trend of an increased proliferation in the Sox2\(^{c/c}\); Glast-Cre\(^{ERT2}\) retinas compared to the Sox2\(^{+/c}\); Glast-Cre\(^{ERT2}\) retinas, there was not a significant difference (p=0.27) Among the 4 individual retinas counted for each genotype, 3 of the 4 of Sox2\(^{c/c}\) retinas and 3 of the 4 Sox2\(^{c/c}\); Glast-Cre\(^{ERT2}\) retinas had highly proliferative areas (defined as >10 EdU-positive cells per slice). These results indicate that hypomorphic levels of SOX2 result in increased Müller cell proliferation. Although the Sox2\(^{c/c}\); Glast-Cre\(^{ERT2}\) retinas had a lower mean and range of EdU-positive cells than Sox2\(^{c/c}\) retinas, they were not significantly decreased (p=0.31). Thus, in the C57BL/6J background, ablation of SOX2 does not enhance the increase in proliferation that occurs as a result of SOX2 hypomorphism. This analysis also (i) illustrates the variability within the proliferative areas of Sox2\(^{c/c}\) and Sox2\(^{c/c}\); Glast-Cre\(^{ERT2}\) retinas due to the wide range of EdU-positive cells per slice, and (ii) strongly suggests that the increased proliferation in these retinas is most likely due to reduced SOX2 levels in progenitors from pre-experimental conditions (i.e., embryonic or early postnatal), rather than SOX2 ablation from Müller glia at P5.

As mentioned above, in contrast to C57BL/6J animals, SOX2 protein levels in Sox2\(^{c/c}\) retinas from mixed strain (B6/CD1) P5 mice were not significantly lower than in Sox2\(^{+/+}\) animals. Given that these animals did not exhibit SOX2 hypomorphism, it was important to determine if a Müller glia-specific ablation of SOX2 would result in Müller glia proliferation. This analysis was performed by again comparing proliferation in Sox2\(^{+/+}\); Glast-Cre\(^{ERT2}\), Sox2\(^{c/c}\), and Sox2\(^{c/c}\); Glast-Cre\(^{ERT2}\) retinas. In contrast to the C57BL/6J retinas, in the B6/CD1 retinas, the number of proliferative areas in both Sox2\(^{c/c}\) and
Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas was not increased relative to Sox2<sup>+/+</sup>; Glast-CreER<sup>T2</sup> retinas (p=0.49 and 0.80, respectively) (Fig. 4.5C). Among the 4 individual retinas counted for each genotype, only 1 of the 4 Sox2<sup>C/C</sup> retinas and 0 of the 4 Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas had highly proliferative areas.

In summary, when SOX2 levels are not significantly different between genotypes in the pre-experimental period (i.e., in the B6/CD1 background), Müller glia maintain quiescence postnatally, regardless of whether SOX2 is ablated. However, when SOX2 levels are decreased pre-experimentally (i.e., in C57BL/6J retinas), the Müller glia proliferate postnatally, and complete ablation of SOX2 does not increase proliferation in the hypomorphic background. These results point to a mechanism in which the SOX2 levels at early developmental time points are critical for regulating Müller glia proliferation during late developmental time points.

4.2.4 Culture stress increases proliferation in Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas

When SOX2 is ablated from C57BL/6J Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas at P5 in vivo, the Müller glia are non-proliferative after 5 days (data not shown), indicating that there must be differences between the in vivo and in vitro environment that inhibit or promote Müller glia proliferation, respectively. Following the removal of retinas from their natural environment to an in vitro culture system, the retinas undergo a great deal of stress. In fact, retinal culture systems have been utilized as injury models in rat and human explants (Osakada et al., 2007; Fernandez-Bueno et al., 2012). Cellular stress is manifested as increased cell death (data not shown), photoreceptor layer rosette formations (Fig. 4.6K, N, P, S), and a gliotic response in Müller glia, indicated by upregulation of glial fibrillary acidic protein (GFAP) in Müller cell processes. One role of Müller glia is to respond to environmental stress by undergoing gliosis, so the culture environment primes the Müller glia to modify their transcriptional profile to respond to stress. For these reasons, we used retinal cultures as an injury model in order to study the role of SOX2 during gliosis and neuronal stress. In our retinal culture system, retinas are
observed to exhibit “stressed” areas, as defined by GFAP upregulation and rosettes, and “non-stressed” areas that lack these characteristics and can act as an internal control within the same tissue.

In order to determine if gliosis is a prerequisite to proliferation in the C57BL/6J SOX2-deficient Müller cells, retinas were exposed to EdU throughout the culture period (Fig. 4.1B), and areas with and without cellular stress were analyzed independently to determine where proliferation was most likely to occur (Fig. 4.6). Only the central retina was examined to exclude the possibility of proliferating progenitor cells in the periphery. In non-stressed areas of control Sox2+/+; Glast-CreER<sup>T2</sup>; Ai9 and SOX2-deficient Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup>; Ai9 retinas, there is a lack of GFAP expression in Müller glial cell processes, and an absence of cells that incorporated EdU (Fig. 4.6A-J). In the stressed areas of these retinas, there is an upregulation of GFAP in Müller glial processes and the formation of rod rosettes (Fig. 4.6K-T). Despite the presence of GFAP in the Sox2<sup>+/+</sup>; Glast-CreER<sup>T2</sup>; Ai9 retinas, the Müller glia do not proliferate, based on their lack of EdU incorporation, indicating that they are in a state of non-proliferative gliosis (Fig. 4.6K-O). In contrast, in the stressed areas of Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup>; Ai9 retinas, many of the Müller glial cells entered the cell cycle, as determined by EdU incorporation in Ai9-tdTomato- and GFAP-expressing cells (Fig. 4.6P-T). In order to quantify the proliferation in stressed areas, Ki67-positive cells were counted in non-stressed and stressed central regions of both Sox- control (includes both genotypes Sox2<sup>+/+</sup>; Glast-CreER<sup>T2</sup> and Sox2<sup>+/+</sup>; Glast-CreER<sup>T2</sup>) and Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas. In the Sox2 controls, there was a modest increase in the stressed regions (mean, 1.56 cells per area) compared to the non-stressed regions (mean, 0.76 cells per area), but the difference was not significant (p=0.56). Only the stressed regions of Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas exhibited an increase in proliferation (mean, 2.6 cells per area) compared to the non-stressed regions (mean, 1.16 cells per area) (p<0.05). The statistical model (random coefficient) with stress level and genotype main effects showed a significant difference in the number of cells for stressed versus unstressed areas (average of 1.12 higher for stressed, p<0.005), and borderline difference in Sox2-control versus Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup>
genotypes (p=0.05). Stratified analyses showed that the difference in non-stressed vs stressed areas was primarily in the Sox2<sup>C/C</sup>; Glast-Cre<sup>E2</sup> retinas. In summary, in both genotypes, the stressed areas had a higher number of proliferating cells, but the difference was only significant in the Sox2<sup>C/C</sup>; Glast-Cre<sup>E2</sup> retinas. Therefore, the external stress of an in vitro environment induced SOX2-deficient Müller glia to switch from non-proliferative gliosis to a more advanced form of proliferative gliosis.

### 4.2.5 Proliferating Müller glia exit the cell cycle, and most undergo cell death

SOX2 has been shown to be important for maintaining progenitor cell identity and for proper differentiation following terminal cell divisions (Pevny and Nicolis, 2010). In order to assess whether SOX2 is important for maintaining Müller glial cell identity and stem cell potential, we determined the fate of the glial cells that proliferated following the ablation of SOX2. This analysis was performed by tracing the fate of these cells after exposing the retinal cultures to a 4 hour pulse of EdU on the fifth day in vitro followed by an additional 2 day culture period (Fig. 4.1C). We compared the identity of the EdU-positive cells that were fixed immediately following the EdU pulse (P5+5 DIV(days in vitro)) to those that were cultured for 2 additional days before fixation (P5+7 DIV) (Fig. 4.7). At P5+5 DIV, EdU-positive cells in Sox2<sup>C/C</sup>; Glast-Cre<sup>E2</sup> retinas had the characteristics of elongated nuclei and colocalization with Glutamine Synthetase (GS) and SOX9, confirming their Müller glial identity (Fig. 4.7A-D, K-M, O). These EdU-positive cells expressed Ki67, indicating that they were in the cell cycle (Fig. 4.7F-H, J). Moreover, these cells were in different stages of the cell cycle, exhibiting varying levels of CyclinD3, which fluctuate with the stages of the cell cycle (Fig. 4.7F-I). These EdU-positive cells lacked neuronal-specific markers, such as the bipolar cell markers CHX10 and ISLET-1 (Fig. 4.7A-C, E, K-N). Two days after the EdU pulse (P5+7 DIV), the EdU-positive cells exited the cell cycle, based on their lack of expression of Ki67 and CyclinD3 (Fig. 4.7U-Y). In addition, most of the EdU-positive cells lost expression of GS and SOX9 (Fig. 4.7P-S, Z-BB, DD) and lacked CHX10 and ISLET-1, indicating that they did not gain neuronal
characteristics (Fig. 4.7P-R, T, Z-CC). Although the EdU cells were negative for cleaved CASPASE-3 and TUNEL, their round morphology and pyknotic nuclei indicate that these cells are most likely dead or dying (Fig. 4.7R, W, BB). However, a small subset of EdU-positive cells survived and maintained a glial identity, which was concluded based on their maintained expression of SOX9 (Fig. 4.7AA’-DD’). From these results, we conclude that most of the SOX2-deficient Müller glia terminally divided, lost their glial identity, and underwent cell death. These results suggest a role for SOX2 in maintaining quiescence and survival in early postnatal Müller glial cells.

4.3 Discussion

In this study, we describe how exposure to low levels of SOX2 in the retina during embryonic or early postnatal stages is critical for maintaining quiescence in Müller glia in later stages of postnatal retinal development. We show that Sox2<sup>C/C</sup> animals have hypomorphic levels of SOX2 at P5 in the C57BL/6J mouse strain, but that in the mixed strain (B6/CD1), Sox2<sup>C/C</sup> animals do not have significantly lower SOX2 levels compared to Sox2<sup>+/+</sup> animals. In addition, C57BL/6J Sox2<sup>C/C</sup> mice have a smaller body size and increased Müller glia proliferation when the retinas are cultured in vitro. The P5 ablation of SOX2 in Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas does not increase the Müller glia proliferation relative to what is observed in Sox2<sup>C/C</sup> mice, indicating that appropriate SOX2 levels are more important before this time point. The proliferating Müller glial cells in Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas were found in areas of high gliosis, and very little to no proliferation was found in areas of low gliosis in Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas or in any area of Sox2<sup>+/+</sup>; Glast-CreER<sup>T2</sup> retinas. Lastly, fate tracing revealed that the majority of proliferating Müller glial cells in the Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas died, while a smaller subset maintained a Müller glial identity. Together, these results provide evidence for a model of Müller glia homeostasis in which the combination of many factors, including precise levels of SOX2 and its activity during specific developmental time points, as well as exposure to environmental stresses, all appear to play a role in determining the outcome of the proliferation and survival of Müller glial cells.
SOX2 has been shown to be critical for eye development in a gene-dosage specific manner (Taranova et al., 2006a; Langer et al., 2012). Mice with modified Sox2 alleles that express 15-40% of wild type SOX2 levels exhibit reduced proliferation, rosette formations, and coloboma in embryonic retinas. In adults, hypomorphic animals display microphthalmia or anophthalmia, thinner retinas, rosette formations, GFAP upregulation, and underdeveloped optic nerves. Consistent with these findings, we have found that a subset of Sox2<sup>C/C</sup> eyes were microphthalmic (C57BL6/J) and exhibited coloboma (C57BL6/J and B6/CD1). In addition, we found that Sox2<sup>C/C</sup> animals have a smaller body weight than their wild type littermates, which has not been previously reported. One of the hypomorphic alleles used in Taranova et al., 2006 is Sox2<sup>Lp</sup>, which resembles the Sox2<sup>C</sup> allele, except for the lack of a LoxP site at the 5’ end. Similar to Sox2<sup>C/C</sup> animals, Sox2<sup>Lp/Lp</sup> animals have 50% SOX2 mRNA levels based on Northern blots from the E14 brain (Taranova et al., 2006b). However, these animals were reported to be born in Mendelian ratios and to be morphologically indistinguishable from wild type mice. The lack of abnormalities in these mice can be explained by differences in the genetic background, since these animals were from a mixed strain of mice from the original chimera (129/SV and C57BL/6J). Perhaps Sox2<sup>Lp/Lp</sup> mice are healthier due to their mixed background, similar to the mixed background B6/CD1 mice analyzed in this study. In contrast to C57BL/6J Sox2<sup>C/C</sup> animals, B6/CD1 Sox2<sup>C/C</sup> mice had a normal body size and were born in expected Mendelian ratios. The results of the analyses in this chapter are consistent with the correlations between SOX2 levels and phenotypes that were observed in previous studies of SOX2 hypomorphs and indicate the severity of the phenotypes is strain-dependent.

SOX2 is critical for neural stem cell proliferation, survival, and neurogenesis (Ellis et al., 2004; Ferri et al., 2004; Favaro et al., 2009; Pevny and Nicolis, 2010). Disruption of SOX2 results in abnormal proliferation and differentiation in both embryonic and adult progenitor cells. In the above experiments, we addressed whether SOX2 was important for the adult stem cell population in the retina, the Müller glia. We found that retinas with hypomorphic levels of SOX2 were more likely to exhibit Müller glial cell
proliferation postnatally *in vitro*. The increased proliferation following downregulation of SOX2 seems counterintuitive for a stem cell factor, but there are plausible explanations. The first possibility is that SOX2 maintains the status of Müller glia as an adult stem cell population, and hypomorphic SOX2 levels are insufficient for maintaining their stem cell potential through quiescence. As a result, the Müller glia that were arrested in G0 phase (i.e., quiescent) proceed through the cell cycle and eventually die. Adult Müller glia represent a population of adult stem cells due to the maintenance of a progenitor cell transcriptional profile (Livesey et al., 2004). Because of their status as a “mature” progenitor cell population, the second explanation is that the proliferating cells in the Sox2<sup>C/C</sup> retinas are derived from retinal progenitors that were unable to properly exit the cell cycle to become mature, quiescent glial cells. To support this latter theory of an increased proportion of cells in the cell cycle, there is evidence that loss of SOX2 in embryonic retinal progenitors causes an increase in cell cycle length (Heavner, in review). These results support previous evidence of the importance of SOX2 for proper maintenance of proliferative and differentiation dynamics in an adult stem cell population (Ellis et al., 2004; Brazel et al., 2005; Driskell et al., 2009; Que et al., 2009; Arnold et al., 2011).

Proper eye formation and retinal progenitor cell maintenance depends on a regulation of SOX2 expression at critical developmental time points and specific dosage levels (Kopp et al., 2008; Langer et al., 2012). SOX2 is critical to retinal development during early embryonic stages and conferring neural competence and proliferation in embryonic retinal progenitor cells (Taranova et al., 2006a; Matsushima et al., 2011). However, when SOX2 is ablated from P5 Müller glia in Sox2<sup>C/C</sup>; *Glast-CreERT<sup>2</sup>* retinas, there is no increase in proliferation compared to the Sox2<sup>C/C</sup> retinas; rather, both of these genotypes exhibit a trend of increased proliferation relative to Sox2<sup>+/+</sup>; *Glast-CreERT<sup>2</sup>* retinas. These results indicate that early hypomorphic levels of SOX2 predispose Müller glia to proliferation after P5. Ablation of SOX2 in Müller glia that already have reduced SOX2 levels has little effect on the number of proliferating cells. In fact, there may be a decrease in proliferation following SOX2 ablation, compared to Sox2<sup>C/C</sup>. This would lead
us to propose a model in which reduced SOX2 levels prevent proper cell cycle exit and continuous expression of cell cycle markers, but complete SOX2 ablation results in cell cycle arrest and eventually death (Fig. 4.7). Further analysis will be needed to determine whether there is a difference in the proliferation in the Sox2\textsuperscript{C/C}; Glast-CreER\textsuperscript{T2} retinas compared to Sox2\textsuperscript{C/C} retinas. Thus, the loss of this critical stem cell factor does not increase the proliferative capacity of the adult stem cell population, which is consistent with previous studies of the requirement of SOX2 for the maintenance of proliferation in a human Müller glial cell line (Bhatia et al., 2011). Furthermore, when SOX2 levels in Sox2\textsuperscript{C/C} animals resemble wild type levels, as is the case in the B6/CD1 strain, there is no increase in proliferation in either the Sox2\textsuperscript{C/C} or Sox2\textsuperscript{C/C}; Glast-CreER\textsuperscript{T2} retinas. These data further support the hypothesis that SOX2 levels in during early retinal development stages determine whether the Müller glia will be proliferative later in development.

Together, the results presented in this chapter shed light on the essential role of SOX2 in Müller glia and of the importance of SOX2 expression at specific developmental time points and appropriate protein levels. In addition to its requirement for Müller glial cell maturation, quiescence, and survival, SOX2 plays an important role following retinal injury by regulating the switch between non-proliferative and proliferative gliosis, which presumably prevents further retinal damage through glial scar formation. Although the precise mechanisms regulating Müller glial cell cycle entry are unknown, this study draws a link between early developmental processes and adult retinal responses to injury. By understanding this connection, we are one step closer to utilizing this adult stem cell population for regeneration purposes in the retina.
Figure 4.1: Alleles and experimental timeline

(A) Description of the alleles used in the study. The Glast-CreER\textsuperscript{T2} allele has a tamoxifen-inducible Cre that is driven by the Glast promoter. The Sox2-conditional allele (Sox2\textsuperscript{C}) contains loxP sites flanking the open reading frame of the endogenous Sox2 locus. The Ai9 transgene expresses tdTomato following a CRE-mediated excision of a STOP codon. (Liu et al.)

Schematic of the experimental timelines. Retinas are dissected at P5 and placed in culture for 5-7 days before fixation. 4-OH-tamoxifen is added to the medium for the first 24 hours to activate CRE. Next, retinas are either fed by medium containing EdU each day (B), or only exposed to EdU on the 5\textsuperscript{th} day in culture, followed by 2 additional days in culture (C).
Figure 4.2: SOX2 levels in retinas containing Sox2<sup>C</sup> alleles

(A, C) Western blots of P5 retinas in which each lane represents a single, isolated retina in the C57BL/6J strain (A) or the B6/CD1 strain (C). (B, D) Quantification of relative SOX2 levels between genotypes, normalized to β-actin band intensities. (B) The level of SOX2 protein in the C57BL/6J strain is significantly reduced in Sox2<sup>C/C</sup> retinas (49%) compared to Sox2<sup>+/+</sup> retinas (p<0.005), but SOX2 levels in Sox2<sup>+/C</sup> remain relatively high (94%, p=0.56). (C) The level of SOX2 protein is not significantly lower in B6/CD1 Sox2<sup>+/C</sup> (92%, p=0.30) or Sox2<sup>C/C</sup> (80%, p=0.06) retinas. n= 4 (8 eyes analyzed separately for each genotype)
**Figure 4.3:** *Glast-CreER<sup>T2</sup>* is expressed in Müller glia and ablates SOX2 in *Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup>* retinas

Retinas were assessed for various markers using immunofluorescence at P5+5 DIV. The Ai9-tdTomato reporter tracks cells with active CRE. (A-C) Ai9-tdTomato-expressing cells are localized to Müller glial cell bodies, as shown by colocalization with CRALBP.Insets depict high magnification images of A-C. (D-L) Immunofluorescence for SOX2 in retinas with the Ai9 allele. (D-F) In control *Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup>; Ai9 retinas, SOX2 is localized to Ai9-tdTomato-expressing Müller glia, as shown by the purple arrowheads in the insets. They yellow arrowhead marks a SOX2-expressing amacrine cell, which lacks CRE activity. Insets depict high magnification images of D-F. (G-L) The *Sox2<sup>C/C</sup>; Ai9 retinas lack CRE activity, and therefore do not have Ai9-tdTomato reporter expression, and SOX2 expression is maintained. (J-L) In *Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> retinas, SOX2 is absent from Ai9-tdTomato-expressing cells, as shown by the purple arrowheads in the insets. The yellow arrowhead marks an amacrine cell that lacks CRE activity, and therefore maintains SOX2 expression. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar: 50 μm in L for A-L.
Figure 4.4: Müller glia proliferation is increased in Sox2^{C/C} and Sox2^{C/C}; Glast-CreERT2 retinas

Retinas exposed to EdU during the P5+5 DIV culture period were stained for EdU and CRALBP using immunofluorescence. (D’-F’) and (G’-I’) are magnified views of the boxed areas in (D-F) and (G-I), respectively. (A-C) In Sox2^{+/C}; Glast-CreERT2 retinas, there were very few EdU-incorporated cells. (D-F’) In the Sox2^{C/C} retinas, there was an increase in EdU-incorporated cells that were identified as Müller glia, as shown by CRALBP expression (arrowheads). (G-I’) The Sox2^{C/C}; Glast-CreERT2 retinas also exhibited an increase in EdU-incorporated Müller glial cells (arrowheads). Scale bars: 50 μm in I for A-F, G-I; 25 μm in I’ for D’-F’, G’-I’.
Figure 4.5: Quantification of proliferation in the central retinas

(A) Experimental setup for proliferation counts. Cryostat sections of retinas stained for EdU and Hoescht were imaged and stitched together to make a composite slice. The 5 most central slices were imaged for each retina, with the optic nerve serving as the marker of the central slice. Two brackets of a 700 μm length defined the peripheral and semi-peripheral areas on each end of the slice (blue brackets). The number of EdU cells were counted in areas excluded from the blue brackets, which is defined as the central retina (green bracket). (B, C) The number of EdU cells counted in the central area of each slice is represented in the box-and-whiskers plot in the C57BL/6J background (B) and B6/CD1 background (C). The middle 50 percentile of data points is represented within the box, the median is the middle line within the box, and the mean is the “x”. *In the C57BL/6J background, p<0.05 for Sox2<sup>C/C</sup>; Glast-CreER<sup>T2</sup> and Sox2<sup>C/C</sup> (mixed modeling analysis, see “Materials and Methods” section). Scale bar: 100 μm in A. n=2 (4 eyes analyzed for each genotype).
Figure 4.6: Culture stress enhances Müller glia proliferation in the Sox2^C/C; Glast-CreER^T2 retinas

Control Sox2^+/+; Glast-CreER^T2; Ai9 retinas (A-E, K-O) and SOX2-deficient Sox2^C/C; Glast-CreER^T2; Ai9 retinas (F-J, P-T) were analyzed by EdU staining and immunofluorescence for GFAP. The boxed areas in A, F, K, and P (10x magnification) indicate the magnified regions shown in each corresponding row (40x). Q'-T’ show magnified views of (Q-T). (Guerra-Junior et al.) Non-stressed retinal areas were absent of rosette formations and lacked GFAP expression and EdU incorporation in both Sox2^+/+; Glast-CreER^T2; Ai9 (A-E) and Sox2^C/C; Glast-CreER^T2; Ai9 (F-J) retinas. (K-T) Stressed retinal areas contained rosette formations and high GFAP expression (K, P, N, S, O, T). While the stressed Sox2^+/+; Glast-CreER^T2; Ai9 retinas do not have EdU incorporation (K-O), the Sox2^C/C; Glast-CreER^T2; Ai9 retinas contain a high number of proliferating cells as determined by EdU incorporation in the stressed areas (P-T’). As EdU is localized to the Ai9-ttdTomato-expressing and GFAP-expressing cells, these EdU-positive cells are gliotic Müller glia. (U) Box-and-whisker plot of the number of Ki67-positive cells in non-stressed and stressed areas of both genotypes, as defined by the presence or absence of GFAP expression. The graph displays the average number of Ki67 cells within each area of a given size (735 μm x 735 μm). Five stressed and five non-stressed areas were counted in each animal, and five animals were counted for each genotype (See Materials and Methods for details). “Sox2 control” refers to both Sox2^+/+; Glast-CreER^T2 and Sox2^+/C; Glast-CreER^T2 retinas. Two extreme outliers were removed from the stressed Sox2 control and stressed Sox2^C/C; Glast-CreER^T2, for the sake of clarity. *p<0.05 for non-stressed and stressed within the Sox2^C/C; Glast-CreER^T2 retinas. Data not significant (d.n.s.) for non-stressed and stressed within the Sox2 control retinas. Scale bars: 200 μm in P for A, F, K, P; 50 μm in T for B-E, G-J, L-O, Q-T; 25 μm in T’ for Q'-T’. n=5 for each genotype.
**Figure 4.7: Fate tracing of proliferating Müller glia reveals cell death in the Sox2\textsuperscript{C/C}; Glast-Cre\textsuperscript{ERT2} retinas**

All panels display the Sox2\textsuperscript{C/C}; Glast-Cre\textsuperscript{ERT2} retinas at either the time point immediately following the 4 hour EdU pulse on P5+5 DIV (A-O), or 2 days after the EdU pulse on P5+7 DIV (P-DD'). All panels are magnified views of the 40x retinas shown in (A, F, K, P, U, Z) in the same row. Purple arrowheads mark EdU cells. (A-E) EdU-positive cells identified as Müller glia due to colocalization with GS but lack expression of the bipolar cell marker CHX10. (F-J) EdU-positive cells were active in the cell cycle and expressed Ki67 and varying levels of CyclinD3. (K-O) In addition, these EdU cells identified with Müller glia marker SOX9, but not with the mature bipolar cell marker ISLET-1. (P-T) At P5+7 DIV, the EdU-positive cells became round, and some had pyknotic nuclei. These cells lost expression of GS and CHX10. (U-Y) EdU-positive cells exited the cell cycle as determined by the absence of Ki67 and CyclinD3. (Z-DD) EdU-positive cells did not gain a neuronal identity (ISLET-1) and lost their Müller cell identity (SOX9). (AA'-DD') A smaller subset of EdU cells maintained a glial identity and SOX9 expression postmitotically. Scale bars: 50 μm in Z for A, F, K, P, U, Z; 25 μm in DD for B-E, G-J, L-O, Q-T, V-Y, AA-DD, AA'-DD'.
Graph 4.1. The Sox2\textsuperscript{C/C} animals of the C57BL/6J strain were underweight
Within the C57BL/6J strain of mice at P5, the Sox2\textsuperscript{C/C} animals weighed less than Sox2\textsuperscript{+/+} and Sox2\textsuperscript{+/C} animals (*p<0.05). In the mixed B6/CD1 mouse strain, the Sox2\textsuperscript{C/C} animals had weights comparable to the Sox2\textsuperscript{+/+} and Sox2\textsuperscript{+/C} animals.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Small body size</th>
<th>%</th>
<th>Unilateral microphthalmia</th>
<th>%</th>
<th>Coloboma</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>Sox2*+/+</td>
<td>1/11</td>
<td>9.1%</td>
<td>0/9</td>
<td>0.0%</td>
<td>0/9</td>
<td>0.0%</td>
</tr>
<tr>
<td>B6</td>
<td>Sox2+/c</td>
<td>3/26</td>
<td>11.5%</td>
<td>1/16</td>
<td>6.3%</td>
<td>1/16</td>
<td>6.3%</td>
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<tr>
<td>B6</td>
<td>Sox2c/c</td>
<td>10/16</td>
<td>62.5%</td>
<td>1/13</td>
<td>7.7%</td>
<td>1/13</td>
<td>7.7%</td>
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<tr>
<td>B6/CD1</td>
<td>Sox2*+/+</td>
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<td>0.0%</td>
<td>0/7</td>
<td>0.0%</td>
<td>0/7</td>
<td>0.0%</td>
</tr>
<tr>
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<td>0.0%</td>
<td>0/4</td>
<td>0.0%</td>
<td>0/4</td>
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</tr>
<tr>
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<td>0.0%</td>
<td>0/12</td>
<td>0.0%</td>
<td>3/12</td>
<td>25.0%</td>
</tr>
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</table>

**Table 4.1. Frequency of physical abnormalities in both mouse strains**

For each genotype, Sox2*+/+, Sox2+/c, and Sox2c/c, and each mouse strain, C57BL6/J and B6/CD1, the incidence of each of the listed physical abnormalities were recorded in P5 animals.
Table 4.2. Relationship of physical abnormalities to P5 SOX2 levels
Any apparent physical abnormalities were noted for each Sox2<sup>c/c</sup> animal in both strain backgrounds at P5 before retinal tissue was digested for Western blots. Then the SOX2 level was calculated by taking the value calculated by the pixel intensity in the Western blot normalized to the pixel intensity in the corresponding β-actin band. This value was normalized to the value of the littermate control Sox2<sup>+/+</sup> on the same Western blot and displayed as a percentage in the chart.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Physical abnormality</th>
<th>SOX2 level (relative to Sox2 +/-)</th>
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</thead>
<tbody>
<tr>
<td>B6</td>
<td>Sox2&lt;sup&gt;c/c&lt;/sup&gt;</td>
<td>normal</td>
<td>54%</td>
</tr>
<tr>
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<td>45%</td>
</tr>
<tr>
<td>B6</td>
<td>Sox2&lt;sup&gt;c/c&lt;/sup&gt;</td>
<td>runt</td>
<td>56%</td>
</tr>
<tr>
<td>B6</td>
<td>Sox2&lt;sup&gt;c/c&lt;/sup&gt;</td>
<td>runt</td>
<td>39%</td>
</tr>
<tr>
<td>B6/CD1</td>
<td>Sox2&lt;sup&gt;c/c&lt;/sup&gt;</td>
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<tr>
<td>B6/CD1</td>
<td>Sox2&lt;sup&gt;c/c&lt;/sup&gt;</td>
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<td>84%</td>
</tr>
<tr>
<td>B6/CD1</td>
<td>Sox2&lt;sup&gt;c/c&lt;/sup&gt;</td>
<td>coloboma</td>
<td>93%</td>
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Chapter 5: bHLH factors regulate cell fate during late retinogenesis

5.1 Introduction

Throughout development, retinal progenitor cells pass through different competency states controlled by intrinsic and extrinsic factors that determine their ability to generate specific cell types at certain times (Cepko et al., 1996). Retinal cell type generation follows a stereotypical temporal order that is consistent among most vertebrate species (Livesey and Cepko, 2001a). During embryonic stages of vertebrate retinal development, progenitor cells exit the cell cycle and differentiate into retinal ganglion cells (RGCs), horizontal cells, cone photoreceptors, and amacrine cells; during postnatal development, progenitors give rise to primarily rod photoreceptors, bipolar cells, and Müller glia (Young, 1985a).

The transformation of proliferating progenitor cells into a mature, functional eye requires coordination and timing of cell cycle exit, proper tissue structural organization, and differentiation into specific cell types. These events are regulated by a complex transcriptional network that is partially controlled by basic helix-loop-helix (bHLH) factors. There are two classes of bHLH factors: transcriptional repressors and transcriptional activators. The bHLH transcriptional repressors interact with cofactors to recruit histone deacetylases to inactivate chromatin (Paroush et al., 1994). These repressors include members of the Hes family, notably Hes1 and Hes5, which are positively regulated by Notch signaling (Jarriault et al., 1995; Ohtsuka et al., 1999). The Hes genes are expressed in proliferating neural stem cells and act to maintain proliferation and prevent neural differentiation by inhibiting the bHLH activators. In Hes1-mutant embryos, cell proliferation is severely impaired in the CNS, including in the retina, and progenitor cells undergo premature neurogenesis (Takatsuka et al., 2004). The bHLH
transcriptional activators include the proneural genes, such as Neurgenin-1 (Ngn1), Neurgenin-2 (Ngn2), NeuroD1/2, Mash1, and Math3/5. Misexpression of proneural bHLH factors induces a neuronal cell fate and upregulation of pan-neuronal gene expression (Kageyama et al., 2005). In addition, proneural factors direct progenitors to differentiate into specific neuronal cell fates. In neural crest stem cells, Ngn1 and Ngn2 promote the generation of sensory neurons, while Mash1 promotes autonomic neurogenesis (Lo et al., 2002). Additionally, Math3 and NeuroD were determined to direct amacrine cell genesis in the retina given that Math3+/−;NeuroD+/− mice lack amacrine cells and have an increased production of Müller glia and retinal ganglion cells (RGCs) (Inoue et al., 2002). An important function of bHLH factors is the regulation of neurogenesis and gliogenesis. In addition to the function of Hes factors in the maintenance of progenitors, they also promote gliogenesis. Hes5-null mice have a 30-40% reduction in the number of Müller glial cells produced during development, and misexpression of Hes5 exhibited an increased the Müller glia population at the expense of neurons (Hojo et al., 2000). In contrast, bHLH activators promote neurogenesis and inhibit gliogenesis. For example, in rat cortical progenitor cells, Ngn1 sequesters complexes from the GFAP promoter in astroglia, thereby preventing glial-specific gene expression and glial differentiation (Sun et al., 2001). Based on these findings, the coordination of a regulatory transcriptional network of bHLH factors is necessary for the proper timing in development of CNS structures, including the retina.

The proneural factor NGN2 is homologous to the Drosophila atonal (Sommer et al., 1996) and is responsible for the specification of neural cell types in the peripheral nervous system (Fode et al., 1998; Ma et al., 1999), spinal cord (Mizuguchi et al., 2001), and brain (Fode et al., 2000). It is also expressed in early retinogenesis, where it is responsible for the progression of the leading edge of the neurogenesis wave that initiates in the dorso-central optic cup and sweeps ventrally and peripherally (Hufnagel et al., 2010). Ngn2 is transiently expressed in retinal progenitors just prior to the differentiation of all of the major retinal neuronal cell types: RGC, amacrine, horizontal, bipolar, and photoreceptors (Ma and
NGN2 has a strong potential to promote neurogenesis, even in non-neural tissue. Gain-of-function experiments show that NGN2 can transdifferentiate RPE cells into photoreceptors and RGCs \textit{in vitro} (Cullinane et al., 2001). Although NGN2 is important for retinal neurogenesis, \textit{Ngn2}−/− mice display minimal retinal defects (Akagi et al., 2004). In these \textit{Ngn2}−/− retinas, \textit{Mash1} and \textit{NeuroD} are upregulated, and it is likely that these factors compensate for loss of NGN2 activity to generate neurons and normal retinal development.

bHLH factors are important for the generation of all retinal neurons, including amacrine cells, which are interneurons that synapse in the inner plexiform layer and modulate information transmitted from bipolar cells to RGCs. There are over 30 different amacrine cell types that have distinct morphologies and functions, making them the most diverse retinal cell type (MacNeil et al., 1999). Amacrine cells can be distinguished into two physiologically distinct categories based on whether they use GABA or glycine as their primary inhibitory neurotransmitter (Fig. 5.1)(Wassle et al., 1998; Schubert et al., 2008). These two categories are further divided based on their expression profiles, including the cholinergic group (i.e. “starburst amacrine”), which express the neurotransmitter acetylcholine; a group that releases the neuropeptide tachykinin; and lastly, the specialized glycinergic AII cells (Fig. 5.1)(Cherry et al., 2009). During murine development, amacrine cells are generated from E8 to P5, with the peak production at E16 (Voinescu et al., 2009). GABAergic amacrine cells are the predominant amacrine cell type generated during embryonic stages, while glycinergic amacrine cells make up the majority produced postnatally. Additionally, there is a small group of amacrine cells that do not identify as either GABAergic or glycinergic subtypes (Fig. 5.1) due to lack of expression of typical markers that define these classes.

In this study, we determined the cell types that were generated from early postnatal mouse retinal progenitor cells as well as the competency of these progenitors to change their cell fate in retinal cultures when proneural bHLH factors were misexpressed. We found that NGN2-misexpressing cells

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gave rise to the same neuronal cell types as the controls, namely, amacrine cells and rod photoreceptors; yet, there was an increase in cells resembling amacrine cells in the inner nuclear layer (INL) compared to the controls. While a subset of progenitors in control retinas gave rise to Müller glia, NGN2-misexpressing progenitors evaded a Müller glia fate. These data provide evidence for a general function of NGN2 in the inhibition of gliogenesis and promotion of neurogenesis, which manifests as amacrine cell production in early postnatal progenitor cells.

5.2 Results

5.2.1 bHLH factors induce a shift in cell fate

During late retinogenesis, retinal progenitors undergo terminal cell divisions to generate primarily rod photoreceptors, bipolar neurons, Müller glia, and a relatively small number of amacrine neurons (Marquardt and Gruss, 2002; Ohsawa and Kageyama, 2008; Voinescu et al., 2009). In order to determine the capacity of late progenitors to generate specific retinal cell types, we misexpressed proneural bHLH factors in the postnatal retina. Since electroporation permits DNA entrance only into dividing progenitor cells (Matsuda and Cepko, 2004), we were able to track the fate of progenitors from a specific developmental time point (P0) following bHLH factor misexpression. We used constructs containing the cDNA of the bHLH factors Neurogenin-1 (NGN1), Neurogenin-2 (NGN2), NeuroD2, or Mash1. In these constructs, the gene sequences were followed by an IRES-EGFP expression cassette and were under the control of a ubiquitous promoter (CIG2) (Hand et al., 2005). The control plasmid was the CIG2 promoter followed by an IRES-EGFP sequence (CIG2-EGFP) (Addgene). P0 retinas were isolated and electroporated with the constructs and cultured for 5 days. In the control CIG2-EGFP retinas, progenitors gave rise to various cell types throughout the retinal layers, as shown by the presence of EGFP in the INL and outer nuclear layer (ONL), but absent from the ganglion cell layer (GCL) (Fig. 5.2A). In contrast, rather than EGFP-positive cells occupying locations throughout the retinal width, the
misexpression of each of the proneural bHLH factors resulted in an increased density of EGFP-expressing cells in the vitreal (basal) side of the INL, where amacrine cells typically reside (Fig. 5.2B-E, arrowheads in C). Interestingly, the same general pattern was observed for all of the bHLH factors, despite their diverse roles during development (Bertrand et al., 2002; Hatakeyama and Kageyama, 2004). These labeled cells extended neurites into the synaptic layers of the IPL (arrows in Fig. 5.2E`). Since NGN2 is critical to many aspects of neurogenesis, such as the perpetuation of the differentiation wave during retinogenesis and its transient expression in progenitors prior to the generation of all the retinal neurons (Ma and Wang, 2006; Hufnagel et al., 2010), we chose to focus on NGN2 for the remainder of our analyses.

5.2.2 NGN2-misexpressing cells were amacrine and rod photoreceptors

As noted, CIG2-NGN2-EGFP-electroporated retinas contained a cell layer in the INL that exhibited relatively high EGFP levels relative to both other retinal layers and control retinas. Due to their localization to the vitreal side of the INL, we hypothesized that they were amacrine cells. We first investigated whether amacrine cells were normally produced by P0 progenitors without bHLH influence using immunohistochemistry. The transcription factor PAX6 is expressed in all of the amacrine cell subtypes, as well as ganglion cells, Müller glia, and retinal progenitor cells (Cherry et al., 2009). In the control CIG2-EGFP-expressing cells in the INL, several cells expressed PAX6 in the INL (arrowheads in Fig 5.3A-D). For increased specificity to amacrine cells, we examined AP-2α, which marks a majority of amacrine cells exclusively of both the glycinergic and GABAergic subtypes but is not pan-amacrine, unlike PAX6 (Bassett et al., 2007). A subset of the CIG2-EGFP-expressing cells were AP-2α-positive (arrowheads, Fig. 5.3E-H), confirming their identity as amacrine cells. To determine the subtype of amacrine cell produced by the P0 progenitors, we examined the cytoplasmic marker GAD-67 for GABAergic amacrine cells, and the nuclear marker Islet-1 for cholinergic amacrine cells (Fig. 5.3I-P). The CIG2-EGFP-expressing cells lacked both of these GABAergic amacrine markers, indicating that postnatal
progenitors do not normally give rise to this subtype of amacrine cell. This result of the lack of GABAnergic cells produced postnatally is consistent with previous evidence that GABAergic cells are primarily produced embryonically (Cherry et al., 2009; Voinescu et al., 2009).

In order to determine the identity of the INL cells with high levels of EGFP in the CIG2-NGN2-EGFP-expressing retinas, we repeated the immunohistochemical analyses on this experimental group. Nearly all of these EGFP-positive cells were localized to the amacrine cell layer, where PAX6- and AP-2α-expressing cells normally reside, and a subset of EGFP-positive cells colocalized with these markers (arrowheads in Fig. 5.3A’-H’). However, a majority of CIG2-NGN2-EGFP-expressing cells lacked GAD-67 and ISLET-1 staining, indicating that these cells are not GABAergic, similar to the control CIG2-EGFP-expressing cells. Yet a small subset of CIG2-NGN2-EGFP-positive cells expressed GAD-67 staining (data not shown), a marker not found in any of the examined CIG2-EGFP-expressing cells. Therefore, in the NGN2-electroporated retinas, the P0 progenitor cells gave rise to mostly amacrine cells that were either 1) a subtype other than GABAergic or 2) were not yet fully differentiated GABAergic cells by the examined developmental age (P5). From these data, it can be concluded that the presence of high EGFP-positive cells in the amacrine cell layer in the CIG2-NGN2-EGFP-expressing retinas may be due to an increase in total amacrine cells compared to CIG2-EGFP-expressing retinas; however, further analysis is needed to determine the relative number of cells in the different amacrine cell populations.

Since retinal progenitor cells give rise to other neurons, primarily rod photoreceptors, during the postnatal developmental period (Young, 1985a), we wanted to investigate the presence of these cell types generated from P0 progenitors. By staining with RHODOPSIN in CIG2-EGFP-expressing retinas, we confirmed that a subset of EGFP-positive cells were rods (arrowheads in Fig. 5.3Q-T). However, a majority of the EGFP-positive cells did not colocalize with RHODOPSIN, possibly due to the young developmental age of the retinas and late onset of the production of RHODOPSIN, which begins expression around P4-P5 (Hicks and Barnstable, 1987). However, given the localization of EGFP-positive
cells to the ONL and their distinct morphology, we were able to classify them as photoreceptors with an immature transcriptional profile. Similarly, following electroporation with CIG2-NGN2-EGFP, a number of EGFP-expressing cells were located in the ONL, and a subset were RHODOPSIN-positive (arrowhead in Fig. 5.3Q′-T′), indicating that NGN2 misexpression permits rod generation postnatally. Additional analyses will be necessary to determine any potential cell fate shift that may occur in rod photoreceptors following NGN2 misexpression.

5.2.3 NGN2-misexpressing cells avoided the Müller glia fate

Since NGN2 is known to be a proneural factor (Bertrand et al., 2002; Hufnagel et al., 2010) (Cullinane et al., 2001), we wanted to determine whether gliogenesis is possible following NGN2 misexpression in progenitors. Many of the control CIG2-EGFP-expressing cells were SOX9- (arrowheads in Fig. 5.4A-D) and CRALBP-positive (arrowheads in Fig. 5.4E-H). The SOX9-expressing Müller glia made up approximately 10.6% of the total EGFP population in the CIG2-EGFP-expressing retinas (Fig. 5.4Q). In contrast, there were very few, if any, Müller glia within the CIG2-NGN2-EGFP-expressing population, as shown by the lack of SOX9 and CRALBP in the EGFP-positive cells (Fig. 5.4I-P). SOX9-positive cells made up 0.1% of the total EGFP-positive population in CIG2-NGN2-EGFP-misexpressing retinas (Fig. 5.4Q).

However, SOX9 and CRALBP expression was still maintained in EGFP-negative cells lacking NGN2 misexpression. These results support the model that NGN2 prohibits postnatal retinal progenitor cells from differentiating into Müller glia.

5.3 Discussion

Through the misexpression of proneural bHLH factors, we have shown that the fate of postnatal retinal progenitor cells can be altered. Following NGN2 misexpression, the progenitors that were fated to be glial cells switched to a neurogenesis program. Additionally, NGN2 not only promoted
neurogenesis but also influenced the generation of specific cell types by directing progenitors to become amacrine cells.

Forced expression of NGN2 caused progenitor cells to avoid a Müller glial fate and to adopt a neuronal fate, which is consistent with previous studies of the role of NGN2 in neural progenitors. Ngn2−/−; Mash1−/− mice show a loss of neural progenitors, inhibition of neuronal differentiation, and premature production of astrocytes in various brain regions, indicating that NGN2 promotes a neural fate over a glial fate determination (Tomita et al., 2000; Nieto et al., 2001). Proper timing of neurogenesis and gliogenesis in neural progenitor cells is controlled by a balance between activator bHLH factors that promote neurogenesis, including NGN2, and repressor bHLH factors that promote gliogenesis, such as Hes1/5 (Hojo et al., 2000). The data in the current analysis suggest that NGN2-misexpressing progenitors overcame inhibition of neural differentiation by Hes1/5, generating amacrine and rod photoreceptors instead of Müller glia.

Misexpression of NGN2, as well as NGN1, NeuroD2, and MASH1, induced a distinct localization of EGFP-positive cells, characterized by an increase in the number of cells in the INL with neurites extending into the IPL (Fig. 5.2). These cells exhibited very high EGFP levels relative to the rest of the EGFP population and expressed PAX6 and AP-2α (Fig. 5.3). Their localization in the INL, inverted orientation, distinct morphology, and marker expression are evidence for their amacrine cell identity. However, most of these high EGFP-expressing cells lacked colocalization with many of the amacrine cell markers tested, including PAX6 and AP-2α. Therefore, it was difficult to definitively quantify whether there was an increase in amacrine cells in NGN2-misexpressing retinas. There are several explanations for the lack of amacrine cell marker colocalization in these cells. The first is that due to the early developmental age of the retinas in our experiments, many postmitotic cells have not yet upregulated their differentiated transcriptional profile. Therefore, it is possible that the EGFP-positive cells did not have sufficient time to upregulate cell type-specific genes given that the total experimental time course
was only 5 days due to *in vitro* culture constraints. Alternatively, these EGFP-positive cells may be an amacrine cell subtype that was not investigated due to a lack of functional antibodies that recognize other amacrine subtypes such as glycinergetic cells. Additional investigations will be needed for more specific amacrine cell type identification and cell fate analyses.

Consistent with the hypothesis that bHLH factors induce amacrine cell genesis is the work done by (Cherry et al., 2011), in which P0 retinas were electroporated *in vivo* with constructs misexpressing NeuroD factors containing *ires-GFP* sequences. By P21, the retinas misexpressing NeuroD2 and NeuroD6 exhibited a 2-fold and 3-fold increase in amacrine cells, respectively, as shown by the expansion in the number of PAX6-positive, SOX9-negative cells. Specifically, these authors identified the increased amacrine cell subtype as glycinergetic cells, mostly of the AII subtype. Additionally, in NeuroD2-null mice, there was a reduction in the number of AII amacrine cells. Since the expression of NeuroD is controlled by NGN2 in both *Xenopus* and mice (Lee et al., 1995; Ma et al., 1996; Ma et al., 1999), it is possible that the apparent increase in the number of amacrine cells generated by NGN2 misexpression in this study was due to upregulation of NeuroD factors. It is also conceivable that the amacrine cells induced by NGN2 were AII glycinergetic cells, a subtype not analyzed in this study. Interestingly, from single cell microarrays of various amacrine cell populations, (Cherry et al., 2011) found that NGN2 was expressed in 50% of GABAergic amacrine cells, including all cholinergic cells, but was completely absent from glycinergetic or other amacrine cell types. Since the amacrine cells induced by NGN2 in this study did not express GABAergic or cholinergic markers, perhaps NGN2 plays a different role in cell fate specification following misexpression compared to its endogenous role in the retina.

Given that the peak production of amacrine cells is during embryonic stages (Cherry et al., 2009; Voinescu et al., 2009), it is interesting that amacrine cell production is favored in postnatal stages following NGN2 misexpression. Because NGN2 promotes neurogenesis in many different retinal neurons and throughout the CNS (Ross et al., 2003; Ma and Wang, 2006; Hufnagel et al., 2010), it is unlikely that
NGN2 functions specifically in amacrine cell fate choice, but rather as a pro-neuronal differentiation factor that may give rise to different neurons under different conditions.

Although the role of NGN2 in promoting neurogenesis and inhibiting gliogenesis has been well characterized in the developing CNS (Cullinane et al., 2001; Bertrand et al., 2002; Hufnagel et al., 2010), the present results show for the first time that NGN2 is capable of inducing the production of amacrine cells from postnatal progenitors and preventing progenitors from adopting a Müller glia cell fate. Further study is needed to characterize the mechanisms controlling the fate of retinal progenitor cells. Through the increased understanding these developmental processes, we can hopefully develop methods to recapitulate adult neurogenesis in diseased tissue for improved stem cell therapeutics in the future.
Figure 5.1: Amacrine cell subtypes
Amacrine cell subtypes are divided into a hierarchical chart based on shared gene expression between the groups, as determined by single cell microarray analysis (Cherry et al., 2009). The major groups are classified according to the primary neurotransmitter expressed in the subtypes.
Figure 5.2: bHLH factors induce a distinct pattern of neuronal organization
Postnatal retinas were electroporated with various constructs and cultured for 5 days. (A) The control CIG2-EGFP-expressing cells comprised a diverse range of cell types as determined by the presence of EGFP-positive cells throughout all retinal layers. Retinas were electroporated with the following constructs containing bHLH factors: (B) CIG2-NGN1-EGFP, (C) CIG2-NGN2-EGFP, (D) CIG2-NeuroD2-EGFP, and (E-E’) CIG2-MASH1-EGFP. (E’) High magnification image of (E). In these bHLH-misexpressing retinas, there was an increase in the number of cells with high levels of EGFP in the INL (arrowheads in C). Additionally, these EGFP-positive cells extended their neurites into the IPL (arrows in E’).
Abbreviations: GCL- ganglion cell layer, IPL- inner plexiform layer, INL- inner nuclear layer, ONL- outer nuclear layer. Scale bars: 50 μm in E for A-E; 25 μm in E’.
Figure 5.3: NGN2-misexpressing retinas give rise to amacrine and rod photoreceptors

Retinas were electroporated with CIG2-EGFP (A-T) or with CIG2-NGN2-EGFP (A’-T’). The three right-hand images in each row are high-magnification views of the boxed areas in the left-most image. A subset of CIG2-EGFP-expressing cells and CIG2-NGN2-EGFP-expressing cells identified as amacrine cells due to their expression of PAX6 (A-D, arrowheads, A’-D’, arrowheads) and AP-2α (E-H, arrowheads, E’-H’, arrowheads). These cells were not GABAergic or cholinergic due to the lack of GAD-67 (I-L, I’-L’) or ISLET-1 (M-P, M’-P’), respectively. Gray arrowheads in (I-L and I’-L’) show GAD-67-positive cells. Both CIG2-EGFP-expressing cells and CIG2-NGN2-EGFP-expressing cells were located in the ONL, and a subset expressed RHODOPSIN (arrowheads in Q-T, arrowheads in Q’-T’).

Abbreviations: INL- inner nuclear layer, ONL- outer nuclear layer.

Figure 5.4: NGN2-misexpressing cells did not become Müller glia
Retinas were electroporated with CIG2-EGFP (A-H) or CIG2-NGN2-EGFP (I-P). The three right-hand images in each row are high-magnification views of the boxed areas in the left-most image. CIG2-EGFP-expressing cells were identified as Müller glia due to expression of SOX9 (A-D, arrowheads) and CRALBP (E-H, arrowheads). CIG2-NGN2-EGFP-misexpressing cells were determined not to be Müller glia based on the lack of expression of SOX9 (I-L) and CRALBP (M-P). (Q) Quantification of the number of SOX9-expressing cells within the EGFP-positive population (SOX9+EGFP+)/ (EGFP+). See “Materials and Methods” for a detailed description of the analysis. Scale bars: 50 μm in M for A, E, I, M; 25 μm in P for B-D, F-H, J-L, N-P. n=2 for each condition.
Chapter 6: Discussion

6.1 Brief Summary of Findings

The work in this dissertation describes the role of transcription factors, namely, SOX2 and NGN2, in the regulation of proliferation and differentiation in postnatal retinal progenitor cells and Müller glia. When SOX2 is ablated from early postnatal progenitor cells at P0, the number of Müller glial cells is decreased and their structural morphology becomes degraded by P5, leading to disruption of the retinal architecture. Additionally, Müller glia proliferate in a stereotypical manner in SOX2-deficient retinas by translocating their soma to the apical surface to divide. Notably, coincident with SOX2 loss, there is a downregulation of Notch signaling components. Conversely, when NICD is overexpressed in SOX2-deficient retinal progenitors, the structural morphology of Müller glia is rescued, yet these cells aberrantly continue to proliferate. We next investigated if the effects following SOX2 loss in retinal progenitors were due to a requirement for SOX2 in Müller glia. To address this question, we specifically ablated SOX2 in Müller glia at P5 using Glast-CreERT2, which resulted in an increase in Müller glia proliferation between P5 and P10. Additionally, when retinas were stressed due to in vitro culturing conditions, SOX2-deficient Müller glia proliferated almost exclusively in areas where they were in a state of active gliosis. In contrast, Müller glia in the control retinas exhibited gliosis due to culture injury but did not proliferate. Fate-tracing experiments revealed that the cell division event in SOX2-deficient Müller glia resulted in a loss of Müller glia identity in the daughters, followed by cell death. Furthermore, we found an increase in Müller glia proliferation in the control, Sox2+/− retinas, similar to the phenotype observed in Sox2C/C; Glast-CreERT2 retinas. During subsequent analyses, we determined that the Sox2C/C animals had 50% SOX2 levels prior to the ablation protocol compared to Sox2+/− controls, which is likely the explanation for the observed proliferation phenotype in the late postnatal
Müller glia. Consistent with this hypothesis, some Sox2°C/C animals also exhibited eye defect phenotypes that are characteristic of SOX2 hypomorphic mice and humans (Male et al., 2002; Taranova et al., 2006a) and were significantly smaller in weight than their wild type litter mates. When we used a mixed mouse strain (B6/CD1) for experiments, we found that the SOX2 levels in the Sox2°C/C retinas were not significantly different from the control Sox2+/+ retinas, and had proliferation levels that were comparable to the controls, indicating that hypomorphism of the Sox2C allele is strain-dependent. These results indicate that SOX2 dosage is important in retinal progenitor cells for promoting Müller glia quiescence during late development.

The study in Chapter 5 addresses the competency of postnatal retinal progenitors to generate specific cell types when influenced by exogenous bHLH factors. The misexpression of several proneural bHLH factors in P0 progenitors resulted in the cells’ final location being in the INL, where amacrine cells reside. Moreover, whereas the progenitors that expressed the control EGFP gave rise to amacrine cells, rod photoreceptors, and Müller glia, progenitors that misexpressed NGN2 gave rise to cells that were identified as amacrine cells and rod photoreceptors, but not Müller glia. This result indicates that progenitors cannot become Müller glia when the neurogenic differentiation program has been initiated by NGN2.

6.2 SOX2 maintenance of progenitor cell identity in Müller glia

Müller glia have characteristics that significantly overlap with those of progenitor cells, both in their radial morphology and gene expression profile (Roesch et al., 2008; Trimarchi et al., 2008), and retain the ability to enter the cell cycle when injured (Braisted et al., 1994; Fischer and Reh, 2001; Ooto et al., 2004). Several of the genes that are expressed in Müller glia also define progenitor cells, including Sox2, Chx10, Pax6, Notch1, Hes1, and Dkk3 (Roesch et al., 2008; Trimarchi et al., 2008). Due to the nature of Müller glia the maintenance of progenitor genes is expected, but it is surprising that late (postnatal) retinal progenitors also express genes that are characteristic of mature Müller glia, such as
Rlbp1 (CRALBP), carbonic anhydrase 2, and clusterin. A large subset of late retinal progenitors that express glial genes may be fated to become mature Müller glia, yet they are still a proliferating population that will also give rise to other cell types. For example, using fate tracing techniques we found that among progenitors that were electroporated with pCRALBP-CreEGFP at P0, a majority gave rise to Müller glia, but around 43% of them gave rise to photoreceptors, bipolar cells, and amacrine cells by P5 (Fig. S3.7). It is well established that progenitor cells divide throughout retinogenesis and then, in a strict, temporally controlled manner, exit the cell cycle to differentiate into neurons. Considering this together with the numerous results indicating Müller glia to be similar to progenitor cells, leads to a model in which, rather than undergoing an irreversible cell fate determination, Müller glia arise from a population of progenitor cells that remain after all other cells have undergone neurogenesis, thereby retaining their progenitor transcriptional profile throughout adulthood.

SOX2 expression defines proliferating retinal progenitors and is downregulated as cells exit the cell cycle, yet it is maintained in the population of cells that does not irreversibly differentiate—the Müller glia (Fig.3.1). Since a function of SOX2 is to maintain NPC identity (Pevny and Nicolis, 2010), perhaps its role in Müller glia is to maintain its status as a quiescent “progenitor” that retains a potential for neurogenesis throughout adulthood. Injury-induced Müller glia in birds can proliferate only once (Fischer and Reh, 2001; Fischer et al., 2002), and it is likely that mammalian Müller glia can only proliferate once because their regenerative capacity is even more limited than birds (Ooto et al., 2004). Thus, a quiescent Müller glia can be viewed as a progenitor cell that is arrested in a G0 phase of a terminal cell division, awaiting release from cell cycle regulators that will result in a final cell division. When SOX2 is ablated in Müller glia, perhaps they lose their “progenitor” identity as they proceed through their terminal cell division and ultimately die (Fig. 4.7).

The discovery that the Sox2<sup>C/C</sup> mice were hypomorphic for SOX2 has led us to another theory to explain the role of SOX2 in Müller glia quiescence. SOX2 hypomorphism has been shown to have
profound effects on the development of the brain and eye, in both mice and humans (Taranova et al., 2006a; Schneider et al., 2009; Langer et al., 2012). Likewise, we observed an increase in Müller glia proliferation in retinas that were hypomorphic for SOX2 (Fig. 4.3 and 4.4). Since these retinas had compromised levels of SOX2 throughout embryogenesis and early postnatal periods, it is likely that SOX2 is critical for cell cycle dynamics in retinal progenitors during these earlier periods and that even the partial loss of this regulation leads to the observed proliferation phenotype in the Müller glia during late development. To test this hypothesis, we would first determine whether there is indeed increased proliferation in Sox2<sup>C/C</sup> retinas prior to P5. We would then determine if any such proliferating cells, as suggested by our post-P5 results, a) identify as CRALBP- or GLAST-expressing late progenitor cells or early Müller glia and b) stain for markers of cell cycle progression. The co-expression of proliferation and Müller glia markers past the appropriate developmental period would strongly suggest that SOX2 is important for permitting the appropriate timing of cell cycle exit in retinal progenitors. In Chapter 4, the abnormally proliferating Müller glia we observed after P5 could be remnants of proliferating progenitors that failed to exit the cell cycle from earlier time periods. Indeed, previous studies in SOX2 have shown that it is involved with regulating proper cell cycle exit. For example, when SOX2 was overexpressed with a lentivirus in E17 mouse explants, progenitor cells aberrantly exited the cell cycle (Lin et al., 2009). In another study, when SOX2 is overexpressed in neural progenitors in the developing chick spinal cord, progenitors are unable to exit the cell cycle and therefore fail to differentiate into neurons (Graham et al., 2003). Although these different experimental paradigms gave differing results (due to the context-dependent nature of SOX2), they both support the idea that SOX2 is important for proper timing of cell cycle exit.

### 6.3 SOX2 regulates proliferation in adult progenitor cells

A majority of the research on the role of SOX2 in adult neural progenitor cells has been in populations that are active centers of neurogenesis, such as the subventricular zone (SVZ) in the brain,
and the dentate gyrus in the hippocampus (Ellis et al., 2004; Suh et al., 2007; Kriegstein and Alvarez-Buylla, 2009). In adult mice with hypomorphic levels of SOX2, a 65% reduction of proliferation was observed in the dentate gyrus and a 55% reduction of proliferation in the SVZ (Ferri et al., 2004). Consistent with this, there was a decrease in neurogenesis, as shown by a reduction in the number of new neurons generated in these areas. In proliferating human Müller glia cell lines (hMGCs), SOX2 inhibition by RNAi resulted in a significant reduction in the number of proliferating cells, from 15% to 1% (Bhatia et al., 2011). In this study, we show the opposite result in that a reduction or ablation of SOX2 results in an increase in the number of proliferating Müller glial cells. One clear distinction between the present and previous studies is that earlier analyses focused on populations of actively proliferating adult progenitor cells that generate neurons during homeostasis (brain) or experimental conditions (hMGCs). In this study, we analyze a population of adult progenitor-like cells that are quiescent during homeostasis and which have very limited abilities to proliferate and generate neurons. However, SOX2 has a role in all of these cell populations; its downregulation results in decreased proliferation in actively proliferating cell (i.e., SVZ, dentate gyrus, hMGCs) and induces proliferation in quiescent cells (i.e., Müller glia).

6.4 SOX2 maintenance of quiescence and a possible link to the cell cycle regulator p27Kip1

Hypomorphic levels of SOX2 result in Müller glia proliferation past the normal developmental timeframe. One possible mechanism by which SOX2 regulates Müller glia quiescence and progenitor cell cycle exit is through interactions with cell cycle regulators, such as p27Kip1. Two primary lines of evidence support this, including the data that describe the role of p27Kip1 in regulating quiescence, cell cycle exit, and gliosis, as well as evidence that SOX2 directly regulates p27Kip1 expression.

The tumor suppressor protein p27Kip1 prevents cell cycle progression by binding to cyclin/cyclin-dependent kinase complexes, thus inhibiting entry into S phase (Besson et al., 2008). Moreover, p27Kip1 facilitates cell cycle exit in cycling progenitor cells, which is supported by the finding that the
overexpression of p27Kip1 in retinal progenitor cells results in cell cycle arrest (Levine et al., 2000). In cycling mouse retinal progenitor cells, p27Kip1 is not expressed at detectable levels, but is upregulated in the final cell cycle prior to exit and is highly expressed in newly postmitotic differentiating neurons (Levine et al., 2000; Dyer and Cepko, 2001a; Green et al., 2003). Thus, an important role for p27Kip1 has been established in controlling cell cycle exit during development of the retina.

Evidence for a link between SOX2 and p27Kip1 in the regulation of quiescence comes from a study in the mouse auditory sensory epithelium of the inner ear (Liu et al., 2012). The cochlea contains a population of cells called the supporting cells (SC) that function as a quiescent stem cell population in the auditory epithelium. Comparable to the function of retinal Müller glia, in non-mammalian vertebrates, SCs are capable of responding to injury by proliferating and differentiating into auditory hair cells (Stone and Cotanche, 2007; Brigande and Heller, 2009). While mammalian SCs are incapable of proliferating in vivo, they can divide and differentiate into hair cells in vitro (White et al., 2006). This divergence of regenerative capacity among vertebrate species is analogous to the differing proliferation capabilities of Müller glia between vertebrate species. Additionally, similar to Müller glia, mammalian SCs maintain expression of SOX2 and p27Kip1 (Liu et al., 2012). When SOX2 was conditionally ablated from cochlear SCs in early postnatal stages, p27Kip1 was downregulated, and a subset of the SCs proliferated (Liu et al., 2012). Similarly, conditional ablation of p27Kip1 also resulted in SC proliferation, yet SOX2 expression was maintained. This proliferative capacity declined with age; SOX2 ablation at adult stages resulted in very few proliferative cells, representing another trait in common with mammalian Müller glia. Lastly, chromatin immunoprecipitation and luciferase assays showed that p27Kip1 is directly regulated by SOX2. Together, the results suggest that SOX2’s regulation of this cell cycle regulator is a mechanism for SOX2’s control of the quiescence of auditory SCs in the neonatal cochlea.
Consistent with the results in the inner ear, the data outlined in this dissertation are consistent with a model for SOX2 regulation of Müller glia quiescence through p27^{kip1}. Similar to SOX2 expression, p27^{kip1} is expressed in the nuclei of quiescent Müller glia in the mature retina (Dyer and Cepko, 2000; Levine et al., 2000; Taranova et al., 2006a). Moreover, p27^{kip1}-deficient retinas display proliferation beyond the normal period of histogenesis (cell type generation) in the retina, specifically, between P7-P11 (Levine et al., 2000). Consistent with these observations, we show that retinas with hypomorphic levels or ablated SOX2 also exhibit proliferation during late development, from P5 to P10. Additionally, Heavner (in review) showed that when SOX2 was ablated from the optic cup at E10.5, the embryonic retinal progenitors lost p27^{kip1} expression. The downregulation of p27^{kip1} coincides with a failure to exit the cell cycle, as indicated by an increase in CyclinD1-expressing cells (Heavner, in review). However, when SOX2 was ablated at P5 in Müller glia in the present study (Sox2^{C/C}; Glast-CreER^{T2}), we found that p27^{kip1} expression was maintained in most Müller glial cells at P10 (n=3, data not shown). The maintenance of p27^{kip1} expression in SOX2-ablated Müller glia can be explained by the observation that despite an increase in aberrant proliferation, a vast majority of Müller glia remained quiescent, possibly due to the maintenance of p27^{kip1} expression. Therefore, in quiescent Müller glia, there must be factors other than SOX2 involved in maintaining quiescence. However, among the very small subset of SOX2-ablated Müller glia that are actively proliferating, it is likely that p27^{kip1} is significantly downregulated, but further analysis is necessary to determine whether this is the case.

In addition to its role in controlling postnatal progenitor cell cycle exit and quiescence, p27^{kip1} has been implicated in gliosis in Müller glia. Retinas that are deficient for p27^{kip1} show a 10-20x upregulation of GFAP expression in nascent Müller glia as well as retinal dysplasia due to Müller glia gliosis and mislocalization of their soma to the photoreceptor layer (Nakayama et al., 1996; Dyer and Cepko, 2000; Levine et al., 2000). Similarly, our data show a role for SOX2 in 1) regulating the retinal architecture through the maintenance of the structure of Müller glia and 2) regulating the progression
of gliosis from a non-proliferative to proliferative state. These results indicate that both p27\textsuperscript{Kip1} and SOX2 prevent the initiation/progression of gliosis and maintain Müller glia homeostasis, thus preventing retinal tissue degradation. The overlapping roles between p27\textsuperscript{Kip1} and SOX2 during gliosis provide further evidence for their molecular link.

Our results show the phenotypes following loss of SOX2 are similar to those associated with p27\textsuperscript{Kip1} loss: increased proliferation in the postnatal retina, Müller glia gliosis and disorganization, and disruption of the structural integrity of the retinal tissue. Interestingly, studies in a population of SC with progenitor characteristics in the inner ear showed that quiescence was regulated through a SOX2-p27\textsuperscript{Kip1}-mediated mechanism. Although further evidence is necessary to conclusively determine the mechanism that underlies the results described in this dissertation, we propose a model by which SOX2 regulates quiescence of nascent Müller glia through a p27\textsuperscript{Kip1}-dependent pathway.

6.5 Conclusion

The studies presented in this dissertation highlight the importance of SOX2 in regulating cell cycle dynamics both in late postnatal retinal progenitor cells and in Müller glia. The results also support previous evidence that neural progenitors are sensitive to slight variations in SOX2 dosage levels, showing that small increases or decreases can have drastic effects on developmental functions. We also show that SOX2 is involved in other functions, such as the maintenance of Müller glia structural morphology, preventing the injury response from transitioning from a non-proliferative to proliferative gliosis, as well as promoting survival through the regulation of quiescence. Together, these results highlight a critical role for SOX2 in the maintenance of quiescence and homeostasis in nascent Müller glial cells.
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