USING THE MOUSE RETINA TO MODEL THE ROLE OF SOX2 IN NEURAL INDUCTION

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ABSTRACT

WHITNEY E. HEAVNER: Using the Mouse Retina to Model the Role of SOX2 in Neural Induction
(Under the direction of Dr. Larysa Pevny)

Neural competence is the ability of a progenitor cell to generate a neuron. The eye is one of the few tissues derived from the neural ectoderm that contains both neurogenic and non-neurogenic cells, all of which arise from a common progenitor pool. Therefore, the eye is a particularly useful model to study the molecular mechanisms that confer neural competence. Moreover, this cell fate dichotomy is highly reminiscent of the earlier process of neural induction, or the decision of an ectoderm precursor cell to become neural plate or epidermis. The HMG-box transcription factor SOX2 is crucial for both of these processes. Little is known about the role of SOX2 in neural induction, and what is known has been worked out primarily in lower vertebrates. Humans and mice with mutations in *SOX2* exhibit a range of neural defects; therefore, from the perspective of human health, it is important to understand SOX2's function in mammalian neuroepithelium. This project takes advantage of the accessibility of the embryonic mouse eye to identify pathways that SOX2 regulates to maintain neural competence.

Chapter I gives an overview of neural induction from an historical perspective, with particular attention to the role of SOXB1 factors. A detailed description of

mammalian eye development and the similarities between optic cup regionalization and neural induction are described.

Chapter II investigates one potential mechanism of how SOX2 specifies neural fate: antagonism of *Pax6*. Using mouse genetics, we show that ablation of *Sox2* in the OC leads to increased *Pax6* expression and loss of neural competence. This phenotype is partially rescued by lowering *Pax6* in $Sox2^{cond/cond}; \alpha P0^{CreiresGFP}$ cells. Chapter III investigates the increased WNT signaling activity observed upon SOX2 ablation. Using mouse genetics, we show that removal of *β-Catenin* from *Sox2*-negative cells rescues some aspects of the *Sox2*-mutant phenotype.

Chapter IV offers some ideas of how these studies potentially relate to the treatment of degenerative eye disease, focusing on identifying candidate genes that could rescue neurogenesis in a *Sox2*-depleted background. To put these ideas into context, an overview of the current state of gene therapy for the treatment of eye disease is offered.

This work is dedicated to my beloved advisor, Larysa, who taught me to revere mouse genetics and adore the developing embryo.

ACKNOWLEDGMENTS

Scientific inquiry is often a solitary endeavor. Discoveries made in isolation can be thrilling and deeply satisfying. However, science by nature is collaborative, and its aims, ideally, are selfless. The work presented here stands on the shoulders of intellectual giants. Indeed, it often seems that eye development has not gone untouched by any major development biologist in the past few decades. Although I could never do adequate justice to those who came before me, directly contributed to this project or shared in the trials and joys associated with my six years in graduate school, I hope this work does some honor to their efforts and accomplishments.

I owe a tremendous amount of gratitude to the members of the Pevny lab. There was never a day that I did not look forward to being in the lab and working with my "Pevny" colleagues and friends. I can't imagine future lab meetings ever being as enjoyable as our weekly 8th floor conference room science tell-all hour. I am particularly indebted to the lovely and talented Danielle Matsushima. Without her this thesis would have never materialized, at least not in its present form. Her pioneering work on the genetic relationship between *Sox2* and *Pax6* allowed me to contribute to a "working" project as soon as I joined the lab. This rarity in graduate school gave me the freedom to spend a lot of time "experimenting" (messing up), learning and growing as a scientist without the risk of lost time.

I am also particularly indebted to Dr. Scott Hutton, who took on much more responsibility than any senior graduate student or post-doc should ever have to. And if he ever resented this fact, he never showed it. From how to deal with lab safety compliance to running qPCR experiments and culturing neurospheres, if Scott didn't know how to do it, then no one did. But most of all, Scott stepped in as an unofficial advisor when I needed it the most. For that I am extremely grateful.

I never cease to be amazed by the amount of support one can receive in a time of need. For the help and encouragement coming from members of the Neuroscience Center and my committee, I am particularly thankful. Dr. Bill Snider, Dr. Eva Anton, Dr. Steve Crews and Dr. Bob Duronio were all instrumental in helping me finish the thesis and find a post-doc. I am also eternally thankful for the support and enthusiasm of Dr. Raluca Dumitru. If I was ever unsure about something, Raluca would give me the confidence and positive energy to go for it. And for the many belays and good advice from Dr. JrGang Cheng.

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For community, colleagues and training I owe the Fundamental Issues in Vision Research course supported by the NEI and the UNC Curriculum in Genetics and Molecular biology.

Lastly and most of all, I am grateful and truly lucky to have been a student of Larysa's. It's very rare in life to meet someone who not only meets your expectations but constantly defies them. In her humble demeanor and quiet perseverance, Larysa was full of surprises. She didn't speak often, but when she spoke, it was captivating and meticulous.

Larysa welcomed me into her lab with enthusiasm and warmth. I knew beyond a doubt that I had found my lab home when I hadn't even finished my third rotation and she had already cleared off a bench for me. Her lab and she were just perfect. In fact, I knew before even meeting her that she is who I wanted to work for. Her name on my list of possible rotations had asterisks and circles around it. I joined her lab with an exclamation point and was not disappointed. The science was fascinating, the lab was fun and the PI was fantastic. She encouraged me, a timid but endlessly curious young graduate student, to speak at international conferences, and she would work with me tirelessly until my talks were perfect. She was genuinely excited to see me do well, like a coach for an athlete, and this was the best encouragement I could receive.

Larysa's passion for science was contagious. She once told me that when she was young, she would collect antique Erlenmeyer flasks and other glassware. It was like the allure of the laboratory, the mysteries and the challenges therein, drew her to the life of a scientist. Indeed, she could see great beauty where others just saw a retina or a neural

tube or an antique Erlenmeyer flask. This was the type of excitement that Larysa embodied. And she would get frustrated when others didn't appreciate what she saw. Her science world was rich and full, and often no one appreciated it more than she did.

I don't say that Larysa was caring in passing. I think it is quite rare for someone to be as empathetic as she was, in science or elsewhere. Her passion for her work was so pure, almost dutiful. It seems like during the last months of her life, she felt extra pressure to pursue her ideas in the lab. Like she only had so much time left to paint this beautiful picture for the world. Like she had to get it out there, what she saw under the microscope. And I would get angry with her for working so hard when her body was obviously so weak. But she had so much drive, and nothing would make her happier than to be in the lab. This is the most visible irony of Larysa's life: that she put so much effort into the health of her cells and her mice and her lab while in the end, no one could do anything to save her. But also how typical of Larysa's life; she gave everything she could and never asked for anything in return. You couldn't help but love her.

Larysa excelled as a scientist. To watch her at her best was awe-inspiring. She also excelled at making me feel better about the world. She had a firm belief in the beauty of the embryo, the possibilities of science and the sanctity of truth. Larysa has probably been the most influential figure in my adult life, and I am lucky for that.

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PREFACE

The section on eye development in Chapter I is largely from a review that Larysa and I published with the following citation:

> Heavner, W. and Pevny, L. (2012). Eye development and retinogenesis. *Cold Spring Harb Perspect Biol* 4.

Although the text and figures are of my own creation (with the exception of Figure 1-7), Larysa made significant contributions to the sections on Muller Glia and Retinogenesis. Permission to use this review in a PhD thesis was granted according to our copyright transfer agreement. The section on gene therapy in Chapter IV is also from this review.

Chapter II was published prior to the writing of this thesis with the following citation:

> Matsushima, D., Heavner, W. and Pevny, L. H. (2011). Combinatorial regulation of optic cup progenitor cell fate by SOX2 and PAX6. *Development* 138, 443-54.

Danielle Matsushima, co-first author on the paper, and I made equal contributions to each experiment represented in the manuscript. Permission to use this work in a PhD thesis was retained from Danielle Matsushima and from the publisher according to http://dev.biologists.org/site/misc/rights_permissions.xhtml.

Chapter III is a draft of a manuscript being prepared for publication. Although I performed the vast majority of the experiments, Danielle Matsushima and Jane Hartung, a rotation student during Spring 2012, contributed to mouse breeding, genotyping and staining.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANP – Anterior neural plate

AVE – Anterior visceral endoderm

bHLH – basic helix loop helix transcription factor

BMP – Bone morphogenetic protein

BrdU - Bromodeoxyuridine

CB – Ciliary body

CD – Circular dichroism

Chx10 – C. elegans ceh-10 homeodomain containing homolog (see also VSX2)

CE – Ciliary epithelium

CNS – Central nervous system

E – Embryonic day

EFTF – Eye field transcription factor

EGF – Epidermal growth factor

EMSA – Electrophoretic mobility shift assay

ESC – Embryonic stem cell

FGF – Fibroblast growth factor

IdU - Iododeoxyuridine

IF - Immunofluorescence

IHC - Immunohistochemistry

ILM – Inner limiting membrane

INL – Inner nuclear layer

IOP – Intraocular pressure

IPL – Inner plexiform layer

iPSC – induced pluripotent stem cell

ISH – *in situ* hybridization

LCA – Leber's congenital amourosis

LEF1 – Lymphoid enhancer binding factor 1

LHX2 – LIM homeobox protein 2

MATH – Mouse atonal homolog

MASH – Mouse achaete-scute homolog

NPC – Neural progenitor cell

NR – Neural retina

OC – Optic cup

OCM – Optic cup margin

OCPC – Optic cup progenitor cell

OCPTF – Optic cup progenitor transcription factor

OCT4 – POU domain-containing transcription factor 4

ON – Optic nerve

ONL – Outer nuclear layer

OPL – Outer plexiform layer

OS – Optic stalk

OV – Optic vesicle

P – Postnatal day

PAX6 – Paired box gene 6

POAG – Primary open angle glaucoma

PNR – Peripheral neural retina

PR – Photoreceptor

RAX – Retina and anterior neural fold homeobox

RGC – Retinal ganglion cell

RPE – Retina pigment epithelium

SEM – Scanning electron microscopy

Sey – Small eye

SOX2 – SRY-box containing gene 2

SRY – Sex-determining region Y

TCF – T-Cell specific transcription factor family member

TEM – Transmission electron microscopy

TGFβ - Transforming growth factor β

TSS – Transcription start site

VSX2 – Visual system homeobox 2

WNT – Wingless-type MMTV integration site family member

CHAPTER I: INTRODUCTION

Overview

This chapter summarizes what is known about eye development in mammals and describes the classical studies that inform the current questions surrounding neural induction. The role of the SOXB1 family of transcription factors in these processes is referenced throughout. The function of the SOXB1 family member SOX2 as a transcriptional activator, and sometimes a transcriptional repressor, is described in terms of neural development. Finally, the usefulness of the optic cup as a model of neural induction is explained.

Eye Development in Mammals

For generations of biologists, the eye has offered an accessible model for investigating the mechanisms that coordinate the development and morphogenesis of diverse cell types. At the beginning of the twentieth century, embryologists studying eye development in amphibians described the concept of *induction* for the first time after discovering that tissues of different origins must interact in order to generate a lens (Spemann, 1901). A century later, with significant advances in molecular biology, the eye has provided a system for studying gene interactions. Because vision is not essential for life, eye malformations have revealed that a single mutation can lead to disease. Currently, the ability to profile gene expression in single retinal cells, which has revealed extensive heterogeneity among retinal progenitors, enables the study of eye development at the systems level (Byerly and Blackshaw, 2009; Kim et al., 2008; Roesch et al., 2008; Trimarchi et al., 2008). Genes that coordinate eye development are highly conserved across species, so while this section focuses principally on mammalian eye

development, the underlying cellular and molecular paradigms can be applied to lower vertebrates as well. Indeed, the eye development field thrives on a rich history of developmental biologists working in organisms from flies to humans.

Briefly, the visible stages of mouse eye development, which are described in detail below, include optic sulci (E8.0), optic vesicle evagination (E9.0), optic cup invagination (E10.0), and retinal neurogenesis (E11.0 – P7).

The eye field: establishing the optic primordia

Shortly after gastrulation, the eye primordium, or eye field, is specified in the medial anterior neural plate and contains all the progenitors of the neural-derived eye structures (Li et al., 1997; Wilson and Houart, 2004; Zaghloul et al., 2005). In mice, the first morphological sign of the eye field is the formation of bilateral indentations in the prospective forebrain termed the optic sulci, or optic pits, at embryonic (E) day 8.0 (Adelmann, 1929; Li et al., 1997; Wilson and Houart, 2004) (Figure 1-1A). Molecularly, the eye field can be identified by the overlapping expression domains of a set of highly conserved genes termed the eye field transcription factors (EFTFs) (Lee et al., 2006; Moore et al., 2004; Zaghloul et al., 2005) (Byerly and Blackshaw, 2009; Zuber et al., 2003). In mammals, the EFTFs, which include *Pax6*, *Rax*, *Six3* and *Lhx2*, constitute a regulatory network required for eye development (Byerly and Blackshaw, 2009; Zuber et al., 2003). The identification of the upstream signaling pathways that specify the eye field is challenging given that these genes also play a role in forebrain development (Hagglund et al., 2011).

A recent study identified an 11kb genomic region in the *Lhx2* promoter that specifically directs *Lhx2* expression to the eye field, thus defining a distinct eye-committed progenitor cell population in the forebrain (Hagglund et al., 2011). The identification of factors controlling the

activity of this *Lhx2* regulatory element may reveal specific pathways required for eye field specification. In fact, the conditional inactivation of *Lhx2* in this cell population has no effect on the activity of the *Lhx2* eye field enhancer, suggesting that *Lhx2* itself is not essential for eye field priming (Hagglund et al., 2011). However, the finding that eye development is arrested in mice lacking *Lhx2* corroborates a study in which ectopic expression of eye field transcription factors can generate eyes in *Xenopus* only when endogenous *Lhx2* expression is induced. (Cavodeassi et al., 2005; Fuhrmann, 2008; Rasmussen et al., 2001; Zuber et al., 2003). Moreover, this study demonstrated that OTX2, a transcription factor essential for forebrain development, and Noggin, a BMP antagonist, potentiate EFTF expression in the anterior neural plate (Zuber et al., 2003). Similarly, *in vitro* data suggest that OTX2 cooperates with SOX2 to activate *Rax* expression, even though *Otx2* becomes down-regulated in the *Rax* expression domain of the early eye field (Andreazzoli et al., 1999; Danno et al., 2008; Zuber et al., 2003). SOX2 in coordination with a POU-factor may directly antagonize OTX2 in this region (see Discussion). These data demonstrate that SOX2 plays an integral role in the earliest stages of eye development and support a model of "progressive induction" (Zuber et al., 2003). This model predicts that the anterior forebrain must be primed for eye field formation such that SOX2 and OTX2 activate EFTF expression, and EFTFs then work in a feedback network to maintain the eye field (Figure 1-2).

Heterozygous mutations in human *OTX2* can cause a range of ocular phenotypes from bilateral anophthalmia to retinal dystrophy (Ragge et al., 2005a). In contrast to *SOX2* mutations (discussed below), *OTX2* mutations are commonly associated with impaired retinal function, perhaps owing to the role of OTX2 in RPE development (Chase, 1944; Grindley et al., 1995; Hanson et al., 1993; Martinez-Morales et al., 2003; Mathers et al., 1997; Medina-Martinez et al.,

2009; Ragge et al., 2005a; Tabata et al., 2004; Tucker et al., 2001; Voronina et al., 2004; Wawersik and Maas, 2000; Zhang et al., 2000)

Eye field transcription factors

Genetic studies in flies (Wawersik and Maas, 2000), mice and humans illustrate that the EFTFs are essential for proper eye development and function in a conserved network to regulate eye formation.

a. Pax6 and Lhx2

An initial genetic demonstration of EFTF function was the identification of heterozygous mutations in the *Pax6* locus that cause the mouse *small eye (Sey)* haploinsufficient phenotype (Hill et al., 1991; Hogan et al., 1988). *Pax6* is a member of the evolutionarily conserved family of paired domain-containing transcription factors (further discussed in Chaper II) (Walther and Gruss, 1991). Humans with mutations in one copy of *PAX6* often have aniridia, a severe ocular malformation characterized by abnormal iris development and, more rarely, microphthalmia, corneal cataracts and macular and foveal hypoplasia (Glaser et al., 1994; Glaser et al., 1992; Hever et al., 2006). Mice with one copy of the *Sey* allele exhibit reduced eye size and variable abnormal development of the retina, iris, lens and/or cornea (Hever et al., 2006; Hill et al., 1991). Homozygous loss of *Pax6* function in humans and mice causes anophthalmia, or the complete lack of eyes (Glaser et al., 1994; Hill et al., 1991).

Like $Pax6^{Sey/Sey}$ mouse mutants, $Lhx2^{-/-}$ embryos generate optic vesicles but never form optic cups (Porter et al., 1997). Conditional inactivation of *Lhx2* in the eye field leads to developmental arrest of the optic vesicle just prior to optic cup formation, but the expression of *Pax6*, *Rax* and *Six3* persists in the optic vesicle (Hagglund et al., 2011) (Tetreault et al., 2009).

The maintenance of *Pax6* in *Lhx2*$^{-/-}$ mutants, and the maintenance of *Lhx2* in *Pax6*$^{Sey/Sey}$ mutants, suggests that these two EFTFs are independently essential but separately insufficient for proper eye development (Porter et al., 1997). Moreover, *Pax6* may cooperate with *Lhx2* to induce the expression of *Six6*, a retinal determinant gene, in the optic vesicle (Tetreault et al., 2009).

b. Rax

Like the *Sey* mouse line, the spontaneous mutant mouse strain *eyeless*, first discovered in the 1940s, carries a hypomorphic mutation in the *Rax* (retina and anterior neural fold homeobox) locus (Chase, 1944; Tucker et al., 2001). Mutations in human *RAX* are associated with anophthalmia (Hanson et al., 1993; Voronina et al., 2004). The role of *RAX* has been further elucidated using mouse model systems where it was shown that *Rax*$^{-/-}$ mice fail to upregulate EFTF expression in the presumptive eye field and do not develop optic vesicles (Grindley et al., 1995; Mathers et al., 1997; Zhang et al., 2000). In chimeric mice containing wild-type and *Rax*$^{-/-}$ cells, the *Rax*-negative cells segregate together and are never found in eye field-derived tissues. This result suggests that RAX is involved in the sorting of cells to form a distinct eye territory, perhaps through the action of cell surface molecules (Medina-Martinez et al., 2009). Conversely, overexpression of *Rax* in mouse embryonic stem cells co-cultured with a host retina promotes retinal cell fates (Tabata et al., 2004).

c. Six3

Six3 encodes a homeobox-containing transcription factor homologous to the *Drosophila* sine oculis gene (Oliver et al., 1995). Genetic inactivation of *Six3* in presumptive eye tissue has demonstrated that it is essential for eye development in mammals (Liu et al., 2010; Marquardt et al., 2001). Conditional inactivation of *Six3* in the eye field abrogates neural retina development, while misexpression of *Six3* in the midbrain-hindbrain region of mouse embryos

causes ectopic optic vesicles (Lagutin et al., 2001; Liu et al., 2010). Mutations in human *SIX3* are associated with holoprosencephaly, or a failure of the cerebral hemispheres to separate (described below) (Geng et al., 2008).

These studies collectively illustrate the importance of the EFTFs in regulating the network of events that control early eye development, from the specification of the eye field to the maintenance of progenitor multipotency.

Division of the eye field

Developmental biologists working in the 1920s observed that both eyes arise from a single eye field that is divided into bilateral hemispheres (Adelmann, 1929; Li et al., 1997; Mangold, 1931). At least two molecules have been clearly demonstrated to be involved in this morphogenetic process. The first is *sonic hedgehog* (*Shh*), which is expressed in the ventral forebrain and prechordal mesoderm (Echelard et al., 1993). Targeted disruption of *Shh* in mice results in the failure of the eye field to split, resulting in cyclopia and a single *Pax6*-positive optic vesicle (Chiang et al., 1996). The second player is *Six3*, which is expressed throughout the anterior neural ectoderm before becoming restricted to the ventral forebrain and eye field (Oliver et al., 1995). In humans, loss-of-function mutations in either *SHH* or *SIX3* result in midline defects that frequently include cyclopia (Belloni et al., 1996; Muenke and Cohen, 2000; Roessler et al., 1996). In fact, SIX3 was shown to regulate *Shh* expression in the ventral midline of the rostral diencephalon via an upstream enhancer element (Geng et al., 2008; Jeong et al., 2008).

Establishing boundaries in the optic vesicle

Ocular development begins with the formation of the optic vesicles. From E8.5-9.0 of mouse development, the walls of the diencephalon evaginate (the optic vesicles) and come into

close contact with the surface ectoderm (Figure 1-1D) where the lens placode is formed. Each optic vesicle (OV) consists of the retinal stem cells (RSCs) that give rise to all neuroectoderm-derived cells of the eye. RSC patterning occurs along the dorso-distal/proximal-ventral axis of the OV prior optic cup formation. Regions along this axis correspond to the presumptive neural retina (NR -- distal OV), retinal pigment epithelium (RPE -- dorsal/proximal OV) and optic stalk (OS -- ventral/proximal OV) (Figure 1-3, Figure 1-4A).

Several cell-intrinsic signaling pathways are involved in patterning the OV. Each compartment of the OV expresses a specific set of transcription factors that are important for the development of the cell type in which they are expressed. The presumptive neural retina specifically expresses the homeodomain protein *Vsx2* (formerly *Chx10*), the future RPE expresses the basic helix-loop-helix transcription factor *Mitf* and the prospective OS expresses the paired domain protein *Pax2* (Hodgkinson et al., 1993; Liu et al., 1994; Nornes et al., 1990). SOX2 is maintained in the prospective NR and OS but is down-regulated in the prospective RPE. There is evidence to suggest that OTX2 directly represses SOX2 in the RPE (Nishihara et al., 2012). Many studies have revealed that *Vsx2*, *Mitf* and *Pax2*, in combination with the EFTFs, have cell intrinsic roles in compartmentalizing the future optic cup, often through reciprocal transcriptional repression of one another (Figure 1-3). Collectively, these studies also suggest that the RSCs of the OV are competent to become NR, RPE or OS when provided with the appropriate combination of signals.

Neural retina versus retinal pigment epithelium versus optic stalk

Mutual antagonism between *Vsx2* and *Mitf* serves to establish the boundary between the future NR and RPE (Horsford et al., 2005; Nguyen and Arnheiter, 2000). *Mitf* is initially expressed throughout the dorsal OV but becomes down-regulated distally upon the expression

of *Vsx2* (Nguyen and Arnheiter, 2000). The function of *Mitf* in boundary formation is supported by the observation that mice with loss of function mutations in *Mitf* exhibit a conversion of RPE to NR (Bumsted and Barnstable, 2000; Nguyen and Arnheiter, 2000). A similar RPE-to-NR conversion phenotype occurs in mice that are deficient in both *Otx1* and *Otx2*, transcription factors that are normally expressed in the dorsal OV/presumptive RPE (Martinez-Morales et al., 2001). Conversely, mice with loss-of-function mutations in *Vsx2*, termed *or*^{J} or ocular retardation mice, exhibit ectopic expression of *Mitf* and *Mitf* target genes in the NR. Fate mapping analyses suggest that this phenotype is a direct transdifferentiation of the neural retina to RPE (Horsford et al., 2005; Rowan et al., 2004).

A similar antagonistic relationship exists between *Pax2* and *Pax6*. *Pax2*$^{-/-}$ mice exhibit ventral expansion of the *Pax6* expression domain and subsequent expansion of the NR and RPE at the expense of the OS (Schwarz et al., 2000). Conversely, *Pax6*-deficient mice exhibit a dorsal expansion of the *Pax2* expression domain and fail to develop a neural retina or RPE, maintaining only the *Pax2*-positive optic stalk. This reciprocally repressive relationship appears to involve a direct molecular interaction, as PAX2 can bind the *Pax6* retina-specific enhancer, α, (Figure 1-4A), and PAX6 can bind the *Pax2* OS-specific enhancer (Schwarz et al., 2000). Similarly, humans with *PAX2* mutations have optic nerve coloboma caused by the failure of the ventral optic fissure to properly close during development (Torres et al., 1996). Therefore, *Pax2* and *Pax6* likely establish the boundary between the optic stalk and the neural retina through mutual repression of one another.

The EFTF *Lhx2* appears to act upstream of the above described genetic interactions. In *Lhx2* loss-of-function mutants, the OV fails to become regionalized, exhibiting ventral expansion of *Pax6* but lacking *Vsx2*, *Mitf* and *Pax2* (Yun et al., 2009). However, in embryos with specific

ablation of *Lhx2* in the eye field, *Pax2* expression persists in the ventral optic vesicle, but *Mitf* is downregulated, and *Vsx2* is absent (Hagglund et al., 2011).

Signaling networks in the optic vesicle

Adding complexity to the system of early eye development is the understanding that these cell intrinsic transcription factors modulate extrinsic signals to functionally compartmentalize the OV. The extrinsic pathways involved in OV patterning include BMP, FGF, Wnt and *Sonic Hedgehog* signaling. Early Lhx2 activity, for instance, is required to transduce *BMP7* to activate *Pax2* expression in the ventral OV, while later in development, Lhx2 is required to maintain *BMP4* expression in the OV (Yun et al., 2009). Similarly, FGF1 or FGF2 from the surface ectoderm activates *Vsx2* in the presumptive NR, which in turn represses *Mitf* (Horsford et al., 2005; Nguyen and Arnheiter, 2000).

FGF9, which is normally confined to the distal OV, promotes NR fate when ectopically expressed in the presumptive RPE, and mice with targeted deletion of *Fgf9* exhibit expansion of the RPE into the NR domain (Zhao et al., 2001). Moreover, OV-specific deletion of the protein phospatase *Shp2*, which mediates the FGF signaling cascade via sustained activation of Ras, causes a cell fate conversion from NR to RPE (Cai et al., 2010). Conversely, inactivation of canonical Wnt signaling in the presumptive RPE causes it to transdifferentiate to NR (Westenskow et al., 2009). Lastly, in addition to its role in splitting the eye field, midline-secreted *Shh* plays an additional role in ventralizing the OV. The optic vesicle of *Shh* mutant mice shows expanded *Pax6* expression at the expense of *Pax2*, while *Otx2*, a presumptive RPE (dorsal) marker, persists in the cyclopic *Shh* mutant eye (Chiang et al., 1996).

Humans with mutations in some of these genes exhibit ocular malformations. Mutations in *BMP4*, for instance, have been described in patients with anophthalmia/microphthalmia,

coloboma and retinal dystrophy (Hayashi et al., 2008). Similarly, mutations in *VSX2* are associated with microphthalmia, iris abnormalities, coloboma and retinal dystrophy (Ferda Percin et al., 2000; Iseri et al., 2010). Although rare in comparison to cyclopia, anopthalmia/microphthalmia and coloboma can result from mutations in human *SHH* (Bakrania et al., 2010).

Optic cup morphogenesis

After the formation of the OV, a coordinated invagination of the lens placode and the OV forms the lens vesicle and the bi-layered optic cup (OC) (Figure 1-1B, C, E, F). The OV folds into itself creating two nested cups; the distal OV becomes the inner layer of the OC -- the presumptive NR -- while the proximal OV becomes the outer layer of the OC -- the presumptive RPE (Figure 1-1F, Figure 4B). An additional invagination occurs in the ventral OV where the optic stalk meets the ventral retina to generate the optic or choroidal fissure (Figure 1-4B). The optic fissure provides an exit for retinal axons and an entrance for the hyaloid artery, which supplies blood to the retina (Saint-Geniez and D'Amore, 2004). The OC grows circumferentially until it closes over the optic fissure (Figure 1-4C).

Crosstalk between the presumptive lens and retina may be necessary for the proper invagination of these tissues *in vivo* (Ashery-Padan et al., 2000; Bassett et al., 2010; Grindley et al., 1995; Smith et al., 2009). SOX2 is expressed in both of these tissues and is necessary for their specification (discussed further in Chapter 3). The optic vesicle and lens placode are tightly apposed at the initiation of OV invagination. However, the necessity of the presence of the lens placode for the formation of the two-walled optic cup remains unclear. A recent study used three-dimensional culture of mouse embryonic stem cell aggregates to derive hollowed spheres of neuroepithelium containing *Rax*-positive domains. Many of these regions spontaneously

invaginated, in the absence of a lens or ectodermal tissues, to form the optic cup (Eiraku et al., 2011). This autonomous morphogenetic process appeared to be driven by forces within the retinal anlage, suggesting that, at least *in vitro*, the OV can form the OC without instruction from other structures (Eiraku et al., 2011). This finding is relevant to the cell-autonomous role of SOX2 in patterning the OC independently of lens-derived signals (discussed in Chapter II).

Conversely, the apposition of the lens placode and the OV appears to be important for placode invagination. The LP arises from the preplacodal region (PPR), an ectoderm-derived bilateral structure that forms discrete thickenings called placodes, the precursors to various vertebrate sensory structures (Streit, 2007). The LP is identified as the group of thickened columnar cells in the head surface ectoderm that arises in response to OV proximity. The LP and OV become physically tethered through cytoplasmic processes, or filopodia, originating from the base of the lens (Chauhan et al., 2009).The LP invaginates to form the lens cup or lens pit which eventually separates from the surface ectoderm to form the lens vesicle (Graw, 2010).

Placode thickening is associated with local changes in cell shape without associated changes in cell volume. LP formation appears to be mediated by adhesion between the optic vesicle and the surface ectoderm, where lens precursors continue to proliferate but cannot expand beyond the region of adhesion (Hendrix and Zwaan, 1975; Huang et al., 2011). The increase in cell number in this fixed area is sufficient to account for the increase in cell length observed during placode formation (Hendrix and Zwaan, 1975). Adhesion between the optic vesicle and the surface ectoderm is mediated by the extracellular matrix protein fibronectin1 (*fn1*) (Huang et al., 2011). Indeed, *fn1* expression is lost when *Pax6*, a master regulator of lens development, is deleted in the surface ectoderm (Huang et al., 2011) (Figure 1-5).

Lens and cornea

In addition to *Pax6*, several other transcriptional regulators are important for lens specification. *Six3* and *Sox2*, for example, are both essential for proper lens development. *Six3* expression precedes that of *Pax6* in the presumptive lens ectoderm and activates *Pax6* transcription (Liu et al., 2006b). While *Sox2* expression in the LP is induced by signals from the OV, it may also be mediated by SIX3 in the surface ectoderm (Furuta and Hogan, 1998; Kamachi et al., 1998). Thus, much like the cell-intrinsic regulation of OV patterning, transcription factors involved in lens induction transduce signaling cascades to activate downstream targets. One such signal is BMP4 from the OV, which may activate *Sox2* but not *Pax6* expression in the LP. Another is BMP*7* in the head ectoderm, which may activate *Pax6* expression in the LP (Furuta and Hogan, 1998; Wawersik et al., 1999).

SOX2 and PAX6 also function in cross- and self- regulatory feedback loops in lens development. Sustained *Sox2* expression appears to depend on PAX6 only after lens placode stages (Ashery-Padan et al., 2000; Smith et al., 2009). Moreover, SOX2 cooperates with PAX6 in its own upregulation in lens precursor cells via the N3 enhancer upstream of the *Sox2* coding sequence (Inoue et al., 2007). Likewise, PAX6 and SOX2 synergistically activate *Pax6* expression via the head surface ectoderm enhancer element LE9 (Aota et al., 2003). SOX2 and PAX6 may also co-regulate other genes important for lens development, such as those encoding lens crystallins (Kamachi et al., 1998; Kamachi et al., 2001). The relationship between SOX2 and PAX6 in lens development is consistent with a strong genetic interaction in optic cup development, as described in detail in Chapter II.

The lens vesicle remains transiently attached to the surface ectoderm via the lens stalk. Once detached from the lens, the surface ectoderm proliferates to restore the exterior,

eventually giving rise to the corneal epithelium. The corneal endothelium is composed of migrated forebrain and midbrain neural crest-derived mesenchymal cells (Kanakubo et al., 2006; Trainor and Tam, 1995). These cells invade the newly formed space between the lens vesicle and the surface ectoderm and condense into a multilayered structure connected by a loose extracellular matrix. The cells between the corneal epithelium and endothelium differentiate into karatocytes and make up the corneal stroma (Cvekl and Tamm, 2004). The space between the future cornea and lens, called the anterior chamber, becomes fluid filled. A second group of mesenchymal cells migrates into the angle between the presumptive cornea and peripheral edge of the optic cup to become the stroma of the iris and ciliary body (described below). Around the same time that mesenchymal cells migrate into the future cornea (E11.5), other POM cells invade the space between the lens and the retina, called the hyaloid, giving rise to the hyaloid vasculature (Gage et al., 2005)

Axes in the neural retina

The inner layer of the OC, which gives rise to the neural retina, is patterned along its dorsal-ventral (D-V) and nasal-temporal (N-T) axes (Figure 1-6). By E10.5, the optic stalk (ventral) has begun to elongate and will eventually give rise to the optic nerve. The axons of retinal ganglion cells from the innermost layer of the NR begin to enter the optic stalk around E11.5 and intersperse with the PAX2-positive optic stalk cells, the glial precursors that will develop into the astrocytes that make up the mature optic nerve (Torres et al., 1996).

Proper optic nerve placement depends on signals that pattern the OC along the D-V axis. These signals are mediated in part by members of the VAX family of homeodomain transcription factors. At E13.5, *Vax1* is expressed in the optic stalk, while *Vax2* exhibits a steep ventral^{high}-to-dorsal^{low} gradient of expression (Mui et al., 2002; Mui et al., 2005). Both *Vax1* and *Vax2* are

expressed in the optic stalk and ventral retina from E9.5 to E11.5, which may explain why *Vax1*$^{-/-}$; *Vax2*$^{-/-}$ double mutant mice exhibit severe ventral eye defects. The optic stalk of these mice becomes dorsalized, developing into RPE and NR instead of optic nerve (Mui et al., 2005). *In vitro* and *in vivo* data suggest that VAX1 and VAX2 cooperatively specify the ventral optic cup and optic stalk by directly repressing *Pax6* expression via the *Pax6* retina-specific enhancer α (Mui et al., 2005). Conversely, the T-box transcription factor *Tbx5*, a BMP4 target, is normally expressed in the dorsal NR (Behesti et al., 2006). Its expression is lost when *Pax6* is ablated from the developing NR, further demonstrating *Pax6*'s role in specifying the dorsal OC (Baumer et al., 2002; Behesti et al., 2006). These genetic studies illustrate the precise regulation of PAX6 localization in the OC, which in turn helps to establish the dorsal-ventral axis.

N-T patterning of the optic cup ensures that the axons of retinal ganglion cells will correctly map to their targets in the superior colliculus (SC) and the dorsal lateral geniculate nucleus (dLGN). In mammals, axons from the nasal retina project to the caudal SC, and axons from the temporal retina project to the rostral SC. The earliest transcriptional regulators of N-T identity in the OC are the forkhead transcription factors FOXD1 (BF-2) and FOXG1 (BF-1). *Foxg1* is expressed in the nasal retina, but appears to play an additional role in D-V patterning, as *Foxg1*$^{-/-}$ mutants lose *Shh* signaling and have dorsalized optic vesicles (Huh et al., 1999). However, *Foxd1* functions in N-T patterning to specify the retinal ganglion cells (RGCs) of the temporal retina (Carreres et al., 2011). Indeed, RGCs of *Foxd1*$^{-/-}$ mutants lose topographic specificity, mapping indiscriminately to the entire extent of the SC (Carreres et al., 2011). Expression of both *Foxd1* and *Foxg1* is lost in *Pax6*$^{-/-}$ mutants, demonstrating an additional role for PAX6 in establishing the naso-temporal region of the retina (Baumer et al., 2002).

Ciliary body and iris

A third axis along which the OC is patterned is the central-peripheral axis (Figure 1-4D, Figure 1-6). The distal tip of the OC, where the presumptive NR meets the RPE, is termed the "ciliary margin" in mice and contains non-neurogenic progenitors that give rise to the epithelia of the ciliary body (CB) and iris (Beebe, 1986; Davis-Silberman and Ashery-Padan, 2008). The CB epithelia are continuous with the RPE and NR, while the iris epithelia are distal to the CB. The outer layer of the ciliary margin gives rise to the pigmented epithelium of the CB and the anterior pigmented layer of the iris, whereas the inner layer gives rise to the non-pigmented epithelium of the CB (herein referred to as the ciliary epithelium or CE) and the posterior pigmented layer of the iris. Non-neurogenic progenitor cells of the optic cup margin can be identified as early as E12.5 by their specific expression of transcription factors, including *Msx1* and *Otx1*, lack of expression of neuronal markers and a slower proliferation rate relative to the NR (Beebe, 1986; Cho and Cepko, 2006; Martinez-Morales et al., 2001; Monaghan et al., 1991; Trimarchi et al., 2009). Indeed, CB development is absent in mice that lack *Otx1* (Acampora et al., 1996).

Several cell-intrinsic and extrinsic pathways have been identified to play a role in specifying the ciliary margin in mammals. CE-specific genes can be induced in embryonic mouse retina when cultured adjacent to an explanted chick lens (Thut et al., 2001). The inductive power of the lens may in part involve BMP signaling, as transgenic expression of the BMP antagonist Noggin in the developing mouse lens abrogates expression of *Bmp4* and *Bmp7* in the presumptive CE at postnatal stages (Zhao et al., 2002). Without BMP signaling, the CE-specific genes *Otx1* and *Msx1* fail to be expressed, and neural retinal cells develop in place of the CB after birth (Zhao et al., 2002).

The appearance of the NR at the expense of the CE in the BMP-deficient ciliary margin suggests that progenitor cells at the boundary of the NR and CE remain competent to take on one fate or the other after these tissues have begun to be specified. Transcriptional control of this binary cell fate decision is mediated by SOX2 and PAX6 (Figure 1-4D). This relationship is discussed at length in Chapter III. Briefly, in the early optic cup, SOX2 and PAX6 exhibit inverse gradients of expression, with SOX2 high in the central OC but low in the periphery where PAX6 is maintained at a high level due in part to its retina-specific enhancer α (Baumer et al., 2002; Matsushima et al., 2011). Specific ablation of SOX2 in OC progenitor cells results in elevated *Pax6* expression and cell fate conversion from NR to CE, a phenotype described in detail in Chapter II. (Matsushima et al., 2011). This cell fate conversion can be partially rescued by reducing *Pax6* (Matsushima et al., 2011). Indeed, several studies have demonstrated the importance of PAX6 to the development of the ciliary margin. Mice that are haplo-insufficient for *Pax6* exhibit reduced ciliary margin size, and humans with mutations in *PAX6* have aniridia (no iris) and small ciliary bodies (Davis-Silberman et al., 2005; Hanson et al., 1993; Okamoto et al., 2004). Further evidence suggests that Wnt signaling plays a role in specifying CE fate. Activated Wnt signaling in the peripheral NR induces expression of CE-specific genes (Liu et al., 2007b). Moreover, the NR-to-CE cell fate conversion caused by the loss of SOX2 is associated with centrally expanded Wnt signaling prior to the ectopic expression of CE genes (Matsushima et al., 2011). Chapter IV focuses on the genetic relationship between SOX2 and Wnt signaling in OC patterning and CE fate specification.

Retinal neurogenesis

Retinal Progenitor Cells (RPCs) are established in the optic vesicle as early as E9.0, each giving rise to a clone of more than 1000 post-mitotic neural retina cells (Goldowitz et al., 1996).

Neurogenesis in the retina proceeds in an ordered and predictable fashion, beginning in the central optic cup and moving toward the periphery. Specification of RPC fate is coordinated with cell cycle exit to generate the correct numbers and types of neurons that make up the mature retina. Neuronal differentiation begins at E11.0 and cell types are generated in overlapping phases, beginning with retinal ganglion cells (RGCs), which are the projection neurons of the retina. The initiation of RGC production is followed in succession by horizontal interneurons, cone photoreceptors and amacrine interneurons in an early wave of neurogenesis (E11.0 – E18.0). A temporal switch in RPC competence then produces rod photoreceptors, bipolar interneurons and Muller glial cells in a late wave of neurogenesis (P0 – P7) (Young, 1985).

The architecture of the mature retina is as follows: The cell bodies of rod and cone photoreceptors are localized to the apical side of the retina in a layer abutting the RPE known as the outer nuclear layer (ONL). Just basal to the ONL is the inner nuclear layer (INL), which contains the cell bodies of the retinal interneurons -- the bipolar, amacrine and horizontal cells – as well as the cell bodies of the Muller glia. The ganglion cells are located in the ganglion cell layer (GCL) on the basal side of the retina, nearest to the lens. Separating the ONL and the INL is the outer plexiform layer (OPL) where photoreceptors synapse onto horizontal cells and bipolar cells. The more elaborate inner plexiform layer (IPL) is located between the INL and the GCL and contains the dendrites of amacrine cells and RGCs (Figure 1-7). All together, this structure illustrates the first neurological steps of visual processing: the photoreceptors receive light input and transmit the signal through bipolar cells, whose axons terminate in the IPL, where RGCs then transmit the signal to visual processing centers in the brain. SOX2 expression remains only in a subset of amacrine cells and in the Muller glia (Surzenko et al., 2013) (Figure 1-7).

A number of transcription factors that regulate RPC fate decisions have been identified. These are members of the homeodomain family or bHLH family of transcription factors. (Figure 1-7). The homeodomain transcription factors include those that are expressed in the forebrain and eye field (OTX2, RAX, SIX3, SIX6, PAX6 and LHX2) as well as those that are expressed early in the prospective retina (VSX2) or play a role in specifying retinal subtypes (PROX1, CRX, LHX1, DLX1 and BRN3). The bHLH factors include repressors of neuronal differentiation, HES1 and HES5, and activators of neuronal differentiation, including the Atonal homologs MATH5, MATH3 and NEUROD and the Acheate-Schute homologs NGN2 and MASH1. RPCs that express one or a combination of these bHLH factors are biased toward a particular cell fate (Hatakeyama and Kageyama, 2004). Achaete-Schute homologs generally play dual roles in regulating the onset of differentiation and cell fate specification. Similarly, atonal homologs are expressed in RPCs at the onset of neuronal differentiation and often identify the fate of the progenitor cell. MATH5 expression in early RPCs, for example, predicts RGC development, while NEUROD and MATH3 each predict amacrine cell development. Late RPCs are competent to give rise to bipolar cells or Muller glia. Math3 or Mash1 expression in these cells predicts bipolar fate (Hatakeyama et al., 2001). Homeodomain and bHLH factors can also act in combination to specify cell fate, with the former regulating layer specificity and the latter regulating subtype within the layer (Hatakeyama and Kageyama, 2004).

Neural Induction

The development of the central nervous system (CNS) in vertebrates begins with neural induction, or the process in which an ectodermal cell is specified as neural (neural plate) or non-neural (epidermis). The mechanisms directing this cell fate decision have come to be understood primarily through experiments using gastrulating frog and chick embryos. Nonetheless, certain neural induction paradigms hold true for mammals as well: secreted factors, namely BMPs and FGFs, specify whether an ectoderm progenitor cell maintains neural competence, becoming neural plate, or acquires epithelial identity, thus losing neurogenic capacity (Delaune et al., 2005; Hemmati-Brivanlou and Melton, 1997; Kuroda et al., 2005; Linker and Stern, 2004; Reversade et al., 2005; Stern, 2006; Wawersik et al., 2005; Wills et al., 2010).

Neural development in mice

At E5, the mouse embryo consists of two layers, the primitive endoderm and the epiblast. The epiblast gives rise to all the cells of the adult mouse. The naïve state of the epiblast is ectoderm. Gastrulation begins at E6.5 when the ectoderm invaginates to produce the primitive streak, which moves anteriorly to produce the mesoderm and endoderm. The anterior end of the primitive streak is the organizing center or node, which secretes signals important for specifying neural progenitor cells, which arise from the anterior distal tip of the ectoderm (Zernicka-Goetz, 2002). Nodal signaling from the primitive streak stabilizes the epiblast state, while Nodal antagonists expressed from the anterior visceral endoderm (AVE), an extraembryonic tissue, disrupt Nodal signaling. The earliest derivative of the mouse epiblast is the anterior neural plate (ANP). It is thought that Nodal, a member of the TGFβ family, functions to specify the AVE, which in turn functions to specify anterior identity in the adjacent epiblast through secretion of the Nodal antagonists Lefty, Cerberus-like and Dickkopf1 (Brennan et al.,

2001; Perea-Gomez et al., 2001). In the absence of *Nodal*, epiblasts ectopically express neural plate markers, including *Sox1* and *Hesx1*, suggesting that Nodal antagonizes neural fate (Camus et al., 2006).

The default model

The classical model of neural induction predicts that neural is the default or ground state of the ectoderm (Hemmati-Brivanlou and Melton, 1997; Weinstein and Hemmati-Brivanlou, 1999). Mouse embryonic stem (ES) cells develop into neural progenitor cells (NPCs) without the addition of exogenous factors (Ying and Smith, 2003). The anterior ectoderm of the mouse will express neural genes in the absence of Nodal (Camus et al., 2006). Similarly, cultured *Xenopus* ectodermal explants, which express *BMP4*, develop into epidermis when kept intact, but become neural when dissociated. Non-neural fate in these cells can be rescued with the addition of BMP4. Moreover, intact explants become neural with the addition of BMP inhibitors (Munoz-Sanjuan and Brivanlou, 2002). These experiments informed the idea that ectoderm becomes epidermis in response to BMP signals and neural plate -- "by default" -- when BMP signals are inhibited.

According to this model, non-neural fate must thus be imposed upon the ectoderm. Nodal-deficient tissues exhibit little-to-no BMP and Wnt activity, suggesting that all three of these pathways are inhibited during anterior neural fate specification. In other words, Nodal, BMP and/or WNT must be active for non-neural fate to be specified (Camus et al., 2006). In *Xenopus*, BMP signaling through BMP receptor (BMPR)-mediated activation of the transcription factor SMAD1 promotes non-neural fate. The BMP antagonists Noggin, Chordin and Follistatin collectively prevent BMPR from phosphorylating SMAD1, thus allowing the ectoderm to generate neural plate. These extracellular signals are produced by Spemann's organizer, a region

of the dorsal mesoderm that is thought to maintain neural fate in the ectoderm (Reviewed in (De Robertis and Kuroda, 2004)).

However, numerous studies have suggested that the default model is an over-simplification of neural induction. For one, it does not fully explain neural fate specification in avian embryos. In chicks, the organizer is sufficient for neural fate, but BMP inhibition is not; only cells at the border of neural and non-neural ectoderm are competent to respond to BMP inhibition, suggesting that these cells are exposed to additional neural-provoking cues (Streit et al., 1998; Streit and Stern, 1999). Similarly, BMP inhibition is sufficient to suppress epidermis but insufficient to induce neural fate in regions of the ectoderm distant from the endogenous neural plate (Delaune et al., 2005). Experiments in *Xenopus* suggest that FGF and IGF, which are potent inducers of neural fate, may be required for neural ectoderm specification and are potential candidates for "neural-inducing cues" (Hardcastle and Papalopulu, 2000; Pera et al., 2001; Streit et al., 2000; Wilson et al., 2000). These growth factors function through SMAD1 regulation as well: activation of MAPK by FGF or IGF phosphorylates SMAD1 in a highly conserved linker region, inhibiting its neural-antagonizing ability. Mutation of the target serines in this linker region results in a strongly ventralized embryo, indicating loss of neural fate. Conversely, BMPR phosphorylates the C-terminus of SMAD1. Mutating these C-terminus serine residues in addition to those in the linker region rescues ventralization, demonstrating that BMPR activity is required to antagonize neural fate (Pera et al., 2003). Therefore, a fine balance between FGF, IGF and BMP signaling regulates neural ectoderm development via SMAD1.

The roles of BMP and FGF signaling differ between mammals and lower vertebrates. In *Xenopus*, as described above, neural induction requires both BMP inhibition by Chordin and the combined activity of FGF and IGF. Over-expression of *Chordin*, *Fgf* or *Igf* alone can expand the

neural plate, induce ectopic sensory neurons and suppress epidermal genes while inducing anterior neural genes. Moreover, constitutive activation of BMPR in a dorsal animal blastomere of *Xenopus* embryos suppresses neural fate, resulting in loss of *Sox2* and *Sox3*, and induces epidermis, expressing *cytokeratin81* (Delaune et al., 2005). Likewise, inhibition of FGF signaling by mutation of the SMAD1 linker region results in loss of neural fate (Pera et al., 2003). In mouse embryos, however, the role of SMAD1 is more complex. Mutation of the linker region does not abrogate neural fate. Rather, mice with targeted disruption of these SMAD1 sites exhibit defects of the gastric epithelium and actin cytoskeleton (Aubin et al., 2004). This relatively mild phenotype may be due to the compensatory function of SMADs 5 and 8. Nonetheless, the regulation of neural fate in mammals is exceedingly more complex than in lower vertebrates. And despite the evolutionary conservation of neural induction genes throughout all vertebrates, further investigation into the function of these genes in mammals is needed.

WNT signaling and neural induction

The studies described above demonstrate that BMP and FGF signaling converge at the regulation of SMAD1 activity and have opposing effects on neural fate. In chick embryos, however, activation of FGF alone or in combination with BMP antagonists is insufficient to induce neural fate. Indeed, even in *Xenopus*, complete removal of *Smad1* only results in a mild ventralization phenotype, suggesting that additional pathways are required for neural induction. A third pathway implicated in neural induction is WNT/β-Catenin signaling. WNTs are secreted glycoproteins that bind transmembrane Frizzled receptors, initiating a signaling cascade that culminates in the de-repression of β-CATENIN, a transcription factor that complexes with members of the TCF/LEF family of transcription factors to regulate the expression of target genes (Nusse, 2012).

WNTs are generally thought to promote non-neural fate in the ectoderm or epiblast: WNT signaling must be inhibited for mouse ESCs to become neural, and antagonism of WNT and Nodal signaling increases generation of telencephalic (neural) precursors (Aubert et al., 2002; Watanabe et al., 2005). Conversely, WNT1 and lithium chloride, which stimulates WNT signaling, can inhibit neural fate in mouse ESCs. In the chick epiblast, Wnt signaling can block the ability of ectoderm cells to respond to FGF, and antagonism of Wnt signaling permits cells to respond to FGF, placing WNTs at the hinge of BMP and FGF antagonism (Wilson et al., 2001). In contrast to its role as an antagonist of anterior neural fate, WNT signaling has been demonstrated to regulate posterior neural plate development in mice through direct activation of the *Sox2* enhancer N-1 (Takemoto et al., 2006). This study provides a direct link between WNT signaling and SOXB1 factors in neural fate specification, and thus begins to piece together the relationship between cell-extrinsic signaling molecules and cell-intrinsic gene expression.

The roles of SOXB1 factors in neural induction

Little is known about the *transcriptional* regulation of neural induction. Members of the SOXB1 family of transcription factors are highly conserved early makers of neural fate in many species from Drosophila (Ma et al., 1998; Nambu and Nambu, 1996; Soriano and Russell, 1998) to fish (Vriz et al., 1996) and avians (Uwanogho et al., 1995) (Figure1-9). In chick embryos, SOX2 is expressed in pre-gastrula cells that are competent to undergo neural induction upon node transplantation (Streit et al., 1997). Similarly, in *Xenopus*, SOX2 is a specific early readout of neural identity (Mizuseki et al., 1998). BMP antagonists, including Chordin, induce SOX2 expression, and SOX2 synergizes with FGF signaling to initiate neural fate (Mizuseki et al., 1998). Moreover, inhibition of SOX2 function combined with expression of a dominant negative BMPR

in animal caps prevented neural induction, but SOX2 expression itself was unaffected (Kishi et al., 2000).

These experiments prompted further investigation into the role of SOXB1 genes in neural induction and questioned the use of SOX genes as definitive neural markers. β-Catenin-depleted *Xenopus* embryos become ventralized and lack an organizer, thus de-repressing BMP signaling. *Sox2* is still expressed around the blastopore lip of these embryos, but a neural plate never forms, and *Sox2*-positive cells never give rise to neurons, which are identified by the expression of β-tubulin and neural cell adhesion marker (NCAM). Moreover, *Sox2* expression disappears with the addition of an inhibitor of FGF receptor. *Sox2* therefore appears to be regulated by FGF signaling, but the presence of *Sox2* does not definitively mark neurogenic tissue, leading some investigators to think of SOX2 as "pre-neural." Conversely, BMP antagonists induce SOX2 expression, even in the absence of FGF signaling, and these SOX2-positive cells *do* give rise to neurons. SOX2 expression is lost in the absence of BMP antagonists, and epidermis develops at the expense of neural plate. Together, these results support three conclusions: 1) Even with the activation of BMP signaling, *Sox2* is still expressed, but these cells never form neural ectoderm, thus supporting the default model of neural induction. 2) SOX2 is induced by both FGF signaling and BMP antagonism independently. 3) Either a more specific marker of neural ectoderm exists downstream of the organizer, or additional pathways are necessary for SOX2-positive cells to give rise to neurons -- perhaps β-Catenin in this instance -- a conclusion that would render the "default model" still questionable (Wills et al., 2010). Indeed, SOX2 may indicate neural fate, rather than "pre-neural" fate, while BMP signaling inhibits neurogenesis (Figure 1-8A vs. B). The controversy, then, would be the definition of neural fate: is "neural" solely the ability to generate neurons, or does "neural" designate the population of non-epidermal cells?

Direct inhibition of SOX2 signaling in early neurula stage *Xenopus* embryos prevents expression of early pan-neural markers but does not affect expression of dorsal BMP antagonists, nor does it induce epidermal markers (Kishi et al., 2000). This result suggests that SOX2 is necessary for neurogenesis but does not repress non-neural fate. However, one caveat of this experiment is that the SOX2-ablated ectoderm peels off at tailbud stages, perhaps not allowing enough time for non-neural markers to become up-regulated, but nonetheless implicating loss of cell adhesion properties. The loss of neural fate in *Sox2*-mutant ectoderm, without manipulating FGF signaling, further supports the conclusion that FGFs do not act as neural inducers per se, but rather maintain ectoderm in a neural-competent state (Hongo et al., 1999). Collectively, results from studies of neural induction in *Xenopus* suggest that SOX2 is necessary but not sufficient to induce neural fate.

In mice, SOX1 is the earliest neural ectoderm-specific marker, becoming expressed in the neural plate at around E7.75, coincident with neural induction (Pevny and Placzek, 2005; Pevny et al., 1998; Rex et al., 1997; Takemoto et al., 2006; Wood and Episkopou, 1999) (Figure 1-9E). SOX2 and SOX3 expression precede gastrulation: SOX2 is expressed in the oocyte and inner cell mass of the blastula, and SOX3 expression initiates in the egg cylinder (Avilion et al., 2003; Kamachi et al., 1998; Pevny and Placzek, 2005). Although it is difficult to manipulate SOX gene expression in mouse epiblasts, experiments in mouse ESCs give insight into the roles of these genes in neural induction, suggesting that they function similarly in mammals and lower vertebrates. Indeed, SOX1 and SOX2 over-expression in mouse ESCs induces neuroectoderm at the expense of mesoderm (Zhao et al., 2004).

In human embryos, it is not a SOX gene, but PAX6 that is the definitive marker of neural plate identity (Zhang et al., 2010). In human embryonic stem cells, *PAX6* expression is induced

when FGF and TGFb signaling are inhibited. Conversely, the presence of FGF2 inhibits *PAX6* expression, suggesting that FGF may have an opposite role in human neural induction than in that of lower vertebrates, maintaining cells in a self-renewing state rather than inducing neural fate. Indeed, pharmacological inhibition of SMAD2 but maintenance of FGF2 results in decreased *NANOG* and *OCT4* but increased *SOX2*, reminiscent of early neuroectoderm formation. Without *PAX6*, however, these cells are not able to produce neural tissue and instead continue to cycle. FGF signaling may antagonize *PAX6* expression in part via OCT4 and NANOG, which are found to occupy the *Pax6* promoter. Moreover, OTX2 positively regulates *PAX6* expression in hESCs, and *Otx2* up-regulation correlates with FGF inhibition. Therefore, in human embryos, FGF signaling may antagonize neural fate by promoting self-renewal genes, while OTX2 promotes neural fate by activating *PAX6* (Greber et al., 2011).

The roles of SOXB1 factors in neural progenitor cell fate

In addition to regulating the early stages of neural fate, SOXB1 genes are implicated in neural progenitor cell (NPC) maintenance in embryos and adults. In mouse embryos, SOX2 is expressed in neuroblasts of the neural retina (Taranova et al., 2006), olfactory epithelium (Ellis et al., 2004) and inner ear sensory epithelium (Uchikawa et al., 1999). In adult mice, SOX2 is found in persistent progenitor cells of the subventricular zone of the later ventricles, the rostral migratory stream, the subgranular zone of the hippocampus and the ependyma surrounding the central canal of the spinal cord (Ellis et al., 2004). Persistent *Sox2* expression is also found in supporting cells of the retina (the Muller Glia) and the auditory sensory epithelium (the inner pillar cells) (Liu et al., 2012; Surzenko et al., 2013).

Although SOXB1 genes are necessary for proper specification and maintenance of NPCs, they become down-regulated in newly generated neurons. SOX2, for example, is expressed in a

ventral^{HI}-dorsal^{LO} gradient in the mouse neural tube consistent with neuronal differentiation, eventually becoming restricted to the ventricular zone (Graham et al., 2003). SOX1 may play a direct role in inducing neuronal differentiation. Over-expression of SOX1, but not SOX2 or SOX3, in E14.0 mouse telencephalon NPCs promotes cell cycle exit and doubles the number of differentiated neurons via activation of *Neurogenin1* and suppression of TCF/LEF signaling and *Hes1* expression (Kan et al., 2004).

However, in chicks, overexpression of SOX2 retains neural progenitor cells in an undifferentiated proliferative state and decreases the number of cells in M-phase of the cell cycle (Bylund et al., 2003; Graham et al., 2003). Conversely, expression of a dominant-negative SOX2 results in premature cell cycle exit and down-regulation of other SOXB1 genes and induction of early but not late neuronal markers (Bylund et al., 2003; Graham et al., 2003). These data suggest that SOXB1 factors must be gradually down-regulated in order for neurons to properly differentiate. SOX3 has also been implicated in cell cycle regulation in *Xenopus* embryos, where it has been shown to promote proliferation of neuroectoderm cells via repression of the cell cycle inhibitor p27^{KIP1} (Hardcastle and Papalopulu, 2000).

The function of SOXB1 proteins in neural progenitor cells is highly conserved. There are two SOXB1 homologs in *Drosophila*, SoxNeuro (SoxB1) and Dichaete (SoxB2-1) (Cremazy et al., 2001) (Figure 1-9A). These are expressed in the early central nervous system of fly embryos and play crucial roles in neural development. *SoxNeuro*-deficient embryos exhibit neural hypoplasia (Overton et al., 2002), and *Dichaete*-deficient embryos suffer loss of midline structures and disorganization of the CNS (Nambu and Nambu, 1996; Russell et al., 1996). Double-mutants show severe neural hypoplasia throughout the CNS and loss of *Achaete*-positive proneural clusters (Buescher et al., 2002; Ma et al., 1998; Overton et al., 2002; Soriano and Russell, 1998).

SOXB1 transcriptional targets in neural progenitor cells

SOX proteins are a subgroup of the Class II family of high mobility group (HMG)-containing transcription factors. HMG proteins were initially identified as a cluster of chromatin non-histone proteins exhibiting high mobility in gel electrophoresis (Grosschedl et al., 1994). SOX genes are grouped based on their greater than 50% amino acid sequence homology to Sex-Determining Region Y (SRY) (Bowles et al., 2000). The SOXB group shares over 85% homology of the HMG domain and consists of SOX1-3 -- the SOXB1 transcriptional activators -- and SOX14 and SO21 -- the SOXB2 transcriptional repressors. SOX genes loosely recognize the DNA sequence T/A T/A GTTT T/A with low affinity *in vitro* (Kamachi et al., 1999). However, *in vivo*, SOX genes appear to bind DNA with strong affinity always in complex with co-regulatory proteins.

Analysis of the secondary and tertiary structures of SOXB1 proteins reveals why these factors are often found in complex with other transcription factors and gives insight into how SOX genes exhibit striking tissue specificity. The HMG box consists of 70-80 amino acids in three α helices stabilized in an L-shaped configuration by two hydrophobic cores. An unfolded (randomly coiled) C-terminus binds DNA in the major groove, inducing a bend toward the minor groove and perhaps inducing the assembly of complex nucleoprotein structures on the DNA. Indeed, SOX genes are known for their ability to quickly switch gene expression, perhaps through changing the local confirmation of DNA (Bianchi and Beltrame, 2000) The SOXB1 homology region is just C-terminal to the HMG domain and participates in protein-protein interactions (Kamachi et al., 1999; Uchikawa et al., 1999). The Serine/Threonine-rich C-terminus is required for transcriptional activation and can function independently of the SOX N-terminus when linked to a heterologous DNA-binding domain (Kamachi et al., 1999; Kamachi et al., 1995;

Uchikawa et al., 1999). The structure of SOX2 bound to DNA in complex with a POU-domain containing transcription factor has been predicted using X-ray crystallography (Remenyi et al., 2003) and NMR (Williams et al., 2004). Circular dichroism of full-length SOX2 confirms the alpha helical structure of the N-terminus and largely unfolded nature of the C-terminus (Figure 1-10).

There have been few well-characterized transcriptional targets of SOXB1 proteins in NPCs: SOX2 in coordination with BRN2 (POU3F2) can bind an enhancer element in the second intron of *Nestin*, and SOX1 can repress *Hes1* expression through direct binding to its promoter (Kan et al., 2004). In mouse retinal progenitor cells, SOX2 can bind an intronic regulatory element of *Notch1*, and reintroduction of the Notch intracellular domain in *Sox2*-ablated Muller Glia partially rescues the gliotic phenotype (Surzenko et al., 2013; Taranova et al., 2006). A chromatin immunoprecipitation screen in ESCs identified a number of targets of the pluripotency factors OCT4, SOX2 and NANOG. In complex, these proteins bind DNA regulatory elements of *Oct4*, Sox2, *Nanog*, *Stat3*, *Zic3*, *TDGF1*, *Lefty2*, *Ebaf*, *Dkk1*, *Frat2* and *Notch1*, keeping these genes active. OCT4, SOX2 and NANOG also have a repressive function in ESCs, inhibiting the expression of *Esx11*, HoxB1, Meis1, Pax6, Lhx5, Lbx1, Myf5 and Onecut1 (Boyer et al., 2005).

Humans with SOX2 mutations exhibit profound neural defects

Heterozygous mutations in human *SOX2* are most often associated with anophthalmia (absence of eye) and have been reported in 10-20% of cases of severe bilateral ocular malformation (Fantes et al., 2003; Ragge et al., 2005b). In cases in which the *SOX2* mutation causes microphthalmia (small eye), the retina remains functional (Fitzpatrick and van Heyningen, 2005). Outside of the eye, there is functional redundancy among SOX1-3. Nonetheless, additional neural defects associated with *SOX2* mutations include hippocampal

abnormalities, epilepsy, pituitary malformation, hypothalamic hamartoma, impaired motor function, and deafness. Cleft palate has also been demonstrated in a hypomorphic mouse model of *Sox2* (Langer et al., 2013). Thus, SOX2 is a key regulator of NPC development throughout the CNS and especially of optic cup progenitor cells, where SOX2 is expressed exclusively of SOX1 and SOX3.

The Optic Cup as a Model of Neural Induction

It has been suggested that SOX2 regulates changes in gene expression necessary to respond to neural-inducing signals (Rex et al., 1997), but its direct transcriptional targets and upstream regulators are as yet unclear. A lack of precise genetic tools to study its role in lower vertebrates and the difficulty of visualizing mouse epiblasts *in vivo* contribute to the challenge of identifying SOX2 target genes in neural ectoderm. A study using mouse epiblast stem cells (EpiSCs), which molecularly resemble the mouse epiblast at E6.5, sought to identify the transcriptional networks involved in neural fate specification (Iwafuchi-Doi et al., 2012). EpiSCs were maintained by Activin (to mimic Nodal) and FGF2, and removal of these exogenous factors resulted in NPC development as identified by expression of OTX2, SOX2, and POU3F1 and down-regulation of POU5F1. The first stage of EpiSC -- NPC development (NP1) resembled the ANP as characterized by the ANP-specific transcription factors *Hesx1*, *Otx1* and *Pax6*. Manipulation of SOX2 levels in this system revealed that SOX2 functions to maintain the epiblast state in cooperation with POU5F1 (OCT4). SOX2 promoted the expression of *Nanog*, *Zic3* and *Otx2*, while ZIC2/3 and POU5F1 participated in a feedback loop to promote *Sox2* expression. In addition, SOX2 was shown to promote ANP fate once POU5F1 was down-regulated, likely participating in the down-regulation of POU5F1 in coordination with OTX2. Over-expression of SOX2 promoted the expression of the ANP transcription factors *Pou3f1*, *Zic3* and *Pax6*, and, in an additional feedback loop, POU3F1 and ZIC3 in coordination with the ANP gene OTX2 promoted *Sox2* expression. SOX2 also promoted expression of the posterior NP marker *Gbx2* and inhibited the expression of the endoderm gene *Sox17*, consistent with a role for SOX2 in maintaining neural competence of progenitor cells throughout the CNS. Collectively, these data begin to piece together a transcriptional network that places SOX2 as a crucial regulator of

epiblast multipotency in cooperation with POU5F1 and neural fate upon the down-regulation of POU5F1 (Iwafuchi-Doi et al., 2012).

Despite the advantages of using EpiSCs to investigate mammalian neural induction, a simple and accessible model is needed to study SOX2's function in neural progenitor cells *in vivo*. Results from this project demonstrate that the mouse retina provides just such a model: ablation of *Sox2* in mouse optic cup progenitor cells (OCPCs) results in complete loss of neural fate and cell fate conversion to non-neural (non-neurogenic) ciliary epithelium. Chapter II describes this phenotype, and Chapters III and IV provide data supporting the notion that SOX2 maintains neural retinal identity and suppresses non-neurogenic CE fate via transcriptional regulation of genes necessary to respond to neural versus non-neural signals.

Neural ectoderm/neural retina is morphologically distinct from epidermis/ciliary epithelium

The presumptive neural plate and epidermis exhibit clear morphological differences (Colas and Schoenwolf, 2001; Lawson et al., 2001). The initial distinction between presumptive neural ectoderm and epidermal ectoderm occurs when the neural plate first forms bilateral ridges at the neural-epidermal junction, and neural cells appear to thicken (Figure 1-11F, F'). Prospective neural cells elongate along the apical-basal axis, a process known as cell palisading, while prospective epidermal cells decrease in height such that the tissue appears to flatten and spread, becoming a single layer of cuboidal epithelium (Lawson et al., 2001). Similarly, the NR and CE can be distinguished morphologically as early as E14.5, when presumptive non-neurogenic cells at the periphery of the eyecup adopt single-layered cuboidal morphology with attachments to both the inner and outer layers of the optic cup. As shown in the scanning and transmission electron micrographs in Figure 1-11A (box), A', and E (box), the distal tips of the eyecup consist of cells attached at both the apical (outer) and basal (inner) surfaces. As optic

cup development proceeds, these prospective CE cells spread into a single-layered epithelial sheet reminiscent of the epidermis. Interestingly, upon SOX2 ablation, retinal tissue in the central part of the eyecup adopts a thin morphology characteristic of presumptive CE and in start contrast to SOX2-positive neural retina (Figure 1-11B, B'). SOX2-ablated cells also appear elongated in contrast to the rounded nature of neural retina progenitor cells (Figure 1-11D vs. C). Therefore, based on morphological characteristics, SOX2 appears to be necessary for neural fate in the optic cup, and loss of SOX2 leads to non-neural epithelial characteristics, making the optic cup an appropriate model to study the role of SOX2 in neural induction.

Proliferation of neurogenic progenitor cells differs from that of non-neurogenic progenitor cells

The neural plate and epidermis have distinct proliferative properties. The thickened morphology of the neural plate arises in part via greater proliferation of neurogenic progenitor cells. This intimate connection between cell proliferation and cell fate raises the question of how regulation of these two processes is linked. Any given tissue requires a certain number of cells of a particular type, so cell fate and cell cycle must be tightly coordinated. It is not surprising, then, that cell cycle regulators control levels of transcription factors, including SOX2, and these in turn control expression of cell cycle regulator genes (Li et al., 2012; Liu et al., 2012; Marques-Torrejon et al., 2013).

In the case of the neural plate, the neural fate-promoting transcription factor XBF1 (FOXG1) controls expression of the cell cycle inhibitor *p27^{Xic1}*. High levels of XBF1 inhibit *p27* expression and convert prospective epidermis into neural plate. This cell fate conversion occurs even when cell division is blocked, suggesting that XBF1 exerts its effect on cell fate independently of its cell cycle-promoting effect. Conversely, a low concentration of XBF1

promotes *p27* expression. But even at low concentrations, XBF1 is pro-neurogenic, leading to ectopic neurons rather than ectopic neural progenitor cells. This "low-dose" effect can be altered to a "high-dose" effect when a low concentration of SOX3 is introduced, suggesting a synergistic relationship between XBF1 and SOX3. Expression of *p27* alone is not sufficient to induce ectopic SOX3-positive neural plate progenitor cells or ectopic N-Tubulin-positive neurons, thus separating the mechanisms by which XBF1 promotes proliferation and imparts neural fate (Hardcastle and Papalopulu, 2000).

The developing NR has a higher proliferation index than the presumptive CE at embryonic stages. The lower proliferation rate of the peripheral optic cup in comparison to the central optic cup is thought to contribute to the thin epithelial structure of the adult ciliary epithelium, thus making the optic cup a useful model for studying the link between cell proliferation and cell fate (Beebe, 1986; Cho and Cepko, 2006). Perhaps complicating the issue, however, is the fact that these two regions express positive and negative cell cycle regulators, albeit seemingly completely different sets in region (Trimarchi et al., 2009). Similar to the neural plate, p27 is expressed in the prospective NR and excluded from the CE. In neurogenic RPCs, p27 promotes cell cycle exit and neuronal differentiation; however, its ectopic expression in the optic cup has not been tested. Therefore, its role in neural retinal fate specification is still unclear. Decreased SOX2 in the developing optic cup causes retinal hypoplasia/proliferation defects; therefore, the optic cup provides a model for addressing the mechanisms through which SOX2 regulates neural fate and cell proliferation. Moreover, given that p27 has been demonstrated to regulate *Sox2* expression (Li et al., 2012), and SOX2 had been shown to regulate p27 (Liu et al., 2012), both of which are expressed in optic cup progenitor cells, this transcriptional/cell cycle network cell cycle can be worked out in the optic cup.

Neural retina and ciliary epithelium are specified by the same pathways that specify neural plate and epidermis

FGF signaling

As in the naïve ectoderm of mice and lower vertebrates, FGF signaling generally promotes neural/neural retina fate. FGFs expressed in the prospective lens activate *Vsx2* in the distal OV, thereby specifying NR fate. Moreover, *Vsx2* in turn represses *Mitf*, thereby suppressing non-neurogenic fate (Horsford et al., 2005; Nguyen and Arnheiter, 2000). FGF9 is also activated in the distal OV, and it promotes NR fate when ectopically expressed in the presumptive RPE. The RPE of mice mutant for *Fgf9* is expanded into the putative NR domain (Zhao et al., 2001).

However, the peripheral OC also expresses FGF ligands, supporting a possible role for FGF signaling in CE fate. To address this question and avoid the functional redundancy of the numerous FGFs and FGF receptors expressed in the OV, an experiment was conducted that ablated the protein phospatase *Shp2*, which mediates the FGF signaling cascade via sustained activation of Ras. This inactivation of all FGF signaling in the OC causes a cell fate conversion of the NR to RPE (Cai et al., 2010). It appears, then, that FGF signaling is necessary for neural retina fate, at least during early stages of eye development. However, it is still unclear what the role of sustained FGF signaling may be at later stages of OC development, and how induction of FGF signaling in the peripheral OC may affect cell fate.

BMP signaling

BMP signaling in the OC is also consistent with its role in promoting non-neural fate. The function of BMP signaling in optic cup cell fate specification has been studied primarily from the perspective of the lens:

Forced expression of FGF4 in the chick RPE causes a cell fate conversion of this tissue to NR, but at a distance from the FGF signal, ectopic CE tissue is observed. It was proposed that where FGF4 overlaps with endogenous BMP in the RPE, a secondary signal is induced that promotes CE fate. A potential candidate for this secondary non-neurogenic signaling pathway is WNT/β-Catenin signaling.

Canonical WNT signaling

WNT signaling is the best-characterized pathway regulating peripheral optic cup cell fates. In chick and in mouse, forced activation of β-Catenin in the optic cup suppresses neural retina fate and promotes ciliary epithelial fate, supporting a role for WNT signaling in the antagonism of neural identity (Cho and Cepko, 2006; Liu et al., 2007b). In the ectoderm, WNT signaling can block the competence of prospective neural cells to respond to FGF signaling, and Wnt down-regulation is necessary for mESCs to give rise to neural cells. However, a more direct look at the function of β-Catenin itself paints a more complex of WNT signaling in mammalian embryonic stem cells. For example, β-CATENIN appears to be inactive transcriptionally in mouse and human ESCs (Davidson et al., 2012; Lyashenko et al., 2011). In human ESCs, OCT4 keeps β-CATENIN inactive, and activation of β-CATENIN inhibits self-renewal and promotes mesoderm formation (Davidson et al., 2012). Similarly, in mouse ESCs, β-CATENIN appears to be important for mesendoderm development (Lyashenko et al., 2011). Mice that are hypomorphic for *β*-

catenin exhibit defective gastrulation and develop according to the neural default hypothesis (Rudloff and Kemler, 2012). Together, these studies suggest that canonical WNT signaling generally antagonizes neural fate in the ectoderm and in the optic cup.

The role of WNT signaling in cell fate specification is often complicated by the additional function of β-CATENIN in cadherin-mediated cell adhesion (Valenta et al., 2011). To address transcription separately from cell adhesion, it is possible to selectively inactivate and add back each functional component of *β-catenin*. One group was able to introduce two mutatations to the *β-catenin* locus -- an N-terminal amino acid substitution and a C-terminal truncation -- to abrogate its transcriptional response to WNTs but maintain its cell-adhesive properties (Valenta et al., 2011). In this manner, it is possible to assess the differences between the functions of the N- and C-termini in transcription and to assess the transcriptional function of the protein as a whole without compromising cell adhesion. It was thereby discovered that β-Catenin transcription is crucial for maintaining neural progenitor identity and neuronal differentiation in the dorsal spinal cord of mice. In β-CATENIN transcriptionally deficient mice, SOX2 expression was strongly reduced in spinal cord neural progenitors, and there was a reduction in the number of sensory neurons and dorsal interneurons as well as the complete absence of the neuronal marker *Dcx* (Valenta et al., 2011). Although these results suggest that β-CATENIN promotes neural identity, it could be a cell-type and stage-specific phenomenon that does not parallel the function of canonical WNT signaling at earlier stages. Indeed, mouse ESCs lacking β-CATENIN exhibit defective neuronal differentiation; however, adding back cell adhesion and not TCF/LEF rescues neuroepithelial formation, suggesting that adherens-junctions and not WNT-mediated transcription is important for neural development of mouse ESCs. Therefore, the majority of studies to date support a role for canonical WNT signaling in the suppression of anterior neural fate in gastrula-stage embryos and in mammalian ESCs.

Summary and scope

Sox2 is specifically expressed in the neural ectoderm and plays a highly conserved role in neural fate specification. Despite its importance for neural progenitor multipotency, little is known of its transcriptional targets. The optic cup resembles the gastrula-stage ectoderm in its morphology and in the signaling pathways that regionalize its neurogenic – non-neurogenic boundary. Moreover, SOX2 is specifically maintained in neurogenic progenitor cells of the optic cup as in the neural ectoderm. Loss of SOX2 in either the ectoderm or the optic cup is associated with loss of neural characteristics. This project uses the mouse optic cup as a model of the gastrula-stage ectoderm to identify the mechanisms whereby SOX2 maintains neural fate. The optic cup thereby provides an accessible method of testing the genetic relationship between *Sox2* and neural induction signaling pathways, thus bypassing the potential lethality of manipulating these pathways in the mouse epiblast and providing a means of confirming results *in vivo* using traditional molecular and developmental biology techniques.

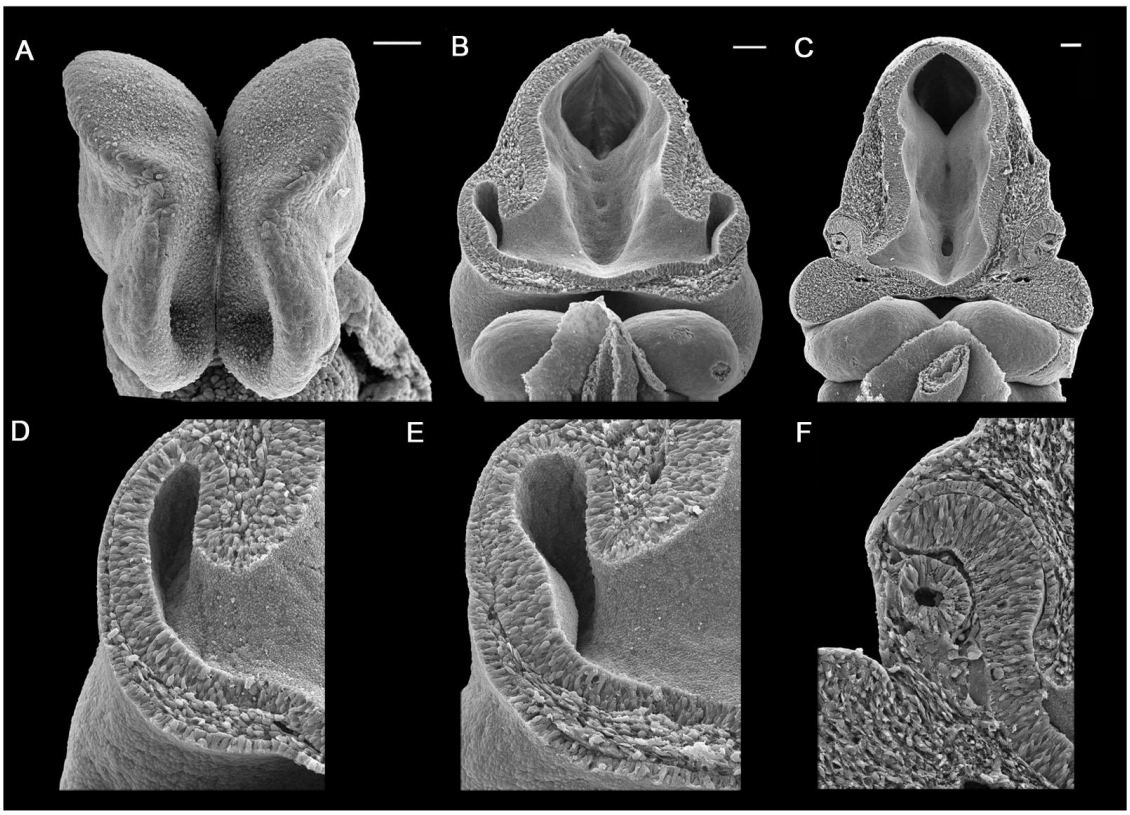


Figure 1-1 Anatomy of embryonic mouse forebrain and eyes. (*A*) Frontal view of embryonic day (E) 8.5 forebrain, just before the eye field splits. The optic sulci are the large pits protruding from the ventral neural ectoderm. (*B, D, E*) Wide (B) and high magnification (D, E) views of frontal sections of the E 9.0 (D) to E 9.5 (D) optic vesicle. The coordinated invagination of the distal optic vesicle and the surface ectoderm, where the lens placode has thickened, begins at E 9.5 (E). (*C, F*). Wide (C) and high magnification (F) views of frontal sections of the E 10.5 optic cup and lens vesicle. The retinal pigment epithelium is the thin layer of cells proximal to the neural retina, which is dorsal to the optic stalk. The optic stalk is continuous with the ventral forebrain. The lens vesicle is distal to the neural retina. Dorsal is to the top (A-F), and proximal is to the right (D-F). Scale bars: *A*, 50 um; *B-C*, 100um. *(Photo Credit: Lee Langer)*

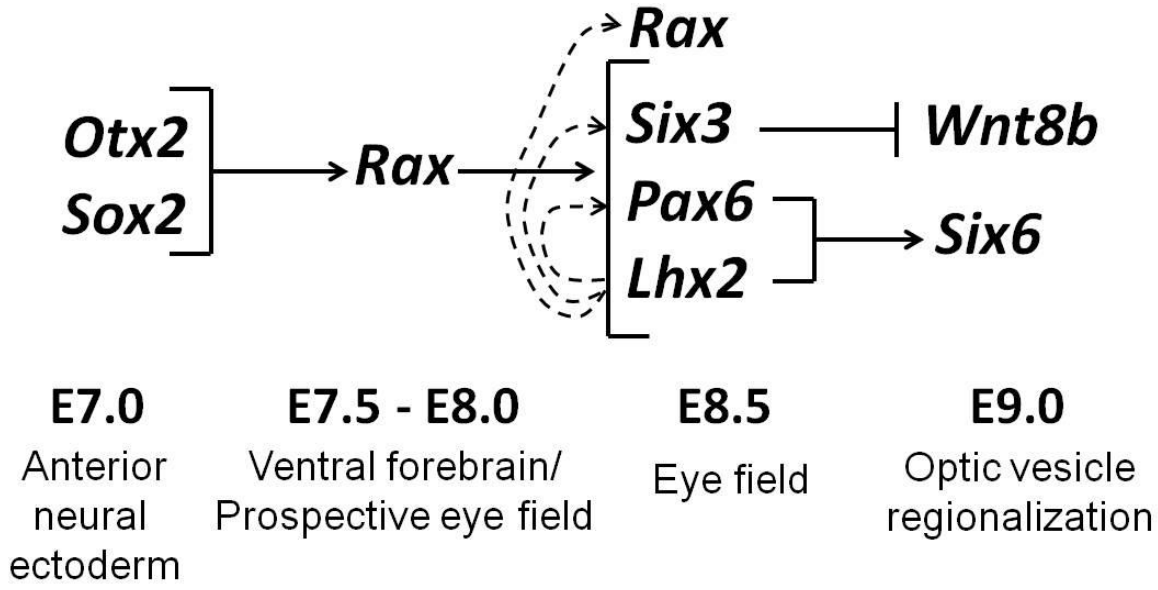


Figure 1-2 Network of transcription factors that establish the eye field. The neural ectoderm transcription factors SOX2 and OTX2 activate *Rax* expression in the prospective eye field, which is located in the ventral forebrain. RAX is required for the upregulation of the EFTFs *Lhx2*, *Pax6* and *Six3* in the eye field. The dashed arrows indicate that LHX2 may co-regulate the expression of *Rax*, *Pax6* and *Six3* in the eye field, as expression of these genes is delayed in *Lhx2*$^{-/-}$ embryos (Tetreault et al., 2009). The EFTFs then coordinate the cell-intrinsic and -extrinsic signaling pathways that regionalize the optic vesicle along its axes.

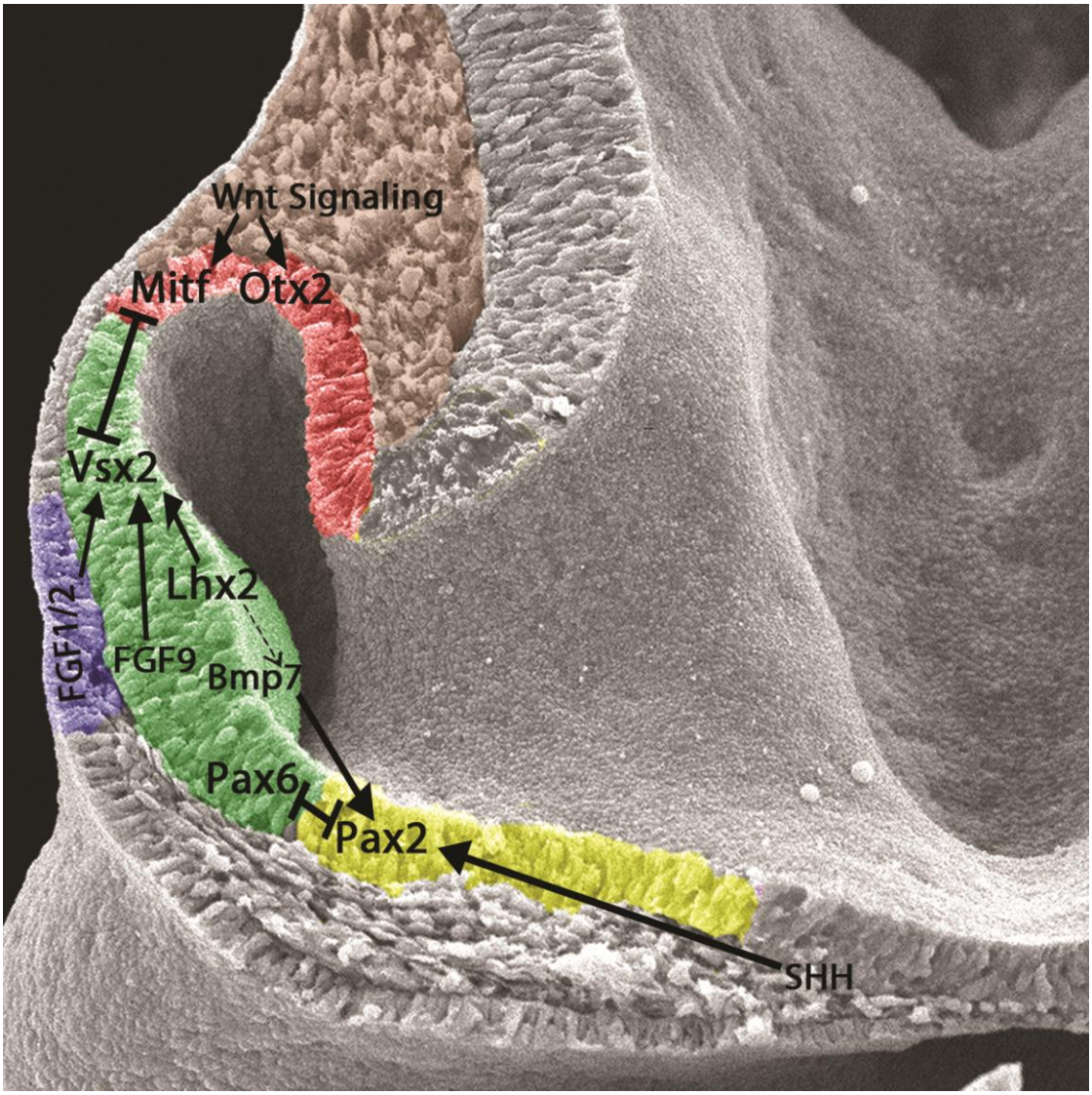


Figure 1-3 Signaling networks establish boundaries in the optic vesicle. Dorsal is to the top, and distal is to the left. The optic vesicle is regionalized into prospective RPE (red, dorsal), neural retina (green, central) and optic stalk (yellow, ventral). Extracellular signals organize the optic vesicle in part through the activation of transcription factors that specify the tissue type in which they are expressed. These transcription factors cell-intrinsically regulate optic vesicle organization through mutual repression of one another. The dotted arrow indicates that early *Lhx2* expression may be required for *Bmp7* expression in the optic vesicle (Yun et al., 2009), but *Bmp7* expression is maintained when *Lhx2* is ablated specifically in the eye field (Hagglund et al., 2011). The lens placode, which expresses FGF ligands important for neural retinal specification, is shown in blue. (Electron microscopy by Dr. Lee Langer)

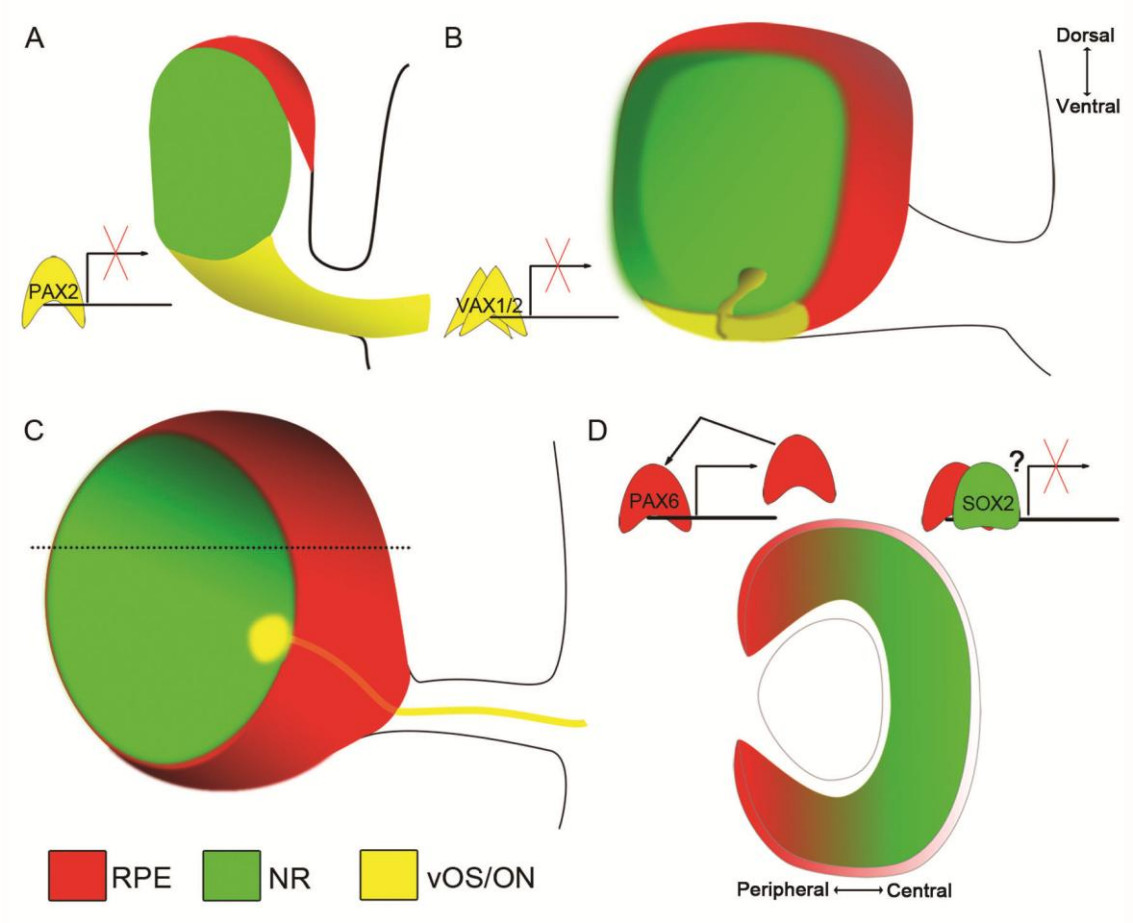


Figure 1-4 Regulation of *Pax6* expression via its eye-specific enhancer α during the early stages of eye development. (*A*) At E9.5, the OV is regionalized into three presumptive tissues: the retinal pigment epithelium (RPE, red), the central neural retina (NR, green) and the ventral optic stalk (vOS, yellow). PAX2 binds α to antagonize *Pax6* expression in the ventral OV. (*B*) At E10.5, the OV invaginates centrally, creating two nested cups -- the RPE and the NR. It also invaginates ventrally, creating the optic fissure. VAX1 and VAX2 suppress ventral *Pax6* expression via α. (*C*) At E11.5, the optic fissure has closed ventrally, leaving a small opening for RGC axons to exit through the optic nerve (ON, yellow). The RPE has completely surrounded the NR. (*D*) Transverse section through the optic cup at the dotted line in *C*. SOX2 (green) and PAX6 (red) exhibit inversely graded expression patterns, with SOX2 highest in the central OC and PAX6 highest in the periphery. The peripheral part of the OC, the OCM, gives rise to the epithelia of the ciliary body and iris, while the central part gives rise to the NR. The OCM highly expresses *Pax6* due in part to positive auto-regulation via α. In the central OC, SOX2 antagonizes *Pax6* via α (see Chapters II and III).

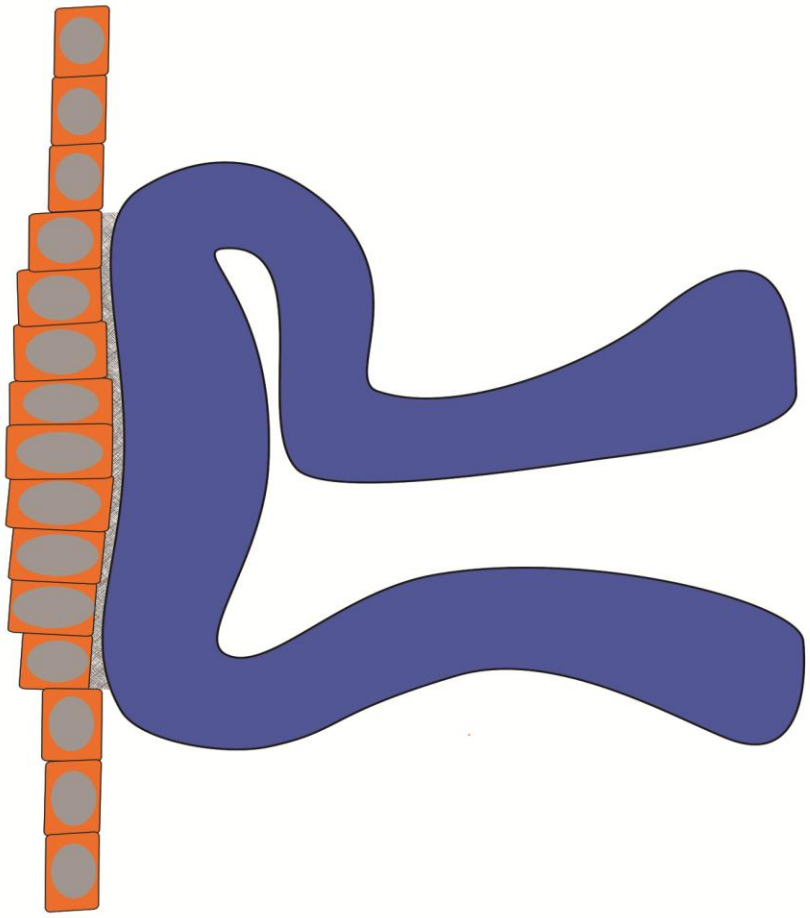


Figure 1-5 Mechanics of lens placode formation. The optic vesicle (blue) contacts the surface ectoderm (orange with gray nuclei) prior to lens placode formation. Adhesion between the optic vesicle and the pre-lens ectoderm is mediated by extracellular matrix components (cross-hatch). The area of contact between the extracellular matrix and the surface ectoderm restricts expansion of the pre-lens domain. Continued cell division in this area leads to cell crowding and cell elongation resulting in placode formation.

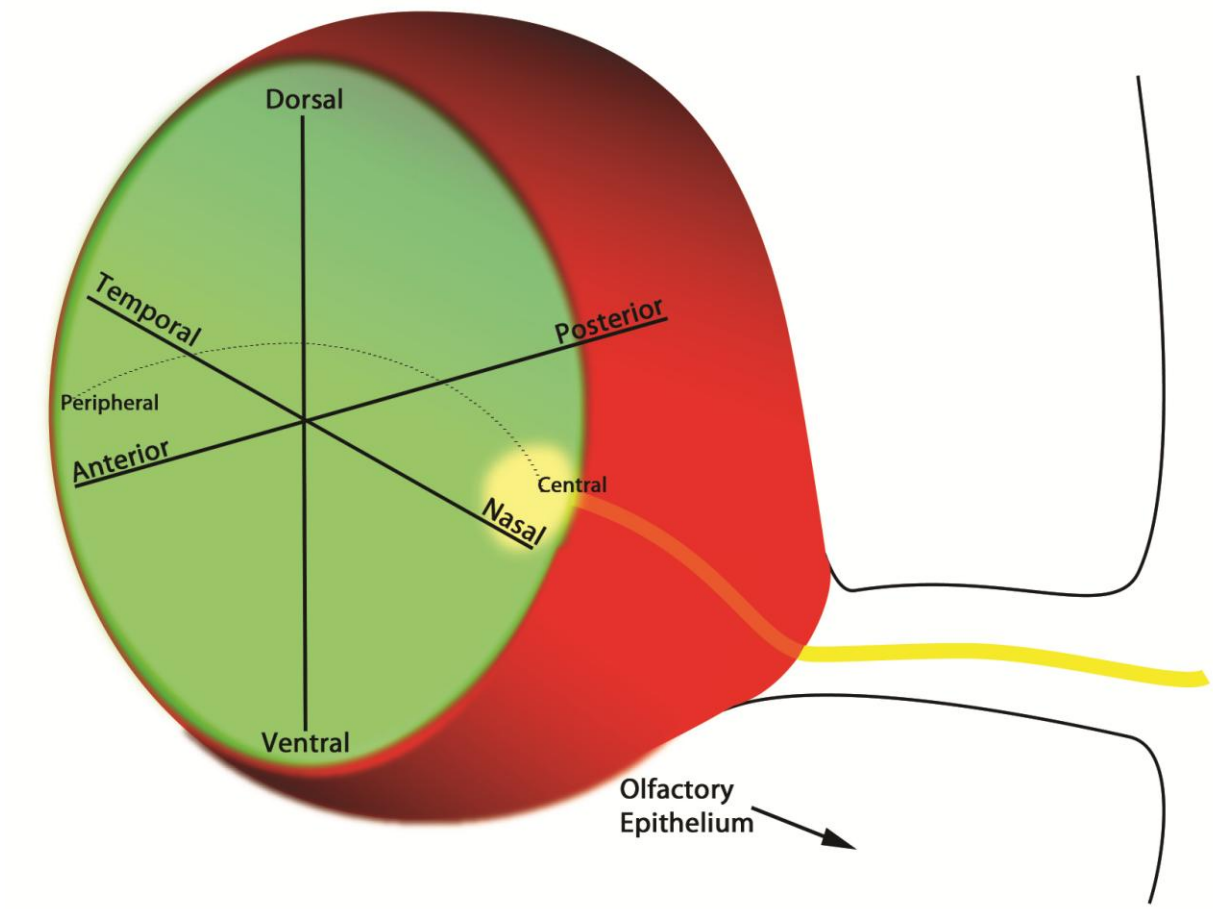


Figure 1-6 Axes in the Optic Cup. The optic cup is regionalized along several axes: dorsal-ventral, anterior (cornea)-posterior (RPE), nasal-temporal and central (central neural retina)-peripheral (ciliary epithelium).

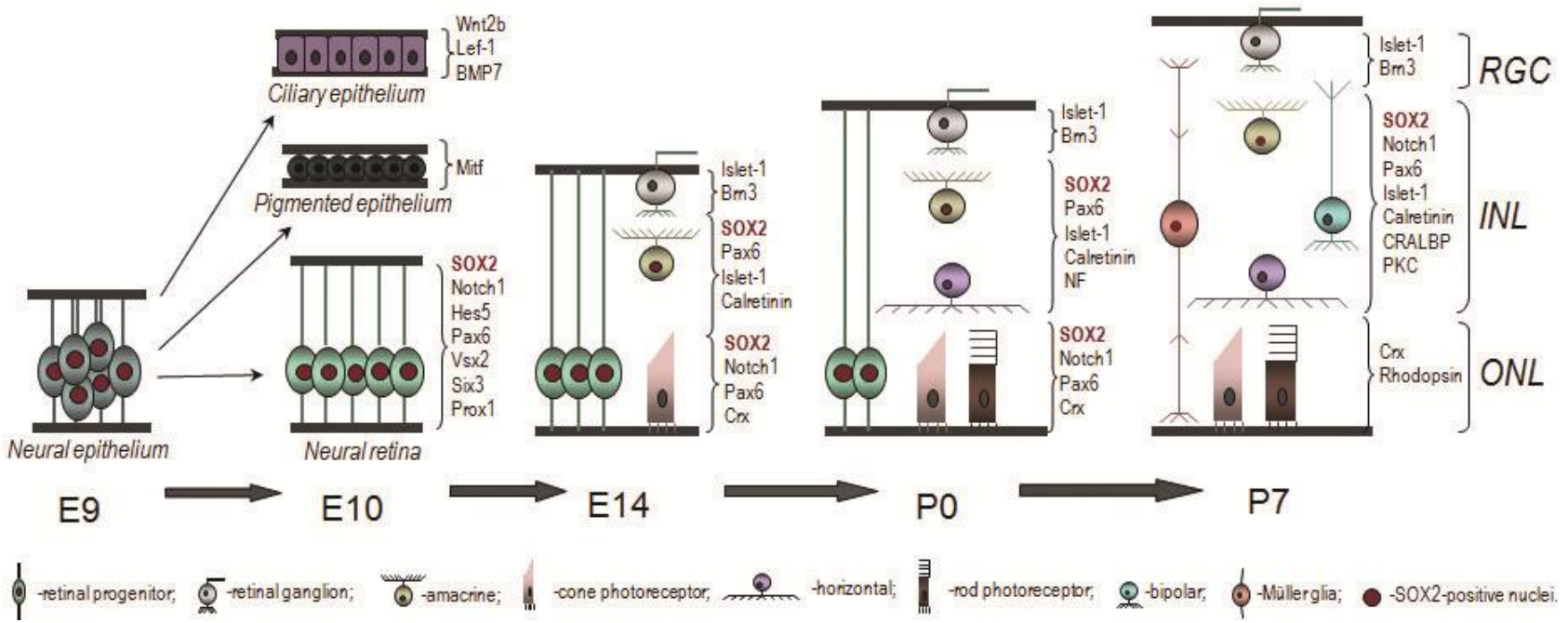


Figure 1-7 Temporal progression of retinogenesis in the mouse. During development of the optic cup, the neural epithelium of the optic vesicle gives rise to pigmented and ciliary epithelia, as well as neural retinal progenitor cells. The wave of neurogenesis in the retina is characterized by production of early born retinal ganglion cells, horizontal interneurons, cone photoreceptors and amacrine interneurons. Postnatal neural progenitor cells predominantly give rise to rod photoreceptors, bipolar interneurons, and Müller glia. In the adult neural retina, neuronal and glial cell types are organized into three distinct cellular layers (GCL – ganglion cell layer, INL – inner nuclear layer, ONL – outer nuclear layer). (Adapted from figure by Dr. Natalia Surzenko)

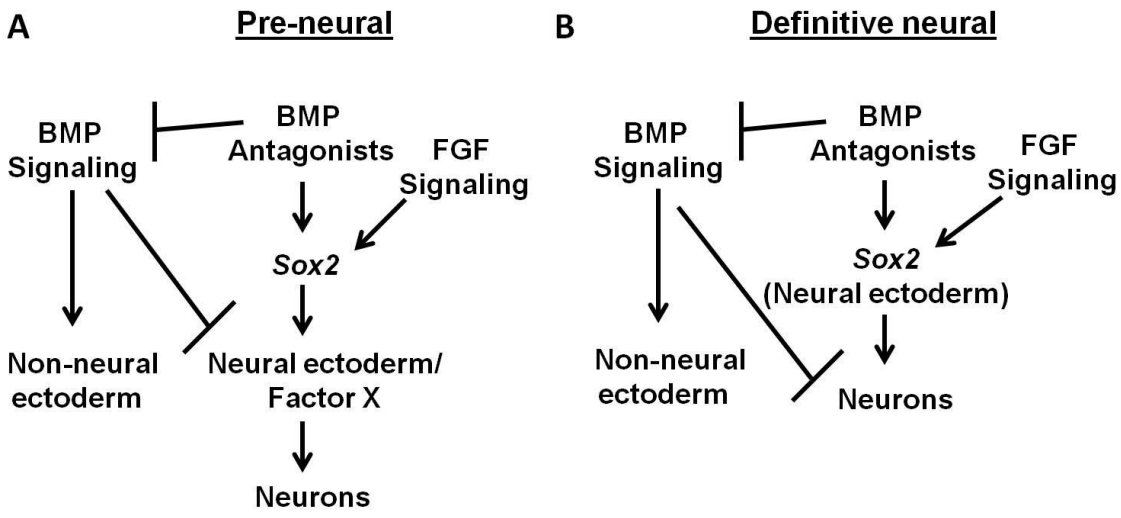


Figure 1-8 Variations on SOX2, being regulated by neural inducing signals, as a marker of neural ectoderm in *Xenopus*. *(A)* SOX2 is often used as a specific marker of neural ectoderm in vertebrates; however, in the presence of BMP signaling, SOX2 is insufficient for neurogenesis. This raises the possibility that SOX2 actually designates "pre-neural" ectoderm, staying consistent with the neural default hypothesis. If neural ectoderm is defined by the ability to generate neurons, then there could be a more specific marker of neural ectoderm progenitor cells ("Factor X"), which is necessary and sufficient for neurogenesis and inhibited by BMPs. *(B)* An alternative possibility is that BMP signaling independently promotes non-neural fate and inhibits neurogenesis in neural ectoderm, such that SOX2 is a specific marker of neural ectoderm as defined by the absence of non-neural identity rather than neurogenesis.

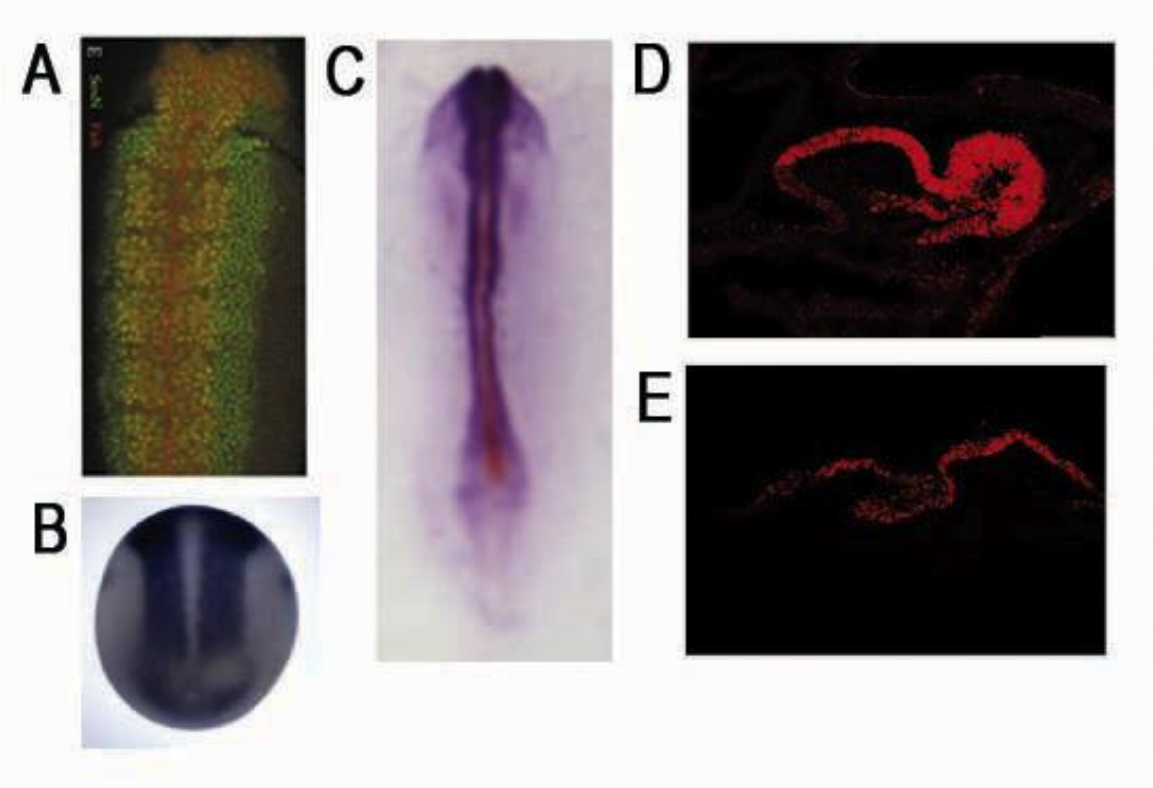


Figure 1-9 SOX2 expression in neural progenitor cells is highly conserved. *(A)* SoxNeuro (green) and Dichaete (red) are co-expressed in the neuroectoderm of the gastrula stage *Drosophila* embryo. *(B-E) Sox2*/SOX2 is specifically expressed in the neural plate of *Xenopus* (B), chick (C), mouse (D) and human (E) embryos. Photos from Cremazy et al. (2000); Streit et al. (2000); Wills et al. (2009) and Zhang et al. (2010) and used with permission.

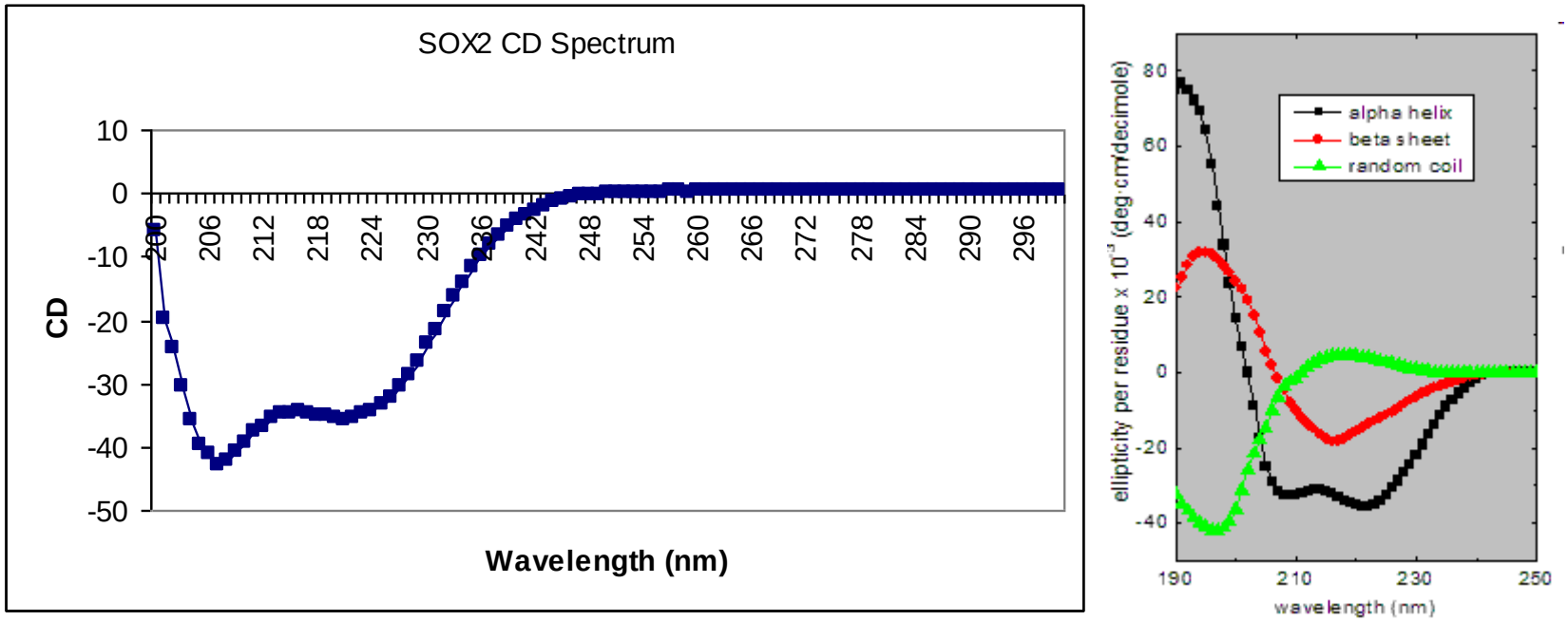


Figure 1-10 Circular dichroism spectrum of purified full-length recombinant SOX2. The two minima around 205 and 225 nm indicate the alpha helical nature of SOX2. Concentration is 12uM SOX2 in 50mM NaPO4 and 200mM NaCl. The legend to the right is from http://www.ap-lab.com/circular_dichroism.htm.

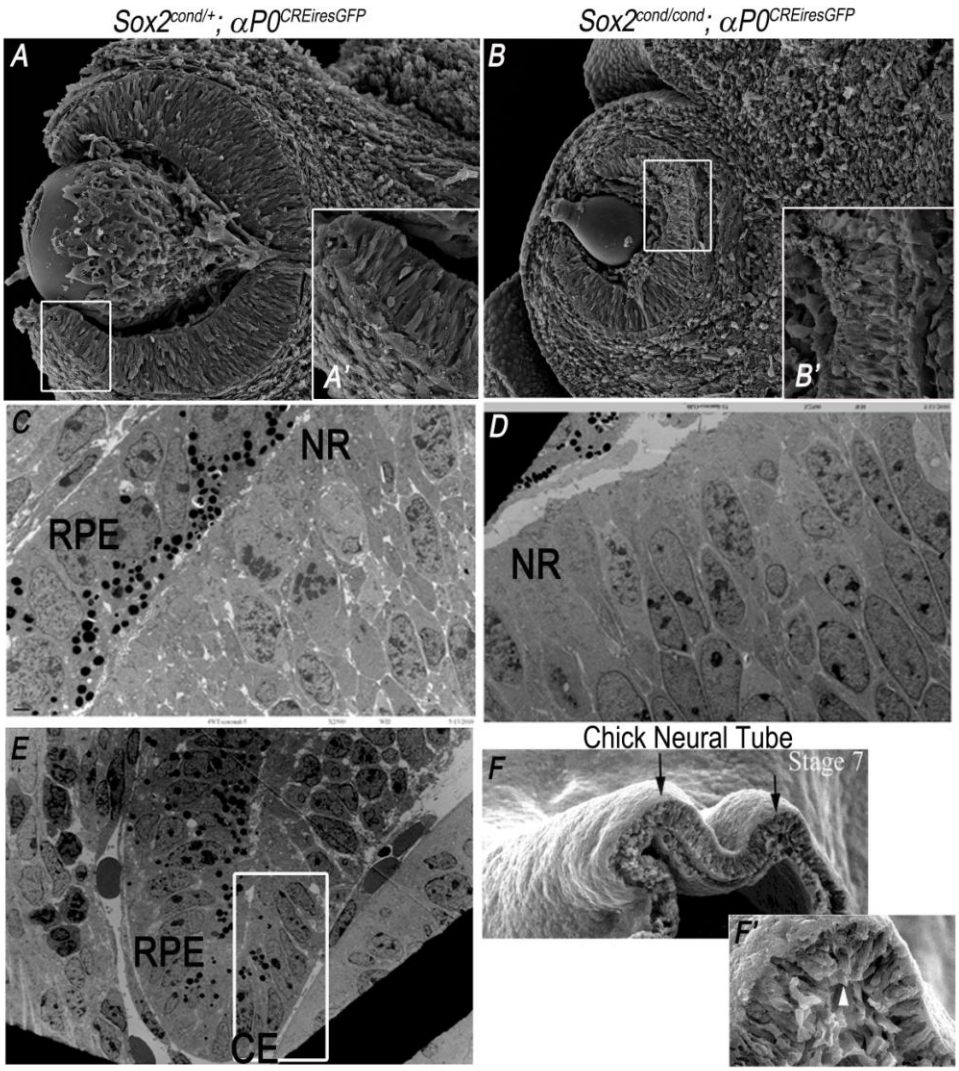


Figure 1-11 The morphology of OC progenitor cells is reminiscent of that of neural tube cells. *(A-A')* SEM of an E14.5 OC of a wild-type embryo showing the difference between the laminar morphology of the central OC and the single-layered morphology of the distal tips (box and A'). *(B-B')* SEM of an E14.5 OC of a *Sox2*-deleted embryo showing the thinning of the central OC (box and B'). *(C-E)* TEMs of E14.5 wild-type (C,E) and *Sox2*-ablated (D) OCs showing the rounded morphology of wild-type central progenitor cells (C "NR",E above box) versus the elongated morphology of peripheral CE progenitor cells (E box) and central *Sox2*-ablated progenitor cells (D "NR"). *(F-F')* SEM of a stage 7 chick neural tube showing the contrast in morphology between the neural ectoderm (between arrows) and the epidermal ectoderm (outside arrows), and the kinking of cells at the border of these two tissue types (F' arrow). F and F' from (Lawson et al., 2001)

CHAPTER II: SOX2 ANTAGONIZES PAX6 TO MAINTAIN NEURAL RETINAL FATE

Overview

In humans, heterozygosity of either SOX2 or PAX6 is associated with microphthalmia, anophthalmia, or aniridia. In this study, through the genetic spatio-temporal specific ablation of SOX2 on both wild-type and *Pax6*-heterozygous backgrounds in the mouse, we have uncovered a transcriptionally distinct and developmentally transient stage of eye development. We show that genetic ablation of SOX2 in the optic cup results in complete loss of neural competence and eventual cell fate conversion to non-neurogenic ciliary epithelium. This cell fate conversion is associated with a striking increase in PAX6, and genetically ablating SOX2 on a *Pax6*-heterozygous background partially rescues the *Sox2*-mutant phenotype. Collectively, these results demonstrate that precise regulation of the ratio of SOX2 to PAX6 is necessary to ensure accurate progenitor cell specification and place SOX2 as a decisive factor of neural competence in the retina.

Introduction

The vertebrate eye, which is composed of neurogenic and non-neurogenic structures, arises from a single progenitor pool in the optic vesicle. The eye therefore provides a useful and accessible model for studying potential interactions of signaling pathways in specifying distinct cell fates. Around embryonic day (E) 10.5 of mouse development, the optic vesicle receives signals from the surface ectoderm telling it to invaginate, forming the bilayered optic cup. The inner layer of the optic cup gives rise to the neural retina (NR), while the outer layer becomes the retinal pigment epithelium (RPE). The interface of these two domains is the peripheral optic

cup margin, which gives rise to the non-pigmented ciliary body epithelium (CE) and the inner iris epithelium. Progenitor cells at the boundary between prospective NR and CE make a binary cell fate decision to become either neurogenic (NR) or non-neurogenic (CE) (Beebe, 1986). The molecular and cellular mechanisms regulating this cell fate decision are poorly understood in part because cells fated to become CE exhibit pervasive expression of the retinal progenitor transcription factors *Rax*, *Chx10* and *Pax6* (Cho and Cepko, 2006; Fuhrmann et al., 2000; Furukawa et al., 1997; Hodgkinson et al., 1993; Kubo and Nakagawa, 2008; Liu et al., 2006a; Liu et al., 2003b; Pittack et al., 1997; Rowan and Cepko, 2004).

A number of cell-extrinsic signaling pathways have been implicated in CE fate specification. One report showed that CE markers are found at the edges of tissue that ectopically expresses FGF, suggesting that the ciliary body is specified in the optic vesicle where BMP and FGF signals overlap (citation). In addition, studies in multiple species have shown canonical Wnt signaling to be a potent regulator of peripheral eye structures (Cho and Cepko, 2006; Liu et al., 2007b; Tomlinson, 2003). A role for Wnt signaling in specifying CE fate in the mouse comes from the observation that constitutive activation of β-catenin in optic cup progenitor cells results in the ectopic expression of CE-specific genes at the expense of NR-specific genes (Liu et al., 2007b). However, these ectopic CE-like cells fail to express *Pax6* and *Chx10*, both of which are normally maintained in the prospective CE of control eyes at early stages. In the adult, *Pax6* is maintained in the CE of the iris and ciliary body.

The reduction of *Pax6* expression upon activated Wnt signaling is surprising given that PAX6 is a positive regulator of peripheral eyecup development (Davis-Silberman et al., 2005). A member of the paired-box and homeobox-containing family of transcription factors, PAX6 has been shown to be required for iris specification, optic cup morphogenesis, lens formation, and

retinal neuronal differentiation (Baumer et al., 2002; Davis-Silberman and Ashery-Padan, 2008; Davis-Silberman et al., 2005; Grindley et al., 1997; Marquardt et al., 2001; Philips et al., 2005; Smith et al., 2009; Xu et al., 1999). These developmental processes require a critical threshold of PAX6 as demonstrated by the fact that heterozygous carriers of *PAX6* deletions (Davis-Silberman et al., 2005; Hill et al., 1991; Hogan et al., 1986; Ton et al., 1991) and transgenic mice with increased levels of PAX6 (Ericson et al., 1997; Schedl et al., 1996) each display eye abnormalities (Favor et al., 2001; Hack et al., 2004; Heins et al., 2002; Kim and Lauderdale, 2008; Manuel et al., 2007 08). Humans with mutations in *PAX6* exhibit aniridia (no iris) and often have smaller ciliary bodies (reviewed by (Hanson and Van Heyningen, 1995; Hayashi et al., 2004; Okamoto et al., 2004; Prosser and van Heyningen, 1998). Mice that are heterozygous for the $Pax6^{Sey}$ mutation exhibit reduced size of the optic cup margin, implicating a shift in the boundary between NR and CE (Davis-Silberman et al., 2005).

Here we address the hypothesis that there is an antagonistic relationship between transcription factors restricted to the prospective NR and those that, like PAX6, span the boundary between prospective NR and CE. One of these potential regulators of NR specification is the high mobility group (HMG)-containing transcription factor SOX2. Conditional deletion of *Sox2* in the developing mouse retina results in the loss of competence to undergo neuronal differentiation, and mice that are hypomorphic for *Sox2* exhibit reduced eye size (Taranova et al., 2006). Moreover, ~10% of human individuals with anophthalmia (lack of eye) or severe microphthalmia (small eye) carry a *SOX2* mutation (Fantes et al., 2003; Hagstrom et al., 2005; Hanson and Van Heyningen, 1995; Ragge et al., 2005a; Ragge et al., 2005b; Zenteno et al., 2005; Zenteno et al., 2006); reviewed by (Hever et al., 2006)).

While both SOX2 and PAX6 have been shown to be essential for the maintenance of multipotent retinal progenitor cells (RPCs) (Marquardt et al., 2001; Taranova et al., 2006; Xu et al., 1999), and studies in mouse illustrate that changes in SOX2 and PAX6 dosage result in developmental defects of the eye, no study has yet addressed their epistatic relationship in the developing optic cup. To examine the relationship between *Sox2* and *Pax6* in the optic cup, we performed genetic analysis in the mouse and uncovered a mechanism through which the eyecup is regionalized into NR and CE. We show that SOX2 and PAX6 are expressed in an inverse gradient in the developing optic cup and find that ablation of SOX2 in multipotent optic cup progenitor cells biases them towards a non-neurogenic CE fate. The immediate molecular readout of this cell fate conversion is the upregulation of PAX6. Accordingly, the deletion of *Sox2* on a *Pax6*-heterozygous background ($Pax6^{Sey/+}$) significantly rescues the *Sox2*-mutant phenotype. Therefore, in the absence of SOX2, multipotent RPCs cannot maintain neuronal differentiation capacity (i.e. NR identity) and undergo cell fate conversion to CE. These results place SOX2 as a critical factor defining multipotent neural retinal progenitor identity (Taranova et al., 2006) and suggest a model of dosage-dependent transcriptional regulation of cell fate in the optic cup.

Materials and Methods

Mouse Breeding

Sox2$^{cond/+}$ mice (Taranova et al., 2006) were crossbred to α*P0*^{CREiresGFP} (Dr. P. Gruss, Max-Planck-Institute of Biophysical Chemistry, Germany (Marquardt et al., 2001)) or *Chx10*^{CREiresGFP}, (Jackson Laboratories (JAX), Bar Harbor, ME (Rowan and Cepko, 2004)) to generate *Sox2*$^{cond/+}$; α*P0*^{CREiresGFP} and *Sox2*$^{cond/+}$; *Chx10*^{CREiresGFP} mouse lines. These lines were then backcrossed to the *Sox2*^{cond} line to obtain homozygous mutant genotypes. Lineage tracing was carried out using *Rosa26Reporter* (*R26R*) mice (JAX (Soriano et al., 1987)). *Pax6*$^{Sey/+}$ mice (Dr. A. LaMantia, The George Washington University (Hill et al., 1991)) were bred to *Sox2*$^{cond/+}$; α*P0*^{CREiresGFP} mice to obtain *Sox2*$^{cond/+}$; α*P0*^{CREiresGFP}; *Pax6*$^{Sey/+}$ mice and then backcrossed to *Sox2*$^{cond/+}$ mice to yield the *Sox2*$^{cond/cond}$; *Pax6*$^{Sey/+}$; α*P0*^{CREiresGFP}double mutant. β-*catenin*^{activated} mice (Dr. R. Wechsler-Reya, Duke University (Harada et al., 1999)) were crossed with the α*P0*^{CREiresGFP} to obtain the constitutively activated genotype β-*catenin*^{activated}; α*P0*^{CREiresGFP}. Primer sequences for all of the alleles mentioned can be found in Table 2-1. It was necessary to genotype for the *Sox2*$^{\Delta cond}$ allele to eliminate animals in which germline recombination occurred. All animal work was carried out in accordance with University of North Carolina at Chapel Hill IACUC and DLAM approval.

Tissue preparation, Immunohistochemistry, and in situ hybridization

Mouse embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). Tissue was immersed sequentially in 10%, 20% and 30% sucrose/DEPC PBS overnight and then embedded and frozen in OCT medium (Tissue-Tek). Horizontal 14μm cryostat sections were blocked in 1% goat serum/0.1% Triton-X in PBS and incubated with primary antibodies at 4°C overnight and secondary antibodies for one hour at room temperature. The following antibodies were used in this study: rabbit anti-Cleaved Caspase 3 (1:300, Cell Signaling), chicken

anti-GFP (1:2000, Abcam), rabbit anti-Ki67 (1:1000, Abcam), mouse anti-PAX6 (1:100, Hybridoma), rabbit anti-SOX2 (1:3000, Millipore), mouse anti-β-tubulin III (1:1000, Covance), Alexa Fluor 488 anti-chicken (1:2000, Molecular Probes (MP)), Alexa Fluor 546 anti-mouse (1:2000, MP), and Alexa Fluor 546 anti-rabbit (1:2000, MP). *In situ* hybridization was performed on 20μm cryostat sections using DIG-labeled antisense probes, followed by enzymatic detection according to manufacture protocols (Roche). The following *in situ* probes were employed in this study: Axin2 ((Dr. F. Costantini (Jho et al., 2002)), Bmp4 (Dr. A LaMantia (Bhasin et al., 2003), Bmp7 (Dr. B. Hogan, (Lyons et al., 1995)), Chx10 (Dr. R. McInnes (Horsford et al., 2005)), Hes5 (Dr. E. Anton (Chenn and Walsh, 2002)), Lef1 (Dr. R. Grosschedl (Galceran et al., 1999)), Msx1 (Dr. Y. Liu (Liu et al., 2006a)), NeuroD1 (Dr. J. Lee (Lee et al., 1995)), Notch1 (Dr. U. Lendahl (Lardelli and Lendahl, 1993)), Rax (Dr. C. Cepko (Furukawa et al., 1997)), Sfrp2 (Dr. J. Nathans (Rattner et al., 1997)), Otx1 (Dr. J.P. Martinez, (Simeone et al., 1992)), Pax6 (Dr. A. LaMantia (Anchan et al., 1997)), and Zic1 (Dr. K. Millen (Aruga et al., 1994)). For *β-galactosidase* (*β-gal)* staining, slides were washed with PBS and immersed in *β-gal* staining solution (final concentrations: 5mM $K_3Fe(CN)_6$ (Sigma), 5mM $K_4Fe(CN)_6 \cdot 3\ H_2O$ (Sigma), 2mM $MgCl_2$ (Mallinckrodt), 0.02% Igepal CA-630 (Sigma), 0.01% Na-Deoxycholate (Sigma) and X-gal (1:50, Promega)) overnight at 37°C. Fluorescent and light microscopy images were taken on a Leica inverted microscope (Leica DMIRB) using a Q-imaging Retiga-4000RV digital CCD camera (Vashaw Scientific, Raleigh, NC, USA).

BrdU labeling

Pregnant mothers were weighed and injected 2 hours before dissection with 6μl/g of 15mg/ml BrdU (Sigma B5002) in PBS. Embryos were sectioned and prepared for

immunohistochemistry as stated above and stained overnight with mouse anti-BrdU (1:500, BD).

PAX6 immunofluoresence intensity analysis

PAX6 immunofluorescence intensity was measured in tissue sections taken from the central-most portion of E14.5 eyes. To exclude intensity variations caused by the appearance of different cell sections (and correspondingly, different volumes) at the focus of the objective, optical sectioning to generate the maximum image projection of the resulting slices was performed. To capture the whole cell volume, initial optical sectioning was conducted to determine the objective position at which the fluorescence intensity in the selected ROI (at least 20 cells per ROI) would be maximal. The ROI was selected at similar locations in each sample. The maximum intensity plane served as a reference point. For the final imaging for intensity calculations, a stack of 13µm (6.5 µm above and below the reference point) was collected. Twenty cells from each region of the eyecup (the lens epithelium, prospective CE, retinal ganglion cells, SOX2-positive neural progenitors and SOX2-ablated neural progenitors) were selected from the final 8-bit image for fluorescence intensity calculations, and the intensity was plotted using the Olympus Fluoview 2.1c. All intensity measurements were conducted on an Olympus Fluoview FV1000 confocal microscope with a 40× NA0.6 objective (Olympus Corp.). Statistics calculations were performed using Excel software (Microsoft Corp., Redmond, Wa).

Results

The central NR and the peripheral optic cup margin are defined by an inverse gradient of SOX2 and PAX6

To establish the functional interaction between SOX2 and PAX6 in the specification of optic cup progenitor cells, we first compared their expression patterns using immunohistochemistry. SOX2 and PAX6 are co-expressed in the anterior neural plate and throughout the optic vesicle prior to optic cup formation (Rex et al., 1997; Uchikawa et al., 2003; Uwanogho et al., 1995). However, in the early optic cup (E10.5), SOX2 and PAX6 begin to exhibit inverse expression gradients. While SOX2 is highly expressed in the prospective NR (Fig. 2-1A,B) in a graded central^{high}-to-distal^{low} pattern, PAX6 is highly expressed in prospective CE and RPE (Fig. 2-1A,C) in a gradient from central^{low}-to-distal^{high} (Baumer et al., 2002; Grindley et al., 1997; Kamachi et al., 2001; Walther et al., 1991). These inverse expression patterns are maintained throughout early optic cup development (Fig. 2-1D,G,J). Consequently, in the adult, SOX2 appears to be excluded from the non-neural CE and RPE (Fig. 2-2A,C), while PAX6 is maintained in the CE (Fig. 2-2B,C).

To determine whether the SOX2-PAX6 gradient is concurrent with the early divergence of cell fate into central prospective NR and peripheral prospective CE, we examined a repertoire of established optic cup markers at E13.5, before the NR and CE become morphologically distinguishable. We found that in the prospective NR, SOX2 and PAX6 (Fig. 2-3A,B) are co-expressed with the NR-specific genes *Notch1* (Lardelli and Lendahl, 1993), *Hes5* (Chenn and Walsh, 2002) and *NeuroD1* (Lee et al., 1995) (Fig. 2-3D,E,F) and with the multipotent optic cup progenitor genes *Rax* and *Chx10* (Fig. 2-3G,H). In contrast, SOX2 is downregulated in the prospective CE (Fig. 2-3A,C,box), where *Msx1* (Liu et al., 2006a), *Otx1* (Simeone et al., 1992),

Bmp4 (Zhao et al., 2002) (Fig. 2-3J,K,L) and *Zic1* (data not shown) (Trimarchi et al., 2009) are preferentially expressed. PAX6 is highly maintained in the prospective CE (Fig. 2-3B,I,box).

Ablation of SOX2 results in neurogenic to non-neurogenic cell fate conversion

We have previously generated mice carrying a conditional floxed allele of *Sox2* (*Sox2*$^{cond/+}$) (Taranova et al., 2006). To conditionally ablate SOX2 from multipotent cells throughout the peripheral optic cup, we used α*P0*^{CREiresGFP} transgenic mice, in which CRE expression is driven by the *Pax6* retina-specific enhancer α and minimal promoter P0 beginning at E10.0 (Kammandel et al., 1999; Marquardt et al., 2001). This mouse line can be used as a transgenic reporter of α enhancer-driven *Pax6* expression in the optic cup (Baumer et al., 2002). We previously crossed *Sox2*$^{cond/+}$ mice with *Sox2*$^{cond/+}$; α*P0*^{CREiresGFP} mice and showed that ablation of SOX2 results in severe ocular deformities, including extremely reduced eye size (Taranova et al., 2006). Here, we determine the fate of *Sox2*-mutant optic cup progenitor cells by comparing gene expression, proliferation and cell death in mutant (*Sox2*$^{cond/cond}$; α*P0*^{CREiresGFP}) eyes with that of control (*Sox2*$^{cond/+}$; α*P0*^{CREiresGFP}) eyes.

We first examined *Sox2* and *Pax6* expression at E16.5, when the prospective NR and CE initially become morphologically distinguishable. In control eyes, the inverse SOX2-PAX6 gradient is maintained throughout the optic cup, with PAX6 expression (Fig. 2-4B) highest in the distal tips and SOX2 expression (Fig. 2-4C) highest in the central optic cup. In contrast, SOX2-ablated cells (Fig. 2-4I) throughout the prospective NR exhibit an increase in α*P0*^{CREiresGFP} reporter (Fig. 2-4G), PAX6 protein (Fig. 2-4H) and *Pax6* mRNA expression (Fig. 2-4U). To confirm this increase in PAX6 protein, we quantified PAX6 immunofluorescence intensity in different regions of the eyecup of control and mutant embryos (Fig. 2-5). PAX6 is significantly upregulated

exclusively in CRE-positive, SOX2-ablated progenitor cells of the central optic cup when compared to wild-type, SOX2-positive central progenitor cells ($p<0.0001$).

To determine the identity of these *Sox2*-mutant cells, we examined the expression of NR-specific genes. *Sox2*-mutant cells fail to express the NR markers *Notch1* (Fig. 3D,J) and *Hes5* (Fig. 3E,K) and markers of postmitotic neurons, including *NeuroD1* (Fig. 3F,L) and *β-tubulin III* (data not shown).

We next examined whether this loss of neural characteristics is specific to the deletion of *Sox2* or is associated with decreased expression of other optic cup progenitor transcription factors, *Rax* and *Chx10*. In contrast to the upregulation of *Pax6* upon SOX2 ablation (Fig. 3H,U) *Rax* (Fig. 3M,S) and *Chx10* (Fig. 3N,T) remain unchanged between controls and mutants. Given that *Rax* and *Chx10* are expressed in CE progenitors, we hypothesized that *Sox2*-mutant cells may gain CE characteristics, so we examined the expression of established CE markers. Our data indicate that some *Sox2*-mutant cells ectopically express a subset of genes normally restricted to the prospective CE, including *Bmp7* (Fig. 3P,V) and *Raldh2* (data not shown). *Otx1* (Fig. 3Q,W), which is normally restricted to the distal tips of the optic cup, exhibits slight central expansion in *Sox2*-mutant eyes. However, most *Sox2*-mutant cells fail to express other prospective CE genes, including *Msx1* (Fig. 3R,X) and *Mitf* (data not shown) at E16.5.

The WNT/β-catenin signaling pathway has been implicated in the specification of CE fate (Cho and Cepko, 2006; Liu et al., 2007a; Liu et al., 2003b). We examined components of this pathway to test the hypothesis that the WNT signaling domain is expanded upon *Sox2* deletion. *Axin2*, an endogenous readout of WNT activity (Fuhrmann et al., 2009; Jho et al., 2002), is increased in *Sox2*-mutant cells compared with wild-type controls (Fig. S3F,M), but *Lef-1* expression appears to be unchanged (Fig. S3E,L). *Sfrp2*, a Wnt signaling antagonist expressed in

NR progenitors (Liu et al., 2003b), is centrally shifted (Fig. S3D,K). Taken together these data suggest that the active Wnt signaling domain is centrally expanded upon SOX2 ablation.

Forced expression of a stabilized form of β-Catenin in optic cup progenitors has been used previously to model the role of WNT signaling in CE induction (Liu et al., 2007b). We therefore compared expression of prospective NR and CE genes between *Sox2*-mutant embryos and embryos with constitutive activation of Wnt signaling. Consistent with the previous results of Liu et al., regions with stabilized β-catenin ($\beta catenin^{activated}$; $\alpha P0^{CREiresGFP}$) exhibit upregulation of *Lef1* and *Axin2* (Fig.2-6S,T) and fail to express the NR markers *Sox2*, *Hes5* and *Sfrp2* (Fig. 2-6O,P,Q,R). However, in contrast to *Sox2*-mutant eyes, which exhibit central expansion of CRE and increased *Pax6* expression, regions that constitutively express β-catenin do not show expansion of $\alpha P0^{CREiresGFP}$ and do not express *Pax6* (Fig. 2-6I,N,P,U). Thus, the loss of SOX2 parallels activation of β-catenin at E16.5 in the expansion of the *Axin2*-positive domain and the loss of NR characteristics.

Based on the central expansion of some genes known to be involved in specifying CE fate, we hypothesized that the loss of SOX2 induces a transient *liminal* or "in-between" state in which a maturation period is required for cells to fully adopt CE identity. To address this hypothesis, we examined the expression of NR- and CE-specific genes in control and *Sox2*-mutant eyes at postnatal day (P) 0. In control eyes, the NR exhibits laminar morphology and $\alpha P0^{CREiresGFP}$ expression in the inner nuclear layer and retinal ganglion cell layer (Fig. 2-7A,A'). The CE contains a single-layer of cuboidal cells with high $\alpha P0^{CREiresGFP}$ expression (Fig. 2-7A box and A' arrow). The NR-specific gene *Hes5* is expressed throughout the laminar NR and marks an abrupt boundary between the neurogenic retina and non-neurogenic CE (Fig. 2-7C,C'). The neurogenic and non-neurogenic regions are also distinguishable by the edge of *β-tubulin III* expression (Fig.

2-7B,B'). In stark contrast to control eyes, *Sox2*-mutant eyes show expanded $\alpha P0^{CREiresGFP}$ expression throughout the central eyecup (Fig. 2-7D,D'). Similar to what was observed in the eyes of mutant embryos, postnatal *Sox2*-mutant cells fail to express genes specific to the NR, including *Hes5* (Fig. 2-7C,C',F,F'), and lose neuronal differentiation capacity as demonstrated by the mutual exclusivity of β*-tubulin III* and $\alpha P0^{CREiresGFP}$ expression (Fig. 2-7B,B',E,E'). In addition to the upregulation of $\alpha P0^{CREiresGFP}$, *Sox2*-mutant cells gain expression of genes that are preferentially expressed in the CE at P0, including *Pax6*, *Msx1* and *Zic1* (Fig. 2-7G-I'). These ectopic CE-like regions exhibit thin single-layered morphology characteristic of wild-type CE. In contrast, the SOX2-positive NR regions, which presumably developed from cells that did not undergo CRE-mediated recombination, exhibit proper thickness and laminar morphology when compared with the NR of control eyes. These results suggest that *Sox2*-mutant progenitors autonomously undergo cell fate conversion from neurogenic retina to non-neurogenic CE.

Previous studies have shown that the development of the CE monolayer results from a decrease in cell division. Indeed, the optic cup margin exhibits a lower proliferation rate than does the prospective NR (Beebe, 1986; Cho and Cepko, 2006; Kubota et al., 2004). Examination for proliferation markers depicts that many SOX2-ablated cells, particularly in the peripheral region of the eyecup, do not incorporate BrdU (Fig. 2-8B,F) or express Ki67 (Fig. 2-8C,G). However, many SOX2-ablated cells in the central eyecup do. These results suggest that upon the deletion of *Sox2* by $\alpha P0^{CREiresGFP}$, there is decrease in the number of proliferating cells, particularly throughout the peripheral eyecup. In contrast, there is no significant change in apoptosis as indicated by Cleaved Caspase-3 expression (Fig. 2-8D,H).

Optic cup progenitor transcription factors PAX6, CHX10 and RAX are not sufficient to maintain neuronal differentiation capacity in the absence of SOX2

The previous data suggest that ablation of SOX2 in multipotent peripheral progenitor cells that can give rise to both NR and CE results in their eventual restriction to CE fate. To further address the hypothesis that cells that are specified to become NR will convert to CE upon loss of SOX2, we used the *Chx10*^{CREiresGFP} mouse line to ablate SOX2 in a mosaic pattern of progenitor cells throughout the whole optic cup beginning at E11.0. Thus, SOX2 is removed from alternating patches of cells that have been specified to become NR, and neighboring wild-type cells can serve as internal controls (Fig. 2-9B,C,H-I') (Donovan and Dyer, 2004; Jadhav et al., 2006; Oron-Karni et al., 2008; Rowan et al., 2004; Zhang et al., 2004). In *Sox2*$^{cond/cond}$; *Chx10*^{CREiresGFP} mutant eyes, SOX2 is specifically ablated in CRE-expressing cells marked by GFP (Fig. 2-9B,G,H,H'). As a consequence of SOX2 loss, mutant cells do not undergo neuronal differentiation, as evidenced by the mosaic expression of β-tubulin III (Fig. 2-9C,I,I') and *NeuroD1* (Fig. 2-9F,L) in a pattern mutually exclusive of *Chx10*^{CREiresGFP} expression (Fig. 2-9I, arrowhead). Moreover, SOX2-ablated cells fail to express the NR-specific genes *Notch1*, *Hes5, and Sfrp2* (Fig. 2-9D,E,J,K,M,S).

To determine if these *Sox2*-mutant progenitor cells undergo cell fate conversion to CE, we examined the localization of genes normally expressed in the prospective CE at E14.5. In regions of SOX2 ablation, *Pax6* is upregulated (Fig. 2-9N,T) and *Chx10* (Fig. 2-9O,U) and *Rax* (Fig. 2-9P,V) are maintained. Moreover, *Sox2*-mutant cells express some prospective CE markers, including *Bmp7* (Fig. 2-9Q,W,W'), but not others including *Msx1* (Fig. 2-9R,X). This expression profile recapitulates that of early *Sox2*$^{cond/cond}$; *αP0*^{CREiresGFP} mutant cells described above.

The upregulation of *Pax6* and *Bmp7* suggest that ablation of SOX2 by *Chx10*^{CREiresGFP} induces a liminal state similar to what was observed in *Sox2*$^{cond/cond}$; α*P0*^{CREiresGFP} mutants. At P0, mosaic regions of *Chx10*^{CREiresGFP} expression/SOX2-ablation are clearly distinguishable from neighboring *Chx10*^{CREiresGFP}-negative/SOX2-postive regions (Fig. 2-10A,A',D,D'). In mutant eyes, SOX2-ablated regions lack expression of the NR gene *Hes5* (Fig. 2-10C,C',F,F') and the post-mitotic neuronal marker *β-tubulin III* (Fig. 2-10B,B',E,E'). Conversely, *Sox2*-mutant regions upregulate *Pax6* (Fig. 2-10G,G',J,J') and cell-autonomously gain expression of the CE markers *Msx1* and *Zic1* (Fig. 2-10H-I',K-L'). These data support our finding that loss of SOX2 in multipotent progenitor cells results in a temporary liminal state typifying their maturation to CE. In addition, at P0, *Sox2*-mutant regions exhibit thin, single-layered morphology that starkly contrasts with neighboring control regions, which exhibit proper NR laminar morphology. *Sox2*-mutant progenitors fated to become NR lose neuronal differentiation capacity and undergo cell fate conversion to CE (Cho and Cepko, 2006; Liu et al., 2007b).

Fate mapping of Sox2 mutant cells depicts loss of neural progenitor capacity in the retina

To directly establish if ablation of SOX2 results in an autonomous cell fate change from NR to CE, we genetically fate mapped *Sox2*-mutant cells using the $\alpha P0^{CREiresGFP}$ mouse line. $Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$ mice were crossed with mice carrying the Rosa26R CRE reporter allele ($Sox2^{cond/+}$; *R26R/+*), which expresses *β-galactosidase* (*β-gal*) following CRE-mediated excision of a translational stop cassette, permanently marking the progeny of CRE-expressing cells (Soriano et al., 1987). Therefore, cells that express CRE at the time of analysis can be detected using GFP fluorescence, while all the progeny of CRE-positive cells express *β-gal*. In control $Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$; *R26R/+* embryos, $\alpha P0^{CREiresGFP}$ is initially expressed throughout the peripheral optic cup (Fig. 2-11A). By E15.5, it becomes restricted to the distal tips (Fig. 2-11B), maintaining high expression in the CE by E17.5 (Fig. 2-11D). However, at E15.5 and E17.5, *β-gal* is detected throughout both the laminar NR ($\alpha P0^{CREiresGFP}$ -negative) and the distal CE (Fig. 2-11C,E), leaving only a portion of central NR cells unmarked. This *β-gal* expression pattern confirms that $\alpha P0^{CREiresGFP}$-expressing progenitor cells can give rise to both NR and CE. In contrast, as previously shown, $Sox2^{cond/cond}$; $\alpha P0^{CREiresGFP}$; *R26R*/+ mutant eyes exhibit expanded $\alpha P0^{CREiresGFP}$ expression into the central optic cup (Fig. 2-11F,G,I), and all $\alpha P0^{CREiresGFP}$-positive cells appear to express *β-gal* (Fig. 2-11H,J). By E17.5, these *β-gal*-positive regions exhibit thin morphology when compared to the SOX2-postive/*β-gal*-negative regions in the same eye (Fig. 2-11J).

***Sox2* and *Pax6* genes interact to coordinate eye development**

Based on the increase in PAX6 expression upon SOX2 ablation, we hypothesized that proper regionalization of the optic cup depends on a fine balance of SOX2 and PAX6 dosage. To directly address this hypothesis, we performed genetic epistasis analysis of SOX2 and PAX6 in the developing optic cup. To modulate PAX6 dosage, we used the $Pax6^{Sey/+}$ mouse line in which a

spontaneous mutation in the *Pax6* gene produces a truncated protein that lacks a DNA-binding homeodomain and the C-terminal transactivation domain. This truncated PAX6 is considered functionally inactive and is widely used as *Pax6*-null allele (Hill et al., 1991; Hogan et al., 1986; Osumi et al., 2008). Compared to $Sox2^{cond/+}$ or $Sox2^{cond/cond}$ mice, which display normal eye development (Taranova et al., 2006), $Pax6^{Sey/+}$ mice exhibit reduced external eye size, iris hypoplasia and small lens (Hill et al., 1991).

To specifically ablate SOX2 in peripheral progenitors on this *Pax6*-heterozygous background, we crossed $Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$ mice with $Sox2^{cond/+}$; $Pax6^{Sey/+}$mice. We then compared resulting $Sox2^{cond/cond}$; $\alpha P0^{CREiresGFP}$; $Pax6^{Sey/+}$ (*Sox2*/*Pax6*-double mutant) embryos with $Sox2^{cond/cond}$; $\alpha P0^{CREireGFP}$ (*Sox2*-single mutant) embryos and $Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$; $Pax6^{Sey/+}$ (*Pax6*-single mutant) control embryos. *Sox2*/*Pax6*-double mutant eyes, which have reduced levels of both SOX2 and PAX6, are significantly normalized compared to *Sox2*-single mutant eyes, which are wild-type for *Pax6* but lose SOX2 upon CRE-mediated ablation (Fig. 2-12J-R,S-AA). The central expansion of $\alpha P0^{CREiresGFP}$ seen in *Sox2*-single mutant eyes is significantly rescued in *Sox2*/*Pax6*-double mutant eyes (Fig. 2-12S-AA,J-R). Lineage tracing analysis using the Rosa26R CRE reporter shows that the domain of $\alpha P0^{CREiresGFP}$ activity, as marked by β-*gal* expression, is peripherally restricted in *Sox2*/*Pax6*-double mutants compared to *Sox2*-single mutants. These data indicate that $\alpha P0^{CREiresGFP}$ expression in *Sox2/Pax6* double mutants is restored to an expression pattern that more closely resembles that of controls (Fig. 2-12B,K). Nevertheless, $\alpha P0^{CREiresGFP}$ expression co-localizes with β-*gal* activity in *Sox2*/*Pax6*-double mutants (Fig. 2-12B,C,K,L), which is consistent with the maintenance of $\alpha P0^{CREiresGFP}$ in SOX2 ablated cells as described above (Fig. 2-11). The ablation of SOX2 in the *Pax6*-heterozygous background significantly rescues neuronal differentiation capacity, as illustrated by normalization of β-*tubulin III* (Fig. 2-12D,M,V), *Notch1* (Fig. 2-12E,N,W), *Hes5* (Fig. 2-12F,O,X)

and *NeuroD1* (Fig. 2-12G,P,Y). Modulating SOX2 or PAX6 levels do not affect *Rax* (Fig. 2-12H,Q,Z) or *Chx10* (Fig. 2-12I,R,AA) expression.

To determine whether this rescue phenotype is maintained postnatally, we examined *Sox2/Pax6*-double mutants at P0, and found a slightly expanded region of $\alpha P0^{CREiresGFP}$-positive thin CE-like cells compared to *Pax6*-single mutant controls (Fig. 2-13A-H, brackets). This result contrasts with the single-layered morphology and CE gene expression present throughout the center of the eyecup in *Sox2*-single mutants (Fig. 2-13I-L). Moreover, the NR of *Sox2/Pax6*-double mutants exhibits proper laminar morphology and neuronal differentiation capacity as indicated by β-*tubulin III* and *Hes5* expression, and *Msx1* expression is restricted to the distal tips (Fig. 2-13F-H). Therefore, *Sox2/Pax6*-double mutants more closely resemble *Pax6*-single mutants than *Sox2*-single mutants.

Discussion

SOX2 maintains neuorgenic fate of proliferating neuorepithelial progenitor cells

Here we have demonstrated for the first time that the genetic ablation of SOX2 causes a neurogenic-to-non-neurogenic cell fate conversion. These results place SOX2 as the critical factor defining neurogenic identity in retinal neuroepithelium. In early stage mouse embryos, SOX2 expression marks the region fated to become neural ectoderm, and its appearance in chicken embryos coincides with the onset of neural fate specification (Wood and Episkopou, 1999). Inhibition of SOX2 function in *Xenopus* embryos blocks neural differentiation, and SOX2 signaling in chick has been shown to promote proliferation and inhibit neuronal differentiation (Bylund et al., 2003; Graham et al., 2003; Kishi et al., 2000). In the retina, unlike in other regions of the CNS, SOX2 is expressed exclusively of the highly related SOXB1 factors, SOX1 and SOX3. Thus, the optic cup, which retains the unique capacity to generate non-neurogenic structures, provides an excellent model for studying SOX2's transcriptional role in maintaining neurogenic identity.

SOX2 ablation initiates gradual cell fate conversion from NR to CE

Secreted molecules known to affect neurogenic versus non-neurogenic optic cup cell fate include FGFs, BMPs and WNTs. A previous report showed that converging FGF and BMP signals may define the prospective NR/CE boundary at optic vesicle stages of the chick (citation). BMP signaling appears to be required for *Otx1* and *Msx1* expression in the prospective CE (Zhao et al., 2002), and *Msx1* expression has been shown to be induced by activated Wnt signaling (Willert et al., 2002). Thus, overlapping FGF, BMP and Wnt signals may coordinate the cell-intrinsic gene expression necessary for CE development. However, at least one study has shown induced ectopic CE marker expression without the onset of Wnt2b expression (citation).

Interestingly, it has been proposed that extrinsic signals that direct neurogenic versus non-neurogenic fate converge at the regulation of SOX proteins (Wilson et al., 2001). Here we investigate the cell-intrinsic role of SOX2 and PAX6 in setting up the NR/CE boundary in the optic cup.

Using genetic fate mapping, we have demonstrated that removal of a transcription factor, SOX2, converts prospective NR to CE. Our results are consistent with previous data showing that neural RPCs maintain multipotent differentiation capacity (i.e. the capacity to form CE) at a developmental time point (E11.0) subsequent to the division of NR and CE fate (Fekete et al., 1994; Turner and Cepko, 1987; Turner et al., 1990). The gradual cell fate conversion upon *Sox2* deletion is characterized by 1) loss of the NR markers *Notch1*, *Hes5*, *NeuroD1*, and *Sfrp2*, 2) maintenance of the multipotent progenitor genes *Chx10*, *Rax* and *Pax6*, 3) expansion of WNT and BMP signals and 4) decreased proliferation with progressive thinning of the neuroepithelium. These characteristics define a liminal or in-between state that culminates in the expression of CE markers, including *Otx1*, *Zic1* and *Msx1* (markers are summarized in Table 2-2). A similar delay in the consolidation of CE identity in response to ectopic Wnt signaling has been previously demonstrated (Cho and Cepko, 2006). In both instances, the delay may be due to the presence of competing signals in the eyecup: WNT signals promote non-neurogenic fate but multipotent progenitor genes and Wnt antagonists promote neurogenic fate. Intringuingly, activation of β-Catenin in the eyecup was shown to produce a stronger and more rapid onset of CE characteristics than did Wnt2b overexpression, suggesting that stabilized β-Catenin bypasses WNT antagonists in the prospective NR to cause a more immediate increase in CE gene expression (Cho and Cepko, 2006). Thus, the presence of WNT antagonists may explain why consolidation of CE fate is gradual upon SOX2 ablation but rapid upon β-catenin activation (Liu et al., 2007a).

Sox2 and Pax6 interact to regionalize the optic cup

A major difference between SOX2 loss-of-function and β-Catenin gain-of-function in the prospective retina is the effect on *Pax6*. Ablation of SOX2 leads to an immediate increase in *Pax6* expression, while activation of β-catenin diminishes *Pax6* expression. In humans, haploinsufficiency of *PAX6* is associated with anterior eye formations, including defects of the iris and ciliary body (reviewed by (Hever et al., 2006; Hill et al., 1991)). In the mouse, deletion of one copy of *Pax6* in the distal optic cup resulted in the loss of CE precursors and a distal shift in the boundary between prospective NR and CE (Davis-Silberman et al., 2005). Moreover, transgenic overexpression of *Pax6* resulted in abnormalities of the ciliary body, the iris and the cornea (Schedl et al., 1996). These studies demonstrate the importance of regulating appropriate levels of PAX6 for the proper development of peripheral eye structures. Here, we have described increased *Pax6* expression in the optic cup upon the removal of a potential repressor.

Our genetic epistasis analyses provide evidence for a mechanism of PAX6 regulation in the developing eyecup. The dramatic increase in PAX6 upon *Sox2* deletion and the subsequent NR-to-CE cell fate conversion suggest that SOX2 normally antagonizes PAX6 signaling to maintain NR identity. Moreover, the genetic rescue of eye development by lowering PAX6 levels while deleting *Sox2* indicates a functional antagonism between the two genes. These data raise the possibility that neurogenic versus non-neurogenic fate in the optic cup is extremely sensitive to the ratio of PAX6 to SOX2.

We propose a model (Fig. 2-14) in which SOX2 levels are high enough to antagonize *Pax6* expression in central optic cup progenitor cells, thereby maintaining neurogenic capacity. Conversely, in the peripheral optic cup, SOX2 levels are low, perhaps allowing PAX6 to activate

its own expression via the α enhancer. In fact, previous studies have shown that PAX6 can directly bind a conserved site in α, and lowering PAX6 levels decreases α-driven CRE-GFP expression (Baumer et al., 2002; Schwarz et al., 2000). Further studies are needed to address whether SOX2 and PAX6 coordinate *Pax6* expression through the α enhancer. Indeed, SOX2 and PAX6 have been shown to co-regulate expression of the *δ-crystallin* gene in the lens, where they form a complex at the DC5 enhancer that is stabilized by both protein-protein and protein-DNA interactions (Kamachi et al., 2001). A similar co-regulation of α enhancer activity may control *Pax6* expression in the optic cup given that the α enhancer has been suggested to mediate the proximal^{low}-to-distal^{high} PAX6 gradient (Baumer et al., 2002; Davis-Silberman et al., 2005).

Whereas the role of secreted signaling molecules in positioning the boundary between prospective NR and CE has been studied extensively, little is known about the cell-intrinsic mechanisms responsible for setting up or maintaining this boundary. Our results suggest a tightly coordinated dosage-dependent transcriptional mechanism directing NR versus CE cell fate. We have shown that the functional antagonism between SOX2 and PAX6 is necessary for proper patterning of the eyecup, such that in the absence of SOX2, PAX6 is not sufficient to maintain NR identity, and cells originally fated to become retinal neurons instead take on a peripheral non-neurogenic cell fate.

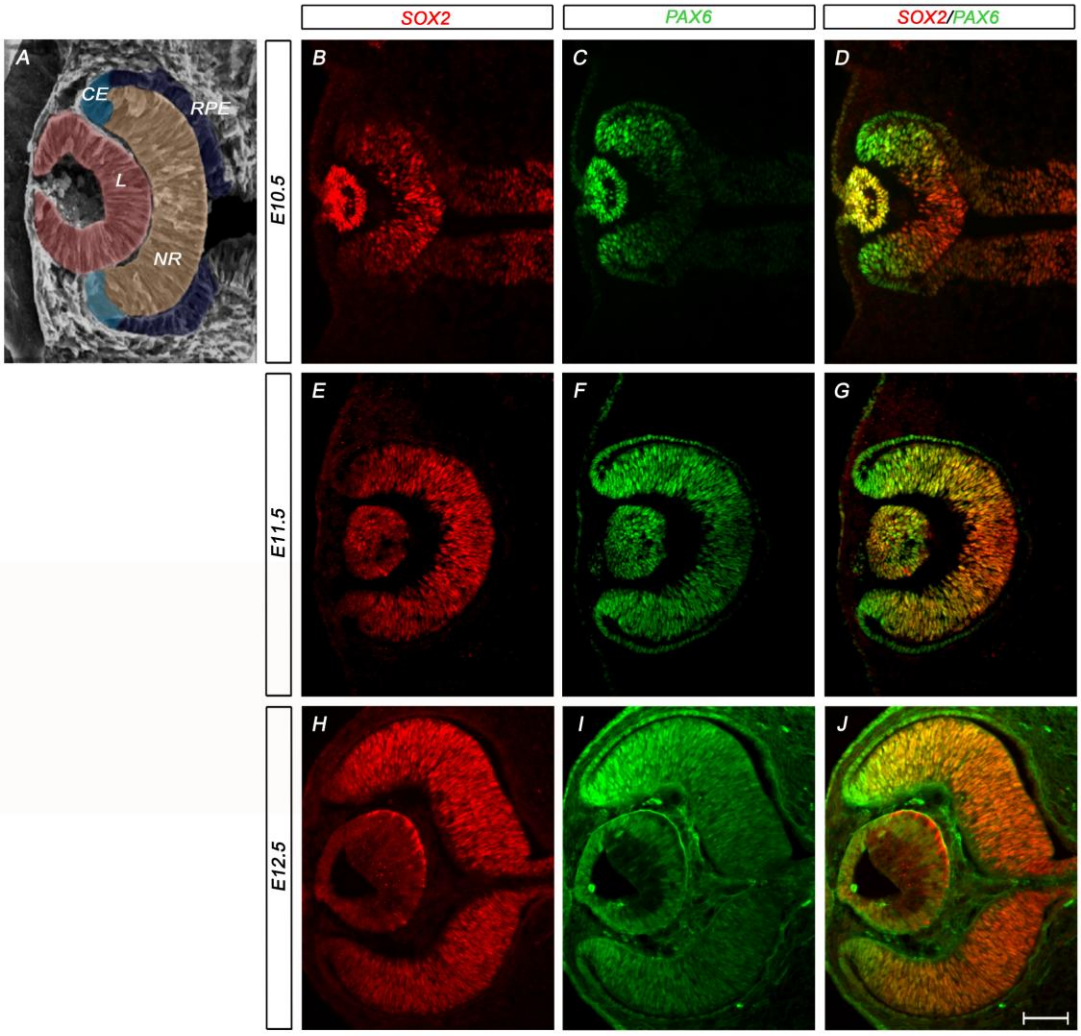


Figure 2-1 The neural retina and optic cup margin are defined by an inverse gradient of SOX2 and PAX6. (A) Schematic of an E12.5 eye indicating the boundaries of the mouse neural retina (NR), lens (L), prospective ciliary body epithelium (CE) and retinal pigment epithelium (RPE). (B-D) Immunohistochemistry on horizontal sections of wild-type embryos shows high SOX2 expression (red) in the central optic cup and high PAX6 expression (green) in the peripheral optic cup margin. (E-J) As the eye develops, the inverse gradients of SOX2 and PAX6 expression are maintained. Scale bar: 100 µm.

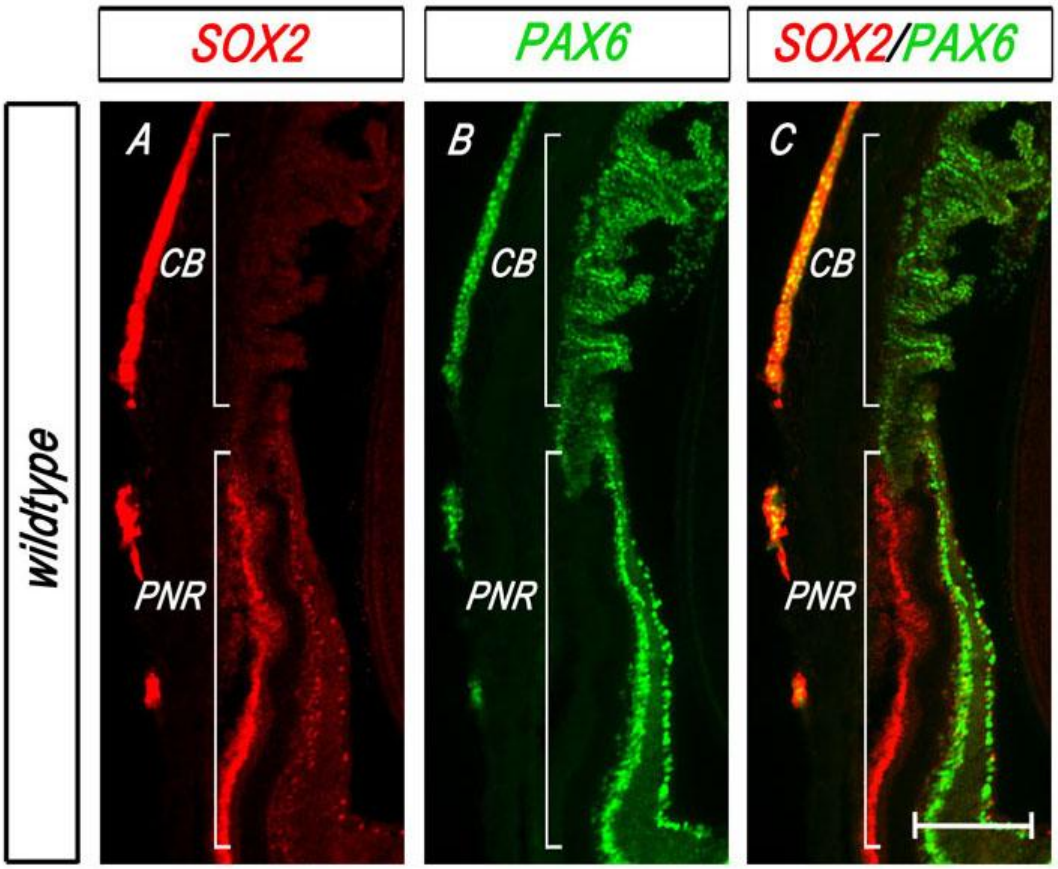


Figure 2-2 SOX2 is expressed in the adult mouse NR but is absent from the ciliary body. *(A,B)* Immunohistochemistry for SOX2 (A, red) in the wild-type adult eye shows expression in the neural retina (NR) but not in the ciliary body (CB), whereas PAX6 (B, green) shows high expression in the adult CB. (C) Merged image of SOX2 (red) and PAX6 (green) illustrates the inverse relationship between the two proteins, with SOX2 restricted to the NR and absent from the CB. PNR, peripheral neural retina. Scale bar: 100μm

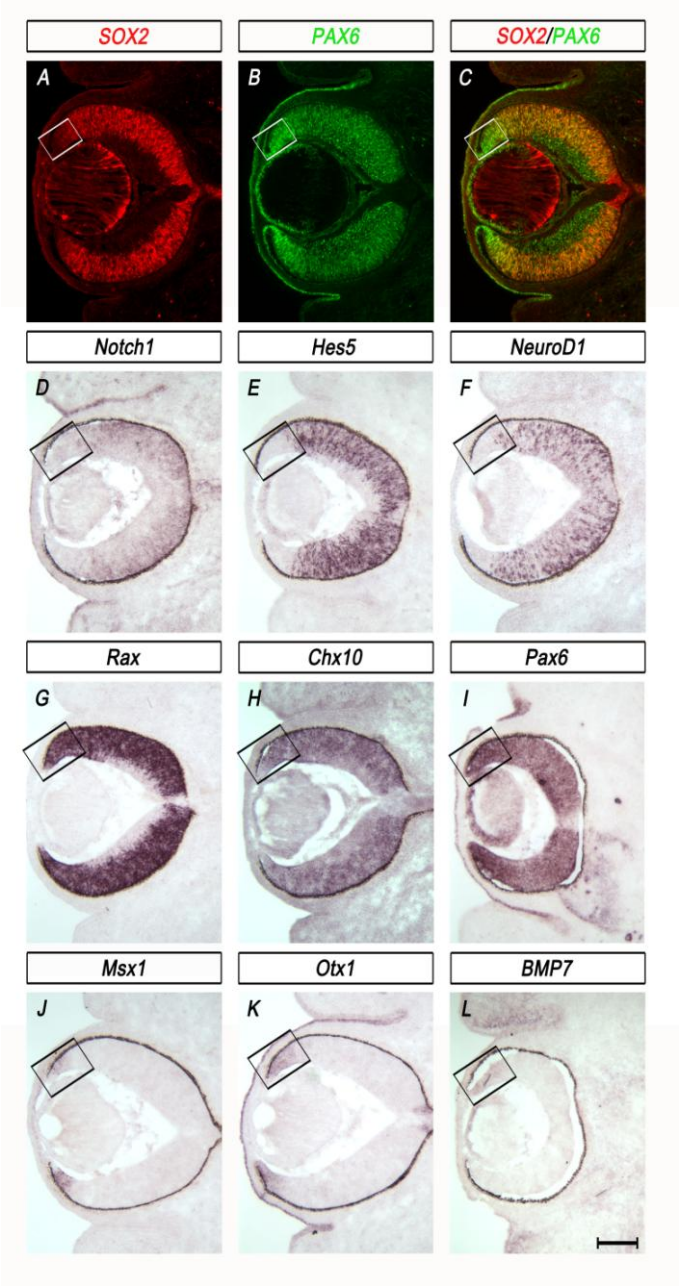


Figure 2-3 Expression profiles of the central NR and peripheral OCM at E13.5 reflect the inverse expression patterns of SOX2 and PAX6. *(A-C)* IHC for SOX2 (red) and PAX6 (green) on horizontal sections of wild-type mouse eyes illustrates inverse gradients of expression, with SOX2 highly expressed in the NR and PAX6 highly expressed in the OCM. *(D-F)* The NR-specific genes *Notch1* (D), *Hes5* (E) and *NeuroD1* (F) are co-expressed with SOX2. *(G-I)* The OC progenitor transcription factors *Rax* (G), *Chx10* (H) and *Pax6* (I) are expressed in both NR and OCM. *(J-L) Msx1* (J), *Otx1* (K) and *Bmp4* (L) are expressed in the OCM. Boxes delineate the OCM. Scale bar: 200 µm.

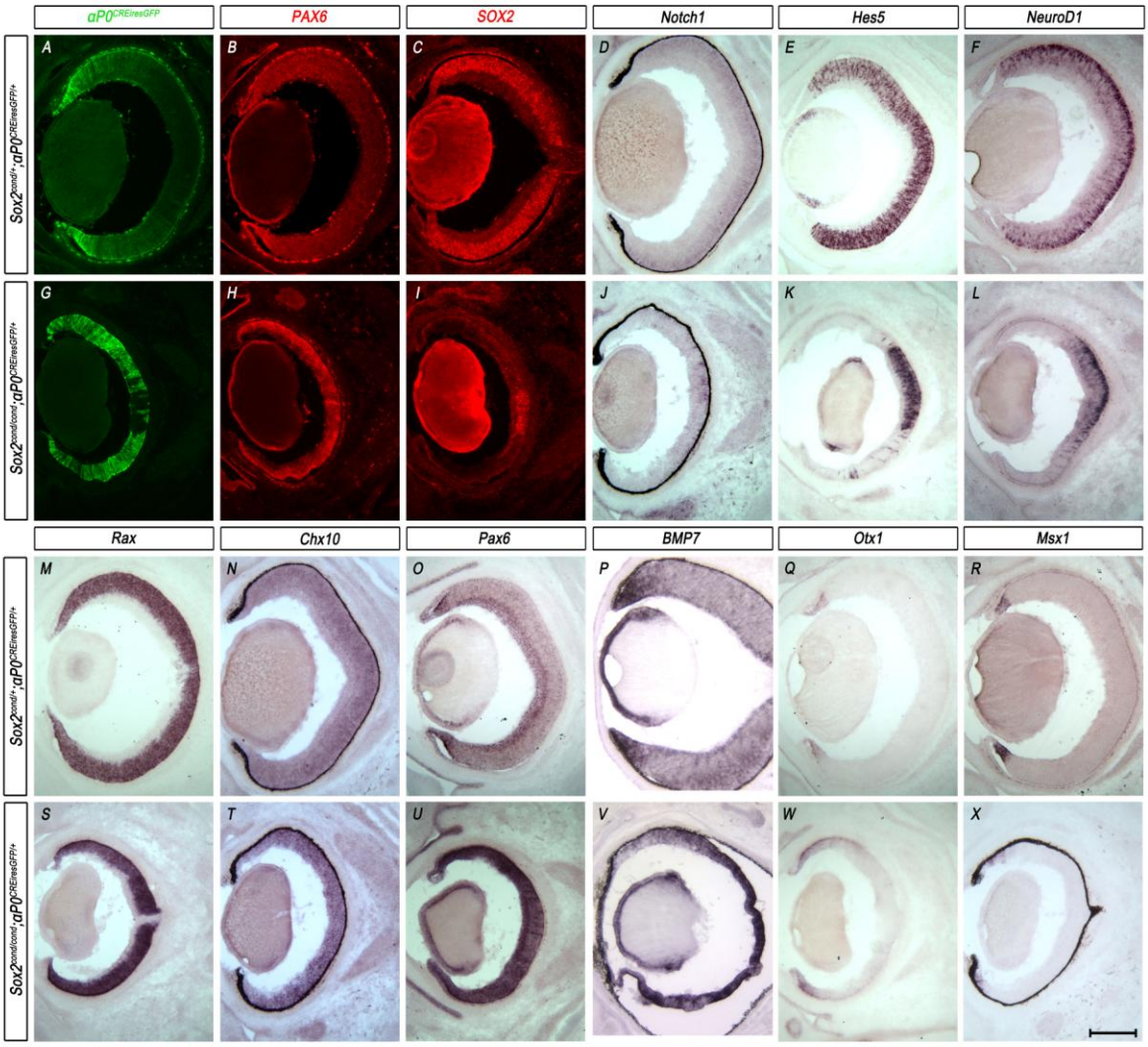


Figure 2-4 Ablation of SOX2 in the peripheral OC results in loss of neural fate and central expansion of the OCM. *(A-X)* Horizontal sections through the eyes of E16.5 mouse embryos. (A,G) $\alpha P0^{CREiresGFP}$ reporter expression as indicated by CRE-GFP (green) depicts central expansion in mutant OCs (G) compared with controls (A). (B,C,H,I) PAX6 is increased in SOX2-negative cells (H,I) when compared with SOX2-positive cells (B,C) of wild-type controls. (D-F,J-L) Expression of the NR-specific genes *Notch1* (N,J), *Hes5* (E,K) and *NeuroD1* (F,L) is lost in SOX2-ablated regions (J-L). (M-O,S-U) *Rax* (S,M) and *Chx10* (T,N) are unchanged, but *Pax6* mRNA is increased (U,O) in *Sox2*-mutant eyes. (P,Q,V,W) *Bmp7* (P,V) and *Otx1* (Q,W) are expanded into the prospective NR of *Sox2*-mutant eyes. (R,X) *Msx1* is unchanged between control (R) and mutant eyes (X). Scale bar: 200 µm.

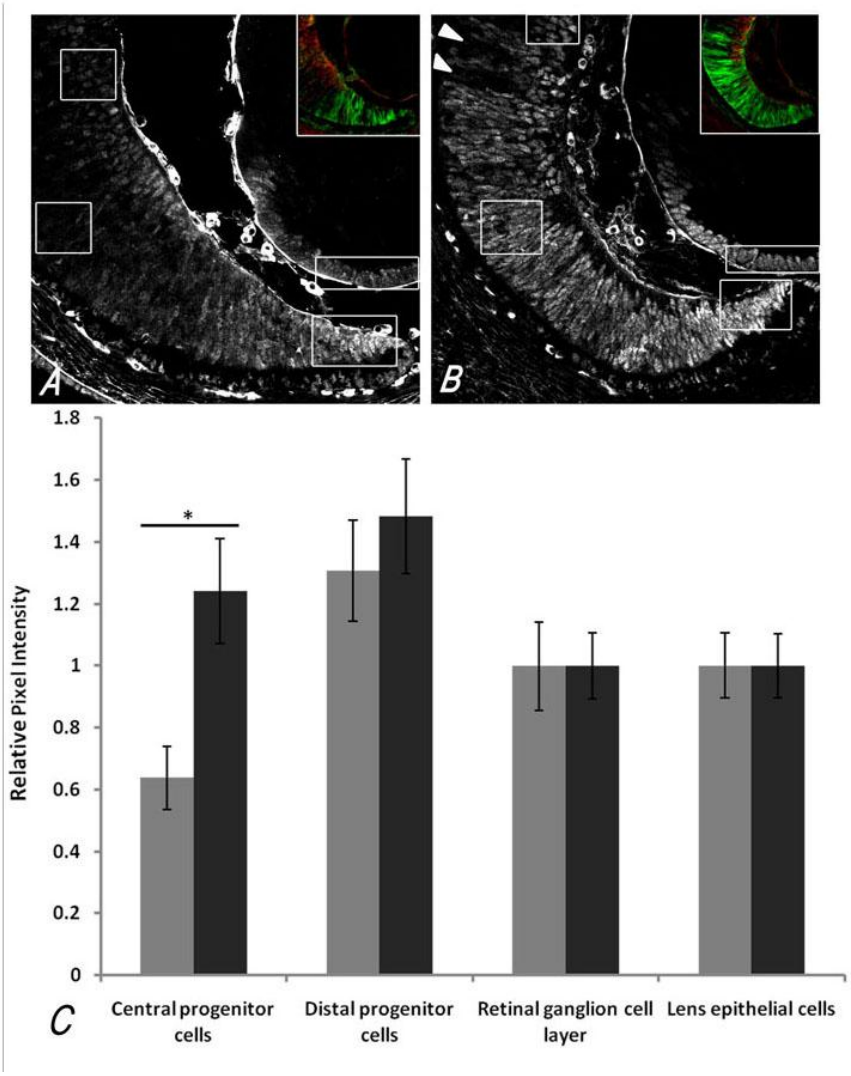


Figure 2-5 PAX6 IF intensity per cell in different regions of the E14.5 OC is increased in central progenitors of mutants. *(A,B)* Squares delineate regions in which cells were chosen to quantify PAX6 IF intensity (clockwise from top): RGC layer, lens epithelium, distal-most OC and prospective NR. PAX6-expressing RGCs were identified by β-tubulinIII expression (red) on adjacent sections (insets). Arrowheads in B indicate regions that do not express $\alpha P0^{CREiresGFP}$ (green, inset) and, therefore, maintain SOX2. (C) Average pixel intensity per cell is not significantly different between distal cells, RGCs and lens epithelial cells of wild-type and *Sox2*-mutant eyes. By contrast, the average pixel intensity per cell is significantly increased in central OC cells of *Sox2*-mutants compared with that of controls ($P<0.0001$). For each region, $n=20$ cells. Error bars indicate s.e.m.

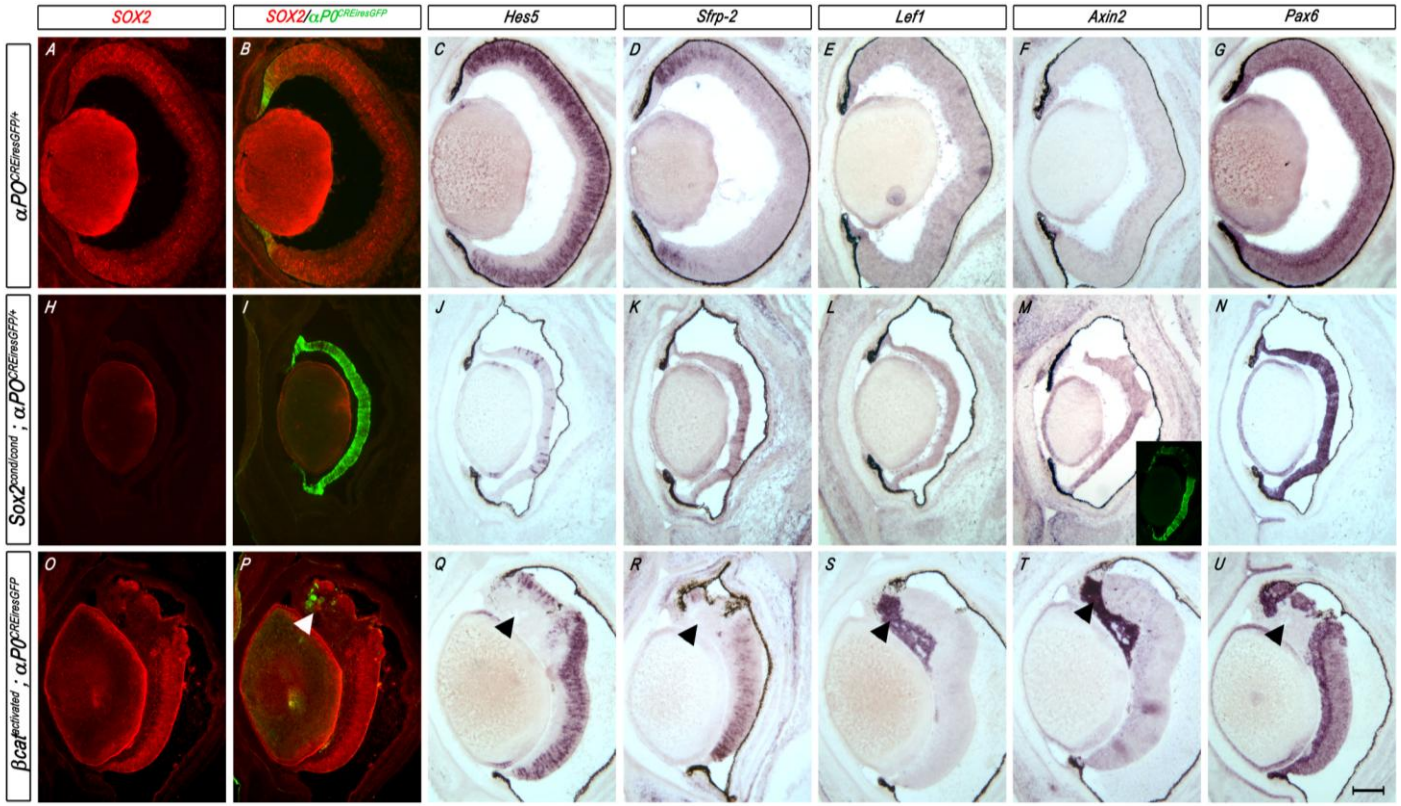


Figure 2-6 WNT signaling is expanded in *Sox2*-mutant eyes at E16.5. (**A**,**B**,**H**,**I**,**O**,**P**) IHC for SOX2 (red) and α*P0*^{CREiresGFP} reporter expression (green) in horizontal sections of control (α*P0*$^{CREiresGFP/+}$), *Sox2*-mutant (*Sox2*$^{cond/cond}$*;* α*P0*^{CREiresGFP}) and stabilized β-Catenin (β-*catenin*^{activated}*;* α*P0*^{CREiresGFP}) eyes. (**C-G**,**J-N**,**Q-U)** ISH for *Hes5* (C,J,Q) and *Sfrp2* (D,K,R) reveals no expression in α*P0*^{CREiresGFP}-positive mutant cells. ISH for *Lef1* (E,L,S) and *Axin2* (F,M,T) indicates restriction to the prospective CE in control eyes. *Axin2* but not *Lef1* is up-regulated in *Sox2*-mutant cells, which are designated by α*P0*^{CREiresGFP} expression (inset in M), and both are highly expressed in regions with stabilized β-Catenin (S,T). ISH for *Pax6* (G,N,U) shows expression in the prospective CE as well as in the inner nuclear layer and retinal ganglion cell layer of control eyes, whereas *Pax6* is upregulated in *Sox2*-mutant cells but absent from cells with constitutively activated β-catenin. Arrowheads indicate areas where stabilized β-catenin is localized. Scale bar: 200 µm.

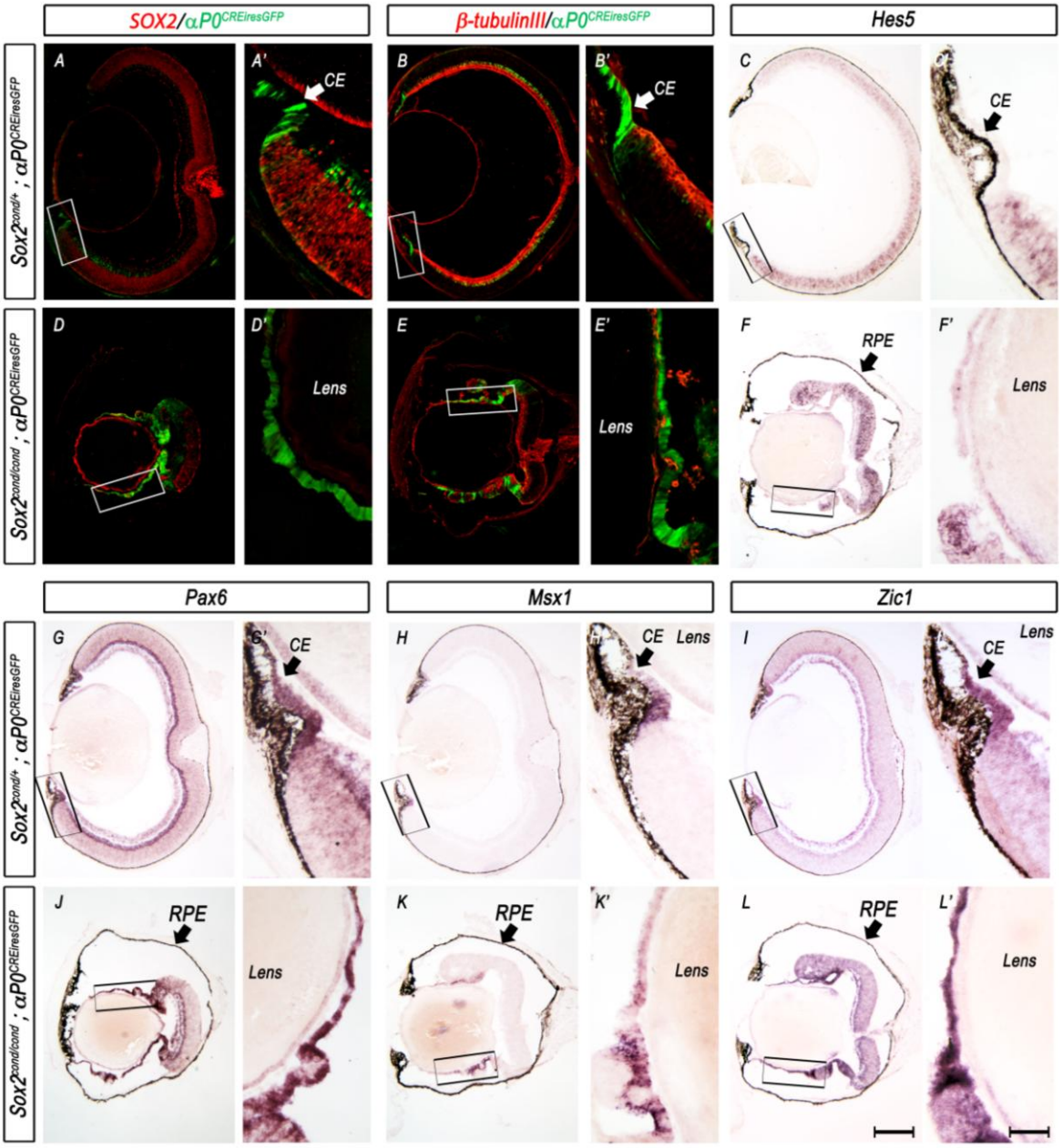


Figure 2-7 Ablation of SOX2 by αP0^{CREiresGFP} results in cell fate conversion of the NR to CE. (**A-L**') Low (A-L) and high magnification (A'-L') images of sections through the eyes of P0 control and mutant pups. Boxes indicate magnified regions. αP0^{CREiresGFP} is expressed in the distal tips as well as in the INL and RGC layer of control eyes (A,A'). By contrast, αP0^{CREiresGFP} expands into the putative NR of *Sox2*-mutant eyes (D,D'). β-tubulinIII (B,B',E,E') and *Hes5* (C,C',F,F') are present only throughout the SOX2-positive NR of mutant eyes and not in SOX2-ablated regions. CE-specific genes, including *Pax6* (G,G',J,J'), *Msx1* (H,H',K,K') and *Zic1* (I,I',L,L'), are ectopically expressed in mutant eyes when compared with controls. CE, ciliary epithelium; RPE, retinal pigment epithelium. Scale bars: in L, 400 µm for A-L; in L', 100 µm for A'-L'.

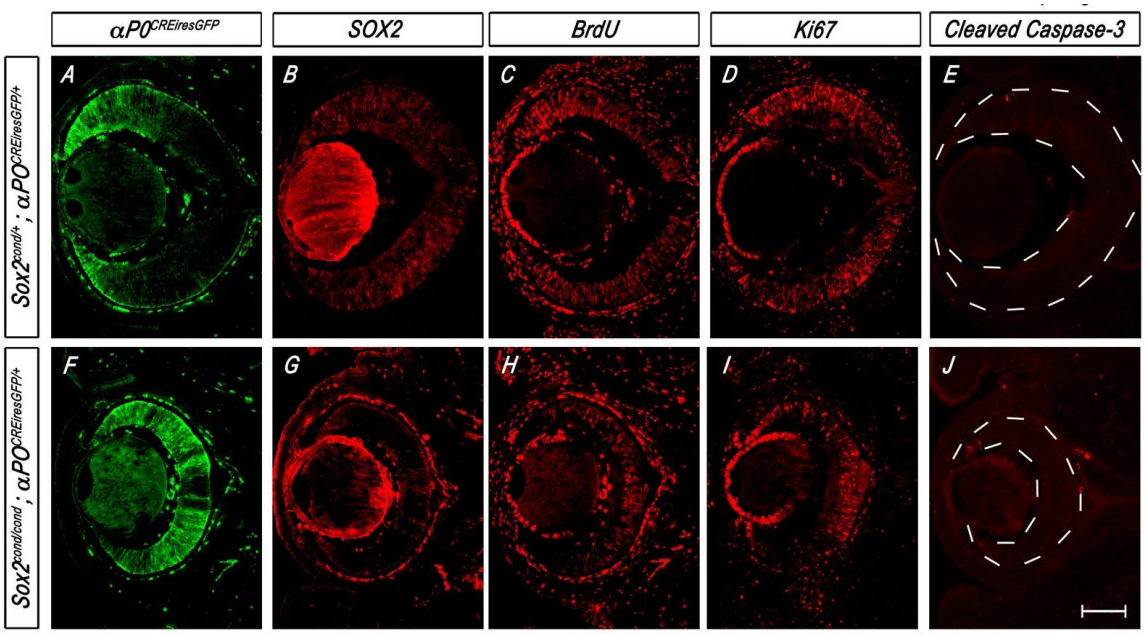


Figure 2-8 *Sox2*-mutant eyes contain fewer proliferating cells. ***(A,E)*** $\alpha P0^{CREiresGFP}$ reporter expression (green) in horizontal sections of E14.5 eyes shows peripheral restriction in control ($Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$) eyes compared with central expansion in mutant ($Sox2^{cond/cond}$; $\alpha P0^{CREiresGFP}$) eyes. ***(B-D,F-H)*** IHC for the proliferation markers BrdU (B,F) and Ki67 (C,G) reveals cycling cells in central regions and very few cycling cells in peripheral regions of mutant eyes. IHC for Cleaved Caspase-3 (D,H) does not depict increased apoptosis in *Sox2*-mutant eyes. The dotted lines outline the eyecup. Scale bar: 200 µm.

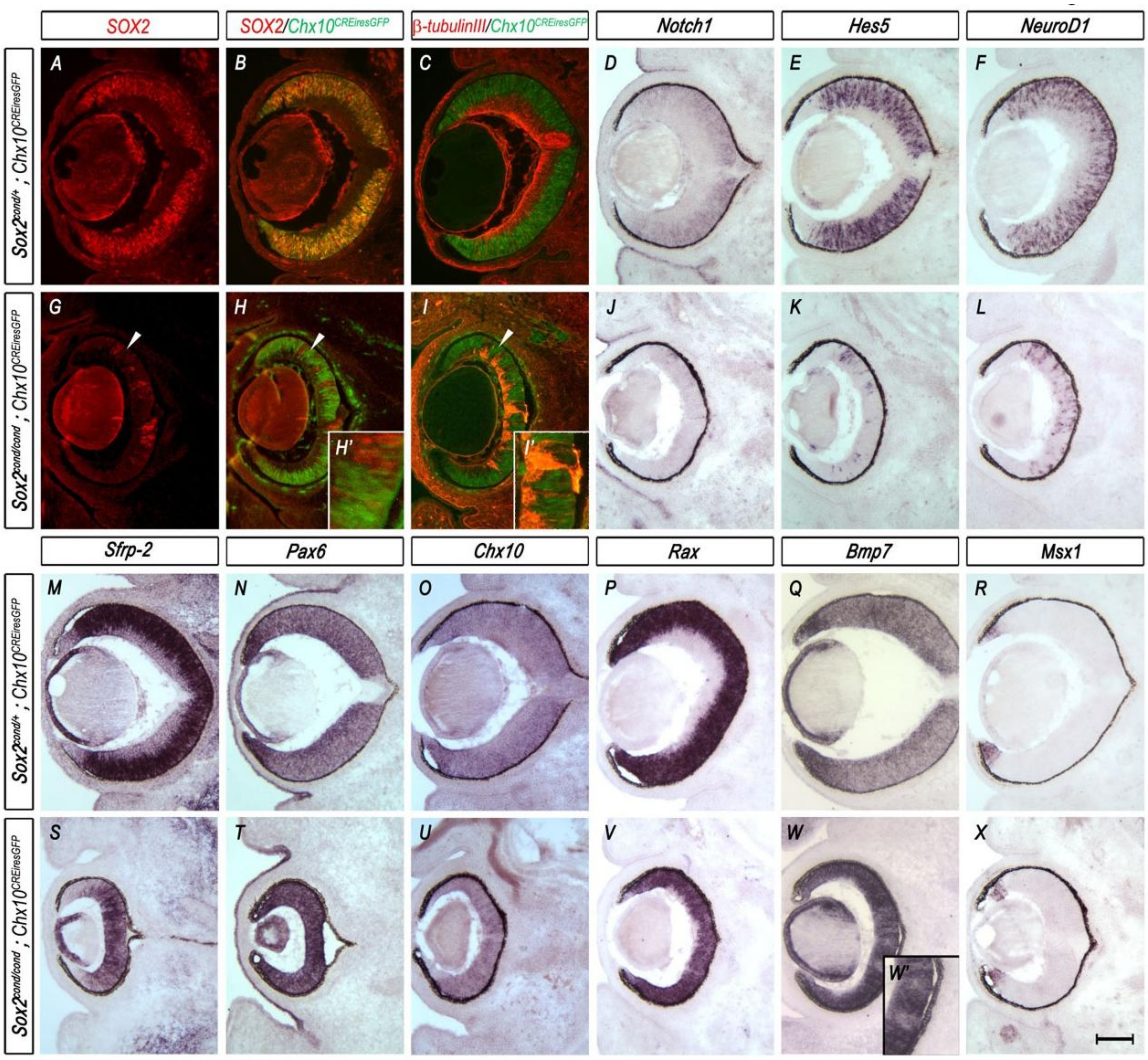


Figure 2-9 Mosaic ablation of SOX2 in neural progenitors results in loss of neuronal fate and central expansion of the OCM. ***(A-X)*** Horizontal sections through the eyes of E14.5 control (*Sox2*$^{cond/+}$*; Chx10*^{CreGFP}) and mutant (*Sox2*$^{cond/cond}$*; Chx10*^{CreGFP}) mouse embryos. (A-C,G-I') *Chx10*^{CreGFP} reporter (B,C,H-I'; green) double labeled with SOX2 (A,B,G-H'; red) or β-tubulin III (C,I,I'; red) shows expression throughout the whole OC in control eyes. In mutant eyes, CRE-GFP is mutually exclusive of SOX2 (H) and β-tubulin III (I). Arrowheads indicate regions in which SOX2 has been ablated. (D-F,J-X) Expression of *Notch1* (D,J), *Hes5* (E,K) and *NeuroD1*(F,L) is lost, *Sfrp2* (M,S) expression is decreased, *Pax6* (N,T) expression is increased, and *Chx10* (O,U) and *Rax* (P,V) are maintained in regions where SOX2 has been ablated when compared with wild-type controls. *Bmp7* (Q,W,W') is ectopically expressed in the prospective NR of mutant embryos, whereas *Msx1* (R,X) shows little difference in expression between the control and mutant eyes. Scale bar: 200 μm.

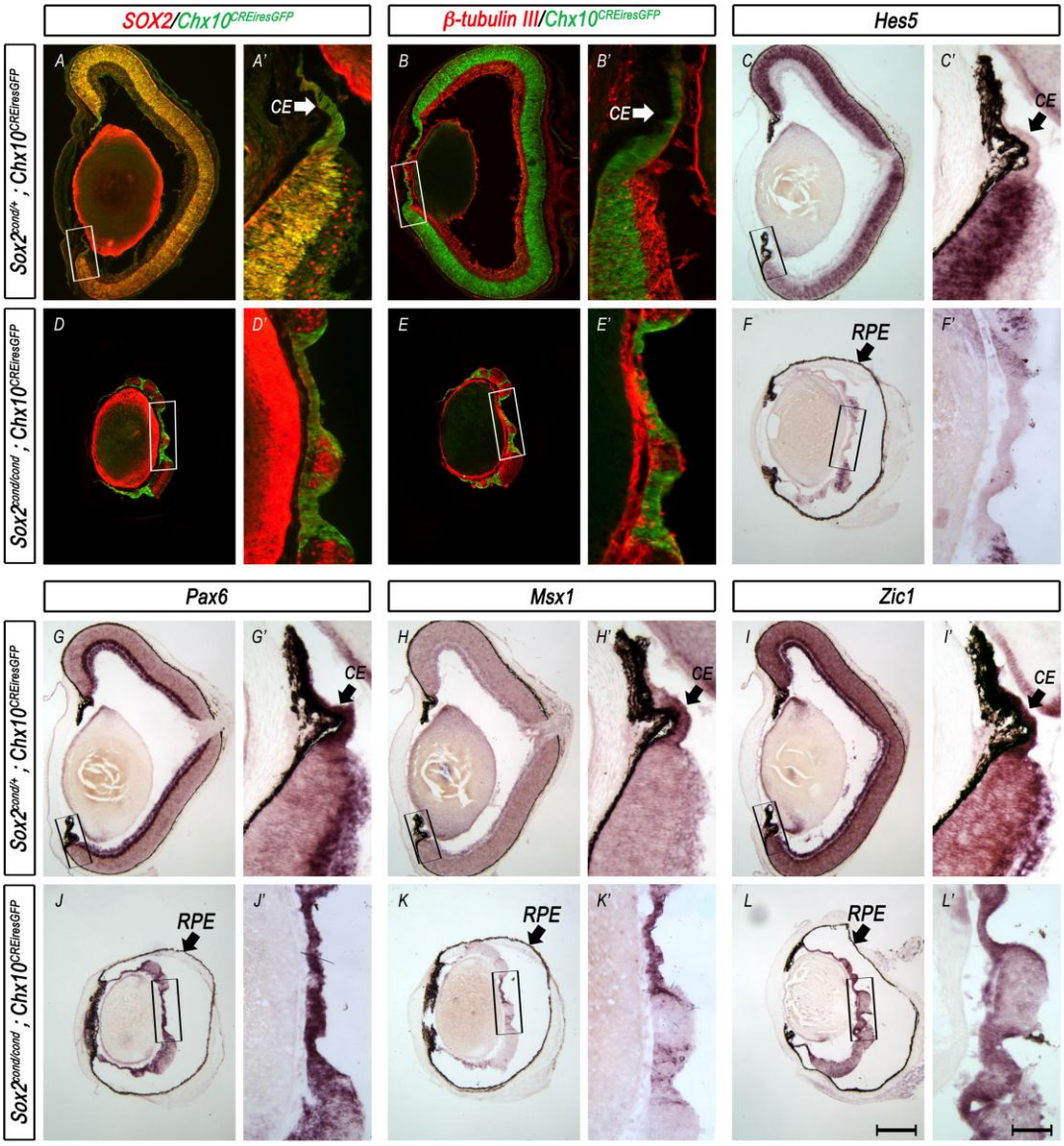


Figure 2-10 At P0, mosaic regions of SOX2 ablation indicate cell fate conversion of the NR to CE. ***(A-L')*** Low (A-L) and high magnification (A'-L') images of sections through the eyes of P0 mouse pups. Boxes indicate magnified regions. IHC for SOX2 (A,A',D,D'; red), β-tubulin III (B,B',E,E'; red) and GFP (A-E'; green) in control and mutant eyes shows thinning of the retinal neuroepithelium to CE-like morphology (D-E') and loss of neuronal differentiation capacity (E,E') in SOX2-negative regions. *Hes5* (F,F') is lost, *Pax6* (J,J') is increased, and *Msx1* and *Zic1* (K-L') are ectopically expressed in SOX2-ablated regions. CE, ciliary epithelium; RPE, retinal pigment epithelium. Scale bars: in L, 400 µm for A-L; in A', 100 µm for A'-L'.

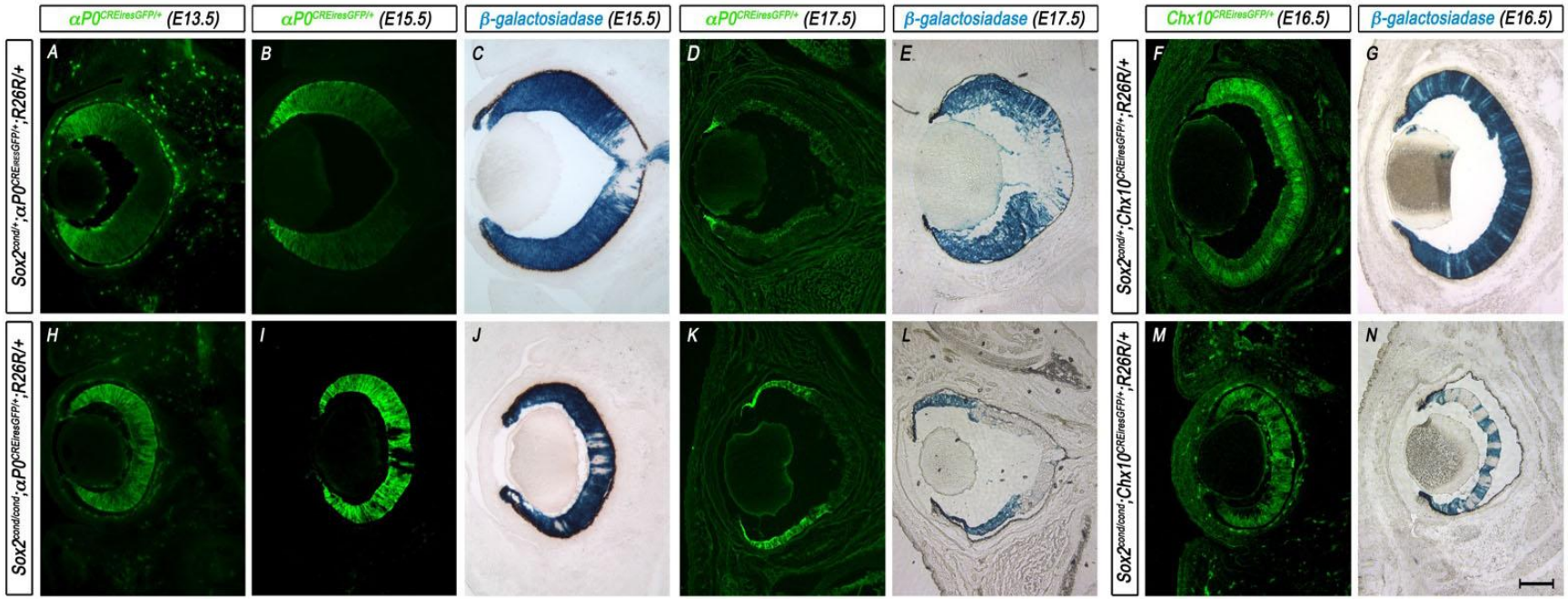


Figure 2-11 Fate mapping *Sox2*-mutant progenitor cells. ***(A, H)*** $\alpha P0^{CREiresGFP}$ (green) is expressed in the CE and in peripheral progenitor cells in the control (*Sox2*$^{cond/+}$*;* $\alpha P0^{CREiresGFP}$*; R26R*) eyes at E13.5, while $\alpha P0^{CREiresGFP}$ reporter expression in the mutant embryo (*Sox2*$^{cond/cond}$*;* $\alpha P0^{CREiresGFP}$*; R26R*) is expanded into the central prospective NR. ***(B, I, D, K)*** As the wild-type eye develops, $\alpha P0^{CRE}$ reporter expression (green) is further restricted to the peripheral retina and eventually serves as a marker of prospective CE by E17.5, while in the mutant eye $\alpha P0^{CRE}$ reporter expression remains an indicator for all recombined cells throughout the NR and prospective CE. ***(C, E, J, L)*** *β-galactosiadase* (*β-gal*) reporter assay on E15.5 as well as E17.5 eyes indicates that $\alpha P0^{CREiresGFP}$-positive progenitor cells are capable of giving rise to both NR and non-neurogenic cells in control eyes but are cell fate-restricted in *Sox2*-mutant eyes. (D, K) By E17.5, $\alpha P0^{CRE}$ reporter expression is only present in the distal tips of the retina as well as in some cells of the inner nuclear layer while $\alpha P0^{CRE}$ reporter expression reflects all recombined cells in the mutant embryo. ***(F, M)*** *Chx10*^{CREiresGFP} fluorescent expression (green) at E16.5 in *Sox2*$^{cond/+}$*; Chx10*^{CREiresGFP}*; R26R* and *Sox2*$^{cond/cond}$*; Chx10*^{CREiresGFP}*; R26R* eyes. ***(G, N)*** *β-gal* reporter assay on E16.5 eyes shows restricted proliferation (expansion) of progenitor cells in the presumptive NR of the mutant *Sox2*$^{cond/cond}$*; Chx10*^{CREiresGFP}*; R26R* eye. Scale bar: 200μm.

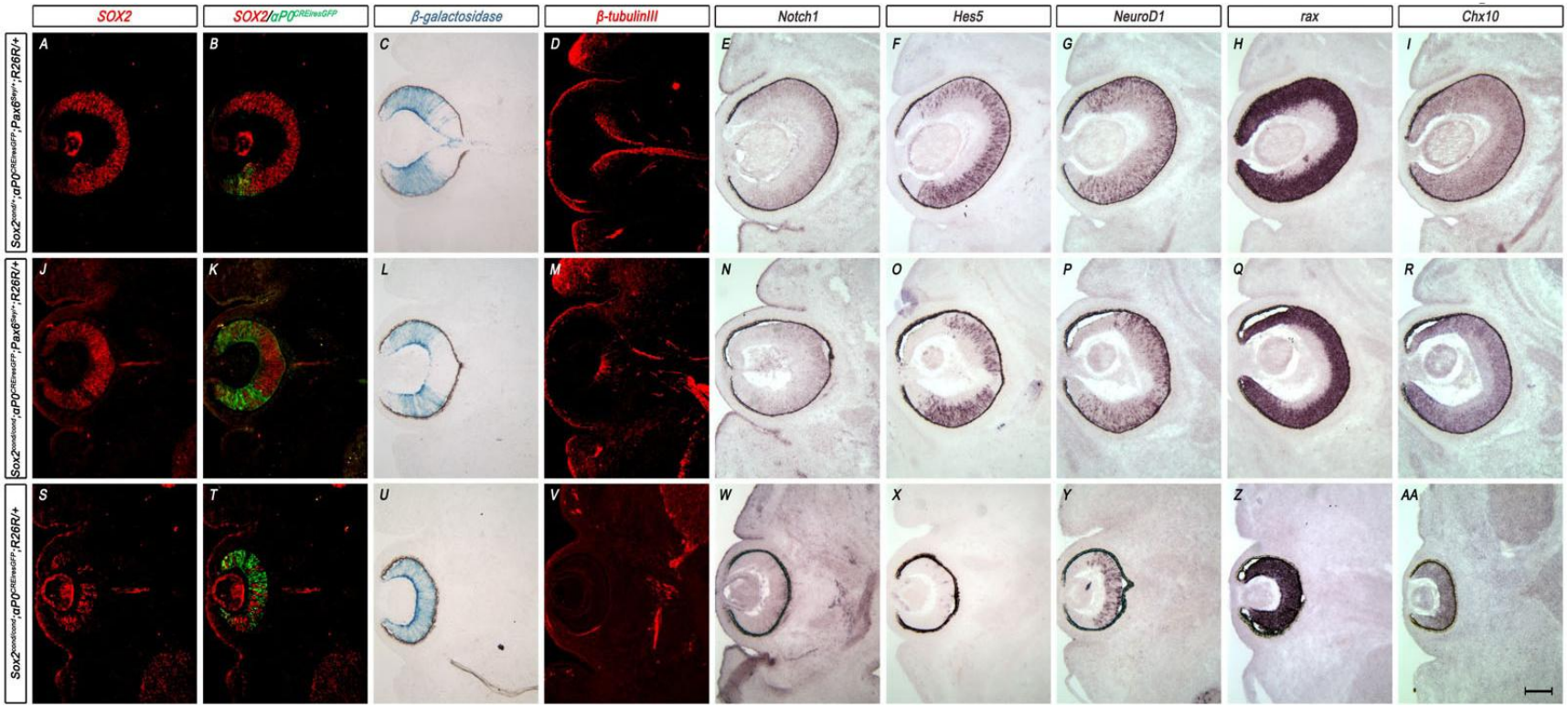


Figure 2-12 *Pax6*-heterozygosity significantly rescues the *Sox2*-mutant NR. (***A,B,J,K,S,T)*** Comparison of SOX (red) with α*P0*^{CREiresGFP} (green) in *Pax6* single mutant, *Sox2 Pax6* double mutant and *Sox2* single mutant eyes indicating little to no SOX2 expression in the *Sox2* single mutant compared with the other two genotypes. (***C,L,U***) β-gal activity illustrating the progeny of all α*P0*^{CREiresGFP}-expressing cells indicates rescue of α*P0*^{CREiresGFP} expression in *Sox2 Pax6* double mutants. (***D,M,V***) β-tubulin III staining (red) shows maintenance of neuronal differentiation capacity in *Pax6* single mutants and *Sox2 Pax6* double mutants but not in *Sox2* single mutants. (**E-I,N-R,W-AA**) *Notch1* (E,N,W), *Hes5* (F,O,X), *NeuroD1* (G,P,Y), *Rax* (H,Q,Z) and *Chx10* (I,R,AA) are maintained in the central OC of *Sox2 Pax6* double mutants. Scale bar: 200 μm.

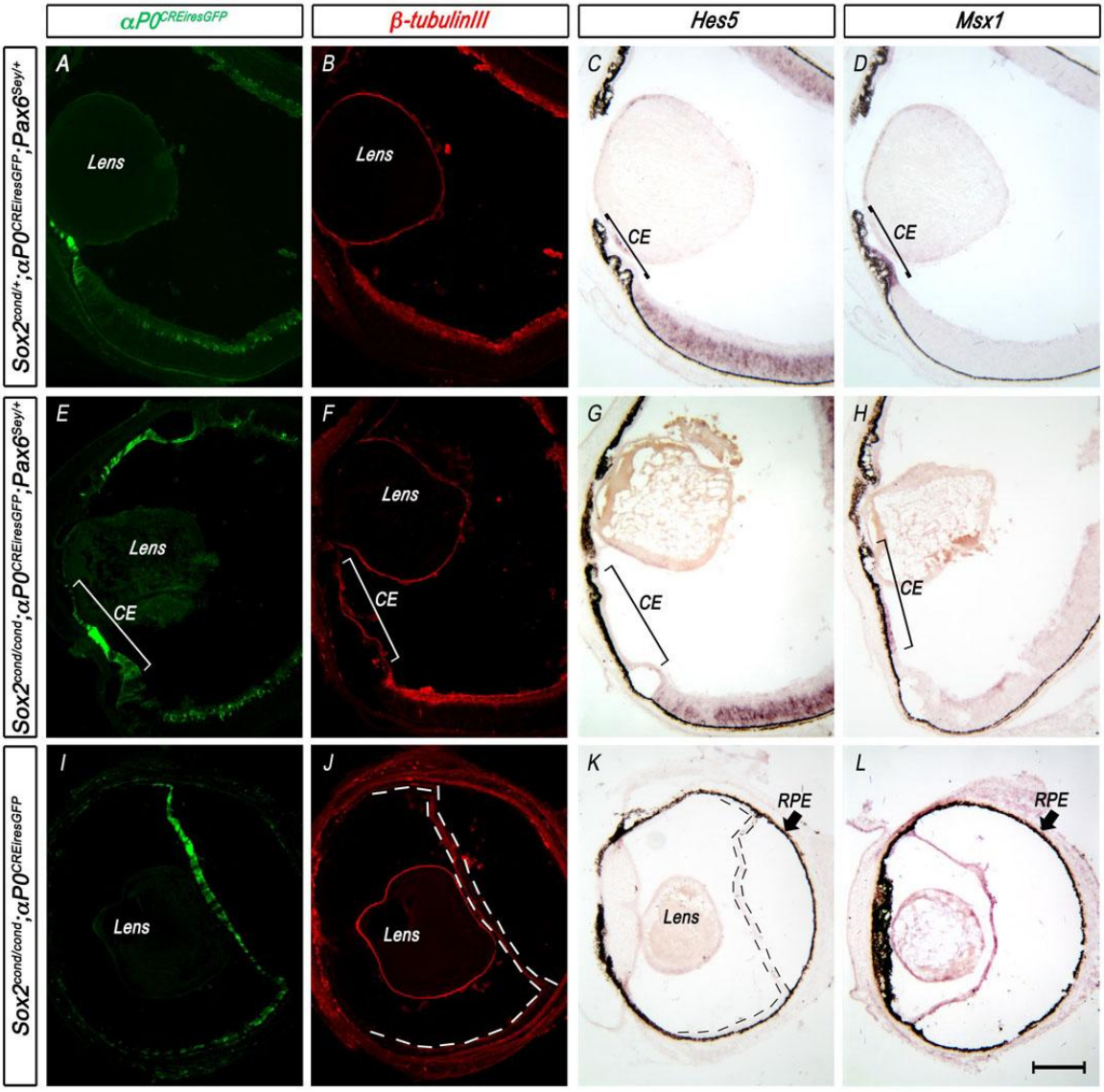


Figure 2-13 The NR is rescued in *Sox2 Pax6* double mutant eyes. (***A-L***) $\alpha P0^{CREiresGFP}$ (A,E,I; green) and (B,F,J) β-tubulin III expression (B,F,G; red) indicate rescued neuronal differentiation capacity in the NR of *Sox2 Pax6* double mutants when compared with *Sox2* single mutants and *Pax6* single mutant controls. ISH of *Hes5* (C,G,K) and *Msx1* (D,H,L) shows proper neurogenic versus non-neurogenic regionalization in *Sox2 Pax6* double mutants, resembling that of *Pax6* single mutants. The dotted lines outline the region converted to CE in the *Sox2* single mutant. Scale bar: 200 µm

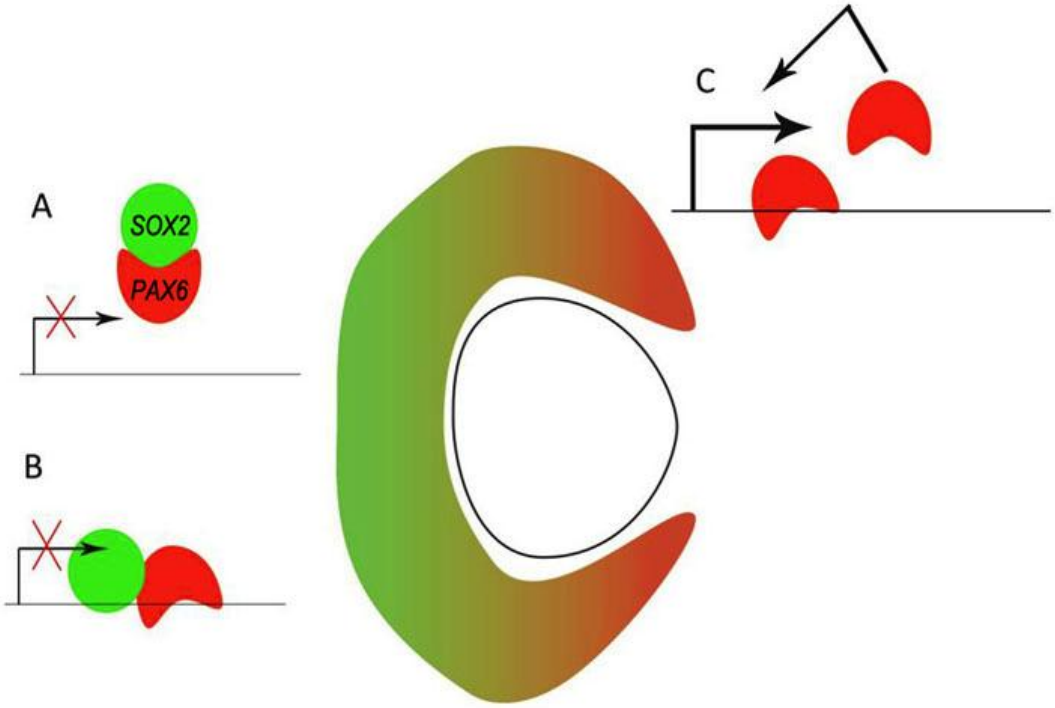


Figure 2-14 Model of how SOX2 expression in the central OC antagonizes *Pax6* expression. In the central optic cup, SOX2 might function as an antagonist to PAX6 at the protein level by binding directly to PAX6 (A) or by cooperatively or competitively binding with PAX6 at its α enhancer (B). In the peripheral optic cup, where SOX2 expression is low and PAX6 expression is high, PAX6 is free to up-regulate its own expression (C).

Allele	Primers (5' to 3')	Product size	Conditions
Sox2cond	Fwd: CAGAGGACTCGTGTTTGGGAAC Rev: TCTTGGATACATAAGGGTGGATGG	Sox2cond: 307 bp wt: 345 bp	4 minutes at 94°C, (15 seconds at 94°C, 30 seconds at 57°C, 30 seconds at 72°C)335, 5 minutes at 72°C
Sox2dcond	Fwd: CTTCTTTCCGTTGATGCTTTCG Rev: ATCTTGGTGGCTGAACAGTTATCC	589 bp	4 minutes at 94°C, (15 seconds at 94°C, 30 seconds at 57°C, 30 seconds at 72°C)335, 5 minutes at 72°C
Cre	Fwd: GCTAAACATGCTTCATCGTCGG Rev: GATCTCCGGTATTGAAACTCCAGC	750 bp	4 minutes at 94°C, (15 seconds at 94°C, 30 seconds at 57°C, 30 seconds at 72°C)335, 5 minutes at 72°C
Rosa26Rep	Primer 1: AAAGTCGCTCTGAGTTGTTAT Primer 2: GCGAAGAGTTTGTCCTCAACC Primer 3: GGAGCGGGAGAAATGGATATG	transgene: 650 bp wt: 340 bp	4 minutes at 94°C, (30 seconds at 93°C, 30 seconds at 58°C, 1 minute at 65°C)335, 10 minutes at 72°C
Pax6-Sey	Fwd: AACTTTTTGGCTTGCTTTGTCATTC Rev: CTGAGCTTCATCCGAGTCTTCTCA	188 bp	1 minute at 95°C, (20 seconds at 92°C, 20 seconds at 63.5°C, 50 seconds at 72°C)334, 4 minutes at 72°C
Pax6-wt	Fwd: GAACACCAACTCCATCAGTTCTAACG Rev: CTTTCCCGGGCAAACACATC	646 bp	1 minute at 95°C, (20 seconds at 92°C, 20 seconds at 63.5°C, 50 seconds at 72°C)334, 4 minutes at 72°C
β-catenin-act	Fwd: AGAATCACGGTGACCTGGGTTAAA Rev: CATTCATAAAGGACTTGGGAGGTGT	transgene: 900 bp wt: 600 bp	90 seconds at 94°C, (30 seconds at 94°C, 1 minute at 60°C, 1 minute at 72°C)335, 2 minutes at 72°C

Table 2-1 PCR primers and protocols.

	E13.5 wildtype		P0 wildtype	
Marker	**NR**	**OCM**	**NR**	**CE**
Neural Retina				
Sox2	+	-	+	-
Sfrp2	+	-	+	-
Notch1	+	-	+	-
Hes5	+	-	+	-
NeuroD1	+	-	+	-
Math5	+	-	+	-
OCPTF				
Chx10	+	+	+	+
Rax	+	+	+	+
Pax6	+	+	+	+
CE				
Bmp4/7	-	+	-	+
Lef1	-	+	-	+
Raldh2	-	+	-	+
Otx1	-	+	-	+
Msx1	-	+	-	+
Mitf	-	+	-	+
Zic1	-	+	-	+

Table 2-2 Expression profiles of the prospective NR and CE. At E13.5 and P0, NR markers are restricted to the central eyecup, OC progenitor transcription factors are expressed in the central and peripheral eyecup and CE markers are restricted to the peripheral eyecup.

CHAPTER III: SOX2 AND WNT SIGNALING COORDINATE THE NEUROGENIC BOUNDARY OF THE RETINA

Overview

Multipotent progenitor cells of the mammalian optic cup generate all the neurons of the mature retina as well as the epithelium of the circumjacent ciliary body and iris. Survival of retinal neurons depends on the proper function of the ciliary body epithelium in both development and adulthood. In this study, we show that precise coordination of SOX2 and canonical Wnt/β-Catenin signaling regulates the boundary between the presumptive retina and ciliary epithelium. Using genetic epistasis analysis and whole-genome expression arrays, we demonstrate that response to Wnt signaling was expanded upon specific deletion of *Sox2* in mouse optic cup progenitor cells. The region of ectopic Wnt signaling correlated with loss of neurogenic capacity, slowed proliferation and cell fate conversion of the neural retina to ciliary epithelium. Removal of *β-catenin* rescued the cell fate conversion; however, the loss of neural competence and the proliferation defect were not restored. Moreover, *Sox2*-mutant optic cup progenitor cells exhibited Wnt-independent up-regulation of D-type cyclins and persistent expression of the progenitor transcription factors *Pax6* and *Chx10*. Collectively, these data show that SOX2 antagonizes Wnt signaling to regulate the boundary of the neural retina, and removal of these two pathways reveals a primitive stem-like population of cells at the optic cup margin. Moreover, our genome-wide expression analysis of the *Sox2*-mutant optic cup provides a resource for understanding ciliary epithelial fate and function.

Introduction

Proper vision relies on the correct specification of the diverse cell types that make up the complex eye. Human eye disease, both congenital and degenerative, can result from improper function of one or more of these cell types. Therefore, the eye is a valuable model to understand the role of developmental pathways in human disease pathogenesis. Moreover, evidence for potential regenerative capacity of mammalian retinal cells *in vitro* (Ahmad et al., 2000; Tropepe et al., 2000), and more recently *in vivo* (Kiyama et al., 2012; Moshiri and Reh, 2004), underscores the importance of investigating the mechanisms involved in retinal progenitor cell multipotency and fate.

All of the cell types that make up the neural-derived components of the eye, including the neural retina (NR), ciliary body (CB) and retinal pigment epithelium (RPE), arise from a common progenitor population located in the eyefield of the ventral forebrain (Li et al., 1997; Zaghloul et al., 2005). In mammals, these pre-neurogenic progenitors give rise to the optic cup (OC), which becomes regionalized along the central-peripheral axis. The central OC consists of neurogenic progenitor cells that give rise to the six types of neurons and one glial cell that make up the mature retina, while the peripheral OC gives rise to the non-neurogenic epithelium of the iris and CB, herein referred to as ciliary epithelium (CE) (reviewed in (Beebe, 1986; Heavner and Pevny, 2012)). Little is known about the molecular mechanisms that specify peripheral OC development, and a number of screens have been performed to identify CE-specific genes (Diehn et al., 2005; Escribano and Coca-Prados, 2002; Ha et al., 2012; Kubota et al., 2004; Thut et al., 2001; Trimarchi et al., 2009).

Canonical WNT signaling, which functions through its downstream transcriptional effector, β-Catenin, has been shown to be crucial for CE fate specification in a number of species

(Agathocleous et al., 2009; Cho and Cepko, 2006; Ha et al., 2012; Kitamoto and Hyer, 2010; Liu et al., 2003a; Liu et al., 2007b; Matsushima et al., 2011; Trimarchi et al., 2009). While β-Catenin is expressed throughout the whole OC, a genetic reporter under the control of β-Catenin/TCF/LEF response elements showed WNT activity to be concentrated in the non-neurogenic peripheral OC (Liu et al., 2003a). Moreover, constitutive expression of *Ctnnb1* in mouse optic cup progenitor cells induces ectopic expression of CE-specific genes at the expense of SOX2-positive NR (Liu et al., 2007b). However, these ectopic CE cells do not express *Pax6* or *Chx10*, two known transcriptional regulators of peripheral OC fate, suggesting that at least two populations of progenitor cells exist in the peripheral OC (Liu et al., 2007b). Further evidence supporting a role for WNT signaling in CE development is that specific ablation of *Ctnnb1* in the developing OC reduces the size of the CE progenitor cell pool, causing hypoplasia of the CE and lamination defects in the neural retina (Fu et al., 2006; Liu et al., 2007b).

SOX genes are known regulators of WNT signaling in a number of developmental systems and disease states (reviewed in (Kormish et al., 2010)). SOX2, a member of the SOXB1 family of transcription factors, is necessary for maintaining neural stem cell competence (Pevny and Placzek, 2005). Moreover, SOX2 is required for retinal progenitor cell multipotency in mice (Matsushima et al., 2011; Taranova et al., 2006). Heterozygous mutations in human *SOX2* are most often associated with anophthalmia (absent eye) and account for 10%-20% of cases of severe bilateral ocular malformation, including microphthalmia (small eye) (Fantes et al., 2003; Fitzpatrick and van Heyningen, 2005; Ragge et al., 2005b). In mammalian development, the presumptive NR maintains SOX2 expression and neurogenic capacity, while the presumptive CE loses both SOX2 expression and neurogenic capacity (Matsushima et al., 2011). A role for SOX2 in specifying NR fate was demonstrated when the ablation of *Sox2* in mouse OC progenitor cells

resulted in loss of neural competence, cell fate conversion of the presumptive NR to CE, and ectopic expression of WNT target genes (Matsushima et al., 2011).

In addition to eye defects, human patients with *SOX2* mutations often exhibit pituitary abnormalities. WNT signaling is known to be involved in hypothalamo-pituitary development, and human SOX2 protein can inhibit β-Catenin-driven reporter expression *in vitro*, but SOX2 proteins containing the human mutations cannot (Alatzoglou et al., 2011; Kelberman et al., 2006). Therefore, it has been suggested that an inability to repress WNT/β-Catenin signaling may be an underlying cause of defects associated with *SOX2* loss-of-function mutations in human patients (Kelberman et al., 2006). Moreover, the complementary eye phenotypes associated with *Sox2* or *Ctnnb1* loss-of-function suggest a genetic interaction between these two pathways in mammalian OC development. In this study, we tested the hypothesis that SOX2 antagonizes canonical WNT signaling to maintain neurogenic fate in the OC of the mouse. We show that loss of *Sox2* in OC progenitor cells resulted in slowed proliferation and aberrant expression of cell cycle regulators and that these defects were independent of β-Catenin. We also demonstrate that removal of *Ctnnb1* from the *Sox2*-mutant OC partially rescued the *Sox2*-mutant phenotype, providing evidence that WNT signaling is a major transcriptional mediator of CE fate, and SOX2 antagonizes CE fate via modulation of WNT signaling. Lastly, using whole-genome expression arrays, we demonstrate that the *Sox2*-mutant OC provides a valuable resource for identifying genes involved in CE fate and function, which has direct relevance to understanding the pathogenesis of diseases associated with the anterior segment, such as glaucoma.

Materials and Methods

Mouse Breeding

All animal work was carried out in accordance with the University of North Carolina IACUC and DLAM approval. Generation of the *Sox2*$^{Cond/+}$ mouse line was described previously (Taranova et al., 2006). *Sox2*$^{cond/+}$ and *Sox2*$^{cond/+}$*;* α*P0*^{CREiresGFP} mice were maintained on a C57BL/6J background, and all others described in this study were maintained on a mixed background containing 129/Sv and C57Bl/6J. α*P0*^{CREiresGFP} mice were a gift from Dr. P. Gruss (Marquardt et al., 2001). *Chx10*^{CreGFP} (Rowan et al., 2004), *Ctnnb1*$^{cond/+}$ (Brault et al., 2001) and *Rosa26Reporter* (Soriano, 1999) transgenic mice were obtained from Jackson Laboratories (Bar Harbor, ME). *Pax6*$^{Sey/+}$ mice were a gift from Dr. A. LaMantia (Hill et al., 1991). Genotyping primers are listed in Table 3-1.

Tissue Preparation, immunohistochemistry and in situ hybridization

Immunostaining was performed as described in *Matsushima et al. (2011)*. Antigen retrieval was used for antibodies against pH3, BrdU, p27^{Kip1}, CyclinD3 and LEF1 as described in *Surzenko et al. (2013)*. Antibody concentrations are provided in Table 3-2 *In situ* hybridization was performed on 14 μm frozen sections (embryo heads or enucleated postnatal eyes) as described in *Matsushima et al. (2011)*. The following probes were used: *Bmp4* (gift from Dr. A. LaMantia) *Msx1* (Gift from Dr. Y. Liu) *CyclinD1 3'UTR* (gift from Dr. C. Cepko) *Shh* (gift from Dr. E. Tucker) *Axin2* (gift from Dr. F. Costantini) *Sfrp2* (gift from Dr. J. Nathans) *Chx10* (gift from Dr. R. McInnes) and *Hes5* (gift from Dr. E Anton). Images were obtained on a Leica inverted microscope (Leica DMIRB) equipped with a Retiga (SRV-1394) camera or on an Olympus laser scanning confocal microscope (Olympus Fluoview FV1000) and processed using Adobe Photoshop software.

BrdU and IdU labeling

Pregnant dams (16.5 dpc) were injected intraperitoneally with 0.12 mg IdU per mg body weight at time (T) 0. After 1.5 hours (T = 1.5), dams were injected with .10 mg BrdU (Sigma B5002) per mg body weight. At T = 2 hours, dams were sacrificed and embryos were removed and fixed as usual (ref). In preparation for cell cycle dynamics analysis, consecutive 12 μm transverse sections were taken through the whole eyecup, and sections through the center of the eyecup were chosen for further analysis. A mouse antibody to BrdU (BD) was used to detect both thymidine analogs, and a rat antibody specific to BrdU (Serotec) was used to detect BrdU alone.

Cell Counting, S-phase and Cell Cycle Measurements

For each eye, a section through the middle of the eyecup was imaged using an Olympus Fluoview FV1000 confocal microscope. The prospective NR was divided into radial degrees in 30° increments from the center to the prospective CE using Olympus Fluoview 2.1c software. All cells in each increment were counted, and the prospective CE was counted as a single unit. Three samples of each genotype were used in the final calculations. The following formulae were used to calculate BrdU index and Ki-67 index:

BrdU index = (BrdU+ cells/Hoechst+ cells) x 100

Ki-67 index = (Ki-67+ cells/Hoechst+ cells) x 100

S-phase and cell cycle times were calculated as described in (ref). The ratio of “S” cells (BrdU and IdU double-positive) to “L” cells (IdU-only) was measured, and the following formulae were used:

S-phase length (T_s) = 1.5 x (S cells/L cells)

Cell Cycle length = T_S x (Ki-67+cells/S cells)

Whole-genome expression analysis

Pairs of eyes were enucleated from six wild-type (*Sox2*$^{cond/+}$; α*P0*^{CREresGFP}) and six mutant (*Sox2*$^{cond/cond}$; α*P0*^{CREresGFP}) embryos on embryonic day (E) 16.5 for a total of six samples (twelve eyes) per genotype. The tissue was immediately placed in RNA-Later RNA stabilization solution and stored at 4˚C for no longer than thirty days. RNA was purified using an Ambion RNaqueous kit following the manufacturer's instructions. 500ng of RNA from each pair of eyes (12 samples total) were amplified and biotin-labeled using an Ambion Illumina TotalPrep RNA Amplification kit. 1.5 micrograms of labeled cRNA were hybridized to Illumina mouse WG-6 expression bead chips. To detect significant changes in gene expression between the control group and the *Sox2*-mutant experimental group, a permutation analysis for differential expression (PADE) was performed. This analysis uses repeated random reassignment of each sample to one of the two groups to determine if the calculated difference in levels of a transcript between groups is non-random. PADE was also used to estimate the false discovery rate for sets of differentially expressed transcripts. A p-value for each transcript was calculated using a two-sample t-test. Transcripts with an accumulated FDR of less than 5%, a p-value of less than 0.05, and a fold change of greater than ± 1.6, totaling 2194 transcripts, were chosen for further analysis.

Electrophoretic Mobility Shift Assay (EMSA)

Full-length HIS-tagged SOX2 (pET28a-SOX2-HIS) was expressed in BL21 (DE3) Codon Plus cells (Invitrogen, Carlsbad, CA) grown in LB at 37°C until an OD600 of 0.6 and then induced. SOX2-HIS was purified on a Sephadex S100 size exclusion column (GE Healthcare). Purification

and folding were assessed by immunoblotting and circular dichroism. For EMSA, purified SOX2 at a final concentration of 50 μM was incubated with 30,000 cpm of end-radio-labeled double-stranded oligonucleotide probe in 2X binding buffer (20 mM Hepes pH 7.9, 20% glycerol, 0.2 mM EDTA, 1mM tetrasodium pyrophosphate, 0.5 mM PMSF) and 50 mM KCl for 30 min. at room temperature. For competition assays, 20-fold molar excess unlabeled probe was added to the reaction. Reactions were run on a 12% non-denaturing polyacrylamide gel. The gel was fixed and exposed to autoradiography film at -80°C. Probe sequences are as follows: *Pax6 α enhancer TOP* (5'-GTG CAG TTC ATT CTC GTC TGA GTG ATC TAC AAA TAG GGA -3') and *Notch1 intron 3 TOP* (5'- CGC AGC ATT GTC AAG GTG GCA TTG TTC CAG TCT GGA ACC CT-3'). Bottom sequences are the reverse complements.

Results

Ablation of *Sox2* in the optic cup causes proliferation defects

Heterozygous mutations in human *SOX2* are often associated with anophthalmia (absence of eye) or microphthalmia (small eye), which suggests a defect in proliferation or survival of optic cup progenitor cells (OCPCs) (Fantes et al., 2003; Fitzpatrick and van Heyningen, 2005; Ragge et al., 2005b). We have shown that mice hypomorphic for *Sox2* exhibit microphthalmia (Taranova et al., 2006). However, ablation of *Sox2* specifically in OCPCs did not result in increased cell death, suggesting that SOX2 may play a role in OCPC proliferation (Matsushima et al., 2011).

To address this question, we used a previously generated conditional floxed allele of *Sox2* (*Sox2*^{cond}) (Taranova et al., 2006). To delete *Sox2* specifically in OCPCs, we used mice transgenic for *αP0*^{CREiresGFP}, in which Cre recombinase is driven by the OC-specific enhancer of *Pax6*, α, and minimal promoter, P0. This Cre is expressed from embryonic day E10.0 in

progenitor cells competent to give rise to both NR and CE (Kammandel et al., 1999; Marquardt et al., 2001). We have previously crossed *Sox2*$^{cond/+}$ mice with *Sox2*$^{cond/+}$; α*P0*^{CREiresGFP} mice to efficiently ablate *Sox2* from most OCPCs and reported that *Sox2*-mutant eyes (*Sox2*$^{cond/cond}$; α*P0*^{CREiresGFP}) were significantly smaller than those of controls (*Sox2*$^{cond/+}$; α*P0*^{CREiresGFP}) (Matsushima et al., 2011; Taranova et al., 2006). Here we measure BrdU incorporation and cell cycle length to compare proliferation of mutant OCPCs with that of controls at E16.5.

We first quantified the percentage of cells that incorporated BrdU over two hours following a single injection (Fig 3-1B,C vs. D, E). Given that the central OC is more proliferative than the periphery, we divided the OC into radial degrees from the center to the prospective CE and analyzed the prospective CE as a single unit. The prospective CE was identified by sudden thinning of the neuroepithelium and a sharp decrease in BrdU incorporation and phospho-Histone H3 expression (Fig 3-1F,G). While central OCPCs of controls steadily incorporated BrdU at an average of 41.2% (range: 36.6 – 45.2%), *Sox2*-ablated central OCPCs showed overall decreased BrdU incorporation at an average of 35.7% (range: 25.9 – 46.5%), and BrdU incorporation decreased in a graded manner from the center to the periphery (Fig 3-1D,E,H). The overall decrease in BrdU incorporation trended towards significance ($p = 0.10$). Consistent with previous reports, the prospective CE of controls was less proliferative than the central OC, with approximately 18% of CE progenitor cells incorporating BrdU (Fig 1H). The prospective CE was expanded upon *Sox2* ablation, as previously shown (Fig 3-1G vs. F) (Matsushima et al., 2011). These “CE-like” progenitor cells incorporated BrdU at an average of 8.7%, and this difference was not significant when compared with controls ($p = 0.12$) (Fig 1H).

***Sox2*-ablated OCPCs gradually increase cell cycle time**

The above data raise the possibility that in the absence of SOX2, OCPCs prematurely exit the cell cycle and/or increase cell cycle length in a graded manner. To address this hypothesis, we quantified the percentage of cells expressing Ki67, a marker of cycling cells, throughout the central OC of controls and mutants. Contrary to our expectations, there were significantly more Ki67-positive cells in *Sox2*-ablated central OCs compared with controls ($p = 0.02$) (Fig 3-2M). Given that *Sox2*-null OC progenitor cells are not competent to differentiate into neurons (Matsushima et al., 2011), this result suggests that they remain in the cell cycle for a prolonged period of time. An increase in the length of the cell cycle upon loss of *Sox2* could therefore underlie the microphthalmia phenotype (fewer cells in mutants).

To address whether cell cycle dynamics were affected by *Sox2* ablation, we quantified the length of the cell cycle of central OCPCs by co-injecting thymidine analogs (see methods and Fig 3-2A-J). *Sox2*-deficient OCPCs exhibited a subtle but graded increase in S-phase and cell cycle lengths from the center to the periphery, whereas control OCPCs maintained a steady rate throughout (Fig 3-2K,L). Prospective CE cells of both controls and mutants did not cycle fast enough to be reliably quantified using this protocol. Together, these results suggest that upon loss of *Sox2*, OCPCs exhibit prolonged proliferation but gradually increased cell cycle time.

Given that the cell fate conversion of the *Sox2*-mutant NR to CE occurs gradually over time and space and does not reliably manifest until postnatal day (P) 0 (Matsushima et al., 2011), we asked whether the increase in cell cycle time culminates in cell cycle exit, which would be consistent with the onset of CE gene expression. To determine whether mutant cells exit the cell cycle to become CE, we examined expression of Ki67 at P0. In control eyes, the most peripheral part of the CE did not express Ki67 (Fig 3-3A- C arrowheads), but some cells of the

more central region did (Fig S2A-C arrows). These cells likely identify the presumptive pars plana, a region that has been shown to retain some proliferative capacity into adulthood in mice (Kiyama et al., 2012). Similarly, in mutant eyes, the most peripheral "CE-like" cells did not express Ki67, and this region was expanded compared with controls (Fig 3-3D-F arrowheads). Moreover, the more central *Sox2*-mutant cells retained Ki67 expression (Fig 3-3D-F). Therefore, upon loss of *Sox2*, OCPCs continued to cycle for an extended period of time, gradually increasing cell cycle length before exiting to become CE. This gradual increase in cell cycle length occurred in a graded manner such that more peripheral OCPCs slowed down and exited first, followed by more central cells.

CyclinD1 protein is up-regulated in SOX2-ablated cells

D-type cyclins are expressed in OCPCs and regulate their proliferation (Das et al., 2009; Das et al., 2012; Tong and Pollard, 2001). A variety of post-transcriptional and post-translational mechanisms regulate Cyclin D expression (Card et al., 2008; Hu et al., 2002; Lin et al., 2000; Spinella et al., 1999). To further investigate the role of cyclins in the proliferation defect, we analyzed protein expression and localization of D-type cyclins in mutant OCPCs versus controls. Cyclin D1 was localized to the prospective NR and excluded from the prospective CE of control retinas, with higher levels appearing to be localized to the boundary of the NR and CE (Fig 3-4B arrowheads). Surprisingly, Cyclin D1 was up-regulated in *Sox2*-ablated OCPCs (Fig 3-4D,E arrowheads). However, *Cyclin d1* mRNA, as analyzed by *in situ* hybridization, was not increased in mutant OCPCs compared with controls (Fig 3-4C vs. F). Moreover, Cyclin D3 protein was confined to the prospective CE of controls and expanded into the central OC of mutants (Fig 3-4G,H vs. J,K). Co-labeling with Cyclin D1 and Cyclin D3 indicated that these two proteins, which

are normally confined to the prospective NR and CE, respectively, were co-expressed in many cells of the *Sox2*-mutant OC (Fig 3-4M-P, arrows) (Das et al., 2012; Sicinski et al., 1995).

D-type cyclins are thought to drive cell cycle progression in part through sequestration of the Cip/Kip family cell cycle inhibitor $p27^{Kip1}$ (Sherr and Roberts, 1999). Moreover, the hypoplastic retina phenotype observed in *Cyclin D1*$^{-/-}$ mice can be fully rescued by deletion of *p27*^{Kip1} (Geng et al., 2001). To determine whether the increase in Cyclin D1 was associated with a concomitant decrease in $p27^{Kip1}$, we analyzed $p27^{Kip1}$ mRNA and protein in wild-type and mutant OCPCs at E16.5 (Fig 3-5). In wild-type OCPCs, $p27^{Kip1}$ was expressed in the developing NR but excluded from the prospective CE (Fig 3-5A, D) (Zhang et al., 1998). By contrast, in the mutant OC, *p27*^{Kip1} mRNA was decreased 2.3-fold, and $p27^{Kip1}$ protein was absent from CRE-positive cells in the central OC (Fig 3-5B,C,E,F).

The increase in Cyclin D1 and decrease in $p27^{Kip1}$ could indicate the mechanism through which *Sox2*-ablated cells persistently divide. Several pathways are known to regulate OCPC proliferation in part through Cyclin D1, including Sonic Hedgehog (SHH) signaling (Moshiri and Reh, 2004; Wall et al., 2009; Wang et al., 2005). To address whether an increase in *Shh* mRNA could explain the increase in Cyclin D1 protein, we analyzed *Shh* mRNA levels by microarray and ISH. *Shh* expression was decreased 4.5-fold in mutants compared with controls ($p = 0.0014$), and the decrease was confirmed by ISH (Fig 3-4I vs. L). Moreover, the primary source of SHH in the developing retina is thought to be retinal ganglion cells (RGCs); however, *Sox2*-ablated OCPCs completely lose neural competence and are not able to produce RGCs (Matsushima et al., 2011; Wang et al., 2002). These data suggest that the persistent proliferation of *Sox2*-mutant OCPCs is most likely driven by an additional mitogenic pathway.

Canonical WNT signaling is activated in *Sox2*-mutant optic cups

The graded manner of decreased proliferation in the mutant OC suggests that a morphogenetic pathway may be activated upon *Sox2* loss (Rogulja and Irvine, 2005). OC development is regulated by a number of morphogens (reviewed in (Heavner and Pevny, 2012)). To determine which of these is regulated by SOX2 signaling, we used Illumina mouse WG-6 expression bead chips to perform a whole genome expression screen of control and mutant eyes from E16.5 embryos. We first performed gene ontology analysis to confirm that genes up-regulated in *Sox2*-mutant OCs were consistent with known functions of the CE and down-regulated genes were consistent with known functions of the NR (which would indicate expansion of the peripheral OC) (Fig 3-6A). We used DAVID to categorize 880 significantly up-regulated genes and 951 significantly down-regulated genes (Huang da et al., 2009a; Huang da et al., 2009b).

Up-regulated genes were enriched for secreted signaling molecules, extracellular matrix and cell adhesion proteins, collagens, and genes involved in organogenesis and the defense response (Fig 3-6B). These categories are consistent with the function of the CE, a highly secretory structure that maintains the intraocular pressure (IOP) through the active transport of fluid via Na^+-K^+ exchange pumps and Cl^- channels (Civan and Macknight, 2004). It is thought that the presumptive CE is functional before it is histologically distinct, and proper IOP during embryonic development is necessary for the normal growth of the eye. The CE produces many of the proteins of the inner limiting membrane and the vitreous body (Beebe, 1986; Coulombre, 1957; Coulombre and Coulombre, 1957). Moreover, the enrichment of defense response genes may suggest a previously unknown role for the presumptive CE in the host immune response, which would begin as early as embryonic stages. Indeed, the most significantly changed gene in

our screen was the defensin *Defb11* (+13.04-fold change in mutants compared with controls). BioGPS confirmed expression of this gene in the mouse ciliary body and iris (Lattin et al., 2008; Wu et al., 2009).

By contrast, down-regulated genes were enriched for molecules involved in neuronal development and function (Fig 3-6B). These categories included axonogenesis, synaptic development and signaling, neuronal differentiation and microtubule cytoskeleton (Fig 3-6B). Collectively, these data confirm that our screen provides a reliable indication of pathways potentially disrupted by *Sox2* ablation.

We used Ingenuity Pathway Analysis of our significantly differentially expressed gene set to identify additional signaling pathways disrupted in *Sox2*-mutant eyes. The most significantly affected pathways included REST, Tgfβ and canonical WNT signaling (Fig 3-6D). The apparent activation of REST, a repressor of neuronal differentiation, is consistent with the loss of neural competence of *Sox2* mutant eyes (Ballas et al., 2005; Schoenherr and Anderson, 1995). Tgfβ signaling and canonical WNT signaling are two morphogenetic pathways known to be involved in CE fate specification (Cho and Cepko, 2006; Dias da Silva et al., 2007; Flugel-Koch et al., 2002; Hung et al., 2002; Liu et al., 2003a; Liu et al., 2007b; Zhang et al., 2007; Zhao et al., 2002). A previous report demonstrated that SOX2 antagonizes WNT signaling in the *Xenopus* OC, and Cyclin D1 is a well-established target of canonical WNT signaling in neural progenitor cells (Agathocleous et al., 2009; Megason and McMahon, 2002). Therefore we chose to investigate whether SOX2 antagonizes canonical Wnt signaling to maintain neurogenic OC fate.

We first scanned our set of significantly changed genes for known OC-specific targets of canonical Wnt signaling and found ten for which the expression was changed at least 1.47-fold consistent with activated β-Catenin (Ha et al., 2012; Kubota et al., 2004; Thut et al., 2001;

Trimarchi et al., 2009) (Fig 3-6E). We have previously observed that *Axin2*, a well-characterized endogenous readout of WNT activity, is sometimes expanded towards the center of the *Sox2*-mutant OC, but this expansion is often variable between embryos, perhaps implicating WNT antagonists (Matsushima et al., 2011). To examine the expression *Axin2* in relation to *Sfrp2*, a WNT antagonist expressed in the peripheral NR, we analyzed the expression of these genes on serial sections of wild-type and mutant OCs at E16.5 (Liu et al., 2003a). *Axin2* was expanded toward the central mutant OC, consistent with our previous report (Fig 3-7A,B vs. F,G, left of the line) (Matsushima et al., 2011). Where *Axin2* staining ended, *Sfrp2* staining began in both wild-type and mutant OCs, consistent with a role for *Sfrp2* in Wnt antagonism (Fig 3-7C,H, right of the line). Moreover, the central restriction of dark *Sfrp2* staining further confirmed expansion of peripheral OC identity into the central OC (Fig 3-7C,H, arrows). Overall, the increase in WNT activity indicated a de-repression of WNT signaling in the absence of SOX2.

We hypothesized that the presence of *Sfrp2* throughout the central mutant OC at E16.5 could inhibit full expansion of WNT signaling at early stages and thus explain the delay in the cell fate conversion. To confirm that WNT signaling targets were up-regulated in mutant cells by postnatal stages, when the cell fate conversion was manifest, we examined expression of the Wnt targets *Axin2* and LEF1, at P1. *Axin2* was expressed in *Sox2*-mutant cells at P1 (data not shown). Moreover, LEF1 was ectopically expressed in all *Sox2*-ablated cells as in the CE of controls (Fig 3-7D,E vs. I,J). Interestingly, LEF1 protein was increased in a gradient from the center (thicker area between the arrowheads) to the periphery (thinner area between the arrows) of *Sox2*-mutant regions (Fig 3-7I,J). Collectively, these data suggest that WNT targets are gradually up-regulated as the retinal neuroepithelium thins and attenuates proliferation associated with cell fate conversion from NR to CE. Moreover, WNT antagonists may contribute to the graded and delayed nature of CE development in mutant eyes.

Genetic ablation of canonical WNT signaling partially rescues the *Sox2*-mutant phenotype

The expansion of WNT signaling activation upon ablation of *Sox2* supports our hypothesis that SOX2 antagonizes the WNT pathway to maintain NR fate. Moreover, WNT signaling can promote or inhibit proliferation in different contexts. Therefore, active WNT signaling could explain the proliferation attenuation and/or the increase in Cyclin D1 observed in *Sox2*-mutant cells.

SOX2 has been shown to directly interact with β-Catenin, the main transcriptional mediator of canonical WNT signaling, in many biological systems and disease states (Chen et al., 2008; Kormish et al., 2010; Mansukhani et al., 2005; Zorn et al., 1999). These data raise the possibility that SOX2 and WNT signaling intersect at the level of β-Catenin in OC development. To address this hypothesis *in vivo*, we performed genetic epistasis analysis of *Sox2* and *β-catenin* in the developing OC. We used *β-catenin*$^{cond/+}$mice, in which exons 2-6 of the *β-catenin* locus are flanked by *loxP* sites (Brault et al., 2001), and transgenic mice carrying *Chx10*^{CreGFP}, a BAC containing Cre recombinase expressed from E11.0 in progenitor cells throughout the whole OC (Rowan et al., 2004). We used *Chx10*^{CreGFP} in order to avoid any potential regulation of *αP0*^{CreiresGFP} by PAX6 and β-Catenin. *β-catenin*$^{cond/+}$ mice were crossed with previously generated *Sox2*$^{cond/+}$*; Chx10*^{CreGFP} mice to generate *Sox2/β-catenin* double mutant OCPCs. We then compared the eyes of *Sox2* single-mutants (*Sox2*$^{cond/cond}$*; Chx10*^{CreGFP}) and *β-catenin* single mutants (*β-catenin*$^{cond/cond}$*; Chx10*^{CreGFP}) with *Sox2/β-catenin* double mutants (*Sox2*$^{cond/cond}$*; β-catenin*$^{cond/cond}$*; Chx10*^{CreGFP}) and age-matched controls (*Sox2*$^{cond/+}$*; β-catenin*$^{cond/+}$*; Chx10*^{CreGFP}) at E14.5 and P0.

We first determined whether *Sox2* and *β-Catenin* could be efficiently ablated with *Chx10*^{CreGFP} by analyzing the localization of these proteins in control and double mutant OCs. In

controls, SOX2 is expressed in a gradient from high in central OC nuclei to low in peripheral OC nuclei as previously reported (Matsushima et al., 2011). β-Catenin is localized to the surface of cells throughout the OC (Fig 3-8A,B). SOX2 and β–catenin were absent from Cre-positive cells localized in a mosaic pattern throughout the OC, demonstrating that *Chx10*^{CreGFP} was able to recombine both loci in the same cells (Fig 3-8C,D). Moreover, deletion of both genes did not result in increased cell death as assessed by activated-Caspase3 staining (data not shown).

The CE is expanded in *Sox2*-mutant eyes, and β-Catenin is an essential positive regulator of CE fate; therefore, the removal of *β-catenin* from *Sox2*-mutant cells should rescue ectopic CE formation. To test this hypothesis, we compared expression of CE and NR markers in the eyes of neonatal single mutants with that of double mutants and controls (Fig 3-9 and 3-10). We used two methods to identify mutant cells: 1) We stained sections with an antibody to β-Catenin to identify β-Catenin-positive and negative cells; and 2) we backcrossed all lines to Rosa26R Cre reporter mice which express *β-galactosidase* from the Rosa26 locus following Cre-mediated excision of a translational stop cassette (Soriano, 1999), resulting in genetic lineage tracing of *Cre*-expressing cells. *BMP4* and *Msx1*, two well-established CE markers, were expressed in the CE and excluded from the NR of wild-type eyes at P0 (Liu et al., 2007b; Monaghan et al., 1991) (Fig 3-9A-H). However, neither of these markers was expressed in the CE of *Ctnnb1*-ablated (Cre- and β-Galactosidase–positive) regions, consistent with previous evidence that *BMP4* and *Msx1* are targets of canonical Wnt signaling in the peripheral OC (Fig 3-9I-P) (Liu et al., 2007b). Conversely, *BMP4* and *Msx1* were ectopically expressed in *Sox2*-ablated regions, as previously reported (Fig 3-9Q-X) (Matsushima et al., 2011). In support of our hypothesis, the ectopic expression of CE genes was rescued in *Sox2/Ctnnb1* double-mutant OC cells (Fig 3-9Y-FF).

We next sought to determine how early in development the rescue of the *Sox2*-mutant phenotype could be observed. Evidence of peripheral OC expansion in *Sox2*-mutants can be identified as early as E13.5 (Matsushima et al., 2011). At this stage, *Zic1*, which is highly expressed in the prospective CE, was expanded into central OCPCs in *Sox2* single-mutant cells. In *Sox2/Ctnnb1* double mutant cells, *Zic1* expression was restricted back to the periphery (data not shown). Therefore, the expansion of peripheral OC identity observed upon ablation of *Sox2* as early as E13.5 is a result of ectopic activation of *Ctnnb1* in the central OC.

Removal of β-Catenin rescued ectopic CE development in *Sox2*-ablated cells, but double-mutant regions remained hypoplastic compared with controls, suggesting that the NR was not fully restored. We therefore examined expression of the NR marker *Hes5* and the pan-OCPC marker *Chx10* to determine the identity of double-mutant cells (Fig 3-10). *Hes5* was expressed in NR progenitor cells throughout the central OC of wild-type controls, but expression was lost in *Sox2*-ablated regions (Fig 3-10E,F vs. K, L). Deletion of *Ctnnb1* in these *Sox2*-mutant regions did not restore expression of *Hes5* (Fig 3-10Q-R). By contrast, *Chx10* was expressed in the CE and NR of wild-type controls and maintained in both single and double-mutant cells of the central OC (Fig 3-10C,D vs. I,J vs. O,P). Interestingly, *Chx10* expression was mosaic in double-mutant eyes in accordance with $Chx10^{CreGFP}$ expression (Fig 3-10M-O). Collectively, these data show that ectopic CE development in *Sox2*-mutant eyes depends on WNT/β-Catenin signaling; however, the loss of neural competence associated with *Sox2* deletion is independent of WNT signaling, and *Chx10* expression persists in the absence of both.

Genetic ablation of canonical WNT signaling does not rescue Cyclin D1 upregulation

Our genetic epistasis analysis uncovered the role of WNT signaling in directing the fate of *Sox2*-mutant cells, but its role in cell cycle regulation was unclear. At P0, *Sox2/Ctnnb1* double-

mutant eyes were generally larger than *Sox2* single-mutant eyes but smaller than those of wild-type and *Ctnnb1* single-mutant controls (n = 4) (Fig 4). This partial restoration of tissue in double-mutant eyes suggested that β-Catenin may have a negative effect on OCPC proliferation. However, in the *Xenopus* OC, Wnt signaling was shown to promote OCPC proliferation (Agathocleous et al., 2009).

To address this discrepancy and define the role of β-Catenin in mouse OCPC proliferation, we examined expression of D-cyclins and SHH signaling in double-mutant, single-mutant and wild-type control eyes at E13.5 (Fig 3-12 and Fig3-13). As expected, Cyclin D1 protein was expressed in the central OC of wild-type and *Ctnnb1* single-mutant eyes and was up-regulated in the center of *Sox2* single-mutant OCs at E13.5 (Fig. 3-12A-C and G-I vs. M-O). Interestingly, Cyclin D1 remained up-regulated in *Sox2/Ctnnb1* double-mutant eyes (Fig. 3-12S-U). By contrast, *Ccnd1* mRNA was not up-regulated in *Sox2* single-mutant or double-mutant eyes compared with wild-type and *Ctnnb1* single-mutant controls (Fig. 3-12V and P vs. D and J). Similarly, removal of *Ctnnb1* from *Sox2*-mutant OCPCs did not restore neurogenesis or, thus, *Shh* expression, both of which were detectable in the central OC of controls (Fig 3-12Q,R and W,X vs. E,F and K,L, arrows). Residual *Shh* was expressed where the few RGCs developed from Cre-negative cells in *Sox2* single-mutants and *Sox2/Ctnnb1* double-mutants (Fig. 3-12Q-R and W,X). This residual *Shh* signaling may account for the prolonged proliferation and Cyclin D1 expression observed upon *Sox2* ablation; however, other reports suggest that the population of RGCs have been depleted too much to maintain the amount of Cyclin D1 expression observed in *Sox2* single-mutants and *Sox2/Ctnnb1* double mutants (Kiyama et al., 2012). Nevertheless, removing *Ctnnb1* from *Sox2*-mutant cells did not rescue Cyclin D1 up-regulation or loss of neural competence at E13.5.

It was still possible that a difference in cell cycle regulation between single and double mutants was delayed until postnatal stages. We therefore examined expression of D-cyclins at P0 (Fig 3-14). Cyclin D1 was expressed in Chx10^{CreGFP}-positive NR progenitor cells in wild-type and *Ctnnb1* single-mutant eyes (Fig 3-13A-B, E-F, I- J). Surprisingly, Cyclin D1 remained up-regulated in the central OC of *Sox2* single-mutants at P0 (Fig 3-13C,G,K). Moreover, deletion of *Ctnnb1* in these cells did not restore Cyclin D1 to wild-type levels, again supporting the idea that the prolonged proliferation observed in *Sox2*-ablated OCPCs is independent of canonical WNT signaling (Fig 3-13D,H,L). In all four genotypes, Cyclin D1 was excluded from the CE (wild-type and *Ctnnb1* single-mutants) and from the thin CE-like converted regions in the far peripheral OC (*Sox2* single-mutants and double mutants) (Fig 3-13A-L). Conversely, Cyclin D3 protein was restricted to the CE of wild-type and *Ctnnb1* single-mutant eyes and was ectopically expressed in *Sox2*-ablated and *Sox2/Ctnnb1*-ablated cells of the central OC (Fig 3-13M-T). Collectively, these results demonstrate that SOX2 antagonizes expression of D-type cyclins in the OC independently of WNT/β-Catenin and SHH signaling. The finding that Cyclin D1 and Cyclin D3 were expressed in OCPCs independently of β-Catenin is consistent with previous reports that β-Catenin does not affect the proliferation of mouse OCPCs (Fu et al., 2006).

SOX2 antagonizes *Pax6* signaling independently of β-catenin

In addition to SOX2, WNT and SHH signaling, retinal progenitor cell proliferation is driven by a number of pathways and transcription factors (Burmeister et al., 1996; Ferda Percin et al., 2000; Humayun et al., 2000). We next sought to identify which of these may support the observed increase in Cyclin D1 protein levels in the absence of SOX2 and β-Catenin. One candidate was the paired-like transcription factor PAX6, which is import for maintaining retinal progenitor cell proliferation and multipotency (Davis-Silberman et al., 2005; Marquardt et al.,

2001). We have previously shown that SOX2 antagonizes *Pax6* to maintain NR fate in the developing OC (Matsushima et al., 2011). Other reports have shown evidence that *Pax6* expression in the OC is suppressed by β-Catenin (Liu et al., 2007b). We therefore asked whether SOX2 and WNT/β-Catenin signaling coordinate *Pax6* levels in the OC, and whether in the absence of these two pathways, PAX6 is responsible for the increased CyclinD1.

We first examined whether the up-regulation of *Pax6* expression previously reported in *Sox2*-mutant OCPCs remained up-regulated in double-mutant cells. At E13.5, *Pax6* mRNA remained up-regulated in double-mutant OCPCs as in *Sox2* single-mutant OCPCs (data not shown). Likewise, at P0, high *Pax6* expression, as assessed by $\alpha P0^{CreiresGFP}$, was expanded into the central OC of both *Sox2* single-mutant and double-mutant eyes when compared with that of wild-type controls (Fig 3-14I,M vs. A). Despite the maintenance of *Pax6*, the ectopic expression of CE markers *Bmp4* and *Msx1* observed in *Sox2*-mutant eyes was rescued in double mutant eyes, as described above (Fig 3-14K,L vs. O,P). Moreover, loss of the NR marker *Hes5* was not rescued in double-mutant eyes (Fig 3-14J vs. N). Interestingly, little $\alpha P0^{CreiresGFP}$ expression was detectable in the CE of *Ctnnb1* single-mutants, reflecting a reduction of CE progenitors upon loss of WNT signaling (Fig 3-14E-H) (Liu et al., 2007b). Nonetheless, the increased *Pax6* expression that resulted from *Sox2* loss occurred independently of *Ctnnb1*, and *Ctnnb1* mediated the transcription of the CE genes *Bmp4* and *Msx1* independently or downstream of PAX6. These data indicate that PAX6 cannot regulate the expression of CE marker genes without the presence of β-Catenin.

We next tested whether SOX2 antagonism of *Pax6* was direct. PAX6 can bind a conserved paired domain site in its OC-specific enhancer, α, to positively regulate its own expression (Baumer et al., 2002; Schwarz et al., 2000). We identified an evolutionarily conserved

SOX2 binding site directly adjacent to this PAX6 site (Collignon et al., 1996) (Fig 3-14A). Using purified recombinant SOX2 protein and electrophoretic mobility shift assay (EMSA), we determined that SOX2 was able to bind to this regulatory element *in vitro* (Fig 3-14B). Purified SOX2 also bound a previously characterized *Notch1* regulatory element, which was used as a positive control, but did not bind a random sequence (Fig 3-14 and data not shown) (Taranova et al., 2006). Moreover, binding to labeled probe was competed away with the addition of unlabeled probe (Fig 3-14B). Circular dichroism indicated that the secondary structure of SOX2 changes, or becomes more alpha-helical, when bound to the α enhancer but not when bound to random DNA (Fig 3-15A-B).

Genetic reduction of *Pax6* does not rescue Cyclin D1 upregulation

The above evidence that SOX2 directly antagonizes *Pax6* expression in the OC raised the possibility that PAX6 drives the increase in Cyclin D1 observed upon *Sox2* ablation. To test this hypothesis, we genetically decreased *Pax6* in *Sox2*-ablated OCPCs using $Pax6^{Sey/+}$ (small eye) mice as previously described (Matsushima et al., 2011). $Pax6^{Sey/+}$ mice contain a nonsense mutation in the *Pax6* locus, resulting in a truncated protein widely used as a *Pax6*-null (Hill et al., 1991; Hogan et al., 1988). We compared Cyclin D1 expression in $Sox2^{cond/cond}$; $Chx10^{CreGFP}$ single-mutant OCs with $Sox2^{cond/cond}$; $Pax6^{Sey/+}$; $Chx10^{CreGFP}$ double-mutant OCs and $Pax6^{Sey/+}$ single-mutant and wild-type controls at E14.5 (Fig 3-14). As expected, Cyclin D1 was upregulated in *Sox2* single-mutants when compared with wild-type and *Pax6* single-mutant controls (Fig 3-14G,H vs. C-F). However, reduction of *Pax6* in the *Sox2*-mutant background did not restore Cyclin D1 to wild-type levels, suggesting that PAX6 does not drive Cyclin D1 up-regulation in the absence of *Sox2*.

Trophic pathways are activated in the *Sox2*-mutant OC

In addition to PAX6, trophic factors have been implicated in OCPC proliferation. For instance, bFGF (FGF2) and EGF were shown to stimulate human fetal retinal progenitors to proliferate *in vitro* (Kelley et al., 1995). Intriguingly, *Bdnf*, a neurotrophin known to be important for neuronal differentiation and survival, is one of the ten most up-regulated genes in the *Sox2*-ablated OC (+6.78-fold change relative to controls). In corroboration with our model, *Bdnf* is expressed in the peripheral OC of developing mouse embryos (Bennett et al., 1999). We therefore hypothesized that growth factors present in *Sox2*-ablated OCPCs may support their prolonged proliferation. We used IPA to identify potentially upregulated trophic pathways. Based on the increased expression of myriad targets known to be regulated by FGF2, IPA predicted that this pathway is activated in the *Sox2*-deficient OC (Fig 3-14K). We also confirmed increased BDNF receptor, *TrkB*, FGF receptor, *Fgfr2* (Fig 3-14I). Like *Bdnf*, some of these have been shown to be expressed in the presumptive CE of mice (de Iongh and McAvoy, 1993; Reneker and Overbeek, 1996). These data suggest that the presence of BDNF and FGF in the optic cup margin (OCM) supports OCPC proliferation in the absence of SOX2 (Fig 3-14K). Given that *Sox2*-single-knockout and *Sox2/Ctnnb1* -double-knockout cells retain stem-like properties, including maintenance of Cyclin D1, Cyclin D3 and the OC progenitor markers *Chx10* and *Pax6*, we propose that removal of SOX2 and β-Catenin reveals a primitive retinal progenitor cell that is capable of prolonged proliferation. However, without either SOX2 or β-Catenin, these cells are not competent to differentiate into retinal neurons or CE.

Discussion

SOX2 and β-Catenin co-regulate the neurogenic/non-neurogenic boundary of the retina

In this study, we have used genetic epistasis analysis in the mouse to show that SOX2 and canonical Wnt signaling coordinately regulate opposing cell fates in the OC: neurogenic neural retina (NR) and non-neurogenic ciliary epithelium (CE), respectively (Modeled in Fig. 3-16). *Sox2* loss-of-function, like *Ctnnb1* gain-of-function, results in restriction of NR progenitor identity and expansion of CE progenitor identity (Fig. 3-16C vs. A). Conversely, *Ctnnb1* loss-of-function results in expansion of NR progenitor identity and restriction of CE progenitor identity (Fig. 3-16B vs. A). Loss of both *Sox2* and *Ctnnb1*causes restriction of NR identity similar to the *Sox2* single-mutant phenotype, but CE-specific genes fail to be up-regulated (Fig. 3-16D vs. C). Together, these data demonstrate that precise coordination of SOX2 and WNT signaling is necessary to ensure proper development of the neural retina and circumjacent ciliary body.

An additional pathway that has been implicated in this cell fate decision is TGFβ signaling, although the specific sources of ocular TGFβ are currently unclear (Dias da Silva et al., 2007; Flugel-Koch et al., 2002; Hung et al., 2002; Veien et al., 2008; Zhang et al., 2007; Zhao et al., 2002). Forced expression of *Noggin*, a TGFβ antagonist, in lens fiber cells, inhibited expression of *Bmp4* and *Bmp7* in the presumptive CE and caused a cell fate shift of these cells from CE to NR. This cell fate conversion was rescued by transgenic co-expression of *Bmp7* (Zhao et al., 2002). Conversely, over-expression of *Bmp7* in the developing lens inhibited neural retinal development, causing widespread apoptosis throughout the retina (Hung et al., 2002). These experiments suggest that the developing lens may provide a source of TGFβ that instructs the peripheral optic cup margin to become CE. However, the CE is correctly specified in the absence of a lens, and there is recent evidence that the lens may be more important for maintaining CE

fate rather than instructing it (Kitamoto and Hyer, 2010; Zhang et al., 2007). Therefore, either another source of ocular TGFβ exists separate from the lens, or TGFβ signaling is downstream of a more potent regulator of CE fate (Zhang et al., 2007). Here we have shown that WNT signaling acts upstream of TGFβ in the regulation of CE fate such that expression of *Bmp4/7* and the BMP target gene *Msx1* is lost in *Ctnnb1* mutant cells.

SOX proteins, including SOX2, have been shown to physically interact with β-Catenin to modulate the expression of WNT target genes (reviewed in (Kormish et al., 2010))(Chen et al., 2008; Mansukhani et al., 2005; Zorn et al., 1999). Our data support a model in which SOX2 and WNT signaling in the OC intersect at the level of target gene expression; however, it is unclear how SOX2 antagonizes these genes (Fig. 3-16E). SOX2 is distantly related to the TCF/LEF family of transcription factors, which also bind DNA through an HMG domain, and thus could compete with TCF/LEF for direct DNA binding (Fig. 3-16E) (Haremaki et al., 2003). However, one report showed that the SOX2 HMG domain was not required to inhibit β-Catenin-induced gene in expression in osteoblasts, suggesting that SOX2 antagonizes Wnt signaling through its C-terminus, most likely via β-Catenin sequestration (Mansukhani et al., 2005). Attenuation of WNT signaling in *Sox2* mutant cells rescued the ectopic expression of CE-specific genes but did not rescue all of the hallmarks of the *Sox2*-mutant phenotype, including the thinning of the retinal neuroepithelium and the loss of NR identity. Therefore, the role of WNT signaling in the *Sox2*-mutant OC is to affect the cell fate conversion by turning on a set of CE-specific genes. This appears to occur in a gradient in which the most peripheral cells exit the cell cycle first and convert to CE followed by a progressive slowing down of central OC cells. The delay in this conversion may be due to the time needed to recruit co-activators to de-repressed WNT target genes, to overcome WNT antagonists such as *Sfrp2*, or to exit the cell cycle.

The observation that OCPCs can proliferate and survive without both SOX2 and canonical WNT signaling is intriguing, given the necessity of these two pathways in specifying NR vs. CE fate. Double-mutant cells do not express NR or CE-specific markers, but maintain the pan-OCPC markers *Chx10* and *Pax6* in addition to up-regulating Cyclin D1 and Cyclin D3. The increase in trophic factors upon *Sox2* ablation, and the maintenance of D-type Cyclins in both *Sox2* single-mutant and *Sox2/Ctnnb1* double-mutant cells, suggests that SOX2 maintains a primitive stem cell population in the mouse OC in a competent state. These "stem-like" cells are revealed and expanded upon SOX2 ablation, and they are maintained even in the absence of WNT signaling. In other words, primitive ciliary margin stem-like cells are expanded in the *Sox2/Ctnnb1* double mutant eye, and although they can proliferate, they are not competent to become either NR (without SOX2) or CE (without β-Catenin) (Fig. 3-16D).

SOX2 and β-Catenin maintain a primitive stem cell niche in the peripheral OC in the mouse

The eyes of lower vertebrates grow throughout adulthood and can regenerate in response to injury. The major source of progenitor cells for regular growth and regeneration of injured retinal neurons is the ciliary marginal zone (CMZ) located at the periphery of the neural retina (reviewed in (Reh and Fischer, 2001). Lineage analysis of the CMZ in *Xenopus* has identified two types of progenitor cells in this population: a more primitive slower cycling stem cell located at the far periphery of the CMZ and a faster cycling more fate-restricted cell located adjacent to the peripheral neural retina (Wetts et al., 1989). It is generally accepted that mammalian eyes do not contain an active CMZ. However, prolonged proliferation of cells in the retinal margin and pars plana (proximal CE) can be stimulated *in vivo* (Kiyama et al., 2012; Moshiri and Reh, 2004), and pigmented CE cells from adult mammalian eyes exhibit properties of neural stem cells *in vitro* (Ahmad et al., 2000; Tropepe et al., 2000).

Here, we have provided further evidence for a limited ciliary margin-like region in the developing mouse OC periphery and implicate SOX2 in its maintenance. We propose a model in which OCPCs give rise to either NR progenitor cells in the central OC, where they maintain SOX2, or CE progenitor cells in the far periphery, where they lose SOX2. In addition, a third progenitor cell type located at the boundary of NR and CE progenitors is capable of giving rise to both NR and CE and exhibits properties of central CMZ cells of lower vertebrates (Fig 6L). In mice, these cells highly express the WNT antagonist *Sfrp2* and retain SOX2 expression in order to retain neural competence. This model is consistent with a role for SOX2 in simultaneously conferring neuronal potential and maintaining progenitor cells in a poised state; SOX2 is necessary for the competence of neural progenitor cells (NPCs) to differentiate into neurons but must be down-regulated in order for NPCs to exit the cell cycle and differentiate (Agathocleous et al., 2009; Graham et al., 2003; Kishi et al., 2000; Taranova et al., 2006).

SOX2 regulates Cyclin D1 independently of PAX6, SHH and canonical Wnt signaling

We have shown for the first time that loss of *Sox2* in a neural progenitor cell population leads to increased Cyclin D1 protein. Although this result is consistent with a role for SOX2 in maintaining stem cell quiescence, it is surprising for two reasons: first, SOX2 has been shown to promote proliferation through the up-regulation of Cyclin D1, particularly in breast cancer cell lines (Chen et al., 2008). Therefore, ablation of *Sox2* would be expected to result in decreased Cyclin D1 protein. Secondly, Cyclin D1 is often used to differentiate the prospective NR from the prospective CE. Except for *Cyclin D1*, all pan-NR markers examined to date have been lost in *Sox2*-ablated cells. These data suggest that *Cyclin D1* expression is regulated by additional pathways.

Given that *Cyclin D1* is a well-characterized positive target of canonical WNT signaling in neural progenitor cells, we examined whether the increase in Cyclin D1 was mediated by β-Catenin. This scenario would fit well with the expansion of Wnt signaling observed in *Sox2*-ablated OCPCs. However, deletion of *β-catenin* in *Sox2*-mutant cells did not rescue Cyclin D1 up-regulation at E16.5 or P0. Moreover, reduction of *Pax6*, a highly conserved regulator of OCPC proliferation, in the *Sox2*-mutant OC did not rescue Cyclin D1 up-regulation. A third regulator of OCPC proliferation is Sonic Hedgehog (SHH) (Moshiri and Reh, 2004; Sakagami et al., 2009; Wall et al., 2009; Wang et al., 2005). In our model, *Shh* was drastically reduced by the near complete elimination of RGC production (Kiyama et al., 2012; Wang et al., 2005). However, OCPCs were able to proliferate and maintain high Cyclin D1 in the near absence of *Shh*.

These results led us to hypothesize additional pathways that could up-regulate Cyclin D1 in the absence of the above-mentioned regulators. One example of SOX2 indirectly antagonizing Cyclin D1 levels occurs in human embryonic stem cells: SOX2 together with OCT4 promotes the expression of miR-302, a cluster of eight microRNAs expressed in pluripotent cells. Specifically, miR-302a was shown to repress *Cyclin D1* post-transcriptionally via its 3'UTR (Card et al., 2008). Such a scenario could occur in OCPCs in which repression of *Cyclin D1* translation becomes de-repressed in the absence of SOX2. Indeed, ablation of *Dicer1*, a major mediator of microRNA biosynthesis, in mouse OCPCs led to expansion of the Cyclin D1 expression domain and a persistent "ciliary margin-like" region in the OC (Davis et al., 2011b). Conversely, degradation or sequestration of Cyclin D1 protein may become inhibited, especially given that only Cyclin D1 protein, not *Ccnd1* mRNA, was increased. The up-regulation of trophic factors observed upon *Sox2* ablation may regulate Cyclin D1 protein post-translationally. In olfactory sensory neuron precursors for example, BDNF elicits a rapid increase in Cyclin D1 independently of both transcription and translation (Simpson et al., 2007).

Lastly, SOX2 could antagonize Cyclin D1 via direct regulation of *p27^{Kip1}* expression. A recent report demonstrated that SOX2 maintains the quiescent state of inner pillar cells of the postnatal mouse auditory sensory epithelium by activating *p27^{Kip1}* transcription (Liu et al., 2012). Likewise, SOX2 has been shown to be down-regulated in some human gastric carcinomas exhibiting increased Cyclin D1 protein and decreased *p27^{Kip1}* expression. Given the absence of *p27^{Kip1}* in *Sox2*-null OCPCs, SOX2 may also promote the transcription of *p27^{Kip1}* in these cells. Moreover, the loss of p27^{kip1} is consistent with the loss of neurogenic fate, which provides a possible link between cell cycle regulation and fate determination in neuroepithelial cells (Bonner et al., 2008; Godin et al., 2012; Hardcastle and Papalopulu, 2000; Liu et al., 2012).

The *Sox2*-deficient OC provides a resource to identify genes important for CE development and glaucoma pathogenesis

Many of the most significantly up-regulated genes following *Sox2* ablation were associated with ion exchange and secretion (Fig. 3-6). These data are consistent with the function of the ciliary body to maintain the intraocular pressure (IOP) through the active transport of fluid via Na^+-K^+ exchange pumps and Cl^- channels (Civan and Macknight, 2004). Elevated IOP is often associated with glaucoma, a blinding disease characterized by the loss of retinal ganglion cells and optic nerve degeneration. One approach to the treatment of glaucoma is to lower IOP through the reduction of aqueous humour inflow. How the ciliary body integrates multiple mechanisms to regulate inflow is still not well understood (Civan and Macknight, 2004). Given that the presumptive ciliary body epithelium significantly expands upon *Sox2* ablation, our genome-wide screen of up-regulated transcripts in these mutants may reveal previously unidentified genes important for the development and function of the CE.

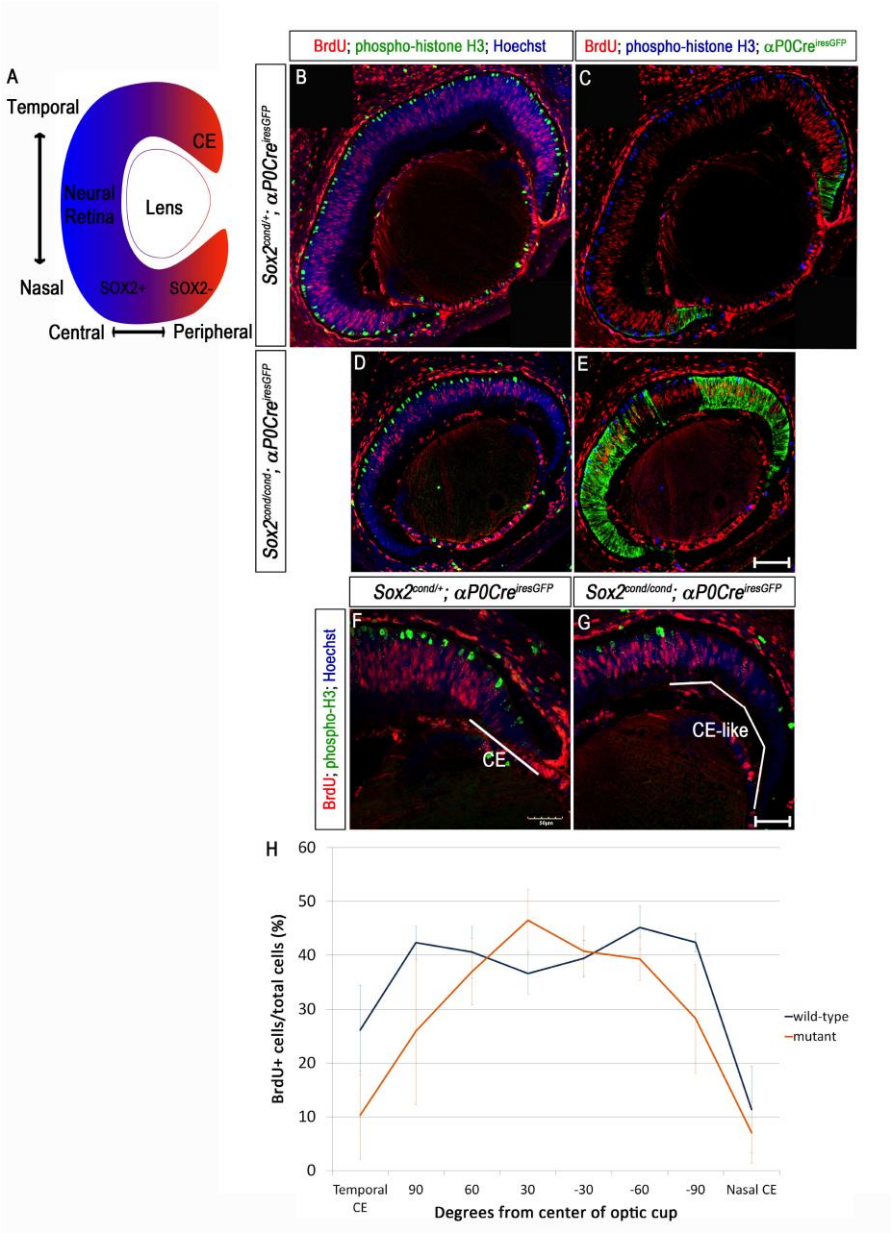


Figure 3-1 *Sox2*-ablated OCPCs gradually decrease proliferation from the center to the periphery at E16.5. (*A*) Diagram of a transverse section through the OC indicating the region of SOX2 expression ***(B-G)*** Staining for BrdU, phosphorylated histone H3 and CRE in control (B,C,F) and *Sox2*-mutant (D,E,G) OCs allowed to incorporate BrdU over two hours following a single injection. Mutant CRE-expressing cells in D and E gradually decrease BrdU incorporation, while central OCPCs of controls (B,C) steadily incorporate BrdU. The prospective CE is expanded in mutants (G) compared with controls (F). (***H***) Quantification of the percent of cells that incorporate BrdU over the total number of Hoechst-positive cells throughout the OC of controls (blue) and mutants (orange). Scale bars: G (for F,G): 50 μm; E (for B-E): 100 μm

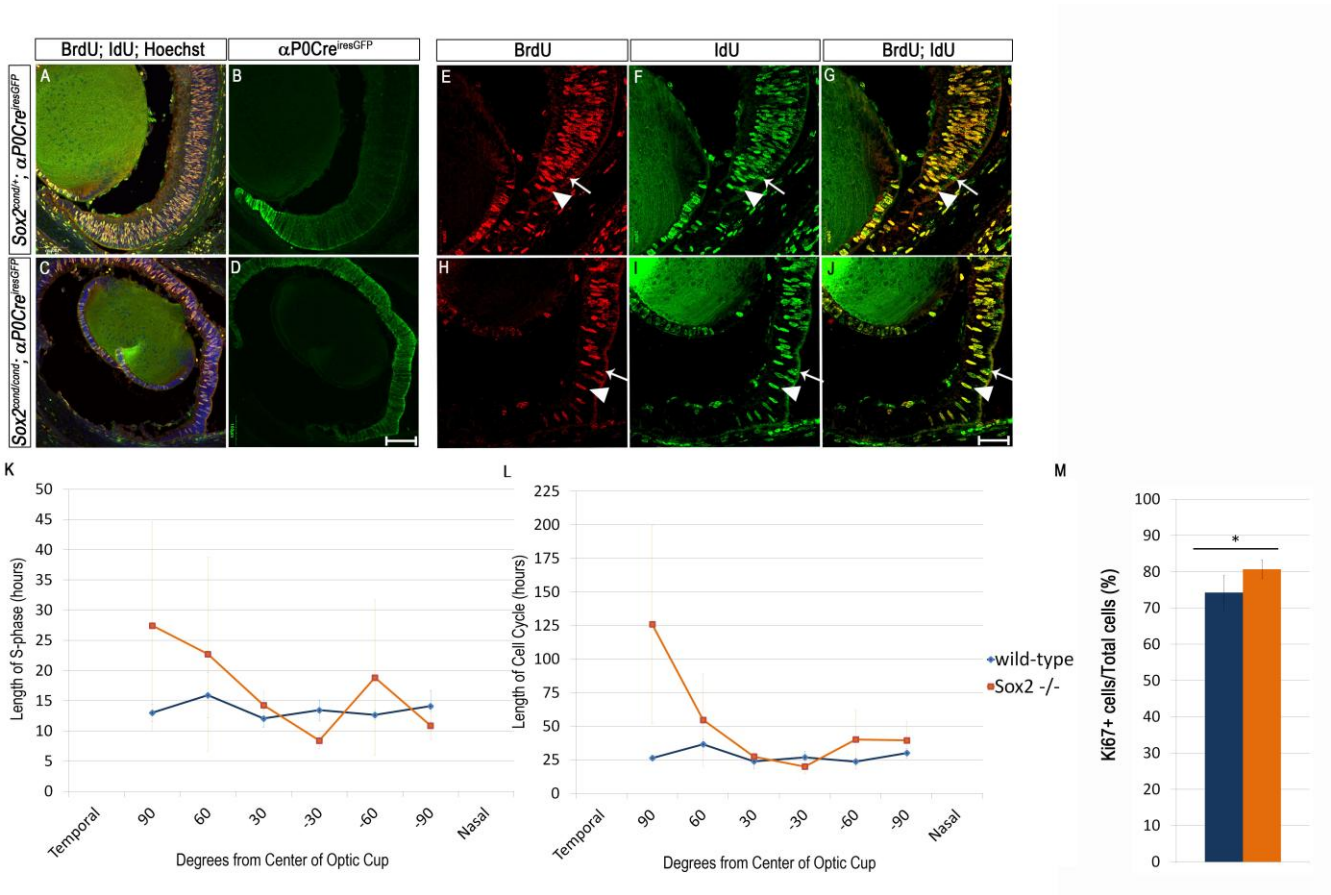


Figure 3-2 The *Sox2*-ablated OC shows a graded increase in the lengths of S-phase and cell cycle at E16.5. ***(A-J)*** Staining for GFP, BrdU and IdU designates cells that leave S-phase (green, arrows) and cells that remain in S-phase (red, arrowheads) in wild-type (A-G) and mutant (C-J) OCs. ***(K,L)*** Quantification of S-phase (K) and cell cycle (L) length in central OCPCs divided into degrees from the center to the periphery in wild-type (blue) and *Sox2*-mutant (orange) OCs. ***(M)*** Quantification of the percentage of Ki-67-positive cells over total Hoechst-positive cells indicates significantly increased percentage of cycling cells in mutants (orange) compared with controls (blue). Scale bars: D (for A-D) 100μm; J (for E-J) 50μm

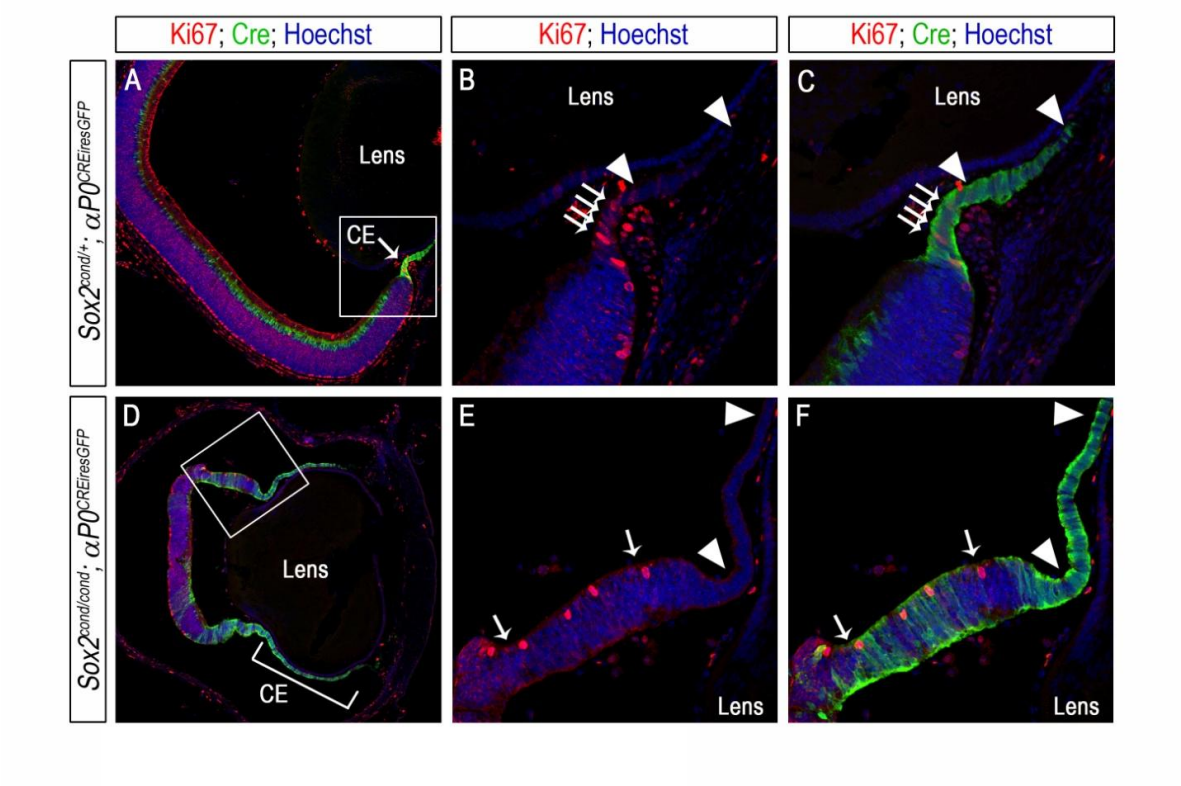


Figure 3-3 Many *Sox2*-deficient OCPCs prematurely exit the cell cycle by postnatal day 0. (*A-F)* Staining for GFP, Ki-67 and Hoechst in wild-type (A-C) and mutant (D-F) eyes at P0. Ki-67 is expressed in NR progenitor cells throughout the central OC (A, red) and in Cre-positive CE cells adjacent to the NR in controls (B,C, arrows) but not in peripheral CE cells (B,C , arrowheads). Similarly, Ki-67 is expressed in centrally located Cre-positive *Sox2*-ablated cells (E-F, arrows) but not in peripheral *Sox2*-ablated cells (E-F, arrowheads).

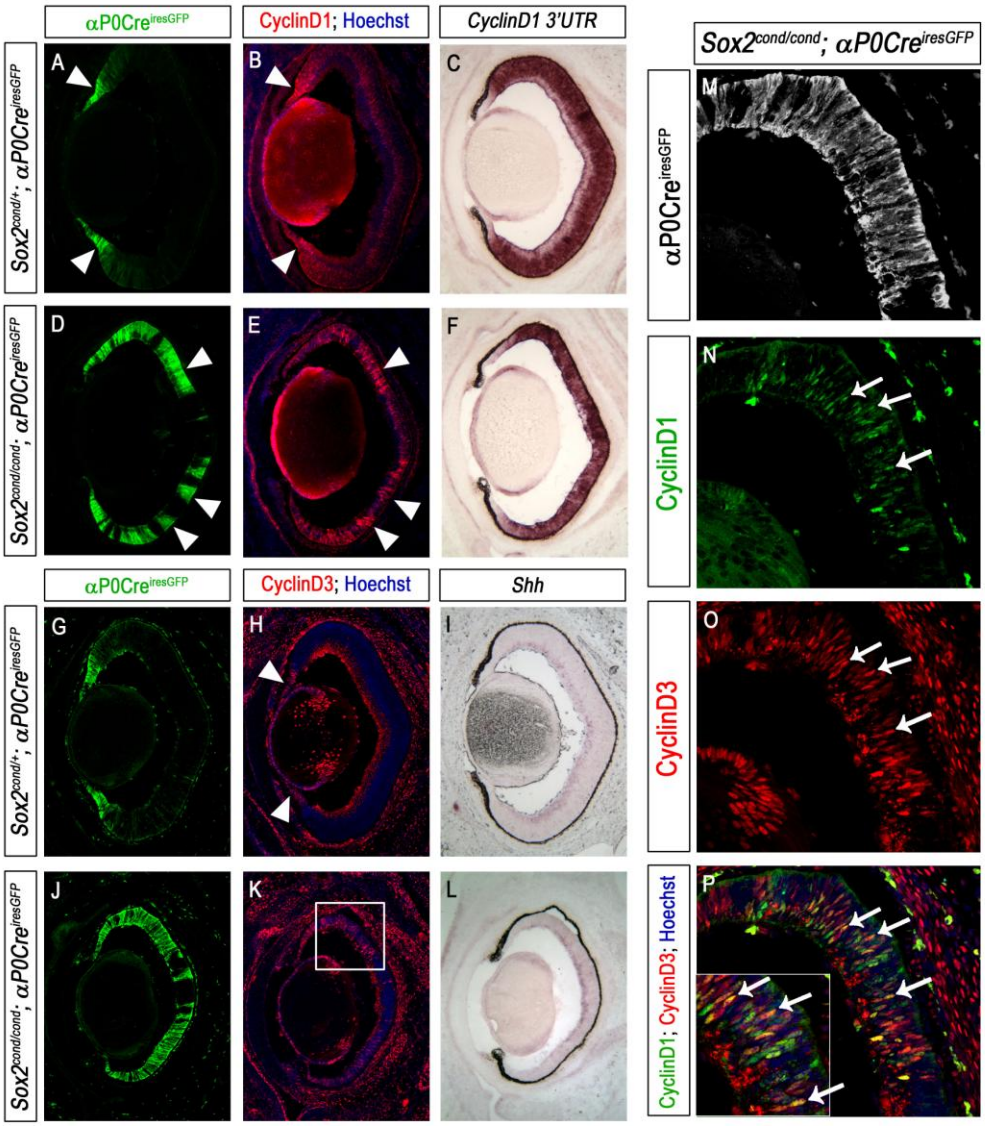


Figure 3-4 D-type Cyclins are aberrantly expressed in *Sox2*-ablated OCPCs at E16.5. ***(A, B, D, E)*** Cyclin D1 protein is up-regulated or stabilized in Cre-positive *Sox2*-ablated cells (D,E arrowheads) compared with controls (A,B). CyclinD1 protein appears elevated in the peripheral NR of controls (A,B arrowheads). ***(C,F)*** ISH of *Ccnd1* indicates that mRNA is not increased in mutants compared with controls. ***(G,H,J,K)*** Cyclin D3 is ectopically expressed in central mutant OCPCs (J,K box) but is restricted to the peripheral OC of controls (G,H, arrowheads). **(*I,L)*** *Shh* is decreased in central OCPCs of mutants (L) compared with controls (I). ***(M-P)*** Magnification of the box in K showing that Cyclin D1 and Cyclin D3 are aberrantly co-expressed in some *Sox2*-mutant central OCPCs (P arrows).

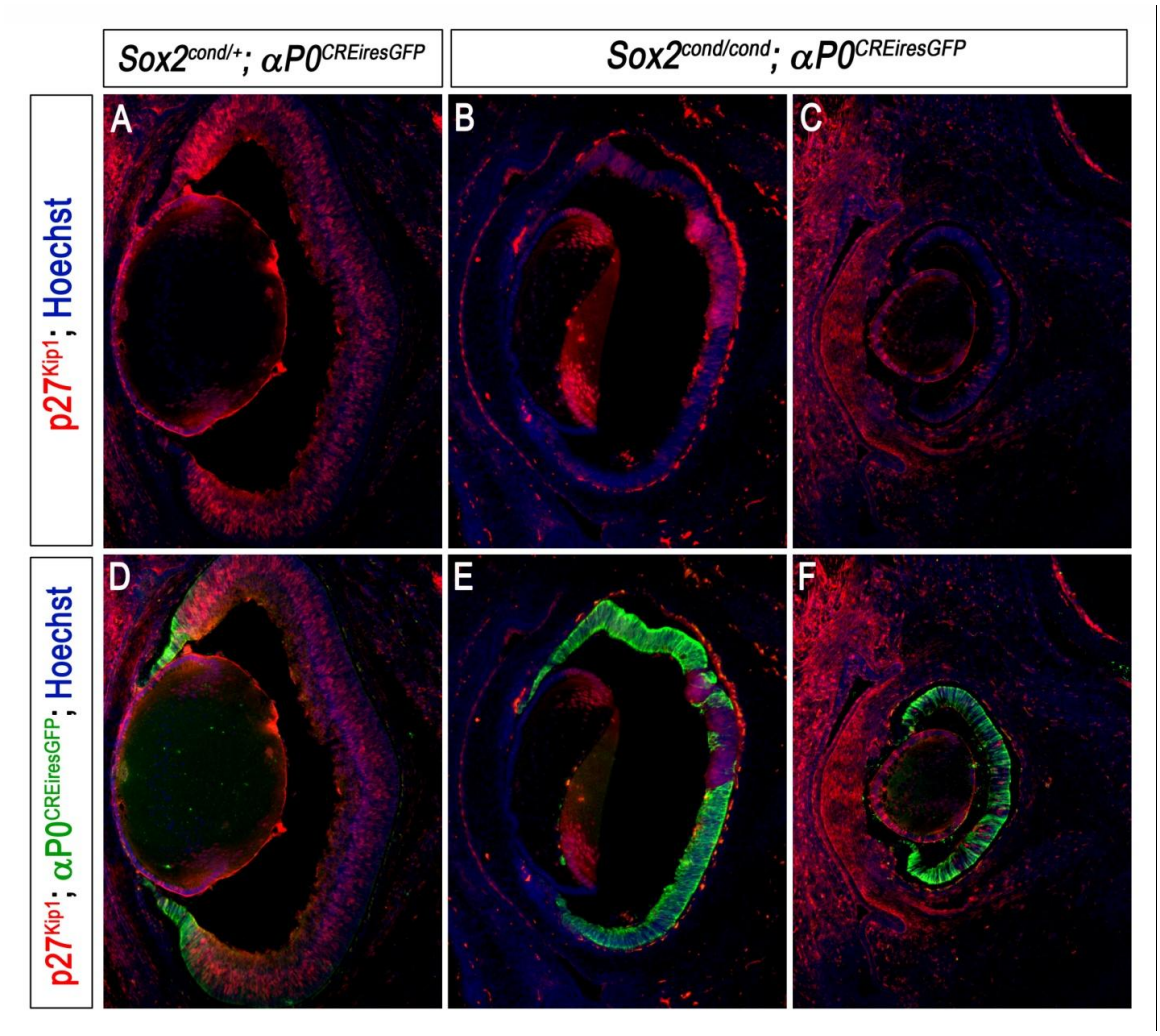
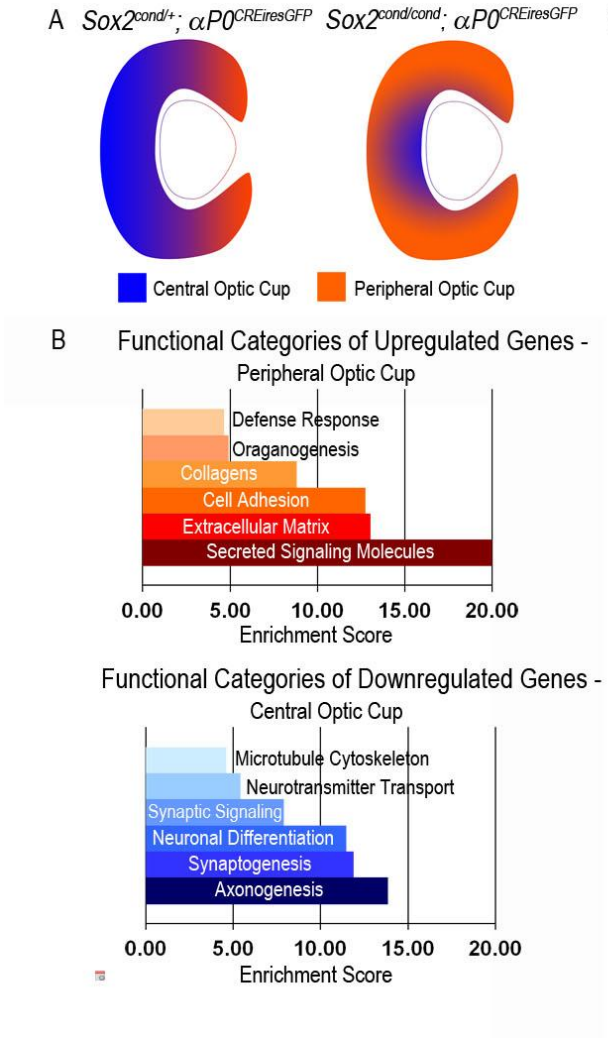


Figure 3-5 P27^{Kip1} is absent from *Sox2*-ablated OCPCs at E16.5. ***(A-F)*** Staining for GFP, p27^{Kip1} and Hoechst in wild-type (A,D) and mutant (B,C,E,F) eyes shows high expression in the central OC of controls (A,D) but reduced or absent expression in the GFP-positive central OC of mutants (B,C,E,F).



C **Potentially activated upstream regulatory pathways**

REST signaling
Chrnb2 (-5.36X) Chb (3.68X) Stmn2 (-3.00X) Syn1 (-2.97X)
Syt1 (-2.89X) Syp (-2.45X) Gap43 (-2.4X) Gria2 (-2.18X) Rab3a (-1.936X)
Tgfβ signaling
Bdnf (6.78X) Csf1r (4.58X) Fstl3 (3.92) Spp1 (3.69) Tfgbr2 (2.76)
Timp3 (2.29X) Tgfb1 (2.26) Has2 (2.13) Pxn (2.10X) Rarg (2.03X)
Bmp7 (1.91X) Ltbp3 (1.87X) Cyba (1.85X) Tgfbi (1.84X) Pdgfb (1.79X)
Thbs1 (1.78X) Hspg2 (1.78X) Wisp1 (1.77X) Col7a1 (1.77X) Fn1 (1.76X)
Igfbp3 (1.72X) Egr1 (1.72X) Emilin1 (1.66X) Itga3 (1.62X) Gas7 (1.61X)
Eomes (-7.76X)
Canonical Wnt signaling
Spp1 (3.69X) Aldh1a1 (2.48X) Cldn2 (2.10X) Zic1 (2.08X) BMP7 (1.91X)
Pitx2 (1.79X) Wisp1 (1.77X) Fn1 (1.76X) Egfr (1.74X) Ephb4 (1.65X)
Shh (-4.54X)

Optic Cup-Specific Canonical Wnt signaling
Foxp2 (6.10X) Wfdc1 (2.98X) Tnfrsf19 (2.60X) Col9a2 (2.49X) Otx1 (2.50X)
Zic2 (2.32X) BMP7 (1.91X) Id3 (1.50X) Cdkn1a (1.47X) Syt13 (1.65X)

Figure 3-6 Genome-wide expression analysis of SOX2-ablated OCs reveals functional characteristics of the peripheral and central OC and indicates increased WNT activity at E16.5. ***(A)*** Diagram of expansion of peripheral OCPCs (orange) in mutant OCs compared with controls. ***(B)*** Gene ontology categories of peripheral OCPCs/CE progenitor cells (orange) and central OCPCs/NR progenitor cells (blue). ***(C)*** Potential pathways activated in the developing OC in the absence of SOX2 include the peripheral OC regulators Tgfβ and canonical WNT signaling.

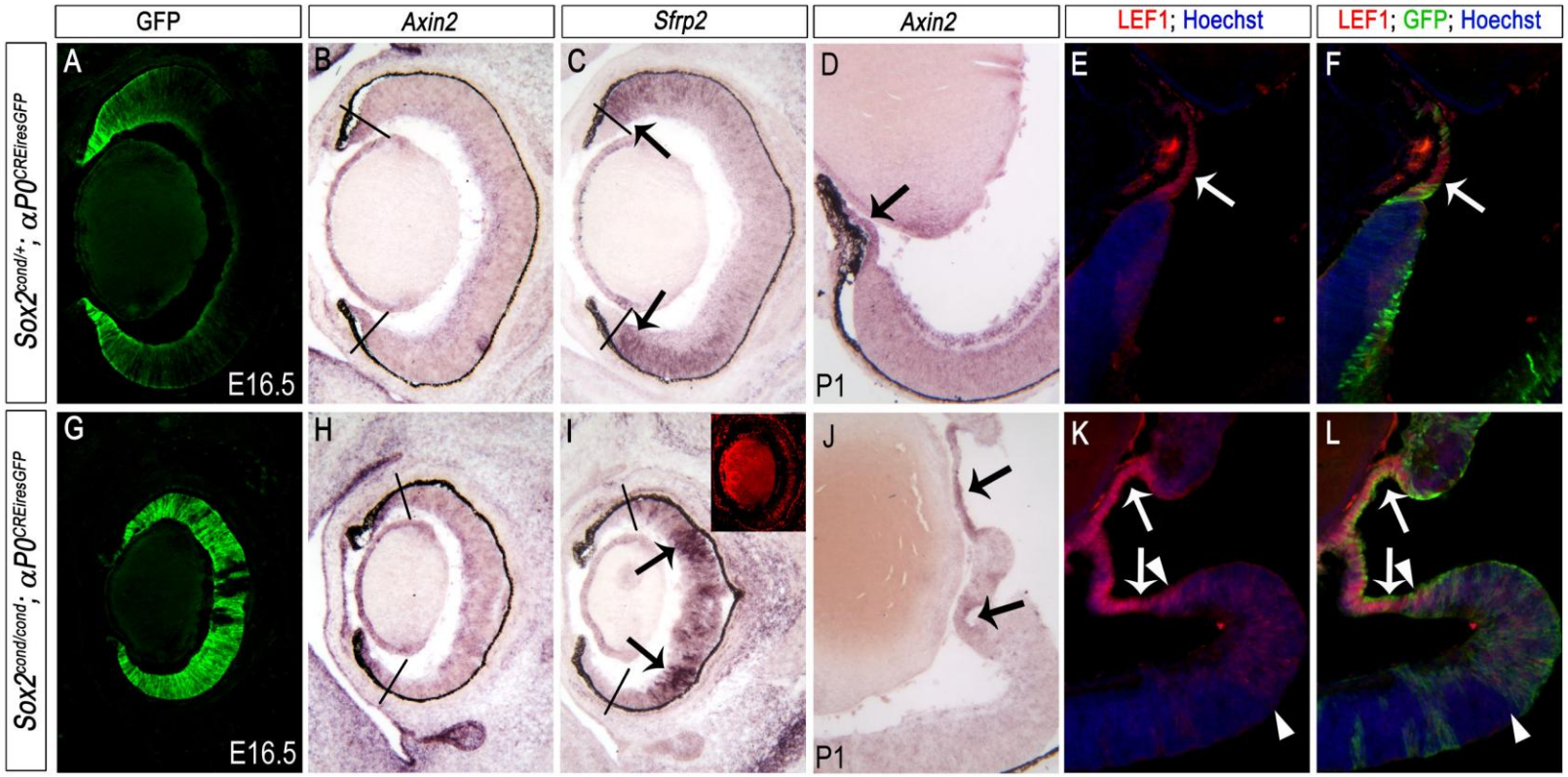


Figure 3-7 WNT activity is expanded in the *Sox2*-ablated OC at E16.5 and P1. ***(A-F)*** *Axin2* is specifically expressed in the prospective CE (left of the line) at E16.5 (A,B) and *Sfrp2* is adjacent to the CE in the peripheral NR (right of the line) (C, arrows). LEF1 is restricted to the CE at P1 (D,E, arrows). ***(G-L)*** *Axin2* is expanded into the central mutant OC (F,G left of the line) and *Sfrp2* is just adjacent to this region (H, right of the line). The areas with highest *Sfrp2* expression (arrows in H) correspond with high BrdU incorporation (inset in H). *Axin2* is expressed in thin *Sox2*-ablated cells, and LEF1 is expressed in GFP-positive CE-like cells at P1 (H-J). Ectopic LEF1 gradually increases as the epithelium thins (K,L, area between arrowheads versus area between arrows).

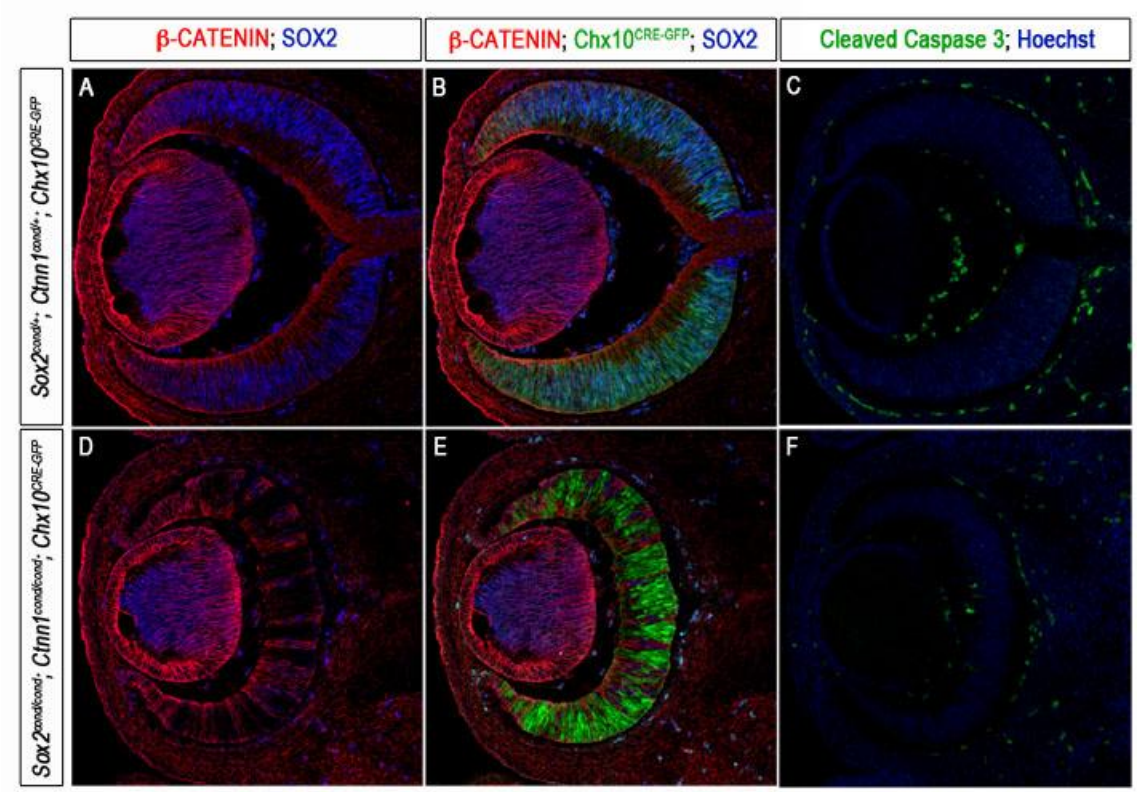


Figure 3-8 SOX2 and β-CATENIN can be efficiently ablated from the OC using *Chx10^{CreGFP}* without causing increased cell death. ***(A,B,D,E)*** Staining for GFP, SOX2 and β-CATENIN indicates efficient ablation of both of these proteins in double-mutants (C,D) compared with controls (A,B). ***(C,F)*** Staining for cleaved Caspase 3 indicates no significantly increased cell death upon ablation of SOX2 and β-CATENIN. .

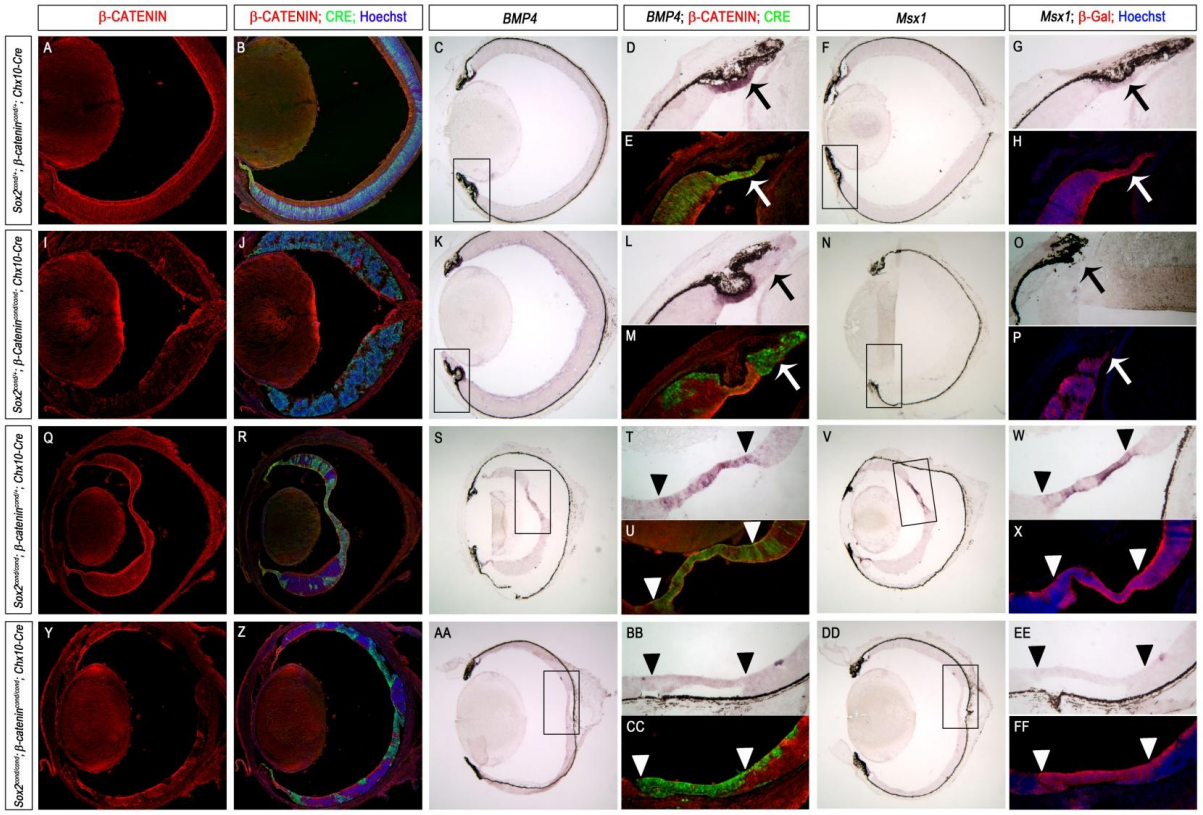


Figure 3-9 Deletion of *Ctnnb1* in *Sox2*-ablated OCPCs rescues the ectopic expression of CE genes *(A,B,I,J,Q,R,Y,Z)* β-CATENIN is efficiently ablated in *Ctnnb1* single-mutants (I-P) and *Sox2/Ctnnb1* double-mutants (Y-FF) compared with *Sox2* single-mutants (Q-X) and wild-type littermates (A-H). ***(C-H)*** *Bmp4* (C-E) and *Msx1* (F-H) are specifically expressed in GFP-positive (E) and β-gal-positive (H) CE. ***(K-P)*** *Bmp4* (K-M) and *Msx1* (N-P) are not expressed in GFP-positive (M, arrow) and β-gal-positive (P, arrow) CE of *Ctnnb1* mutants. ***(Q-X)*** *Bmp4* (S-U) and *Msx1* (V-X) are not expressed in GFP-positive (U, arrowheads) and β-gal-positive (X, arrowheads) central OCPCs of *Sox2* mutants. ***(Y-FF)*** Ectopic expression of *Bmp4* (AA-CC) and *Msx1* (DD-FF) is rescued in GFP-positive (CC arrowheads) and β-gal-positive (FF arrowheads) central OCPCs of *Sox2/Ctnnb1* double mutants.

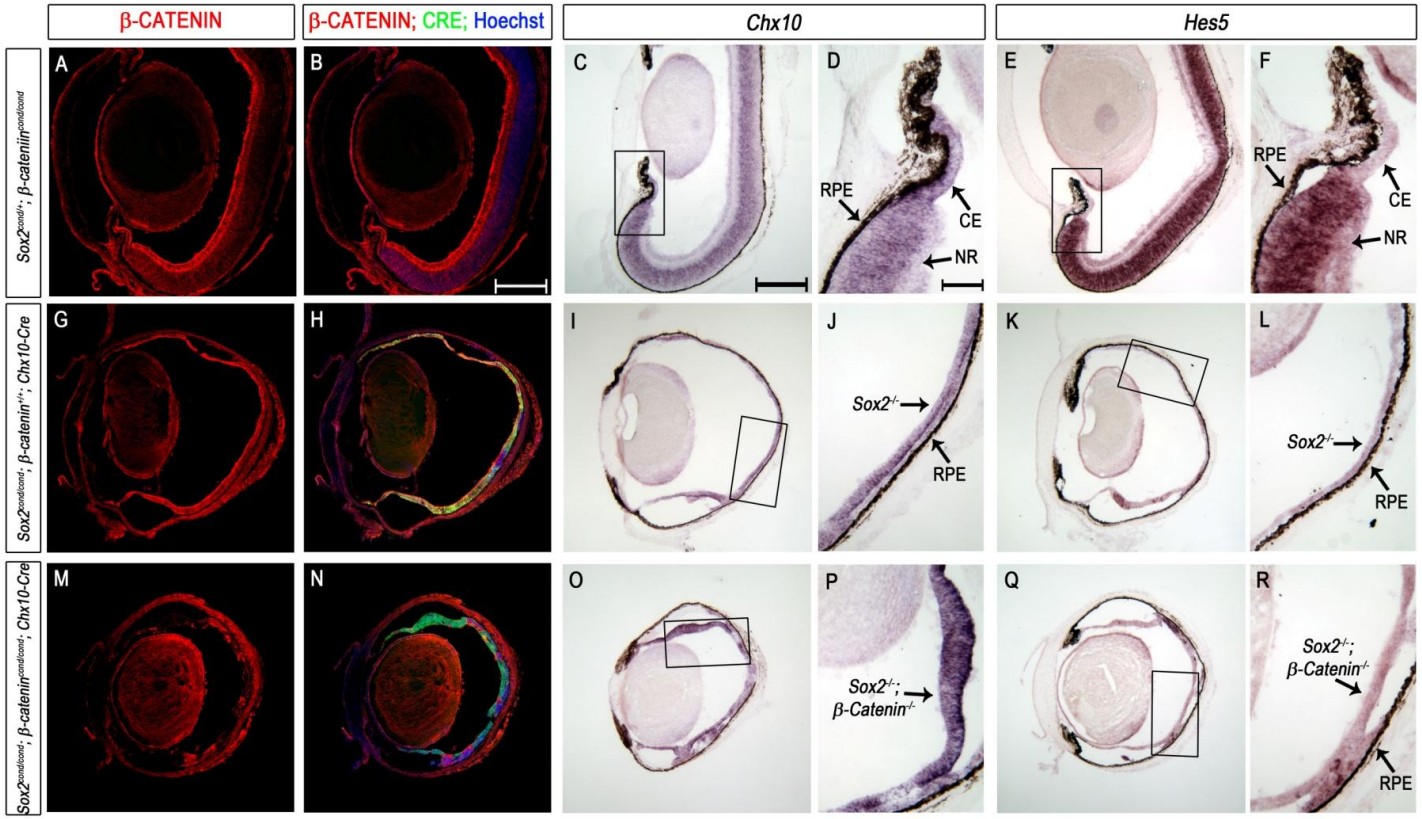


Figure 3-10 Deletion of *Ctnnb1* in *Sox2*-ablated OCPCs does not rescue NR identity. ***(A,B,G,H, M,N)*** HC for GFP, β-CATENIN and Hoechst identifies β-CATENIN-depleted regions in double mutants (M,N) compared with *Sox2* single-mutants (G,H) and controls (A,B). ***(C-F)*** *Chx10* is expressed throughout the whole OC (C,D), and *Hes5* is restricted to NR progenitor cells (E,F) in wild-type controls. ***(I-L)*** *Chx10* is maintained in *Sox2*-ablated cells (I,J) but *Hes5* is not (K,L). ***(O-R)*** Likewise, *Chx10* is maintained in *Sox2/Ctnnb1* double-ablated cells (O,P) but *Hes5* is not expressed in these cells (Q,R).

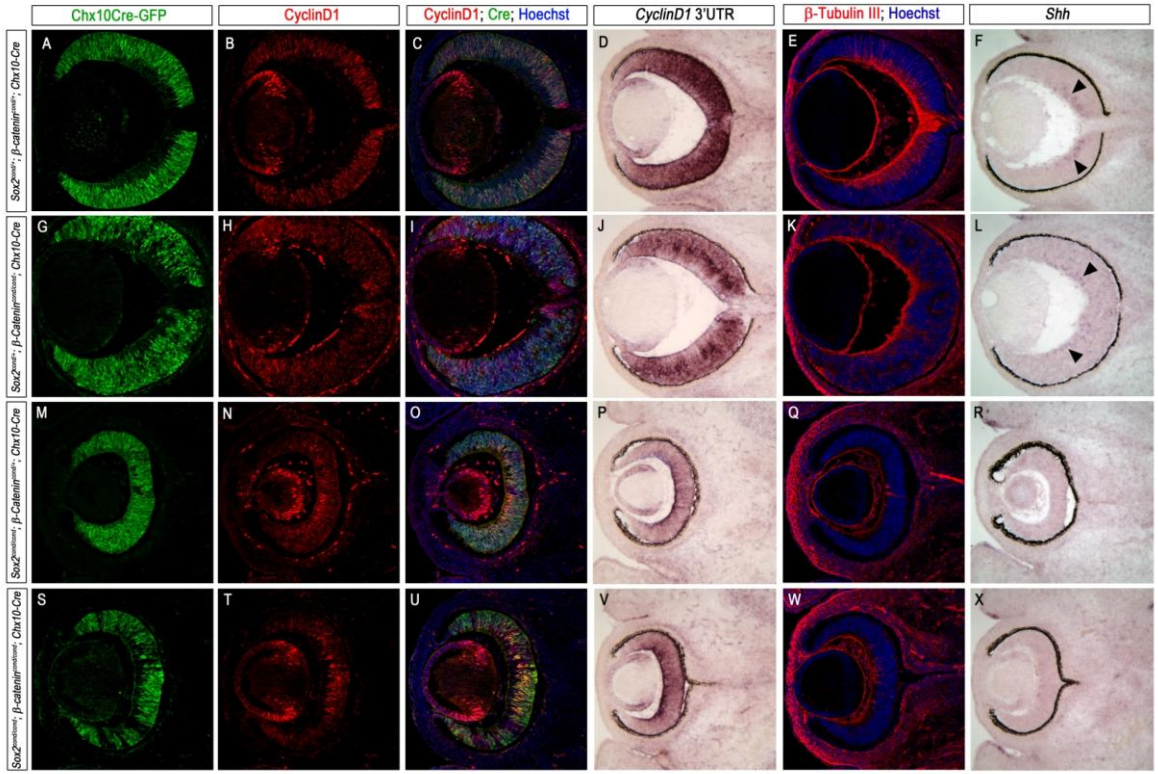


Figure 3-11 Deletion of *Ctnnb1* in *Sox2*-ablated OCPCs does not rescue increased Cyclin D1 at E13.5. ***(A-D,G-J,M-P,S-V)*** Cyclin D1 protein is localized to the central OC of control (A-C) and *Ctnnb1* single-mutants (G-H), and *Ccnd1* mRNA is restricted to the prospective NR of both controls (D) and *Ctnnb1* mutants (J). Cyclin D1 protein is up-regulated in the central GFP-positive OC of both *Sox2* single mutants (M-O) and *Sox2/Ctnnb1* double mutants (S-U). However, *Ccnd1* mRNA expression is not up-regulated (P,V vs. D,J). ***(E,F,K,L,Q,R,W,X)*** Staining for β-TubulinIII (E,K,Q,W) and *Shh* (F,L,R,X) indicate specific expression of *Shh* in the RGC layer of controls (E,F arrowheads) and *Ctnnb1* single-mutants (K,L arrowheads) and residual expression in the few neuronal cells that differentiate in *Sox2* single-mutants (Q,R) and double mutants (W,X).

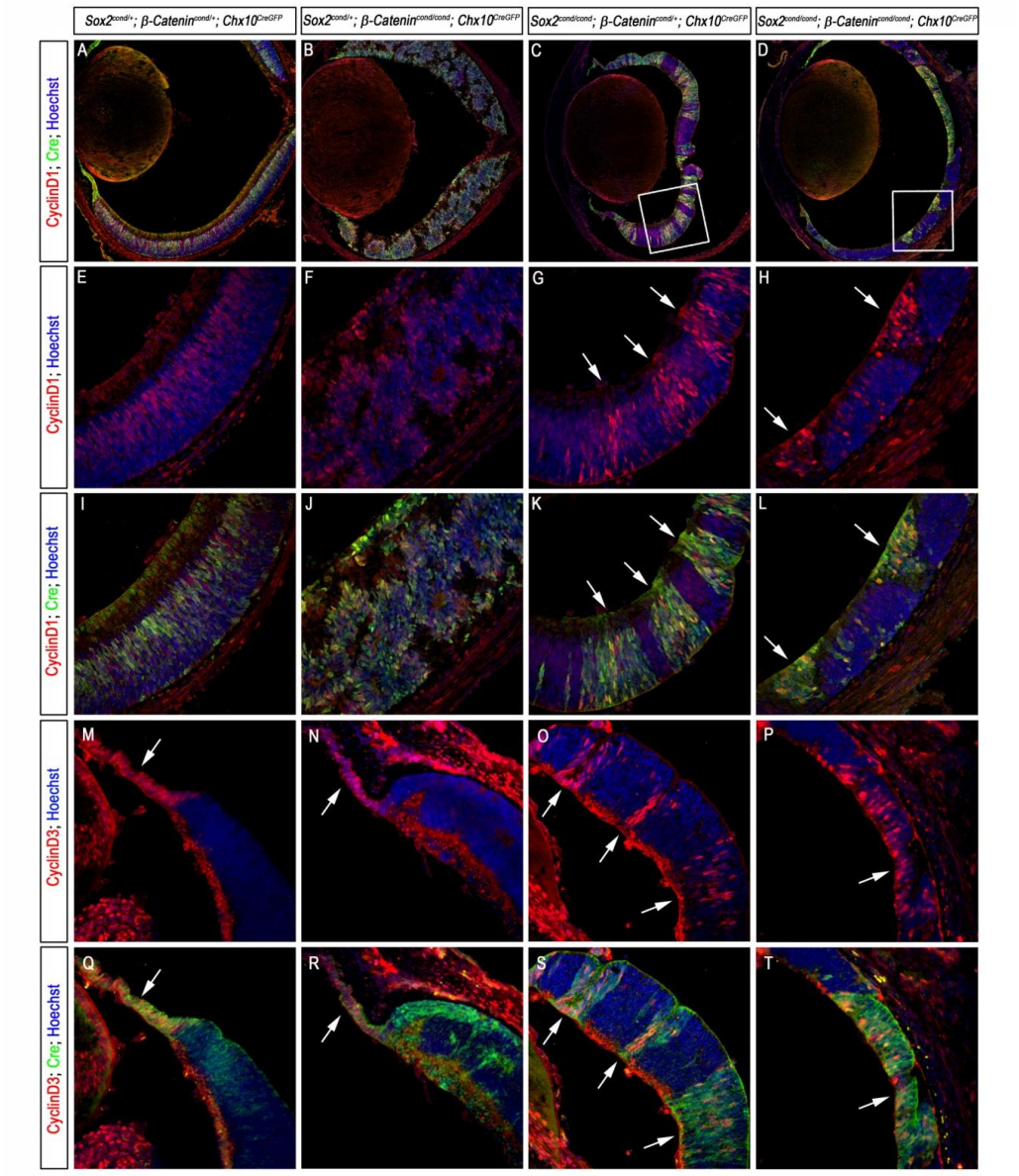


Figure 3-12 Deletion of *Ctnnb1* in *Sox2*-ablated OCPCs does not rescue the aberrant expression of in D Cyclins observed in *Sox2*-deficient cells at P0. *(A-L)* Staining for GFP, Cyclin D1 and Hoechst indicates Cyclin D1 expression restricted to NR progenitor cells in controls (A,E,I) and *Ctnnb1*-mutant eyes (B,F,J). Cyclin D1 is increased in GFP-positive cells in the central OC of *Sox2*-mutant eyes (C,G,K,arrows) and in *Sox2/Ctnnb1* double-mutant eyes (D,H,L, arrows). ***(M-T)*** Staining for GFP, Cyclin D3 and Hoechst indicates Cyclin D3 expression restricted to CE cells in controls (M,N, arrows) and *Ctnnb1* single-mutant eyes (N,R, arrows) but ectopic expression in GFP-positive cells in the central OC of *Sox2*-mutant eyes (O,P, arrows) and *Sox2/Ctnnb1* double-mutant eyes (S,T, arrows).

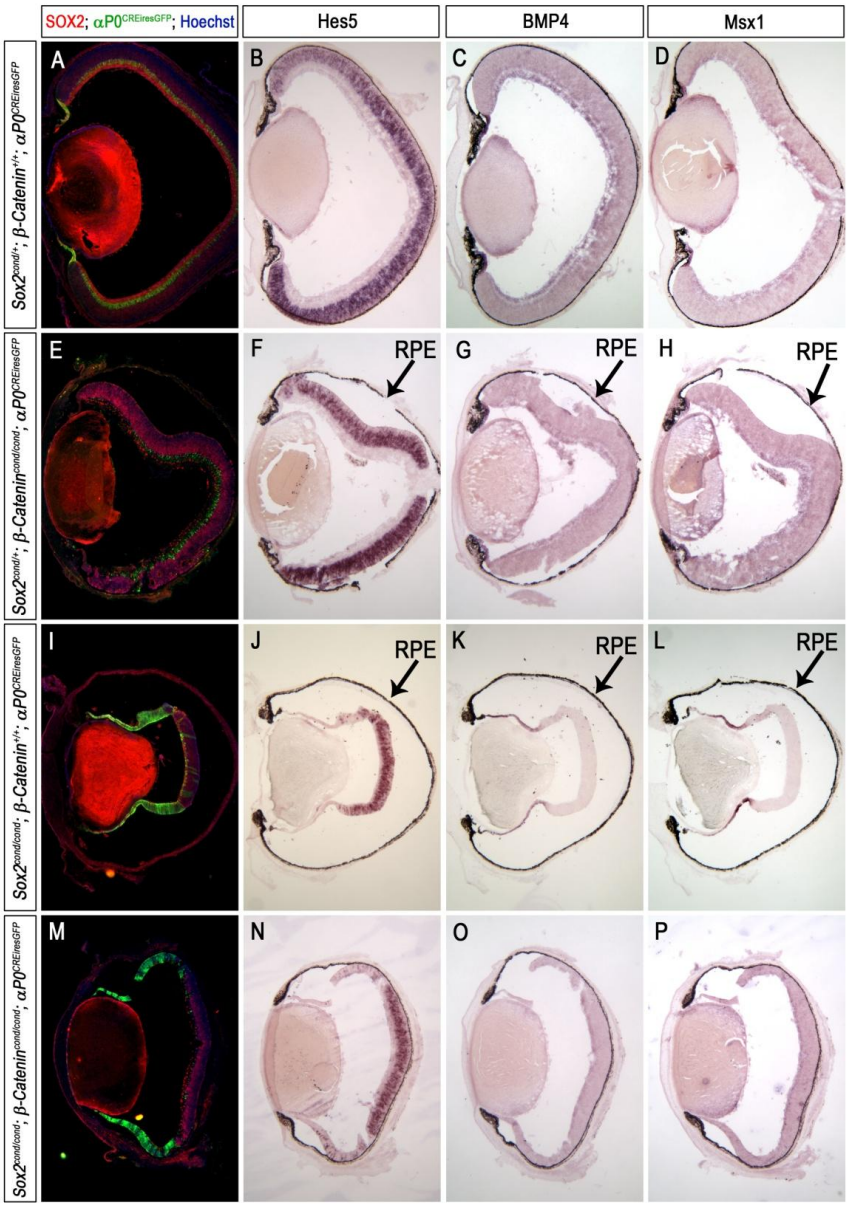


Figure 3-13 Deletion of *Ctnnb1* and *Sox2* in peripheral OCPCs rescues the ectopic expression of CE genes at P0. ***(A,E,I,M)*** Staining for GFP, SOX2 and Hoechst identifies SOX2-ablated regions in *Sox2* single-mutant (I) and double-mutant eyes (M) compared with *Ctnnb1* single-mutant (E) and control eyes (A). ***(B-D)*** *Hes5* is specifically expressed in the NR (B), and *Bmp4* and *Msx1* are specifically expressed in the CE (C,D) of control eyes. ***(F-H)*** ISH of NR and CE genes shows correct specification of these regions in *Ctnnb1*-mutant eyes. ***(J-L)*** *Hes5*-positive NR identity is lost (J), and *Bmp4* (K) and *Msx1* (L) are ectopically expressed in *Sox2*-mutant eyes. ***(N-P)***, ISH of NR and CE genes in double-mutant eyes shows loss of *Hes5*-positive NR identity (N) but rescue of the ectopic expression of *Bmp4* (O) and *Msx1* (P) observed in *Sox2*-mutant eyes.

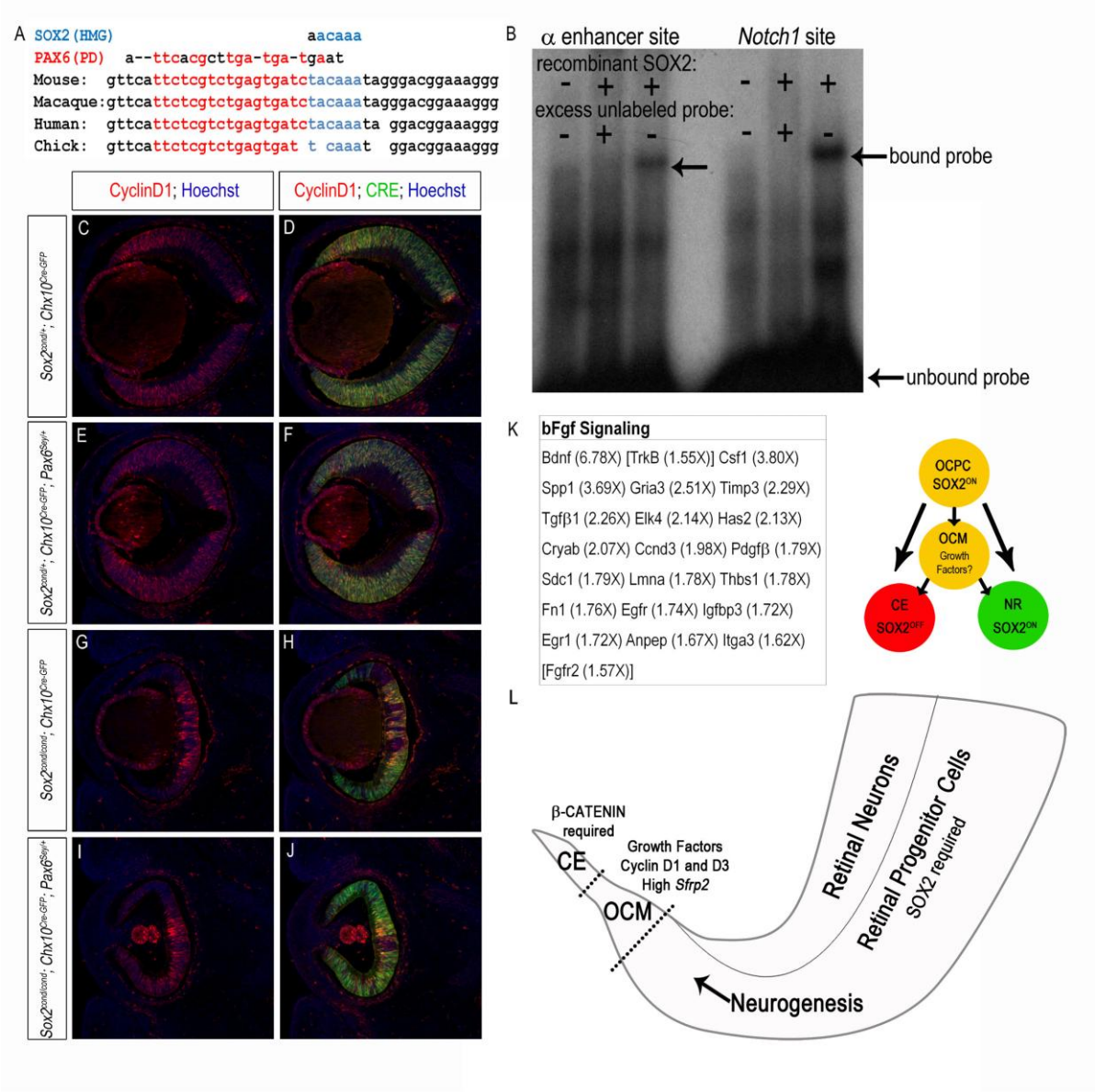


Figure 3-14 SOX2 directly antagonizes *Pax6*, but reduction of *Pax6* in *Sox2*-ablated OCPCs does not rescue increased Cyclin D1 at E14.5. ***(A)*** A conserved SOX2 consensus binding sequence (blue) was located directly adjacent to a previously characterized PAX6 binding site (red) in the *Pax6* OC-specific enhancer α. ***(B)*** Purified SOX2 binds this sequence (left arrow) and a previously characterized regulatory element of *Notch1* (top right arrow). ***(C-J)*** Staining for GFP and Cyclin D1 shows expression in the central OC of control (C,D) and $Pax6^{Sey/+}$ mutants (E,F) and increased protein in the central OC of *Sox2*-mutants (G,H) and *Sox2*/$Pax6^{Sey/+}$ double mutants (I,J). ***(K)*** Many FGF2 targets are increased more than 1.6-fold in the *Sox2*-mutant OC. These data support a novel model in which cells at the boundary of NR and CE proliferate and retain the capacity to become NR or CE for a prolonged period of time. ***(L)*** Illustration of the location of OCPC types: prospective CE cells at the periphery, neurogenic progenitor cells in the center and marginal progenitor cells at the boundary.

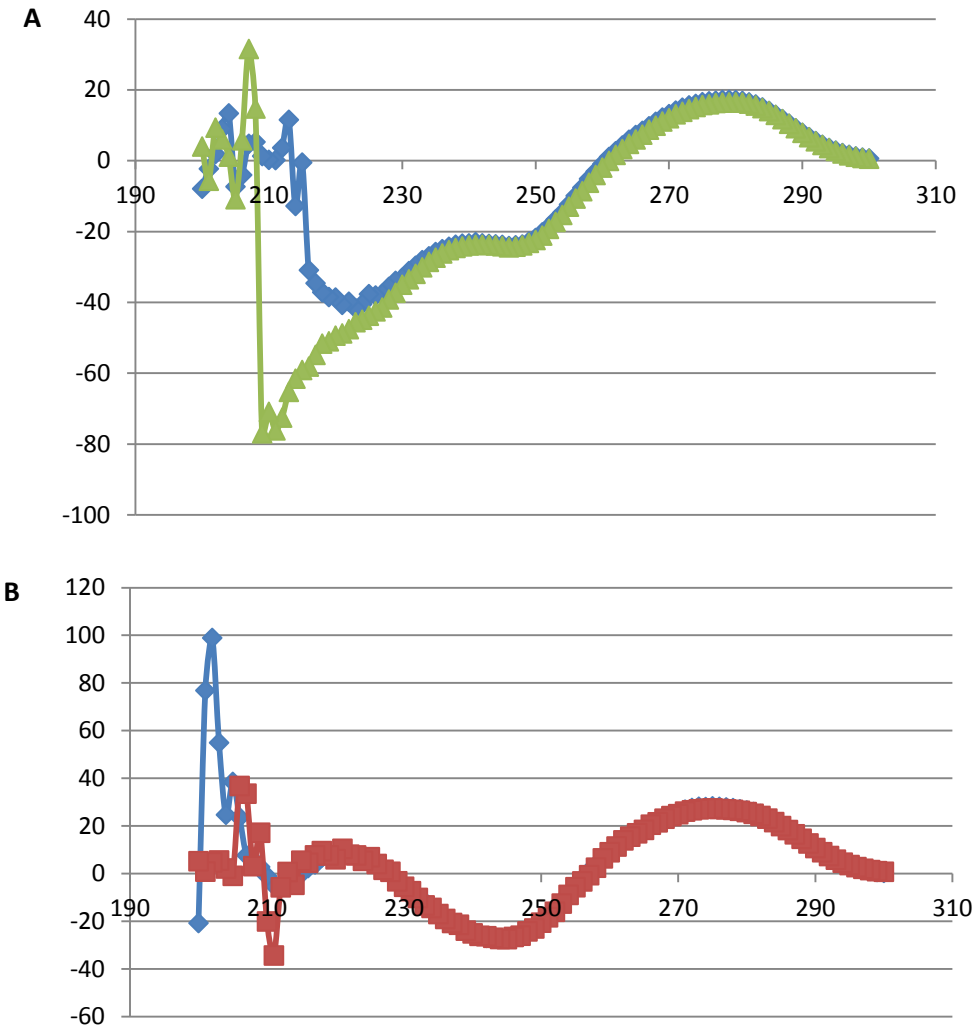


Figure 3-15 CD spectra of SOX2 *with* DNA versus *and* DNA. *A* The CD spectrum of SOX2 mixed with α enhancer DNA (green) shifts to the left when compared with the CD spectrum of SOX2 separated from α enhancer DNA (blue). This shift indicates a gain of α helical structure. ***B*** The CD spectrum of SOX2 mixed with random DNA (red) completely co-localizes with the CD spectrum of SOX2 separated from random DNA.

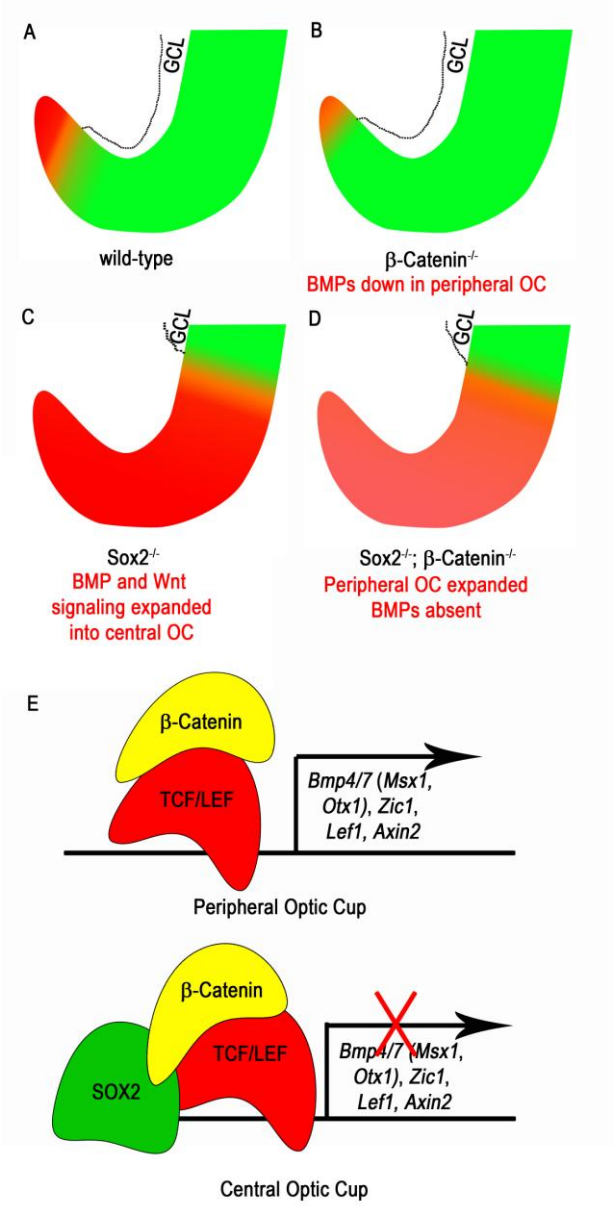


Figure 3-16 Model of how SOX2 and canonical WNT signaling regulate the neurogenic boundary of the OC. ***(A-D)*** The boundary between NR (green) and CE (red) is shifted peripherally in the *Ctnnb1*-mutant OC (B vs. A), supporting a role for canonical WNT signaling in CE specification. BMP signaling is also decreased in these cells. Conversely, the NR-CE boundary is shifted centrally in the *Sox2* single mutant OC (C vs. A), supporting a role for SOX2 in NR specification. WNT and BMP signaling are expanded in SOX2-ablated cells. The NR-CE boundary remains centrally shifted in the *Sox2/Ctnnb1* double-mutant OC (D vs. A). However, BMP signaling and other classical CE markers fail to be expressed in this expanded "CE-like" region. ***(E)*** Model of how SOX2 and β-Catenin coordinately regulate the neurogenic boundary of the retina: In the peripheral OC, β-Catenin (yellow) in complex with TCF/LEF (red) regulates the transcription of classical CE markers, including BMPs. In the central OC, SOX2 (green) may compete with TCF/LEF/β-Catenin for binding regulatory elements of these genes.

Allele	Primers (5' to 3')	Product size	Conditions
Sox2cond	Fwd: CAGAGGACTCGTGTTTGGGAAC	floxed: 307 bp	4 minutes at 94°C, (15 seconds at 94°C, 30 seconds at
	Rev: TCTTGGATACATAAGGGTGGATGG	wt: 345 bp	57°C, 30 seconds at 72°C) x35, 5 minutes at 72°C
Sox2dcond	Fwd: CTTCTTTCCGTTGATGCTTTCG	589 bp	4 minutes at 94°C, (15 seconds at 94°C, 30 seconds at
	Rev: ATCTTGGTGGCTGAACAGTTATCC		57°C, 30 seconds at 72°C) x35, 5 minutes at 72°C
Cre	Fwd: GCTAAACATGCTTCATCGTCGG	750 bp	4 minutes at 94°C, (15 seconds at 94°C, 30 seconds at
	Rev: GATCTCCGGTATTGAAACTCCAGC		57°C, 30 seconds at 72°C) x35, 5 minutes at 72°C
Rosa26Rep	Primer 1: AAAGTCGCTCTGAGTTGTTAT	transgene: 650 bp	4 minutes at 94°C, (30 seconds at 93°C, 30 seconds at
	Primer 2: GCGAAGAGTTTGTCCTCAACC	wt: 340 bp	58°C, 1 minute at 65°C) x35, 10 minutes at 72°C
	Primer 3: GGAGCGGGAGAAATGGATATG		
Pax6-Sey	Fwd: AACTTTTTGGCTTGCTTTGTCATTC	188 bp	1 minute at 95°C, (20 seconds at 92°C, 20 seconds at
	Rev: CTGAGCTTCATCCGAGTCTTCTCA		63.5°C, 50 seconds at 72°C) x34, 4 minutes at 72°C
Pax6-wt	Fwd: GAACACCAACTCCATCAGTTCTAACG	646 bp	1 minute at 95°C, (20 seconds at 92°C, 20 seconds at
	Rev: CTTTCCCGGGCAAACACATC		63.5°C, 50 seconds at 72°C) x34, 4 minutes at 72°C
β-catenincond	Primer 1: AAGGGTAGAGTGATGAAAGTTGTT	floxed: 324 bp	1 minute 30 seconds at 94°C, (30 seconds at 94°C,
	Primer 2: CACCATGTCCTCTGTCTATTC	wt: 221 bp	1 minute at 60°C, 1 minute at 72°C) x35,
	Primer 3: TACACTATTGAATCACAGGGACTT	floxdel: 500	2 minutes at 72°C

Table 3-1 PCR primers and genotyping protocols.

Primary Antibodies	Source	Dilution
mouse anti-BrdU	Becton Dickinson	1:500
rat anti-BrdU	AbD Serotec	1:500
rabbit anti-cleaved caspase 3	Cell Signaling	1:250
chick anti-GFP	Abcam	1:2000
rabbit anti-phospho-histone H3	Abcam	1:1000
rabbit anti-CyclinD1	Thermo Scientific	1:400
mouse anti-CyclinD3	Cell Signaling	1:100
rabbit anti-β-Catenin	Abcam	1:250
rabbit anti-β–Galactosidase	Molecular Probes	1:10000
rabbit anti-Ki-67	Abcam	1:1000
mouse anti-$p27^{Kip1}$	Thermo Scientific	1:100
rabbit anti-LEF1	Cell Signaling	1:100
mouse anti-SOX2	R&D	1:100
rabbit anti-SOX2	Millipore	1:3000
Secondary Antibodies	**Source**	**Dilution**
Goat anti-mouse IgG, IgG2a Alexa Flour 546, 633	Invitrogen	1:1000
Goat anti-rabbit IgG 546, 633	Invitrogen	1:1000
Goat anti-chicken IgG Alexa Fluor 488	Invitrogen	1:1000
Goat anti-rat Alexa Fluor IgM 546	Invitrogen	1:1000
Fluorescent Dyes	**Source**	**Dilution**
Hoechst 33258	Invitrogen	1:10000

Table 3-2 Working dilutions of antibodies for immunohistochemistry.

Chapter IV: DISCUSSION AND FUTURE DIRECTIONS

Re-analysis of the default model of neural induction

The default model of neural induction is based on the finding that *Xenopus* ectoderm will become neural plate in the absence of any inductive cues. However, as discussed in Chapter I, conflicting reports from studies in chick leave the default model up for debate. This thesis project approaches the default model of neural induction from the standpoint of the optic cup and finds that the ground state of OCPCs is neural retina, and NR identity is fully dependent on SOX2. SOX2 maintains neural fate in part via antagonism of PAX6 and canonical WNT signaling.

The optic cup can serve as a model of SOX2 function in neural induction

Given the difficulty of accessing and manipulating the mammalian epiblast, the molecular mechanisms that specify neural fate remain a mystery. The optic cup is an appropriate model to study neural fate specification and the role of SOX2 as a neural ectoderm determinant. Ablation of SOX2 in the OC results in complete loss of neural characteristics and conversion to non-neurogenic epithelium reminiscent of epidermis. This cell fate conversion is associated with classical non-neural features including the loss of the ability to generate neurons, reduced proliferation, differential expression of cell cycle regulators and activated WNT/β-Catenin signaling.

Neurogenic versus Non-neurogenic

Neural fate is defined by the competence to generate neurons (Wills et al., 2010). SOX2 is expressed throughout the epiblast before neural plate and epidermis are specified. Therefore, SOX2 expression itself does not define neural fate in all instances and is not sufficient to bestow neural competence (Wills et al., 2010). The maintenance of SOX2 specifically in neural plate, however, does implicate SOX2 as being necessary for neural competence. Indeed, without SOX2, epiblast cells are not able to generate neurons (Kishi et al., 2000; Wills et al., 2010). Likewise, SOX2 is expressed throughout the optic vesicle prior to the specification of neural retina and ciliary epithelium. SOX2 is specifically maintained in the NR and is required for retinal neurogenesis; however, ectopic expression of SOX2 in the CE is not sufficient to induce neurogenesis (W Heavner unpublished data). Again, in this instance, SOX2 is implicated in neural competence but alone is insufficient to bestow it. The OC therefore, provides a system with which to address the mechanism whereby SOX2 maintains the ability of a progenitor cell to generate a neuron. Given that SOX2 alone cannot induce neural competence, it is crucial to identify its transcriptional co-activators and downstream targets in order to fully understand what it means to be "neural-competent."

Differential Cell Cycle Regulation

A persistent question in developmental biology is how cell cycle and cell fate are linked. Neural fate is associated with the cell cycle inhibitor $p27^{Kip1}$ (Hardcastle and Papalopulu, 2000), and a mechanism for p27 in neuronal differentiation was recently identified (Godin et al., 2012). The loss of neural retinal fate upon ablation of SOX2 is characterized by loss of $p27^{Kip1}$ (Chapter III). In addition to $p27^{Kip1}$, other cell cycle regulators exhibit differential expression between the central and peripheral OC (Barton and Levine, 2008; Schwank and Basler, 2010). In the SOX2-

ablated OC, both positive and negative cell cycle regulators were present in the increased and decreased gene sets. *Cyclin D2*, *Cyclin D3* and *Cyclin E1* all showed increased expression in mutants compared with controls, and each of these positive cell cycle regulators have been shown to be expressed in the peripheral OC (Fig 4-1) (Trimarchi et al., 2009). Moreover, expression of the Cip/Kip family members, $p21^{Cip1}$ (*Cdkn1a*) and $p57^{Kip2}$ (*Cdkn1c*), was slightly increased, 1.5- and 1.4-fold, respectively (Fig 4-1). These two cell cycle inhibitors are normally excluded from the prospective NR, and their increase could indicate a cell fate shift from NR to CE (Zhang et al., 1998). Conversely, *Cyclin D1*, $p27^{Kip1}$ (*Cdkn1b*) and $p19^{Ink4d}$ (*Cdkn2d*), which are associated with neural fate, were decreased in mutants compared with controls (Fig 2C) (Cunningham et al., 2002; Godin et al., 2012; Liu et al., 2007b).

Together, these results demonstrate that overall changes in cell cycle gene expression most likely indicate a shift in cell fate rather than a functional mechanism of proliferation inhibition in the *Sox2*-mutant OC. Nonetheless, the stark contrast in expression profiles between NR and CE supports the notion that cell cycle and cell fate are linked. It remains unclear how much proliferation contributes to cell fate. Indeed, these data do not rule out the possibility that one or more of these cell cycle genes is instrumental in differential OCPC proliferation, and, therefore, cell fate. Experiments to test this question are proposed below.

Extrinsic Signals

The link between cell cycle and cell fate may be a morphogenetic cue (Schwank and Basler, 2010). The classical extracellular neural induction signals -- BMPs and FGFs -- are differentially expressed in the OC, and their expression is affected by the loss of *Sox2* consistent with a loss of neural fate and gain of non-neural fate. The major FGF associated with NR identity, FGF9, is drastically reduced, and the major BMPs associated with CE identity, BMP4 and BMP7,

are ectopically expressed. It remains in question how these morphogenetic cues affect the expression of cell cycle regulators and if these, in turn, are instrumental in affecting cell fate decisions.

Alternatively, the overall in change in the expression of cell cycle regulators could simply be a secondary consequence of cell fate conversion. The data presented in Chapter III uncouple the mitogenic role of WNT signaling from its role in cell fate specification, placing WNT/β-Catenin signaling as a major regulator of non-neurogenic cell fate in the OC. OCPCs continue to cycle and express D-type cyclins in the absence of WNT signaling, but they do not fully differentiate into CE. These data are consistent with the known role of canonical WNT signaling in mammalian neural induction (Aubert et al., 2002) (further discussed below).

However, it is curious that SOX2-ablated cells are not able to fully differentiate into CE until they exit the cell cycle or dramatically reduce their proliferation. Two possibilities for the link between cell cycle and cell fate thus arise: 1) cell cycle regulators, such as Cyclin D3 and $p27^{Kip1}$, are simply part of the cohort of genes that are expressed in response to a neural or non-neural-inducing signal and function to ensure that the correct number of cells are generated for a specific cell type. Or 2) cell cycle regulators somehow control the competence of a cell to become neural or non-neural.

One way to test this question is to prevent a CE progenitor cell from exiting the cell cycle or to force a neural progenitor cell to exit the cell cycle prematurely. Variations of these experiments have been performed: Decreasing SOXB1 factors in chick neural tube progenitor cells forces them to exit the cell cycle and aberrantly differentiate, but they remain neuronal (Bylund et al., 2003). In fact, SOXB1 factors must be down-regulated in order for a neuron to form in the chick neural tube. Conversely, forcing peripheral OC cells to proliferate does not

necessarily make them become neural retina (Kiyama et al., 2012; Moshiri and Reh, 2004). However, these experiments may need to be performed much earlier in optic cup development (E11-E14) in order for the question to be adequately resolved. At any rate, these studies combined with the present thesis project suggest that cell cycle regulators do not directly control cell fate but are nonetheless instrumental in specifying the correct numbers and types of cells in already-specified tissues.

Neural retina is the ground state of the optic vesicle

The specific ablation of *Sox2* in the OC leaves upstream regulators untouched. This method allows for the analysis of SOX2 function in neural fate without manipulating extrinsic pathways. "Artificial" ablation of SOX2 in a neural cell type also offers insight into the default nature of neural fate. The data presented in this project suggest that neural is the ground state of the optic vesicle: all OV progenitor cells initially express SOX2, but SOX2 must be down-regulated in order for non-neurogenic CE to form. Therefore, SOX2 essentially represses CE fate. It must be that non-neural inducing cues repress SOX2; otherwise, neural retina will form by "default" mechanisms anywhere SOX2 is present (Figure 4-2). BMPS and WNTS, in addition to being downstream markers of CE, may also function upstream to down-regulate SOX2 (Figure 4-2).

Re-defining default

The term "default" is mis-leading, because it suggests that a particular cell fate will arise in the absence of all inducing cues. "Ground state" may be a more appropriate term to describe the initial fate of a cell, or the identity upon which other cell fates are imposed, often by inhibitory means (as in the case of sex determination). Indeed, both neural and non-neural signals are required for cells to become NR or CE, respectively. The true default nature of the OV

--- the cell type that develops in the absence of both SOX2 and WNT/BMP activity -- appears to be a progenitor cell type that is neither NR nor CE (Chapter III).

The role of WNT signaling in OC development is consistent with its putative role in non-neural fate specification in the mouse epiblast

β-Catenin-mediated WNT signaling in mouse ESCs has been studied from the perspective of the dual function of β-Catenin in cell adhesion and transcriptional regulation. β-Catenin is required for mESC pluripotency, but its transcriptional function appears dispensable for pluripotency (Davidson et al., 2012; Faunes et al., 2013). Whereas β-Catenin mediates pluripotency through regulating the levels of membrane-bound OCT4, it also transcriptionally promotes mesoderm formation (Davidson et al., 2012; Faunes et al., 2013; Lyashenko et al., 2011; Valenta et al., 2011). Mouse ESCs hypomorphic for β-Catenin follow a neural-default developmental pathway, but this could be due to a loss of pluripotency rather than the suppression of neural fate (Rudloff and Kemler, 2012). All together, these studies place canonical WNT signaling as a dual regulator of ESCs: membrane-bound β-catenin promotes pluripotency while nuclear β-catenin promotes mesoderm. Neither of these functions has been shown to operate through direct repression of neural fate or *Sox2* expression in ESCs.

Similarly, in the OC, the transcriptional function of β-Catenin is to specify non-neural retina fate and appears dispensable for neural fate. In the absence of β-Catenin, CE-specific genes are not expressed, but OCPCs still self-renew and are capable of becoming neural retina. Moreover, the presence of β-Catenin does not normally suppress NR fate. Therefore, in the OC, canonical WNT signaling induces non-neural fate in regions where SOX2 is absent; however, it is still unclear whether WNT signaling could act upstream of SOX2 to antagonize NR fate (Figure 4-2). In this scenario, SOX2 would work in a feedback loop to repress WNT targets in the

prospective NR. The delay in the up-regulation of *Axin2* upon SOX2 ablation suggests that there is an additional mechanism that represses β-Catenin target gene expression. It is possible that this occurs through WNT antagonists, like *Sfrp2*, which are expressed in the prospective NR. However, it would be most informative to use a reporter of WNT activity, such as the *Axin2-LacZ* transgene, as a more sensitive readout of WNT activity upon SOX2 ablation. Nonetheless, the results of this study are consistent with the known role of WNT signaling in mammalian neural induction: β-Catenin transcriptional activity specifies non-neural plate/non-neural retina fates but does not directly repress neural fate.

SOX2 is the neural determinant in the mouse optic cup

In the human epiblast, PAX6 is the earliest known marker of neural plate, whereas in the mouse, the earliest known marker of neuroepithelium is SOX1 (Pevny et al., 1998; Zhang et al., 2010). In the mouse OC, SOX2 becomes restricted to the prospective NR domain and is excluded from non-neuroegenic regions, including the CE and RPE, making it the earliest known specific marker of NR. In contrast, PAX6 is expressed in all three regions of the OC, and high PAX6, especially in the absence of SOX2, is associated with non-neurogenic fate (Chapter II). Therefore, SOX2, not PAX6, is the neural determining factor in the OC. Indeed, as in the epiblast, where SOX2 is initially expressed in all cells but later becomes restricted to neurogenic progenitors; so too in the OC, where SOX2 is initially expressed throughout the whole OV but later becomes restricted to the NR.

Clinical Significance

Is there a "Factor X" that rescues neurogenesis in a *Sox2*-deficient retina?

Knocking out *Sox2* in OCPCs and simultaneously re-expressing it from the *Rosa* locus rescues neurogenesis. However, sustained ectopic expression of *Sox2* in CE progenitor cells is

not sufficient to induce neurogenesis (W Heavner unpublished data). These data raise the possibility that SOX2-positive neural epithelium is competent to generate neurons because of the activation of SOX2 downstream targets and/or the absence of inhibitory factors, which are presumably present in the CE or epidermis. Such inhibitory factors could include inaccessible chromatin in the regulatory regions of neurogenic genes and/or the presence of repressive co-factors. The identification of SOX2 targets that induce or inhibit neurogenesis would give insight into how SOX2 confers neural competence and offer clinical significance.

Cell Cycle Inhibition

OCPCs that lose SOX2 continue to cycle and harbor increased or stabilized Cyclin D1. By contrast, in the mouse neural tube, premature loss of SOXB1 factors induces cell cycle exit and aberrant neuronal differentiation. In fact, down-regulation of SOX2 is necessary for neural progenitor cells to exit the cell cycle and differentiate into neurons, and this is accomplished in part at the protein level (Bani-Yaghoub et al., 2006; Bylund et al., 2003). It would be interesting to test whether forcing *Sox2*-ablated cells to exit the cell cycle would induce them to become neurons. It is likely that this experiment would have to be performed early enough (E11-E14) that SOX2-negative cells have not yet begun to follow the CE lineage. Such an early window comprises when their neuronal genes are most likely accessible and capable of being expressed. The results of this experiment would tell whether SOX2-negative cells have a window of competence when they are still capable of generating neurons (and neural-inhibiting pathways have not taken over) and whether prolonged proliferation (inability to exit the cell cycle) contributes to their loss of neural competence.

Neurogenin1 and the inner ear sensory epithelium

The developing ear sensory epithelium is remarkably similar to the developing NR, especially in regard to SOX2 (Neves et al., 2007). *Sox2* is initially expressed in the neural sensory domain of the otic cup but expression is lost in neuroblasts and sensory hair cells. Over-expression of SOX2 in the chick otic cup induces ectopic expression of *Neurogenin1* without concomitant neurogenesis (Evsen et al., 2013). However, over-expression of *Neurogenin1* alone, or *NeuroD1* alone, is sufficient to induce ectopic neurogenesis. The difference in these two experiments is that *Sox2* expression is sustained in the former but down-regulated in the latter. In fact, both Neurogenin1 and NeuroD1 can repress *Sox2* expression via the *Nop1* enhancer (Evsen et al., 2013). These data suggest that *Neuogenin1* is a direct target of SOX2 and contributes to the competence of neuroepithelial cells to generate neurons. And Neurogenin1 works in a feedback loop to down-regulate *Sox2* and allow for cell cycle exit. However, it is still unknown whether ectopic expression of *Neurogenin1* in a normally non-neurogenic (SOX2-negative) cell type, such as the CE, would confer neural competence.

Identification of SOX2-regulated genes in the OC

Despite Neurogenin1 being such a potent inducer of neurogenesis in an already neural-competent cell type, it still remains to be discovered exactly how SOX2 confers neural identity. Over-expression of Neurogenin1 in the SOX2-depleted OC would reveal whether neural competence can be defined by Neurogenin1. To identity other potential direct targets of SOX2 in the OC, we scanned the regulatory regions of 1030 significantly changed genes in the $Sox2^{cond/cond}$; $\alpha P0^{CreiresGFP}$ OC for SOX2/POU binding sites. Given that SOX2 is always found in complex with transcriptional co-factors, the best characterized being members of the POU family of transcription factors, we searched for the 13-bp SOX2/POU cassette 5000 bp upstream

and downstream of the transcription start sites of genes changed at least 2-fold with an FDR of 0.04 or less (Chakravarthy et al., 2008) (Tables 4-1 and 4-2). These gene lists provide a starting point from which to test putative master regulators of neural fate downstream of SOX2.

Is one of these a "magic bullet" for neural fate or does neural competence require a slew of neuronal genes? Two recent reports suggest that SOX2 regulates a larger network of genes than previously thought, primarily binding to distal enhancers in coordination with POU factors – POU5F1 (OCT4) in mouse ESCs and BRN2 (POU3F2) in mouse NPCs (Wenger et al., 2013) (Lodato et al., 2013). This transition of POU binding partners between ESCs and NPCs contributes to SOX2 tissue specificity and may be a mechanism through which SOX2 keeps neuronal genes poised for expression. In fact, SOX2-bound poised enhancers in NPCs include those for genes involved in neuronal differentiation, such *Atoh1*, *Lhx8*, *Id2* and *Id4*; whereas SOX2-bound active enhancers in NPCs are linked to genes involved in the negative regulation of neurogenesis and WNT receptor signaling (Lodato et al., 2013).

A resource for identifying glaucoma-associated genes

The two most enriched gene ontology categories of transcripts up-regulated following *Sox2* ablation are *secretion* and *extracellular matrix*. These functional categories are consistent with the role of the ciliary body in maintaining IOP via Na^+-K^+ exchange pumps and Cl^- channels (Civan and Macknight, 2004). Elevated IOP is a hallmark of glaucoma, and lowering IOP through the reduction of aqueous humour inflow is often used to treat this disease. However, it is still unclear how the ciliary body integrates multiple mechanisms to regulate inflow (Civan and Macknight, 2004). Given that ciliary body epithelial cells expand exponentially upon *Sox2* ablation, our genome-wide screen of up-regulated transcripts in these mutants may reveal previously unidentified genes important for the development and function of the CE. In fact, the

presumptive CE is considered to be functional before it is histologically distinct, because IOP is necessary for the normal growth of the embryonic eye, and the CE produces proteins of the inner limiting membrane and vitreous body (Beebe, 1986; Coulombre, 1957; Coulombre and Coulombre, 1957).

Abnormal development of the anterior segment is often associated with elevated IOP and congenital glaucoma (Gould et al., 2004). Genes that are known to contribute to anterior segment dysgenesis are often linked to elevated IOP in humans and mice (Chang et al., 2001; Davis et al., 2011a; Gould et al., 2004; Mears et al., 1998; Nair et al., 2011; Nishimura et al., 2001; Nishimura et al., 1998; Semina et al., 2001). Our screen of differentially expressed genes in *Sox2*-ablated eyes reveals up-regulation of genes that have been associated with anterior segment dysgenesis and juvenile-onset and age-related glaucoma (Table 4-3). The significance of these data is two-fold: 1) the *Sox2*-mutant OC provides a valuable resource for identifying previously unknown CE genes, and 2) disruption of the function of many of these genes may contribute to glaucoma.

The current state of using gene therapy to treat eye disease

The mature eye provides a valuable model for cellular replacement and regenerative therapies. It is surgically accessible and non-essential for life, and failed tissue grafts can be ablated or removed with relative ease. Moreover, advanced imaging technologies for evaluating the structure and function of the adult eye, and the wealth of basic knowledge of eye field and retinal development, together have enhanced the field of regenerative medicine for the treatment of currently incurable eye disease.

At present, the only viable option for treating loss of retinal cells in the adult eye is cellular replacement therapy. Emerging technologies have shown promise for improving the

vision of patients suffering from diseases affecting the pigmented (RPE) and photoreceptor layers of the retina. The RPE serves as a barrier between the outer retina, where the photoreceptors reside, and the choriocapillaris, the network of capillaries that provides nutrients to the retina. Because the RPE actively maintains the homeostasis of the outer retina, diseases of the RPE can lead to secondary photoreceptor cell loss. The apposition of the RPE and photoreceptor layers creates the surgically accessible subretinal space where cells can be readily transplanted to treat retinal cell loss. Two diseases that affect these layers and may benefit from transplants to the subretinal space include age-related macular degeneration (AMD) and retinitis pigmentosa (RP).

Retinal transplants have been studied for their therapeutic potential since the 1950s when it was demonstrated that fetal rat retina is viable for months after being transplanted to the anterior chamber of the maternal eye (Royo and Quay, 1959). Subsequent studies have shown that full thickness sheets of fetal retina transplanted intraocularly into the adult rodent eye survive, differentiate into retinal cell types, form synapses with host tissue and perhaps improve vision (del Cerro et al., 1991). The first indication that similar results could be obtained in humans was the temporary visual improvement of patients with RP after microaggregate suspensions of human fetal neural retina were injected into the subretinal space (Humayun et al., 2000). It was subsequently shown that injections of intact human fetal retinal sheets with attached RPE could survive and improve the vision of patients with RP or AMD (Radtke et al., 2008; Radtke et al., 2002).

Immune reactivity, insufficient donor material and persistent difficulty establishing graft-host connectivity present challenges to the use of fetal retinal transplants as a common treatment for degenerative eye disease. An alternative to fetal tissue is the use of healthy

autologous RPE transplanted from the peripheral retina to the damaged central retina of patients with AMD (Binder et al., 2004; Binder et al., 2002; Falkner-Radler et al., 2011). However, the surgical morbidity associated with this therapy, the volume of patients in need of RPE transplants and the genetic defects of autologous RPE cells isolated from patients with AMD highlight a need for alternative therapies to treat degenerative eye disease (MacLaren et al., 2007).

Stem cell biology has rapidly advanced the regenerative medicine field. The ability to generate large quantities of multipotent cells, which can then be directed to become any retinal cell type in culture, greatly expands the potential for new cell replacement therapeutics. Retinal progenitor cells (RPCs) are considered to be the active regenerating component of fetal retinal transplants. In 2004, Young and colleagues demonstrated that RPCs from mouse neonates can self-renew culture, develop into photoreceptors and improve the visual light response after being injected into the subretinal space of adult mice suffering from retinal degeneration (Klassen et al., 2004). Another study suggested that immature rod precursor cells, as opposed to multipotent RPCs, preferentially integrate into the host retina, form synaptic connections and improve visual function in mouse models of retinal degeneration (MacLaren et al., 2006).

This landmark finding that donor cells must be differentiated in order to incorporate into the host retina anticipated future studies that used embryonic stem cells to generate photoreceptor precursors for transplantation. Indeed, both mouse and human embryonic stem cells (ESCs) can be directed to form retinal neurons or RPE in culture, and these differentiated cells improve vision when injected into mouse models of retinal degeneration (Haruta et al., 2004; Ikeda et al., 2005; Kawasaki et al., 2002; Lamba et al., 2009; Lu et al., 2009; Osakada et al., 2008). However, as with fetal retinal transplants, issues of donor-host compatibility and ethics

of tissue isolation have spurred investigation into the utility of adult stem cells and induced pluripotent stem cells for cellular replacement therapy.

Potential adult stem cells have been identified in the ciliary margin of humans and mice, but their ability to self-renew and give rise to all of the retinal cell types remains unclear (Cicero et al., 2009; Coles et al., 2004; Tropepe et al., 2000). An alternative is the reprogramming of adult somatic cells to pluripotency using retroviral transfection of four embryonic transcription factors, *Oct4*, *Sox2*, *KLF4* and *c-Myc* (Takahashi and Yamanaka, 2006). Like ESCs, these induced pluripotent stem (iPS) cells can be directed to produce RPE, RPCs, and retinal neurons in culture, and have demonstrated therapeutic potential in mouse models of retinal degeneration (Buchholz et al., 2009; Carr et al., 2009; Lamba et al., 2010; Meyer et al., 2009; Tucker et al., 2011). One barrier to stem cell therapy for humans is the potential for cells to de-differentiate in the subretinal space, giving rise to teratomas. However, successive rounds of depletion of undifferentiated cells pre-transplantation have been shown to greatly decrease this risk (Tucker et al., 2011). Another challenge is the potential need to replace the damaged Bruch's membrane, the basement membrane of the RPE, to provide a scaffold for injected cells (Lee and Maclaren, 2011).

In addition to cellular replacement therapy, viral delivery of genes to the subretinal space has demonstrated therapeutic potential for a number of diseases. Leber congenital amourosis (LCA) is a group of rare hereditary retinal dystrophies caused by a mutation in one of more than fourteen genes. Gene therapy has been used to treat human patients with a specific form of LCA caused by a mutation in *RPE65*. After receiving subretinal injections of AAV carrying the human RPE65 gene, LCA patients reported improvements in visual sensitivity (Hauswirth et al., 2008). Similarly, viral delivery of the light-activated chloride pump halorhodopsin to cone

photoreceptor cell bodies can restore light sensitivity was restored in mouse models of RP (Busskamp et al., 2010).

Advances in cellular replacement therapy for the treatment of degenerative eye disease have depended on a deep understanding of the basic science of eye field and retinal development. Indeed, protocols for generating retinal neurons and RPE from pluripotent cells have taken into account what is known about the signaling pathways involved in establishing the eye field -- BMP and WNT signaling inhibition and IGF-1 signaling -- and generating specific retinal cell types -- Notch pathway inhibition for photoreceptors, for example (Jadhav et al., 2006; Tucker et al., 2011). Knowledge of the timing and location of tissue-specific gene expression *in vivo* has allowed for the identification and enrichment of specific cell types differentiated from human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) (Vaajasaari et al., 2011). The intersection of basic and clinical science recently materialized in the use of hESC-derived RPE to treat dry AMD and Stargardt's macular dystrophy in humans. The preliminary results of this Phase I/II clinical trial established the safety and tolerability of transplantation of hESC-RPE into the subretinal space (Schwartz et al., 2012). These cells did not appear to hyperproliferate, grow abnormally or cause intraocular inflammation. Moreover, no vision was lost, and there was even some evidence to suggest that vision was slightly improved (Schwartz et al., 2012). The ability to characterize differentiated RPE based on basic science principles, including morphology, gene expression and functional assays, was critical to this therapeutic use of hESC-RPE.

The developing optic cup is an accessible model for understanding SOX2 and disease

The eye has served as an invaluable model for understanding the mechanisms that coordinate human embryogenesis. Developmental genes can be identified readily through

ocular phenotypes, since the eye is not essential for the survival of the organism. Moreover, given that vision is so important for survival, the eye is particularly sensitive to selective pressure and therefore offers insight into the evolution of developmental genes.

Continued characterization of eye development genes and persistent dialogue between basic scientists and clinicians serves as a paradigm for treating degenerative disease. The holy grail of developmental biology today is tissue regeneration. As an absolutely necessary component of neurogenesis and a marker of adult neural stem cells, SOX2 offers a springboard from which to investigate mechanisms that could stimulate neurogenesis in a diseased state. The data presented here confirm that the OC is an appropriate model for studying the role of SOX2 in neural ectoderm determination and investigates two mechanisms whereby SOX2 confers neural competence or specifies neural fate: antagonism of *Pax6* and repression of canonical WNT signaling. The natural next step of this project would be to identify the specific genes necessary for retina neural competence with the long-term goal of using these to treat degenerative diseases.

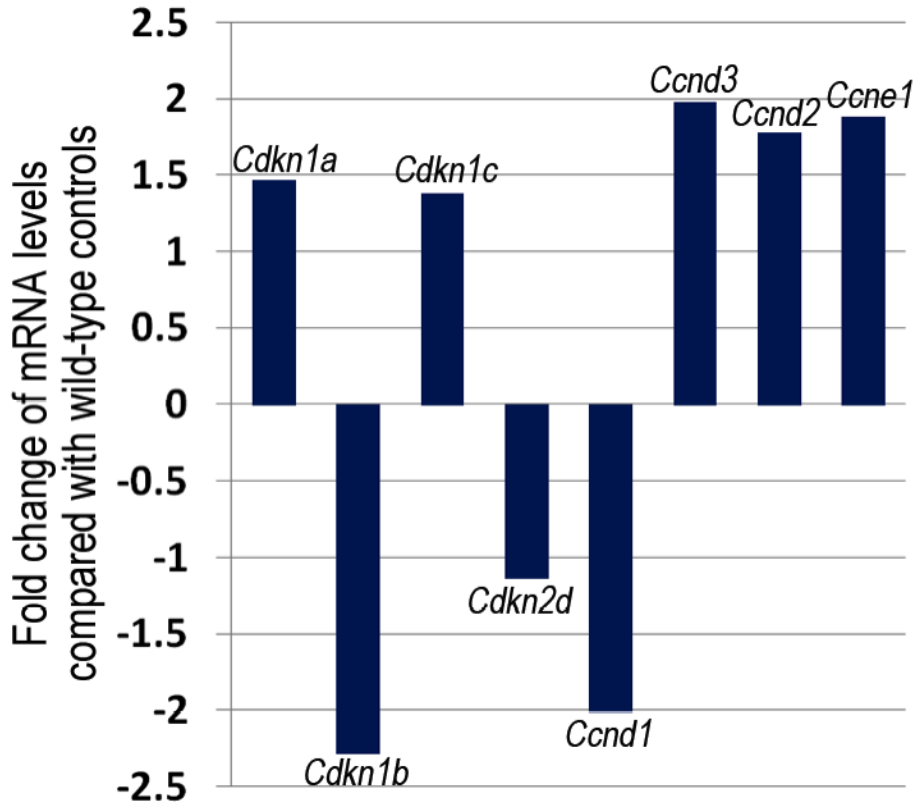


Figure 4-1 Expression of positive and negative cell cycle regulators are changed consistent with a cell fate conversion from NR to CE. The negative cell cycle regulators *Cdkn1b* ($p27^{Kip1}$) and *Cdkn2d* ($p19^{Ink4d}$) as well as the positive cell cycle regulator *Ccnd1* are expressed in the prospective NR. These decrease upon SOX2 ablation. The negative cell cycle regulators *Cdkn1a* ($p21^{Cip1}$) and *Cdkn1c* ($p57^{Kip2}$) as well as the positive cell cycle regulators *Ccnd3*, *Ccnd2* and *Ccne1* are expressed in the prospective CE. These increase upon SOX2 ablation.

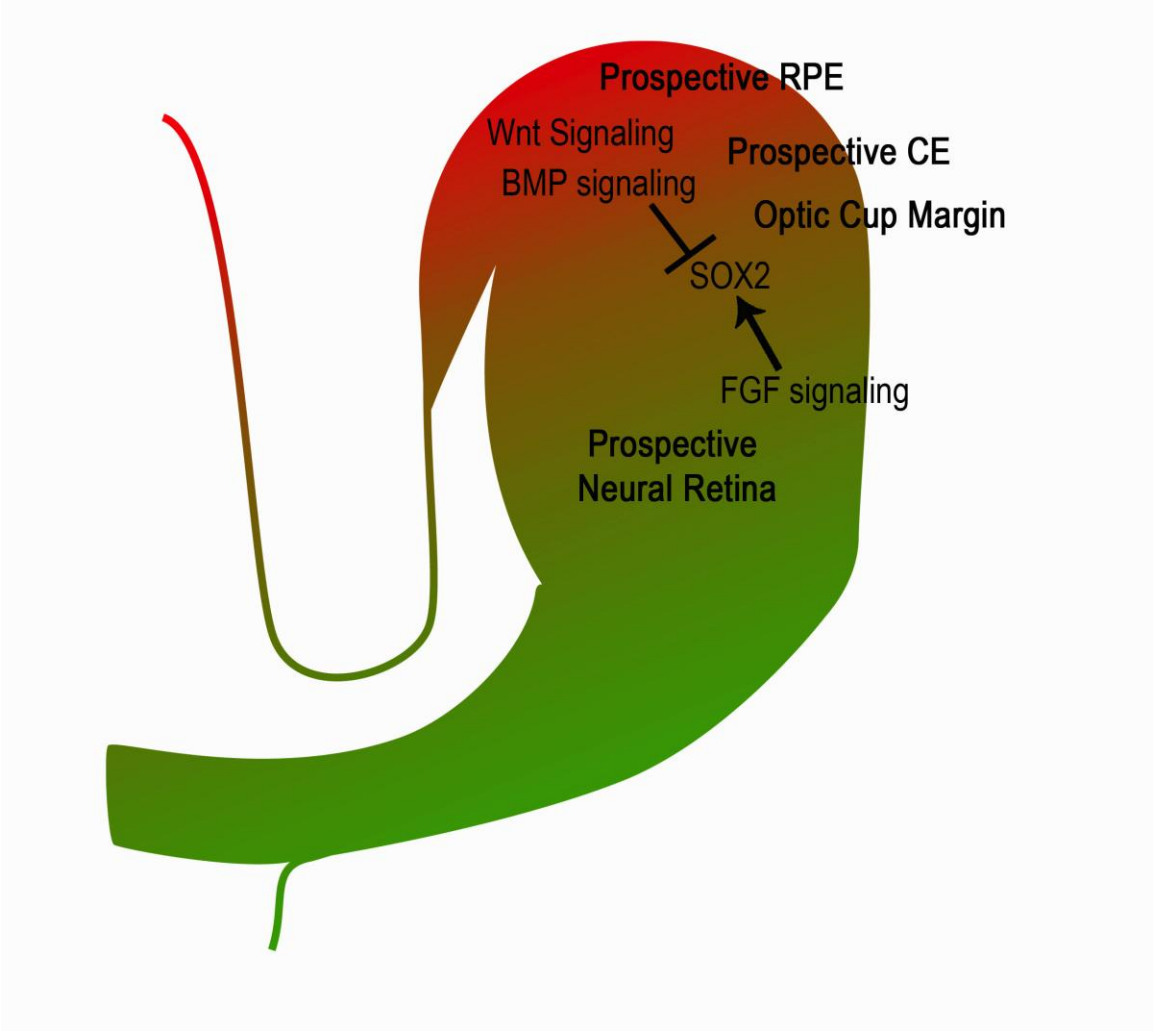


Figure 4-2 Proposal that "neural retina" is the ground state of the OC. Morphogenetic pathways that regionalize the OV converge on the regulation of SOX2. SOX2 is initially expressed throughout the entire OV and may be maintained in the prospective NR by FGF signaling, where it antagonizes non-neurogenic fate. SOX2 must be down-regulated in the prospective CE and RPE in order for these regions to develop as non-NR. WNT and BMP signaling may act upstream of SOX2 to repress this repressor, thus allowing non-NR cell types to be specified.

Gene	window	chromosome	sequence_Start	TSS	strand	regulation	evalue
Evi2a	3	chr11	79341011	79344111	-	up	1.8
Elmo1	5	chr13	20181364	20182464	+	up	1.8
Cpne8	1	chr15	90504819	90509819	-	up	2.8
Smim6	11	chr11	1.16E+08	1.16E+08	+	up	2.8
Frrs1	11	chr3	1.17E+08	1.17E+08	+	up	2.8
Aldh1a1	5	chr19	20675371	20676471	+	up	2.8
Elmo1	1	chr13	20177464	20182464	+	up	4.3
Lgi1	10	chr19	38342921	38339271	+	up	4.3
Olfr1232	3	chr2	89163235	89166335	-	up	4.3
Cldn1	9	chr16	26374625	26371925	-	up	4.3
Cysltr1	9	chrX	1.04E+08	1.04E+08	-	up	4.3
Clcf1	10	chr19	4218041	4214391	+	up	7
Zmym1	11	chr4	1.27E+08	1.27E+08	-	up	7
Lingo2	3	chr4	36895677	36898777	-	up	7
Syngr1	3	chr15	79918663	79921763	+	up	7
Pxmp2	6	chr5	1.11E+08	1.11E+08	-	up	7
Il17d	7	chr14	58144465	58143665	+	up	7
Defb11	8	chr8	23018654	23016904	-	up	7

Table 4-1 Up-regulated transcripts in the *Sox2*-ablated OC that contain a consensus SOX2/POU binding site(s) in the proximal regulatory region.

Gene	window	chromosome	sequence_Start	TSS	strand	regulation	evalue
Nr2e1	1	chr10	42298394	42303394	-	less_down	0.055
Ttyh1	11	chr7	4075734	4071134	+	less_down	0.055
Pcsk2	3	chr2	1.43E+08	1.43E+08	+	down	0.23
Rbp3	11	chr14	34771807	34767207	+	down	0.36
Sox2	10	chr3	34552576	34548926	+	down	0.9
Itgbl1	11	chr14	1.24E+08	1.24E+08	+	down	0.9
Ptpro	2	chr6	1.37E+08	1.37E+08	+	down	0.9
Igfbpl1	7	chr4	45840499	45839699	-	less_down	0.9
Nrxn3	7	chr12	89962119	89961319	+	down	0.9
Zfp114	1	chr7	24955063	24960063	+	down	1.8
Stk32b	2	chr5	38104342	38108392	-	less_down	1.8
Pacsin1	3	chr17	27789526	27792626	+	down	1.8
Chrna4	1	chr2	1.81E+08	1.81E+08	-	less_down	2.8
St6galnac5	1	chr3	1.53E+08	1.53E+08	-	down	2.8
AI593442	10	chr9	52491184	52487534	-	down	2.8
Gnat2	10	chr3	1.08E+08	1.08E+08	+	down	2.8
Otx2	10	chr14	49290831	49287181	-	less_down	2.8
Adcy1	11	chr11	6968091	6963491	+	less_down	2.8
Fmn2	11	chr1	1.76E+08	1.76E+08	+	down	2.8
Nr2e1	11	chr10	42307994	42303394	-	less_down	2.8
Pkib	2	chr10	57347736	57351786	+	down	2.8
Gng3	3	chr19	8910636	8913736	-	down	2.8
Kcnrg	5	chr14	62225193	62226293	+	down	2.8
Dnaic1	7	chr4	41517626	41516826	+	less_down	2.8
Glra2	8	chrX	1.62E+08	1.62E+08	-	down	2.8
Ascl1	1	chr10	86951405	86956405	-	down	4.3
Rtn1	11	chr12	73514526	73509926	-	down	4.3

Robo2	3	chr16	74408057	74411157	-	less_down	4.3
Cplx2	4	chr13	54470562	54472712	+	down	4.3
Nap1l3	4	chrX	1.2E+08	1.2E+08	-	less_down	4.3
Itgbl1	5	chr14	1.24E+08	1.24E+08	+	down	4.3
Slc1a2	5	chr2	1.02E+08	1.02E+08	+	down	4.3
Sox4	9	chr13	29048251	29045551	-	less_down	4.3
9330159F19Rik	1	chr10	28926448	28931448	+	less_down	7
Celsr3	1	chr9	1.09E+08	1.09E+08	+	less_down	7
Psmf1	10	chr2	1.52E+08	1.52E+08	-	down	7
Ptf1a	10	chr2	19370939	19367289	+	down	7
AA792892	11	chr5	94773373	94768773	+	down	7
Fgf15	11	chr7	1.52E+08	1.52E+08	+	less_down	7
Ing3	11	chr6	21904214	21899614	+	down	7
Tubb2b	11	chr13	34226823	34222223	-	less_down	7
9330159F19Rik	2	chr10	28927398	28931448	+	less_down	7
Glra2	2	chrX	1.62E+08	1.62E+08	-	down	7
Lmtk3	3	chr7	53036216	53039316	+	less_down	7
Dcx	4	chrX	1.4E+08	1.4E+08	-	less_down	7
Gadd45a	7	chr6	66988201	66987401	-	less_down	7
Pnma2	8	chr14	67531794	67530044	+	less_down	7
Cntn1	9	chr15	91782670	91779970	+	less_down	7
Cxcr4	9	chr1	1.3E+08	1.3E+08	-	less_down	7

Table 4-2 Down-regulated transcripts in the *Sox2*-ablated OC that contain a SOX2/POU consensus binding site(s) in the proximal regulatory region.

Gene Symbol	Fold Change	Glaucoma Association
Foxe3	2.92	Loss-of-function causes anterior segment dysgenesis
PRSS56	2.39	Mutated in posterior microphthalmia in humans; causes angle-closure glaucoma in mice
Foxc2	2.09	Loss of function causes anterior segment dysgenesis
Cyp1b1	1.53	Loss-of-function associated with congenital glaucoma
Rassf3	1.52	Duplicated in primary open angle glaucoma
Sparc	1.50	Increased expression in PACG iris
Foxc1	1.37	Loss of function causes anterior segment dysgenesis
Nphp1	1.36	Duplicated in primary open angle glaucoma
Bmp4	1.31	Heterozygosity causes anterior segment dysgenesis and elevated IOP

Table 4-3 Genes up-regulated upon SOX2 ablation that have been linked to glaucoma, a disease often associated with malformation of the anterior segment.

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