# HOW TO MAKE A ROD PHOTORECEPTOR: THE ROLE OF BZIP TRANSCRIPTION FACTOR NRL IN RETINAL DEVELOPMENT

**by**

**Edwin Oh**

**A dissertation submitted in partial fulfillment**
**of the requirements for the degree of**
**Doctor of Philosophy**
**(Neuroscience)**
**in The University of Michigan**
**2008**

**Doctoral Committee:**

**Professor Anand Swaroop, Chair**
**Professor Margit Burmeister**
**Professor James Douglas Engel**
**Associate Professor Thomas M. Glaser**
**Associate Professor David L. Turner**
**Assistant Professor Scott Barolo**

**For my family:**
**Lawrence, Nancy, Jeffrey, Esther and Eyleen**

## ACKNOWLEDGEMENTS

During this long journey to complete my thesis, I have had the pleasure to work with many wonderful people. My thesis advisor, Anand Swaroop, is the most important person who has guided me along my path. He has provided a rich environment to explore many exciting questions. My thesis committee, Doug Engel, Tom Glaser, Dave Turner, Scott Barolo, and Margit Burmeister, has always been ready to lend a hand with helpful suggestions and constructive criticism. Their input in my thesis work helped get much of the material published, and I am especially grateful that Doug Engel and Tom Glaser wrote so many recommendation letters.

The Neuroscience Program office was very supportive throughout my time in Ann Arbor. If it were not for Rich Hume, I would not be a graduate student at the University of Michigan. His belief in my "potential" gave me the opportunity to move from Singapore and change my way of life within a span of 2 weeks. Peter Hitchcock is also another important person who has provided guidance and encouraged me to apply for a number of Neuroscience awards. I am grateful that Charma Shoemaker, Valerie Smith and Linda Wilson were always there at the Program office to answer my questions.

During my time in the lab, many people within the Swaroop lab have provided guidance and shared their experiences: Hong Cheng guided me in my first staining procedure of a retinal section, James Friedman taught me how to carry out a luciferase experiment, Hemant Khanna shared his skills on EMSAs and protein purification, Ritu Khanna and Matthew Brooks provided assistance on how to carry out a microarray experiment and to analyze the results, Jingyu Yao taught me how to carry out subretinal injections. Also, Sufiya Mandal and Shirley Cohen have provided assistance towards some projects. I am especially grateful that Lin Jia and Cara Elmer played a major role in developing several projects. Sharyn Ferrara has been extremely helpful throughout my time in the lab. She facilitated many projects and provided advice on many issues.

Though I made it a point to focus on my work during my years in Ann Arbor, I always needed to find an outlet for some excitement. Fortunately, this came about in the form of joining various UM intramural and Ann Arbor volleyball leagues. I met many wonderful people during this time: Johanna Abend, Will Brant, Steph Chien, Jeffrey Fogel, Adam Hartigan, Phil Irawan, Abby Thornbladh, and the Underdogs people.

Funding for my education and travel to several conferences has come through a number of sources. I thank the Neuroscience program, Rackham, and the Midwest Eye-Banks and Transplantation Center for funding three years of study. I also thank Gordon Research Conferences, FASEB, ARVO, and the Jackson laboratories for travel awards.

Finally, my family has helped push me on despite the many times I wanted to consider other options during my thesis. My army brothers, Alvin, Desmond, Heng, and Javier have always supported and motivated me. My parents, brother and sister always believed in me and Eyleen has provided the unconditional love and support I have needed during this long journey.

## TABLE OF CONTENTS

## LIST OF FIGURES

**Chapter IV:**



**Chapter V:**



**Chapter VI:**

## LIST OF TABLES

**CHAPTER III:**

## ABSTRACT

During the development of the mammalian retina, neuroepithelial progenitors are proposed to pass through a series of competence states and give rise to different cell types on a predictable schedule. The mechanism(s) by which multipotent progenitors respond to intrinsic factors and/or extrinsic signals and differentiate into mature neurons are poorly understood. The neural retina leucine zipper protein (NRL), a bZIP transcription factor of the Maf subfamily, is a key regulator of developmental processes in the mammalian retina. The goal of my dissertation is to investigate how NRL modulates photoreceptor cell fate decisions.

In the first part of my dissertation, I tested the hypothesis that NRL plays an instructive role in determining rod photoreceptor fate. To accomplish this, I generated a series of transgenic mouse lines that ectopically express NRL in early post-mitotic photoreceptor precursors or in presumptive cone photoreceptors using the *Crx* or the *S-opsin* promoter, respectively. My studies reveal that expression of NRL results in complete transformation of cone precursors to functional rod photoreceptors. In addition, rod photoreceptors — in the absence of cones — can recruit cone-specific

circuitry. This research provided direct *in vivo* evidence of post-mitotic developmental and synaptic plasticity in the developing mammalian retina.

To investigate the epistatic relationship between NRL and NR2E3, an orphan nuclear receptor that appears to be downstream of NRL in transcriptional hierarchy, I generated a transgenic mouse line in which cone precursors express NRL but not NR2E3. This new line also provided a novel tool to examine gene profiles from rods and cones. Interestingly, ectopic expression of NRL can partially repress S-opsin, but not M-opsin, expression even in the absence of NR2E3. These and additional studies suggest that, while the decision of rod versus cone cell fate is dependent on NRL, simultaneous expression of NRL and NR2E3 is required to promote functional rod differentiation and to suppress cone development.

As both rods and cones connect to horizontal cells (HCs) in the retina, we wanted to understand whether the differentiation of HCs is affected by the absence of afferent neurons by examining *Nrl*$^{-/-}$ (rodless) and *Crxp-Nrl* (coneless) mice. Our studies reveal that HCs can develop in the absence of either photoreceptor type; however, the morphology of axon terminal system or dendritic field of the HC is defined respectively by rods or cones.

Finally, in the last part of my dissertation, I wanted to dissect the functional differences among Maf family proteins. I used the *Nrl* promoter to examine whether the expression of NRL or related large Maf proteins in the retina could rescue the *Nrl*$^{-/-}$ phenotype. As predicted, NRL expression in the rod precursors resulted in the expression of rod-specific markers, absence of

S-opsin staining, and normal electrophysiological responses to light. However, ectopic expression of related Maf family proteins in rods could only induce rhodopsin expression *in vivo* and did not suppress S-opsin expression.

The major conclusions of my studies are that NRL plays an instructive role in rod photoreceptor development and that photoreceptor precursors are competent to make binary decisions in acquiring rod versus cone identity.

# CHAPTER 1

# INTRODUCTION

With an aging population, retinal and macular degeneration are now increasingly common blinding eye diseases that afflict millions of people worldwide. The incidence of these conditions is projected to triple over the next 25 years as the "baby boom" generation ages (www.nei.nih.gov). At present, little effective treatment exists to prevent the progressive loss of vision and even lesser possibility of recovering the lost vision. These degenerative diseases have a central effect on the death of photoreceptors, a retinal neuron that is critical for sensing light and forming an image. Below, I review the current status of research in retinal biology, with an emphasis on several key transcription regulatory factors that are involved in the generation of rod and cone photoreceptors.

## Structure and function of the eye and the retina

The highly ordered set of neuronal connections that characterize the adult visual system arises gradually during development. By the time the eyes open, the neural tube (neuroectoderm), mesoderm and the surface

ectoderm develop into the retina proper and its associated pigment cell layer, the corneoscleral and uveal tunics, and the lens, respectively (Pei and Rhodin, 1970). In the mouse, the first detectable anatomical structures that give rise to the retina are the optic pits, which are depressions in the anterior neural plate on day 8 of gestation (Pei and Rhodin, 1970). The optic pit develops into the optic vesicle that comes into direct contact with the overlying surface ectoderm to induce the formation of a lens placode from the surface ectoderm. Invagination of the optic vesicle leads to formation of the optic cup and lens vesicle on day 10 (Figure I-1). As the optic cup develops, the outer layer of the cup becomes the retinal pigmented epithelium and the inner layer of the cup develops into the neural retina. Retinal neurons are formed in a conserved sequential order with extensive overlap (Cepko et al., 1996; Livesey and Cepko, 2001). The retinal ganglion cells, cone photoreceptors, horizontal cells, and amacrine cells are among the first neurons born during prenatal development (Carter-Dawson and LaVail, 1979; Rapaport et al., 2004; Young, 1985). The amacrine and ganglion cells extend processes into the developing inner plexiform layer so that at birth — in rodents — the retina consists of a large outer neuroblast layer (ONBL) and an inner region of more mature cells. Bipolar and Muller glial cells are produced in the early postnatal days, while rods are born both during prenatal and postnatal developmental periods (Carter-Dawson and LaVail, 1979; Rapaport et al., 2004; Young, 1985). By the end of the first postnatal week, the outer retina has clearly divided into the outer nuclear layer of photoreceptors and

an inner nuclear layer of bipolar, horizontal, amacrine, and Müller cells. During the second postnatal week, photoreceptors and ganglion cells connect to interneurons in the plexiform layers through a process called synaptogenesis (Figure I-2).

In the vertebrate retina, rods and cones are the cells that transduce visual information into neural signals. While cones are responsible for color and high acuity vision, rods are essential for night vision as they are more sensitive to light than cones. In most vertebrates, rods outnumber cones, and in rodents this difference is as large as 32:1 (Carter-Dawson and LaVail, 1979; Jeon et al., 1998). Rod photoreceptors are remarkable cells that not only possess all the molecular machinery necessary for generating the light response, but also contain systems for adjusting light sensitivities in accord with the level of background illumination (Nickle and Robinson, 2007). The light response is first initiated by the capture of photons by a complex of rod or cone opsins and 11-cis-retinal. Rhodopsin is the rod opsin and has a peak absorption at 500 nm (Nathans and Hogness, 1983; Nathans and Hogness, 1984). While primates (including humans) have three cone subtypes, rodents have only two types of cones based on the presence of specific opsin pigment: M-cones containing M opsin (medium wavelength, with an absorption peak at 530 nm), and S-cones having S opsin (short wavelength, with an absorption peak at 420 nm) (Ebrey and Koutalos, 2001; Nathans et al., 1986). Although various steps in the phototransduction cascade have been well-characterized, most of the research in this area is based on studies

of rod photoreceptors because of their abundance in most experimental animals.

The activation phase of the phototransduction cascade begins when a photon is absorbed by rhodopsin, specifically its chromophore, 11-cis-retinal. Light exposure causes the isomerization of 11-cis-retinal to all-trans-retinal, which then stimulates the exchange of GDP to GTP in the heterotrimeric G protein transducin. The active form of transducin (Tα) then dissociates from rhodopsin and activates cGMP phosphodiesterase (PDE), another component of the phototransduction cascade. In rods, PDE consists of two equally active catalytic subunits, α and β, and two inhibitory γ subunits. Upon activation, the γ subunits are released permitting the PDE α and β subunits to hydrolyze cGMP to 5'-GMP. A decrease in cGMP levels results in the closure of cGMP-gated channels and hyperpolarizes the cell. The resulting decrease in the steady inward 'dark current' ultimately leads to a decrease in the tonic release of the neurotransmitter glutamate from presynaptic terminals. This final step results in the completion of activation phase. Recent studies suggest that this cascade is shut off and cGMP is resynthesized during the recovery of the photoresponse [reviewed in (Burns and Baylor, 2001; Calvert et al., 2006; Hamer et al., 2005; Pepe, 2001)] (Figure I-3).

## Retinal diseases that affect photoreceptors

Age-related maculopathy (ARM) is the leading cause of severe visual impairment in the elderly population of industrialized countries. There are two

broad types of age-related macular degeneration (AMD): “dry” and “wet.” Approximately 90% of the cases of macular degeneration are the “dry” (atrophic) type. In the “dry” AMD, the deterioration of the retina is associated with the formation of small yellow deposits, known as drusen, in the macular region (www.nei.nih.gov). This phenomena leads to thinning of the macula and results in the death of photoreceptors (Curcio et al., 2000; Curcio, 2001; Reme et al., 2003). In ARM, rod photoreceptors play a major role during the initial progression of the disease; during early stages, rod sensitivity declines and the dark adaptation process is prolonged. Although it is not known why rods die in ARM, several laboratories are trying to understand the role of apoptotic rod cell death in the pathogenesis of the disease.

Retinitis Pigmentosa (RP) is another disease that involves photoreceptor degeneration (www.nei.nih.gov). This disease is characterized by the death of rods, beginning in the mid-periphery and gradually advancing towards the macula. Cone-Rod Dystrophy (CRD) is similar to retinitis pigmentosa in that a decreased visual acuity is observed in the early stages followed by loss of peripheral vision. However, the cone photoreceptor function in CRD patients is first affected, followed by rod function (Birch and Anderson, 1990). There is no early loss of night vision in CRD, and the rate of rod and cone degeneration is fairly close (Birch and Anderson, 1990) (Figure I-4).

## Retinal development

Retinal cell differentiation is initiated in the central optic cup and progresses towards the periphery in a wave-like fashion until reaching the region of the presumptive iris (Hu and Easter, 1999). Retinal progenitors cells (RPCs) are multipotent cells that can give rise to all major types of cells in the retina. The multipotent nature of RPCs was first elucidated in a series of seminal lineage tracing experiments in the embryonic clawed frog *Xenopus laevis* and in the mouse retina (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988). These studies revealed that RPCs retained their ability to generate different cell types up to the final cell division.

The differentiation of RPCs can be influenced by extrinsic signals such as transforming growth factor α (Lillien and Cepko, 1992), epidermal growth factor (Lillien, 1995), sonic hedgehog (Jensen and Wallace, 1997; Wallace and Raff, 1999), nerve growth factor (Frade et al., 1999), brain-derived neurotrophic factor (Frade et al., 1997; Frade et al., 1999), and ciliary neurotrophic factor (Ezzeddine et al., 1997; Hofmann, 1988). RPCs display significant intrinsic changes in their potential to generate different cell types during retinogenesis and exhibit differential response to inductive and mitogenic factors (Cepko, 1999). The intrinsic changes during retinal development have been illustrated by heterochronic co-culture studies that mixed early-born progenitors with late-born cells and vice versa (Alexiades and Cepko, 1997; Morrow et al., 1998). These experiments demonstrated

that cells born early could not be influenced to adopt a late born identity, revealing the relative importance of intrinsic regulation on retinal cell fate. In another significant study, rat retinal progenitors at embryonic day 16–17 (E16–17) were found to develop similarly in serum-free clonal density cultures and in serum-containing retinal explants — in the number of times they divide, the cell types they generate, and the order in which they produce different cell types (Cayouette et al., 2003). Thus, intrinsic mechanisms clearly play a significant role in cell fate decisions in the developing retina.

In an effort to explain retinal cell fate determination, a competence model (Figure I-5) was proposed to integrate recent findings (Cepko et al., 1996; Livesey and Cepko, 2001). In this model, retinal progenitors are predicted to pass through a series of competence states and give rise to specific cell types. It is thought that competence, or the ability to produce an exact cell type, is intrinsically defined and thus cell fate choices can only be made at precise times during development. Within a given competence state, the generation of a particular type of cell is regulated by positive and negative extrinsic signals.

A number of transcription factors play important roles in defining the intrinsic state of mitotic retinal progenitors. In particular, homeodomain (HD) transcription factors such as RX (Mathers et al., 1997), PAX6 (Ashery-Padan and Gruss, 2001; Gehring and Ikeo, 1999) and SIX3/6 (Loosli et al., 1999) have been implicated in an evolutionary-conserved regulatory network driving retinal development (Zuber et al., 2003). Their central role was demonstrated

by the ability to promote the formation of ectopic retinal tissue in fish and frog embryos (Bernier et al., 2000; Chow et al., 1999; Kenyon et al., 2001; Loosli et al., 1999; Zuber et al., 1999). CHX10 and PROX1 are HD proteins that regulate the exit of RPCs from the cell cycle. In mice lacking CHX10, a reduction of retinal precursors and an absence of bipolar cells was observed (Burmeister et al., 1996), while PROX1 knockout mice lacked horizontal cells in the retina (Dyer et al., 2003). In addition to HD transcription factors, the role of basic helix-loop-helix (bHLH) proteins has also been described. Studies on MASH1 (Tomita et al., 2000) and MATH3 (Tomita et al., 2000) have demonstrated that the dual expression of these proteins is essential for the specification of bipolar cells in mice. MASH1 or MATH3 mouse mutants show little to no phenotype in the visual system, yet when the two were intercrossed to create compound animals, bipolar cells were completely absent (Hatakeyama et al., 2001; Tomita et al., 2000). This phenotype was accompanied by an increase in Müller glia cell numbers, suggesting that bHLH proteins play a key role in cell specification and bipolar cell fate determination.

Newly-generated post-mitotic neurons must induce a number of genes necessary for the acquisition of mature functional phenotype. Transcriptional regulators that influence cell fate have been characterized in photoreceptors. The expression of HD factor CRX is closely correlated with the postmitotic differentiation of photoreceptor cells (Chen et al., 1997; Furukawa et al., 1997). In mice deficient for *Crx*, rod and cone photoreceptor cells are

generated but fail to form outer segments (Furukawa et al., 1999). CRX can activate the expression of several phototransduction genes in both rods and cones by potentially recruiting histone acetyl-transferase (HAT)-containing co-activators and promoting histone acetylation (Chen et al., 1997; Peng and Chen, 2007). Interestingly, human mutations in CRX lead to photoreceptor-related retinopathies, such as cone-rod dystrophy-2 and retinitis pigmentosa (Freund et al., 1997; Sohocki et al., 1999; Swain et al., 1997; Swaroop et al., 1999). Nevertheless, CRX appears to be important for photoreceptor maturation and function, but it is not essential for the initial specification of rod or cone photoreceptor fate (Furukawa et al., 1999).

Nuclear receptor proteins also play an important role in the retina by regulating cone photoreceptor maturation. Expression of thyroid hormone receptor TRβ2 is critical for the development of M-cones and down regulation of *S-opsin* gene expression (Ng et al., 2001; Roberts et al., 2006). Transient expression studies revealed that TRβ2 activated M-opsin expression and down regulated S-opsin in the presence of thyroid hormone. Similarly, the loss of TRβ2 resulted in an absence of M-cones and a concomitant increase in S-cones. Retinoid-related receptor γ (RXRγ) is another negative regulator of S-cones (Roberts et al., 2005). RXRγ expression is localized to developing cone photoreceptors, and RXRγ knockout mice revealed an increase in S-opsin throughout the retina (Roberts et al., 2005). Although RXRγ does not have a role in M-cone differentiation, it appears that RXRγ and TRβ2 heterodimerize to suppress S-cone development.

A key transcription factor, NR2E3 was originally identified by its homology to the orphan nuclear receptor tailless (TLX or NR2E1) and demonstrated to regulate photoreceptor development (Akhmedov et al., 2000; Chen et al., 1999; Haider et al., 2000). In mice, the expression of NR2E3 begins around E18 in postmitotic cells and peaks during rod differentiation at around P6 (Cheng et al., 2004). A rod-specific expression pattern was observed in mouse and human cryosections using NR2E3 antibodies (Cheng et al., 2004; Bumsted O'Brien et al., 2004). Insights into NR2E3 function first came with the discovery of a naturally occurring mouse mutant retinal degeneration 7 (*rd7*) that lacked functional NR2E3 (Akhmedov et al., 2000). The mutant mice carry a 380 base pair (bp) deletion due to a splicing defect resulting from an L1-retrotransposon insertion (Chen et al., 2006). Interestingly, the *rd7* mice have features associated with enhanced function of S-cones. *NR2E3* gene mutations were discovered in patients with enhanced S-cone syndrome (ESCS) (Haider et al., 2001). The expression pattern and function of NR2E3 is similar to that of neural retina leucine zipper protein (NRL). In a subsequent section, I will elaborate on this important transcription factor.

**Maf family members**

The proteins of the Maf sub-family of basic motif leucine zipper (bZIP) transcription factors show homology to the v-Maf oncoprotein that was originally identified in the genome of the AS42 chicken musculoaponeurotic

sarcoma retrovirus (Kawai et al., 1992; Nishizawa et al., 1989). Maf proteins have been cloned from many organisms including human, mouse, frog, chick, and zebrafish. The common domains among these proteins include a highly conserved basic region and a bZIP domain. Proteins of the Maf sub-family are divided into two groups: small Maf and large Maf (Figure I-6), based on their structure. The small Maf proteins (MafK, MafF, MafG, MafT and MafS) lack a transactivation domain and act as repressors when they form homodimers (Igarashi et al., 1995; Kataoka et al., 1995). Small Maf proteins can also act as activators of genes after forming heterodimers with Cap'n'collar (CNC) or Bach family bZIP proteins, and regulate erythroid-differentiation and oxidative stress responses (Motohashi et al., 1997). The large Maf proteins (v-Maf/c-Maf, MafB/Kreisler/Valentino, MafA/L-Maf/S-Maf1, S-Maf2/Krml2, NRL and DMaf/Traffic Jam) have a conserved amino-terminal domain that provides a trans-activation function (Kataoka et al., 2002; Kurschner and Morgan, 1995; Li et al., 2003; Swaroop et al., 1992; Wang et al., 1999). This domain is rich in Asp (D), Glu (E), Ser (S), Thr (T) and Pro (P) residues, and recent findings suggest that phosphorylations within this domain regulate the biological activity of Maf proteins (Han et al., 2007; Swain et al., 2001).

The Maf transcription factors are important regulators of differentiation and modulate diverse processes such as hematopoiesis, lens differentiation, and segmentation of hindbrain (Blank and Andrews, 1997; Ogata et al., 2004; Ogino and Yasuda, 1998; Reza and Yasuda, 2004; Ring et al., 2000). In the

avian lens, three large Maf family proteins (L-Maf, MafB, and c-Maf) are expressed in a sequential manner during development and regulate the expression of major lens-specific protein crystallins (Ogino and Yasuda, 1998). Recently, MafA and MafB have been implicated in regulating the transcription of insulin in pancreatic endocrine cells (Matsuoka et al., 2003; Nishimura et al., 2006; Tsuchiya et al., 2006; Zhao et al., 2005). NRL appears to be the only Maf protein expressed in the mammalian retina and plays a defining role in rod photoreceptor differentiation.

## Cloning and characterization of NRL

In 1992, NRL was cloned by a subtractive hybridization approach (Swaroop et al., 1992) from a human adult retina library. NRL encodes a member of the bZIP family of DNA-binding proteins (Figure I-7) and is highly conserved between mouse and man. Northern blot and in situ hybridization assays demonstrated the abundance of *Nrl* transcripts in the retina (Rehemtulla et al., 1996; Swaroop et al., 1992). Early *in vitro* experiments revealed that NRL could form homodimers and heterodimerize with Maf and several members of the c-FOS and c-JUN family (Kerppola and Curran, 1994a; Kerppola and Curran, 1994b). NRL dimers could bind to the consensus sequence TGCN6-8GCA (*Nrl* response element, NRE). Due to the retina-specific expression of NRL, Swaroop and colleagues looked at the promoter elements of several retina-enriched genes that contained NRE-like sequences. This led to seminal experiments showing the presence of a NRE-

like sequence in the promoter of rhodopsin and its regulation by NRL (Kumar et al., 1996; Rehemtulla et al., 1996). Soon thereafter, it was shown that both NRL and CRX can together act synergistically and activate the expression of rhodopsin (Chen et al., 1997; Mitton et al., 2000; Swaroop et al., 1999).

In an effort to understand whether NRL plays a role in human retinal disease, an analysis of patients with retinitis pigmentosa (RP) was carried out. Initial mutation screenings of RP patients did not reveal disease-causing changes in NRL. In the mean time, linkage analysis of a large autosomal dominant RP (adRP) pedigree revealed the disease locus at 14q11, with a maximum lod score of 5.72 for the marker *D14S64*. Interestingly, *D14S64* resided in the genomic region of NRL, and therefore the three *NRL* exons were screened for mutations in affected members (Bessant et al., 1999). All affected individuals were found to have a T to A change, resulting in a serine to threonine substitution at codon 50 of the NRL protein ($NRL^{S50T}$). This mutation was within the putative transactivation domain, and the authors surmised that these amino acid substitutions could alter the activity, specificity or ability of NRL to interact with other transcription factors. To assess the effect of the S50T mutation, an *in vitro* experiment was conducted; the data showed that mutant $NRL^{S50T}$, in presence of CRX, could significantly enhance the synergistic activation of the rhodopsin promoter compared to the wild-type NRL (Bessant et al., 1999) (Figure I-7). Currently, NRL mutation screens have revealed fourteen missense and three frameshift sequence variations in patients with different retinal diseases (Nishiguchi et al., 2004; Kanda et al,

2007) (Figure I-7). Several of these mutations alter NRL phosphorylation and affect *rhodopsin* promoter activation *in vitro* (Kanda et al, 2007). These findings demonstrate that mutations in NRL can have an effect on the regulation of rhodopsin expression. It will be of great interest to examine how altered rhodopsin expression may directly contribute to photoreceptor degeneration *in vivo*.

In order to understand the function of NRL in the retina, additional *in vitro* experiments have been conducted to discover new downstream targets. The promoter of β-subunit of cGMP-PDE (PDE6B) is reported to contain two functionally relevant control elements, an AP1/ NRE binding sequence and a G/C-rich sequence (Lerner et al., 2001). Interactions of these *cis* elements with NRL and SP1/SP4 DNA-binding proteins, respectively, were characterized *in vitro*. In a related study, both NRL and CRX were shown to bind to and transactivate the α-subunit of cGMP-PDE (PDE6A) promoter (Pittler et al., 2004).

**Role of NRL in photoreceptor development**

The first *in vivo* demonstration of NRL function came from the characterization of the *Nrl* knockout mice (Figure I-8). Mears and colleagues replaced almost entire coding region with a neomycin cassette and determined that mice lacking *Nrl* were viable and fertile (Mears et al., 2001). Upon examining retinal histology, the authors discovered a complete loss of rod photoreceptors. This was confirmed by the analysis of rod specific

transcripts at the RNA and protein level. In place of the rod photoreceptors, an increase was observed in S-cones. Electroretinograms (ERGs) were then recorded to evaluate retinal function. Mice lacking *Nrl* did not respond to flash intensities typical of rod function, while light–adapted ERG b-waves were several times larger. These data were consistent with the histological data and suggested that these mice had enhanced cone activity. Additional ERGs were performed to reveal that the amplitude of S-cone responses was six times larger in $Nrl^{-/-}$ mice. More recently, the S-cones in $Nrl^{-/-}$ mice have been shown to be similar to cones in the wild-type retina by ultrastructural, histochemical, molecular, and physiological studies (Daniele et al., 2005; Nikonov et al., 2005). The $Nrl^{-/-}$ mice could therefore serve as a good model to understand cone biology. These mice have been used for microarray gene profiling to characterize cone photoreceptor specific genes (Corbo et al., 2007; Yoshida et al., 2004; Yu et al., 2004) that could be potential candidates for human retinal diseases.

Another advance in understanding the role of NRL came from the generation of mice expressing green fluorescent protein (GFP) in NRL-expressing cells (Figure I-8). Akimoto and colleagues utilized the *Nrl* promoter to express GFP for several reasons (Akimoto et al., 2006). First, the authors wanted to clarify whether NRL was expressed in mitotically-active or post-mitotic cells. In co-labeling studies with various cell cycle markers, the authors show that GFP was absent in mitotic cells. Thus, NRL was indeed a marker of post-mitotic rods. Second, these transgenic mice were

generated to establish whether S-cones in the *Nrl*$^{-/-}$ mice were derived from rod precursors. This question was addressed by crossing *Nrl*$^{-/-}$ mice to *Nrl-GFP* mice. Subsequent studies of the hybrid line demonstrated that S-cones were in fact GFP positive. The *Nrl-GFP* mice provided a source of rod photoreceptor cells that could be purified using flow cytometry. These cells could then be used for a variety of investigations such as microarray analysis and transplantation studies. Recent advances in the transplantation work in mice have revealed that GFP-positive rod precursors can in fact integrate in a degenerating retina, differentiate into rhodopsin expressing rod cells, and exhibit some photoresponse (MacLaren et al., 2006).

The important data generated by Mears and colleagues that NRL is necessary for rod differentiation provided the platform to carry out this dissertation. In addition, the availability of the *Nrl*$^{-/-}$ mice and the characterization of the *Nrl* promoter yielded new tools and information to ask whether NRL or any other gene of interest can directly instruct photoreceptor precursors to become rods. I have evaluated these issues and documented my findings in the following chapters:

1. Through gain of function studies, I demonstrate that NRL expression in cone precursors can drive rod differentiation at the expense of cones. In addition, I show that even S-cones can differentiate into rods. A potential mechanism for this plasticity has been explored by testing whether NRL can regulate cone-specific genes. (Chapter II)

2. I have determined whether *Nr2e3* is a direct transcriptional target of NRL and whether NRL, in the absence of NR2E3, is sufficient to convert cones to rods. To isolate cone specific genes, I have generated gene expression profiles of retina from various transgenic lines. (Chapter III)

3. I have examined whether photoreceptors play a role in defining horizontal cell birth and morphology. In addition, I have carried out quantitative PCR experiments using P5 and P10 retinal tissue from coneless mice to examine genes that may be implicated in specific cellular pathways. (Chapter IV)

4. Using transgenic mice, I have examined whether *Nrl* under the control of *Nrl* promoter can rescue the *Nrl*$^{-/-}$ phenotype. In addition, I have tested whether other large Maf proteins can regulate photoreceptor-specific gene expression *in vitro* and/or *in vivo*. (Chapter V)

**Figure I-1: Eye development.** (A) Scanning electron micrographs of the developing E11 rat embryo optic cup showing the optic vesicles (OV) with a broad attachment to the forebrain vesicle. The midline neural groove has not fully closed at this stage (arrows). (B) At E13, the optic cup (OC) is more obvious and the optic fissure (OF) does not extend into the optic stalk (OS). Illustrations in (C) and (D) highlight early developmental events when indentation of the central region of the optic placode results in the formation of the optic pit. The optic pit develops into the optic vesicle, which comes into direct contact with the overlying surface ectoderm to induce formation of a lens placode from the surface ectoderm. Invagination of the optic vesicle then leads to formation of the optic cup and lens vesicle. (Zhang et al., 2002)

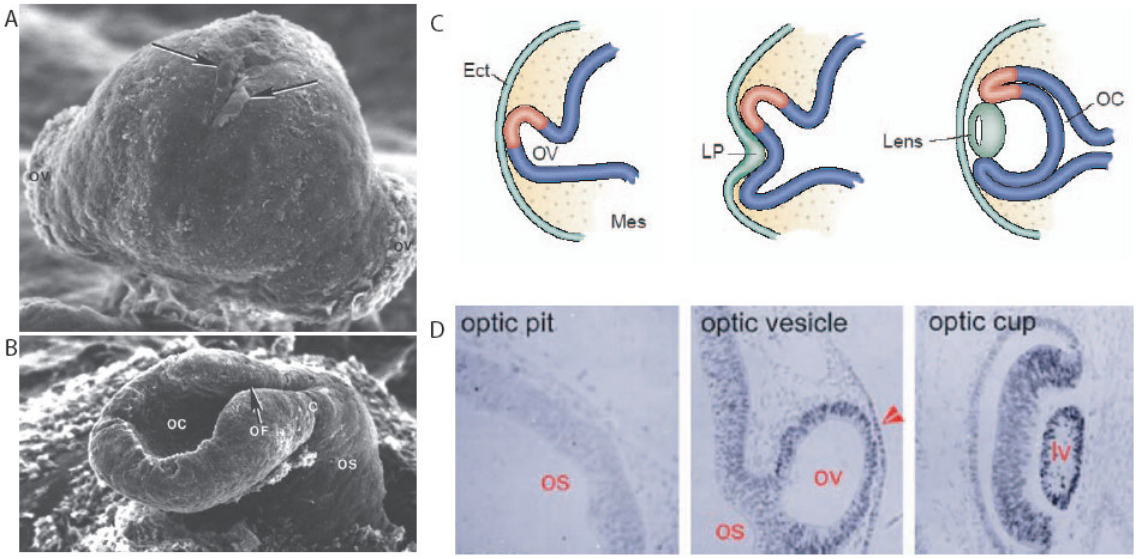


**Figure I-2: The architecture of the retina and major neuronal cell-types in the different layers.** (A) A retinal cross-section from mGluR6-GFP transgenic mouse showing lamination of cell bodies and synaptic terminals. The bipolar cells (green), photoreceptors (magenta), and amacrine and ganglion cells (red) can be readily stained with various antibodies. (B) Schematic illustrating the major neuronal components and their projection patterns in the mature vertebrate retina. The location of rods (R), cones (C), rod bipolars (RB), cone bipolars (CB), horizontal cells (H), amacrine (AII and A), and ganglion cells (G) are marked in the illustration (adapted from the laboratory of Rachel Wong).

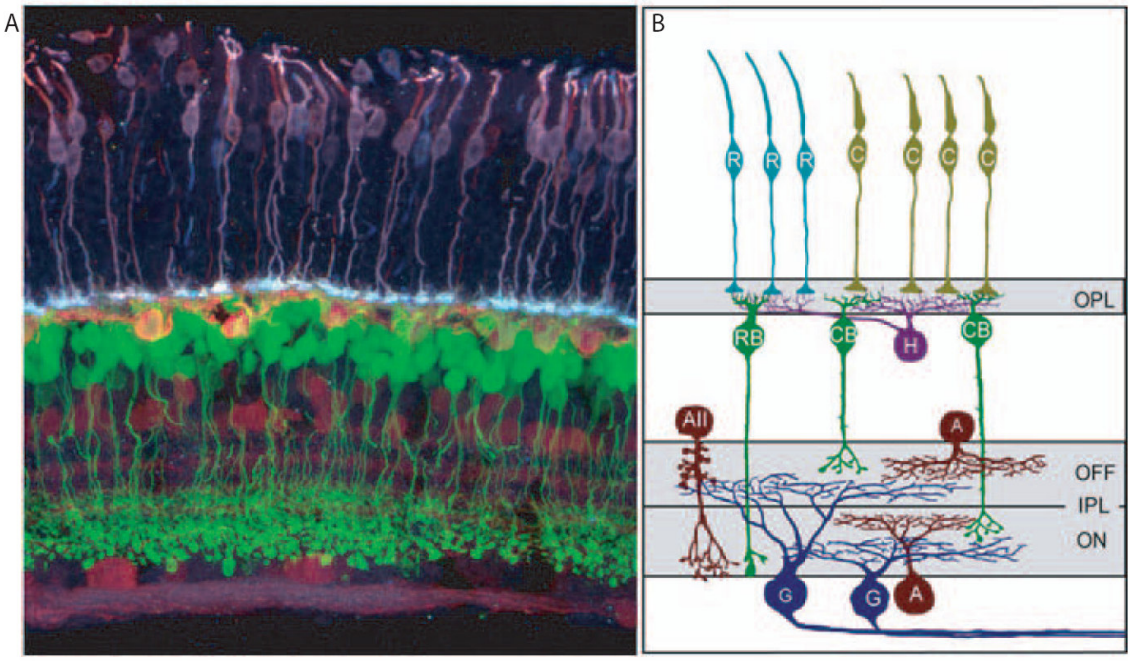


**Figure I-3: The phototransduction cascade.** In the Dark (top): The chromophore molecule, 11-cis-retinal, lies in the pocket formed by rhodopsin (R). Both the G-protein transducin (Gα-GDP-Gβ-Gγ) and phosphodiesterase (PDE6) are in their inactive states; and the intracellular concentration of cyclic GMP is relatively high. cGMP is thus able to bind to and open cyclic-nucleotide-gated (CNG) channels in the plasma membrane, through which Ca2+ and Na+ ions flow into the cell. Activation (bottom): The absorption of a photon isomerizes the chromophore to its all-trans form, and triggers a conformational change of rhodopsin into its activated state (R*). R* then activates transducin by catalyzing the exchange of GDP for GTP, which causes the separation of activated α-transducin (Gα*) from the trimer. Gα* in turn activates the phosphodiesterase enzyme (PDE6*) by exposing a site that catalyzes the hydrolysis of cGMP into GMP. The decreased cGMP concentration results in the loss of cGMP from the CNG channels, which close, blocking the inward flow of Na+ and Ca2+ ions, reducing the circulating electrical current, and hyperpolarizing the membrane voltage (Stockman et al., 2007).

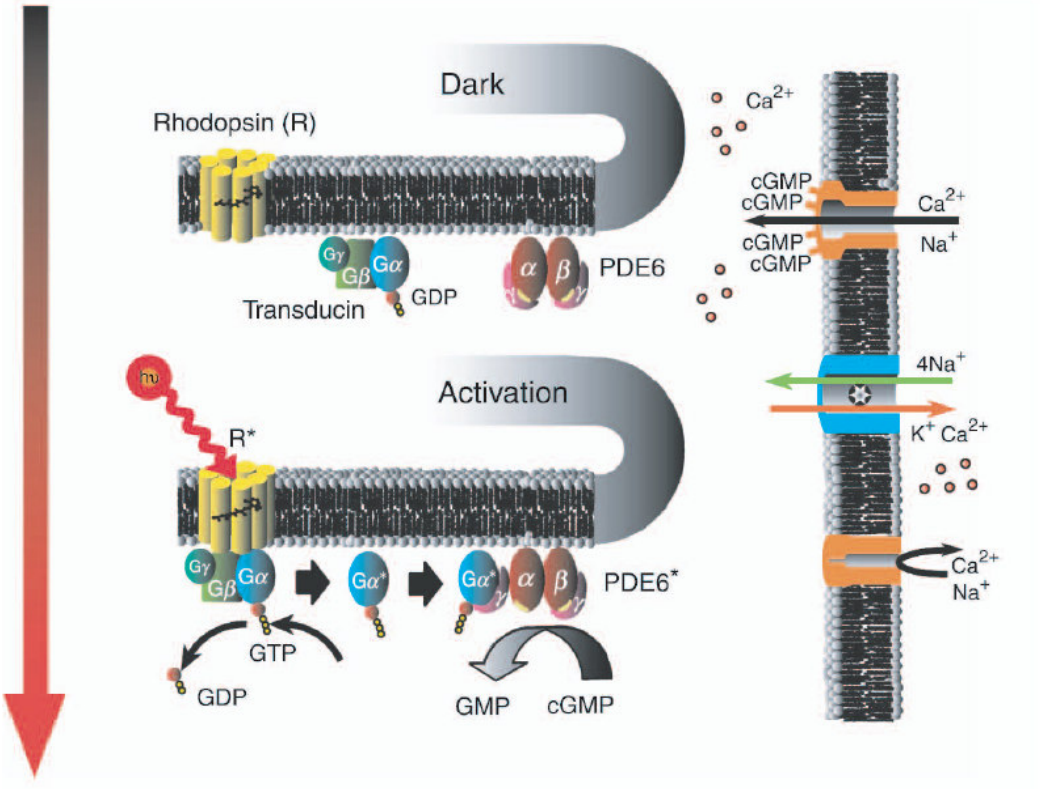


**Figure I-4: Fundus images from human patients with photoreceptor-related diseases.** (A) Figure showing the back of the eye (retinal fundus) in a normal patient. (B) Fundus image from a retinitis pigmentosa patient demonstrates the presence of black pigment flecks in the retina (bone spicules), which is a hallmark sign of this condition. A narrowing of blood vessels and abnormalities of the optic nerve head are also associated findings. (C) and (D) show two clinical presentations of AMD. (C) is taken from a 71 year old male with extensive soft drusen. (D) is taken from a 87 year old woman with geographic atrophy of the retinal pigemented epithelium (figure from www.nei.nih.gov) and (Swaroop et. al, 2007)

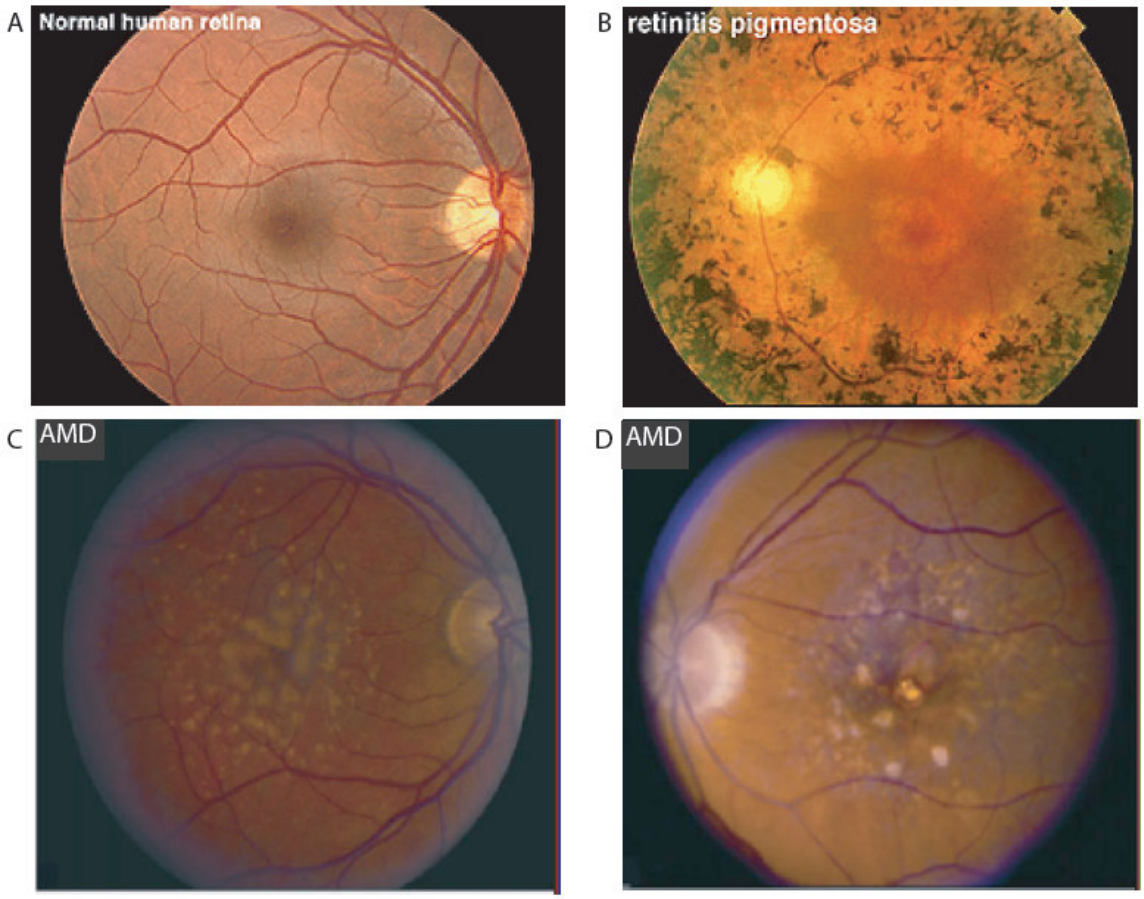


**Figure I-5: The competence model and generation of neurons in the mouse retina.** (A) A progenitor passes through waves of competence, indicated by different colors, during which it is competent to generate only a subset of types of postmitotic cells. A key feature of this model is that cells both acquire and lose the ability to make various cell types. (B) A predicted lineage tree built up by cell divisions of multipotent progenitors over time. The first division shown generates two progenitors, whereas all of the other divisions generate either a progenitor and a postmitotic cell or two postmitotic cells. (C) Predicted timetable when retinal cells are born in the mouse. Ganglion, horizontal, cone and amacrine cells are for the most part born during prenatal stages, while rods, bipolars, and muller glia are predominantly born during the first postnatal week. (Livesey and Cepko, 2001; Marquardt, 2003).

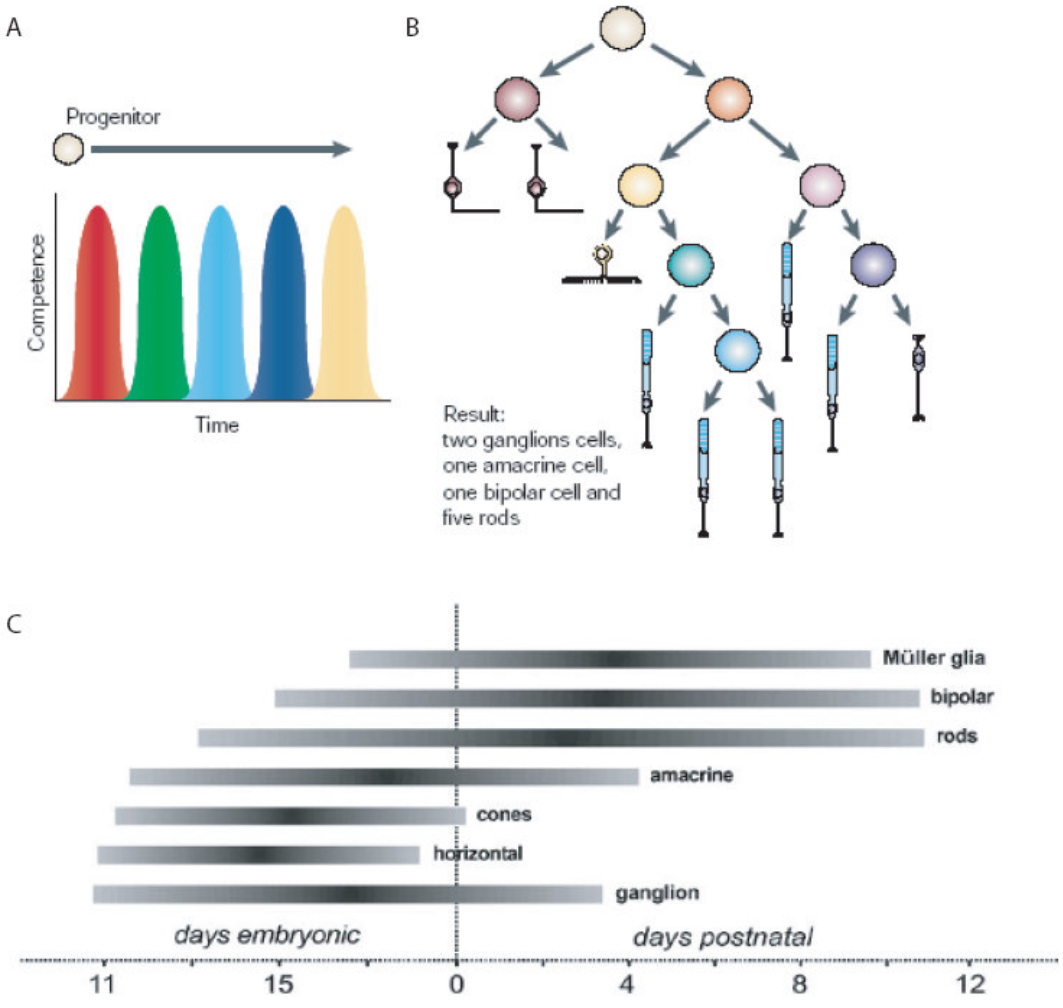


**Figure I-6: The Maf family of transcription factors.** (A) Schematic of the structure and function of small and large Maf proteins. MARE: Maf-recognition element. (B) Members of large Maf family proteins identified in various species (Homo sapiens, Mus musculus, Gallus gallus, Xenopus tropicalis, Danio rerio and Drosophila melanogaster). Note that chicken L-Maf is an orthologue of mammalian MafA, but its function in lens fiber cells to activate crystallin genes seems to be replaced by c-Maf in mammals. (Kataoka, 2007).

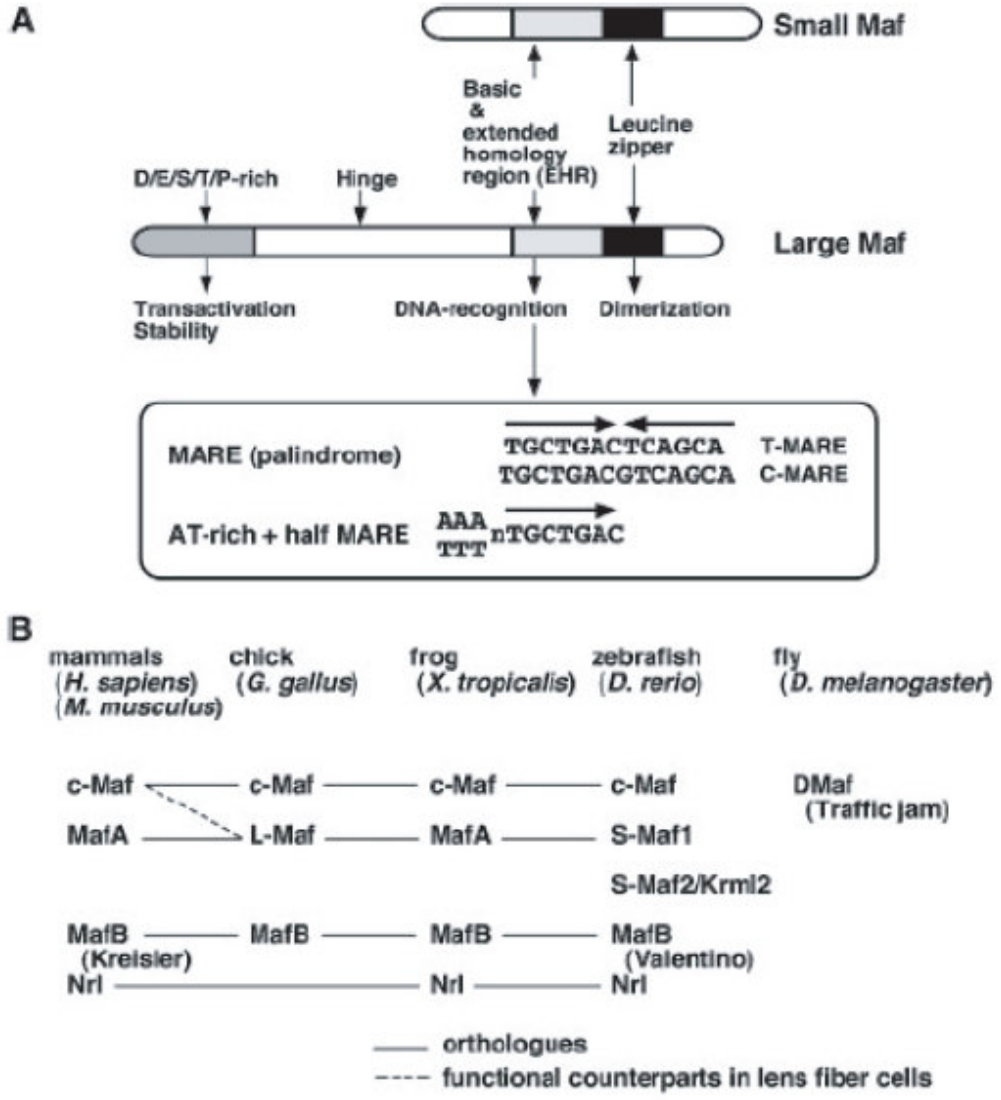


**Figure I-7: Cloning of NRL and analysis of human mutations.** (A) Schematic diagram of the NRL protein, including the minimal transactivation domain (MTD), the basic leucine zipper (bZIP) domain, and the intron/exon structure of the *NRL* gene. The location of 10 different amino acid changes is indicated above the protein structure. (B) Effect of the S50T mutation on NRL-mediated transactivation of r*hodopsin* promoter activity in CV1 cells. Different concentrations of pED-NRL and pED-NRLS50T expression constructs (0.003-0.3 mg) were cotransfected with pBR130-luc (*Rhodopsin* promoter/ luciferase reporter) with and without pCDNA-CRX. An increased transactivation of the r*hodopsin* promoter was observed with NRLS50T compared with NRL. The synergistic transactivation of NRLS50T with CRX was enhanced over that of NRL+CRX (Akimoto et al., 2006; Bessant et al., 1999).

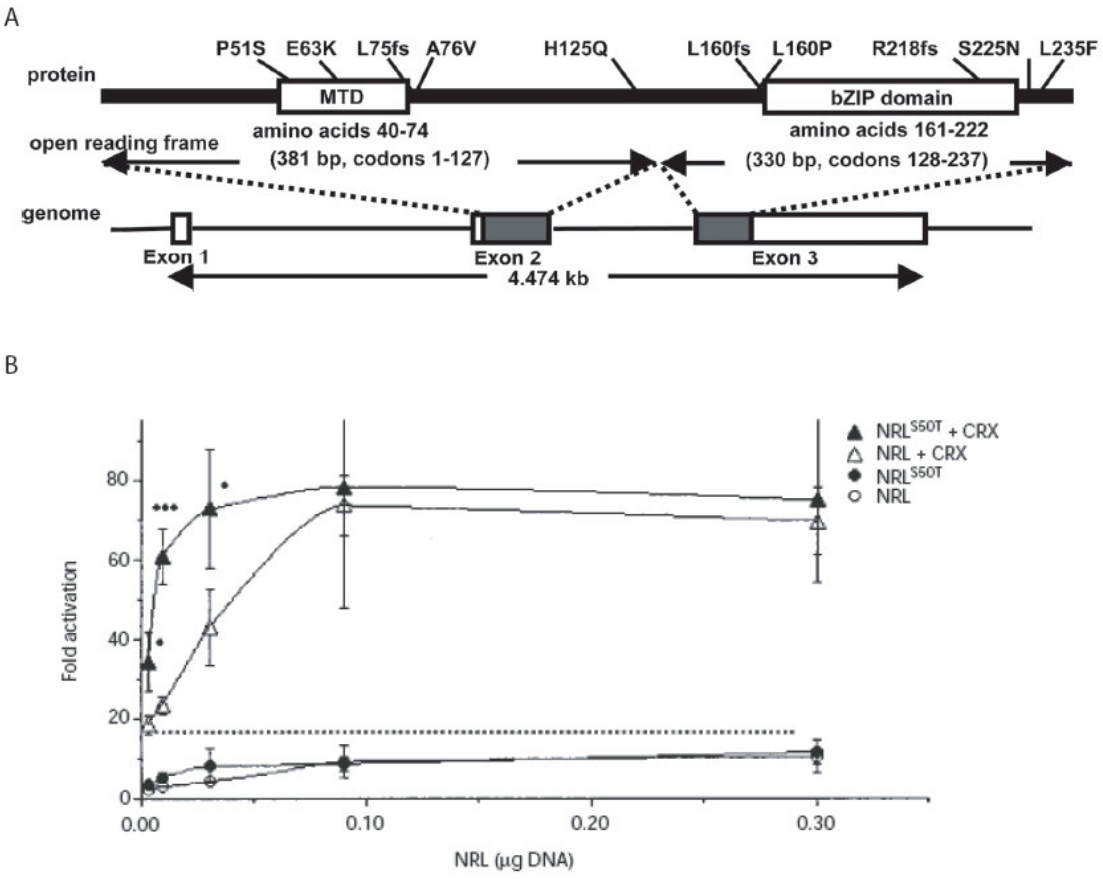
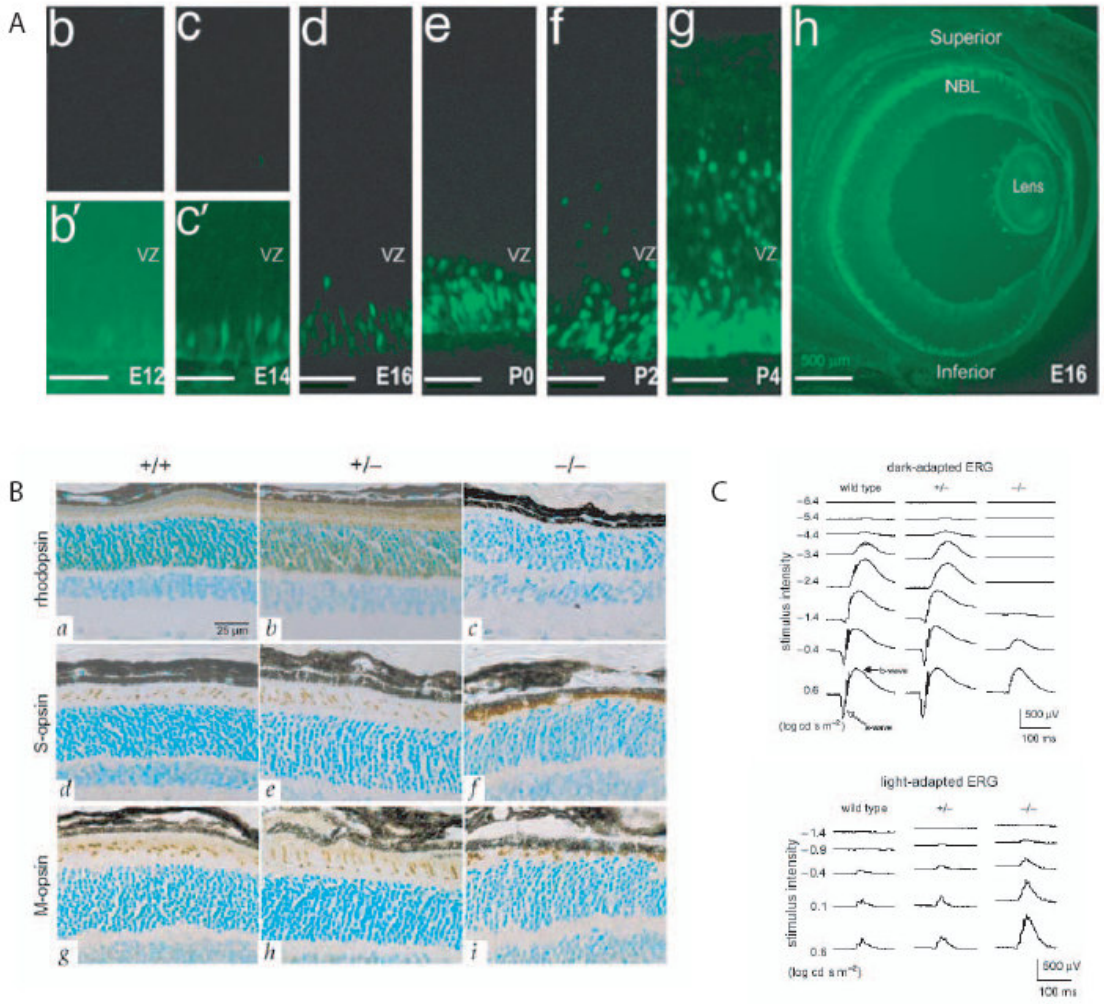


**Figure I-8: Characterization of NRL expression in the retina and *Nrl*$^{-/-}$ phenotype in mice.** (A) Retinal cross sections from Nrl-GFP mice. GFP is expressed under the control of the *Nrl* promoter and is found only in rod photoreceptors. The temporal expression of GFP can be observed from E12 onwards. (B) Retinal cross sections were taken from wild-type (+/+), heterozygote (+/-), and *Nrl*$^{-/-}$ (-/-) mice. There is a lack of rhodopsin staining, and increased S-opsin staining in *Nrl*$^{-/-}$ mice. (C) Electroretinograms demonstrate that the scotopic response (dark-adapted ERG) in *Nrl*$^{-/-}$ mice is impaired, while photopic responses (light-adapted ERG) are enhanced (Akimoto et al., 2006; Mears et al., 2001).



## References


Akhmedov, N. B., Piriev, N. I., Chang, B., Rapoport, A. L., Hawes, N. L., Nishina, P. M., Nusinowitz, S., Heckenlively, J. R., Roderick, T. H., Kozak, C. A.*, et al.* (2000). A deletion in a photoreceptor-specific nuclear receptor mRNA causes retinal degeneration in the rd7 mouse. Proc Natl Acad Sci U S A *97*, 5551-5556.

Akimoto, M., Cheng, H., Zhu, D., Brzezinski, J. A., Khanna, R., Filippova, E., Oh, E. C., Jing, Y., Linares, J. L., Brooks, M.*, et al.* (2006). Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. Proc Natl Acad Sci U S A *103*, 3890-3895.

Alexiades, M. R., and Cepko, C. L. (1997). Subsets of retinal progenitors display temporally regulated and distinct biases in the fates of their progeny. Development *124*, 1119-1131.

Ashery-Padan, R., and Gruss, P. (2001). Pax6 lights-up the way for eye development. Curr Opin Cell Biol *13*, 706-714.

Bernier, G., Panitz, F., Zhou, X., Hollemann, T., Gruss, P., and Pieler, T. (2000). Expanded retina territory by midbrain transformation upon overexpression of Six6 (Optx2) in Xenopus embryos. Mech Dev *93*, 59-69.

Bessant, D. A., Payne, A. M., Mitton, K. P., Wang, Q. L., Swain, P. K., Plant, C., Bird, A. C., Zack, D. J., Swaroop, A., and Bhattacharya, S. S. (1999). A mutation in NRL is associated with autosomal dominant retinitis pigmentosa. Nat Genet *21*, 355-356.

Birch, D. G., and Anderson, J. L. (1990). Rod visual fields in cone-rod degeneration. Comparisons to retinitis pigmentosa. Invest Ophthalmol Vis Sci *31*, 2288-2299.

Blank, V., and Andrews, N. C. (1997). The Maf transcription factors: regulators of differentiation. Trends Biochem Sci *22*, 437-441.

Burmeister, M., Novak, J., Liang, M. Y., Basu, S., Ploder, L., Hawes, N. L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V. I.*, et al.* (1996). Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat Genet *12*, 376-384.

Burns, M. E., and Baylor, D. A. (2001). Activation, deactivation, and adaptation in vertebrate photoreceptor cells. Annu Rev Neurosci *24*, 779-805.

Calvert, P. D., Strissel, K. J., Schiesser, W. E., Pugh, E. N., Jr., and Arshavsky, V. Y. (2006). Light-driven translocation of signaling proteins in vertebrate photoreceptors. Trends Cell Biol *16*, 560-568.
Carter-Dawson, L. D., and LaVail, M. M. (1979). Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. J Comp Neurol *188*, 263-272.

Cayouette, M., Barres, B. A., and Raff, M. (2003). Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. Neuron *40*, 897-904.

Cepko, C. L. (1999). The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. Curr Opin Neurobiol *9*, 37-46.

Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M., and Ezzeddine, D. (1996). Cell fate determination in the vertebrate retina. Proc Natl Acad Sci U S A *93*, 589-595.

Chen, F., Figueroa, D. J., Marmorstein, A. D., Zhang, Q., Petrukhin, K., Caskey, C. T., and Austin, C. P. (1999). Retina-specific nuclear receptor: A potential regulator of cellular retinaldehyde-binding protein expressed in retinal pigment epithelium and Muller glial cells. Proc Natl Acad Sci U S A *96*, 15149-15154.

Chen, J., Rattner, A., and Nathans, J. (2006). Effects of L1 retrotransposon insertion on transcript processing, localization and accumulation: lessons from the retinal degeneration 7 mouse and implications for the genomic ecology of L1 elements. Hum Mol Genet *15*, 2146-2156.

Chen, S., Wang, Q. L., Nie, Z., Sun, H., Lennon, G., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Zack, D. J. (1997). Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron *19*, 1017-1030.

Cheng, H., Khanna, H., Oh, E. C., Hicks, D., Mitton, K. P., and Swaroop, A. (2004). Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors. Hum Mol Genet *13*, 1563-1575.

Chow, R. L., Altmann, C. R., Lang, R. A., and Hemmati-Brivanlou, A. (1999). Pax6 induces ectopic eyes in a vertebrate. Development *126*, 4213-4222.

Curcio, C. A. (2001). Photoreceptor topography in ageing and age-related maculopathy. Eye *15*, 376-383.

Curcio, C. A., Owsley, C., and Jackson, G. R. (2000). Spare the rods, save the cones in aging and age-related maculopathy. Invest Ophthalmol Vis Sci *41*, 2015-2018.

Daniele, L. L., Lillo, C., Lyubarsky, A. L., Nikonov, S. S., Philp, N., Mears, A. J., Swaroop, A., Williams, D. S., and Pugh, E. N., Jr. (2005). Cone-like morphological, molecular, and electrophysiological features of the photoreceptors of the Nrl knockout mouse. Invest Ophthalmol Vis Sci *46*, 2156-2167.

Dyer, M. A., Livesey, F. J., Cepko, C. L., and Oliver, G. (2003). Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. Nat Genet *34*, 53-58.

Ebrey, T., and Koutalos, Y. (2001). Vertebrate photoreceptors. Prog Retin Eye Res *20*, 49-94.

Erkman, L., McEvilly, R. J., Luo, L., Ryan, A. K., Hooshmand, F., O'Connell, S. M., Keithley, E. M., Rapaport, D. H., Ryan, A. F., and Rosenfeld, M. G. (1996). Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. Nature *381*, 603-606.

Ezzeddine, Z. D., Yang, X., DeChiara, T., Yancopoulos, G., and Cepko, C. L. (1997). Postmitotic cells fated to become rod photoreceptors can be respecified by CNTF treatment of the retina. Development *124*, 1055-1067.

Frade, J. M., Bovolenta, P., Martinez-Morales, J. R., Arribas, A., Barbas, J. A., and Rodriguez-Tebar, A. (1997). Control of early cell death by BDNF in the chick retina. Development *124*, 3313-3320.

Frade, J. M., Bovolenta, P., and Rodriguez-Tebar, A. (1999). Neurotrophins and other growth factors in the generation of retinal neurons. Microsc Res Tech *45*, 243-251.

Freund, C. L., Gregory-Evans, C. Y., Furukawa, T., Papaioannou, M., Looser, J., Ploder, L., Bellingham, J., Ng, D., Herbrick, J. A., Duncan, A.*, et al.* (1997). Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (CRX) essential for maintenance of the photoreceptor. Cell *91*, 543-553.

Furukawa, T., Morrow, E. M., and Cepko, C. L. (1997). Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell *91*, 531-541.

Furukawa, T., Morrow, E. M., Li, T., Davis, F. C., and Cepko, C. L. (1999). Retinopathy and attenuated circadian entrainment in Crx-deficient mice. Nat Genet *23*, 466-470.

Gan, L., Wang, S. W., Huang, Z., and Klein, W. H. (1999). POU domain factor Brn-3b is essential for retinal ganglion cell differentiation and survival but not for initial cell fate specification. Dev Biol *210*, 469-480.
Gan, L., Xiang, M., Zhou, L., Wagner, D. S., Klein, W. H., and Nathans, J. (1996). POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells. Proc Natl Acad Sci U S A *93*, 3920-3925.

Gehring, W. J., and Ikeo, K. (1999). Pax 6: mastering eye morphogenesis and eye evolution. Trends Genet *15*, 371-377.

Haider, N. B., Jacobson, S. G., Cideciyan, A. V., Swiderski, R., Streb, L. M., Searby, C., Beck, G., Hockey, R., Hanna, D. B., Gorman, S.*, et al.* (2000). Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. Nat Genet *24*, 127-131.

Haider, N. B., Naggert, J. K., and Nishina, P. M. (2001). Excess cone cell proliferation due to lack of a functional NR2E3 causes retinal dysplasia and degeneration in rd7/rd7 mice. Hum Mol Genet *10*, 1619-1626.

Hamer, R. D., Nicholas, S. C., Tranchina, D., Lamb, T. D., and Jarvinen, J. L. (2005). Toward a unified model of vertebrate rod phototransduction. Vis Neurosci *22*, 417-436.

Han, S. I., Aramata, S., Yasuda, K., and Kataoka, K. (2007). MafA Stability in Pancreatic {beta} Cells Is Regulated by Glucose and Is Dependent on Its Constitutive Phosphorylation at Multiple Sites by Glycogen Synthase Kinase 3. Mol Cell Biol *27*, 6593-6605.

Hatakeyama, J., Tomita, K., Inoue, T., and Kageyama, R. (2001). Roles of homeobox and bHLH genes in specification of a retinal cell type. Development *128*, 1313-1322.

Hofmann, H. D. (1988). Development of cholinergic retinal neurons from embryonic chicken in monolayer cultures: stimulation by glial cell-derived factors. J Neurosci *8*, 1361-1369.

Holt, C. E., Bertsch, T. W., Ellis, H. M., and Harris, W. A. (1988). Cellular determination in the Xenopus retina is independent of lineage and birth date. Neuron *1*, 15-26.

Hu, M., and Easter, S. S. (1999). Retinal neurogenesis: the formation of the initial central patch of postmitotic cells. Dev Biol *207*, 309-321.

Igarashi, K., Itoh, K., Motohashi, H., Hayashi, N., Matuzaki, Y., Nakauchi, H., Nishizawa, M., and Yamamoto, M. (1995). Activity and expression of murine small Maf family protein MafK. J Biol Chem *270*, 7615-7624.

Jensen, A. M., and Wallace, V. A. (1997). Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. Development *124*, 363-371.

Jeon, C. J., Strettoi, E., and Masland, R. H. (1998). The major cell populations of the mouse retina. J Neurosci *18*, 8936-8946.

Kataoka, K. (2007). Multiple mechanisms and functions of maf transcription factors in the regulation of tissue-specific genes. J Biochem (Tokyo) *141*, 775-781.

Kataoka, K., Han, S. I., Shioda, S., Hirai, M., Nishizawa, M., and Handa, H. (2002). MafA is a glucose-regulated and pancreatic beta-cell-specific transcriptional activator for the insulin gene. J Biol Chem *277*, 49903-49910.

Kataoka, K., Igarashi, K., Itoh, K., Fujiwara, K. T., Noda, M., Yamamoto, M., and Nishizawa, M. (1995). Small Maf proteins heterodimerize with Fos and may act as competitive repressors of the NF-E2 transcription factor. Mol Cell Biol *15*, 2180-2190.

Kawai, S., Goto, N., Kataoka, K., Saegusa, T., Shinno-Kohno, H., and Nishizawa, M. (1992). Isolation of the avian transforming retrovirus, AS42, carrying the v-maf oncogene and initial characterization of its gene product. Virology *188*, 778-784.

Kenyon, K. L., Zaghloul, N., and Moody, S. A. (2001). Transcription factors of the anterior neural plate alter cell movements of epidermal progenitors to specify a retinal fate. Dev Biol *240*, 77-91.

Kerppola, T. K., and Curran, T. (1994a). A conserved region adjacent to the basic domain is required for recognition of an extended DNA binding site by Maf/Nrl family proteins. Oncogene *9*, 3149-3158.

Kerppola, T. K., and Curran, T. (1994b). Maf and Nrl can bind to AP-1 sites and form heterodimers with Fos and Jun. Oncogene *9*, 675-684.

Kurschner, C., and Morgan, J. I. (1995). The maf proto-oncogene stimulates transcription from multiple sites in a promoter that directs Purkinje neuron-specific gene expression. Mol Cell Biol *15*, 246-254.

Lerner, L. E., Gribanova, Y. E., Ji, M., Knox, B. E., and Farber, D. B. (2001). Nrl and Sp nuclear proteins mediate transcription of rod-specific cGMP-phosphodiesterase beta-subunit gene: involvement of multiple response elements. J Biol Chem *276*, 34999-35007.

Li, M. A., Alls, J. D., Avancini, R. M., Koo, K., and Godt, D. (2003). The large Maf factor Traffic Jam controls gonad morphogenesis in Drosophila. Nat Cell Biol *5*, 994-1000.

Lillien, L. (1995). Changes in retinal cell fate induced by overexpression of EGF receptor. Nature *377*, 158-162.

Lillien, L., and Cepko, C. (1992). Control of proliferation in the retina: temporal changes in responsiveness to FGF and TGF alpha. Development *115*, 253-266.

Livesey, F. J., and Cepko, C. L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci *2*, 109-118.

Loosli, F., Winkler, S., and Wittbrodt, J. (1999). Six3 overexpression initiates the formation of ectopic retina. Genes Dev *13*, 649-654.

MacLaren, R. E., Pearson, R. A., MacNeil, A., Douglas, R. H., Salt, T. E., Akimoto, M., Swaroop, A., Sowden, J. C., and Ali, R. R. (2006). Retinal repair by transplantation of photoreceptor precursors. Nature *444*, 203-207.

Marquardt, T. (2003). Transcriptional control of neuronal diversification in the retina. Prog Retin Eye Res *22*, 567-577.

Mathers, P. H., Grinberg, A., Mahon, K. A., and Jamrich, M. (1997). The Rx homeobox gene is essential for vertebrate eye development. Nature *387*, 603-607.

Matsuoka, T. A., Zhao, L., Artner, I., Jarrett, H. W., Friedman, D., Means, A., and Stein, R. (2003). Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells. Mol Cell Biol *23*, 6049-6062.

Mears, A. J., Kondo, M., Swain, P. K., Takada, Y., Bush, R. A., Saunders, T. L., Sieving, P. A., and Swaroop, A. (2001). Nrl is required for rod photoreceptor development. Nat Genet *29*, 447-452.

Mitton, K. P., Swain, P. K., Chen, S., Xu, S., Zack, D. J., and Swaroop, A. (2000). The leucine zipper of NRL interacts with the CRX homeodomain. A possible mechanism of transcriptional synergy in rhodopsin regulation. J Biol Chem *275*, 29794-29799.

Morrow, E. M., Belliveau, M. J., and Cepko, C. L. (1998). Two phases of rod photoreceptor differentiation during rat retinal development. J Neurosci *18*, 3738-3748.

Motohashi, H., Shavit, J. A., Igarashi, K., Yamamoto, M., and Engel, J. D. (1997). The world according to Maf. Nucleic Acids Res *25*, 2953-2959.
Nathans, J., and Hogness, D. S. (1983). Isolation, sequence analysis, and intron-exon arrangement of the gene encoding bovine rhodopsin. Cell *34*, 807-814.

Nathans, J., and Hogness, D. S. (1984). Isolation and nucleotide sequence of the gene encoding human rhodopsin. Proc Natl Acad Sci U S A *81*, 4851-4855.

Nathans, J., Thomas, D., and Hogness, D. S. (1986). Molecular genetics of human color vision: the genes encoding blue, green, and red pigments. Science *232*, 193-202.

Ng, L., Hurley, J. B., Dierks, B., Srinivas, M., Salto, C., Vennstrom, B., Reh, T. A., and Forrest, D. (2001). A thyroid hormone receptor that is required for the development of green cone photoreceptors. Nat Genet *27*, 94-98.

Nickle, B., and Robinson, P. R. (2007). The opsins of the vertebrate retina: insights from structural, biochemical, and evolutionary studies. Cell Mol Life Sci.

Nikonov, S. S., Daniele, L. L., Zhu, X., Craft, C. M., Swaroop, A., and Pugh, E. N., Jr. (2005). Photoreceptors of Nrl -/- mice coexpress functional S- and M-cone opsins having distinct inactivation mechanisms. J Gen Physiol *125*, 287-304.

Nishiguchi, K. M., Friedman, J. S., Sandberg, M. A., Swaroop, A., Berson, E. L., and Dryja, T. P. (2004). Recessive NRL mutations in patients with clumped pigmentary retinal degeneration and relative preservation of blue cone function. Proc Natl Acad Sci U S A *101*, 17819-17824.

Nishimura, W., Kondo, T., Salameh, T., El Khattabi, I., Dodge, R., Bonner-Weir, S., and Sharma, A. (2006). A switch from MafB to MafA expression accompanies differentiation to pancreatic beta-cells. Dev Biol *293*, 526-539.

Nishizawa, M., Kataoka, K., Goto, N., Fujiwara, K. T., and Kawai, S. (1989). v-maf, a viral oncogene that encodes a "leucine zipper" motif. Proc Natl Acad Sci U S A *86*, 7711-7715.

Ogata, A., Shimizu, T., Abe, R., Shimizu, H., and Sakai, M. (2004). Expression of c-maf and mafB genes in the skin during rat embryonic development. Acta Histochem *106*, 65-67.

Ogino, H., and Yasuda, K. (1998). Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. Science *280*, 115-118.

Pei, Y. F., and Rhodin, J. A. (1970). The prenatal development of the mouse eye. Anat Rec *168*, 105-125.

Peng, G. H., and Chen, S. (2007). Crx activates opsin transcription by recruiting HAT-containing co-activators and promoting histone acetylation. Hum Mol Genet.
Pepe, I. M. (2001). Recent advances in our understanding of rhodopsin and phototransduction. Prog Retin Eye Res *20*, 733-759.

Pittler, S. J., Zhang, Y., Chen, S., Mears, A. J., Zack, D. J., Ren, Z., Swain, P. K., Yao, S., Swaroop, A., and White, J. B. (2004). Functional analysis of the rod photoreceptor cGMP phosphodiesterase alpha-subunit gene promoter: Nrl and Crx are required for full transcriptional activity. J Biol Chem *279*, 19800-19807.

Rapaport, D. H., Wong, L. L., Wood, E. D., Yasumura, D., and LaVail, M. M. (2004). Timing and topography of cell genesis in the rat retina. J Comp Neurol *474*, 304-324.

Rehemtulla, A., Warwar, R., Kumar, R., Ji, X., Zack, D. J., and Swaroop, A. (1996). The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. Proc Natl Acad Sci U S A *93*, 191-195.

Reme, C. E., Grimm, C., Hafezi, F., Iseli, H. P., and Wenzel, A. (2003). Why study rod cell death in retinal degenerations and how? Doc Ophthalmol *106*, 25-29.

Reza, H. M., and Yasuda, K. (2004). Roles of Maf family proteins in lens development. Dev Dyn *229*, 440-448.

Ring, B. Z., Cordes, S. P., Overbeek, P. A., and Barsh, G. S. (2000). Regulation of mouse lens fiber cell development and differentiation by the Maf gene. Development *127*, 307-317.

Roberts, M. R., Hendrickson, A., McGuire, C. R., and Reh, T. A. (2005). Retinoid X receptor (gamma) is necessary to establish the S-opsin gradient in cone photoreceptors of the developing mouse retina. Invest Ophthalmol Vis Sci *46*, 2897-2904.

Roberts, M. R., Srinivas, M., Forrest, D., Morreale de Escobar, G., and Reh, T. A. (2006). Making the gradient: thyroid hormone regulates cone opsin expression in the developing mouse retina. Proc Natl Acad Sci U S A *103*, 6218-6223.

Sohocki, M. M., Malone, K. A., Sullivan, L. S., and Daiger, S. P. (1999). Localization of retina/pineal-expressed sequences: identification of novel candidate genes for inherited retinal disorders. Genomics *58*, 29-33.

Stockman, A., Smithson, H. E., Michaelides, M., Moore, A. T., Webster, A. R., and Sharpe, L. T. (2007). Residual cone vision without alpha-transducin. J Vis *7*, 8.

Sullivan, L. S., Bowne, S. J., Birch, D. G., Hughbanks-Wheaton, D., Heckenlively, J. R., Lewis, R. A., Garcia, C. A., Ruiz, R. S., Blanton, S. H., Northrup, H.*, et al.* (2006). Prevalence of disease-causing mutations in families with autosomal dominant retinitis pigmentosa: a screen of known genes in 200 families. Invest Ophthalmol Vis Sci *47*, 3052-3064.

Swain, P. K., Chen, S., Wang, Q. L., Affatigato, L. M., Coats, C. L., Brady, K. D., Fishman, G. A., Jacobson, S. G., Swaroop, A., Stone, E.*, et al.* (1997). Mutations in the cone-rod homeobox gene are associated with the cone-rod dystrophy photoreceptor degeneration. Neuron *19*, 1329-1336.

Swain, P. K., Hicks, D., Mears, A. J., Apel, I. J., Smith, J. E., John, S. K., Hendrickson, A., Milam, A. H., and Swaroop, A. (2001). Multiple phosphorylated isoforms of NRL are expressed in rod photoreceptors. J Biol Chem *276*, 36824-36830.

Swaroop, A., Wang, Q. L., Wu, W., Cook, J., Coats, C., Xu, S., Chen, S., Zack, D. J., and Sieving, P. A. (1999). Leber congenital amaurosis caused by a homozygous mutation (R90W) in the homeodomain of the retinal transcription factor CRX: direct evidence for the involvement of CRX in the development of photoreceptor function. Hum Mol Genet *8*, 299-305.

Swaroop, A., Xu, J. Z., Pawar, H., Jackson, A., Skolnick, C., and Agarwal, N. (1992). A conserved retina-specific gene encodes a basic motif/leucine zipper domain. Proc Natl Acad Sci U S A *89*, 266-270.

Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F., and Kageyama, R. (2000). Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. Embo J *19*, 5460-5472.

Tsuchiya, M., Taniguchi, S., Yasuda, K., Nitta, K., Maeda, A., Shigemoto, M., and Tsuchiya, K. (2006). Potential roles of large mafs in cell lineages and developing pancreas. Pancreas *32*, 408-416.

Turner, D. L., and Cepko, C. L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature *328*, 131-136.

Turner, D. L., Snyder, E. Y., and Cepko, C. L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833-845.

Wallace, V. A., and Raff, M. C. (1999). A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. Development *126*, 2901-2909.

Wang, P. W., Eisenbart, J. D., Cordes, S. P., Barsh, G. S., Stoffel, M., and Le Beau, M. M. (1999). Human KRML (MAFB): cDNA cloning, genomic structure, and evaluation as a candidate tumor suppressor gene in myeloid leukemias. Genomics *59*, 275-281.

Wetts, R., and Fraser, S. E. (1988). Multipotent precursors can give rise to all major cell types of the frog retina. Science *239*, 1142-1145.

Xiang, M. (1998). Requirement for Brn-3b in early differentiation of postmitotic retinal ganglion cell precursors. Dev Biol *197*, 155-169.

Yang-Feng, T. L., and Swaroop, A. (1992). Neural retina-specific leucine zipper gene NRL (D14S46E) maps to human chromosome 14q11.1-q11.2. Genomics *14*, 491-492.

Yoshida, S., Mears, A. J., Friedman, J. S., Carter, T., He, S., Oh, E., Jing, Y., Farjo, R., Fleury, G., Barlow, C*., et al.* (2004). Expression profiling of the developing and mature Nrl-/- mouse retina: identification of retinal disease candidates and transcriptional regulatory targets of Nrl. Hum Mol Genet *13*, 1487-1503.

Young, R. W. (1985). Cell differentiation in the retina of the mouse. Anat Rec *212*, 199-205.

Yu, J., He, S., Friedman, J. S., Akimoto, M., Ghosh, D., Mears, A. J., Hicks, D., and Swaroop, A. (2004). Altered expression of genes of the Bmp/Smad and Wnt/calcium signaling pathways in the cone-only Nrl-/- mouse retina, revealed by gene profiling using custom cDNA microarrays. J Biol Chem *279*, 42211-42220.

Zhang, S. S., Fu, X. Y., and Barnstable, C. J. (2002). Molecular aspects of vertebrate retinal development. Mol Neurobiol *26*, 137-152.

Zhao, L., Guo, M., Matsuoka, T. A., Hagman, D. K., Parazzoli, S. D., Poitout, V., and Stein, R. (2005). The islet beta cell-enriched MafA activator is a key regulator of insulin gene transcription. J Biol Chem *280*, 11887-11894.

Zuber, M. E., Gestri, G., Viczian, A. S., Barsacchi, G., and Harris, W. A. (2003). Specification of the vertebrate eye by a network of eye field transcription factors. Development *130*, 5155-5167.

Zuber, M. E., Perron, M., Philpott, A., Bang, A., and Harris, W. A. (1999). Giant eyes in Xenopus laevis by overexpression of XOptx2. Cell *98*, 341-352.

## CHAPTER II

## TRANSFORMATION OF CONE PRECURSORS TO FUNCTIONAL ROD PHOTORECEPTORS BY BZIP TRANSCRIPTION FACTOR NRL

### Abstract

Networks of transcriptional regulatory proteins dictate specification of neural lineages from multipotent retinal progenitors. Rod photoreceptor differentiation requires the basic motif-leucine zipper (bZIP) transcription factor NRL, as loss of *Nrl* in mice (*Nrl*$^{-/-}$) results in complete transformation of rods to functional cones. To examine the role of NRL in cell fate determination, we generated transgenic mice that express *Nrl* under the control of *Crx* promoter in post-mitotic photoreceptor precursors of wild type and *Nrl*$^{-/-}$ retina. We show that NRL expression, in both genetic backgrounds, leads to a functional retina with only rod photoreceptors. The absence of cones does not alter retinal lamination, though cone synaptic circuitry is now recruited by rods. Ectopic expression of NRL in developing cones can also induce rod-like characteristics and partially suppress cone-specific gene expression. We show that NRL is associated with specific promoter sequences in *Thrb* (encoding TRβ2 transcription factor required for M-cone differentiation) and *S-opsin*, and may therefore directly participate in transcriptional suppression of cone development. These studies establish that NRL is not only essential but is sufficient for rod differentiation and that post-

mitotic photoreceptor precursors are competent to make binary decisions during early retinogenesis.

## Introduction

Neuronal cell fate is determined by a hierarchical, stepwise process of binary decisions, commencing with multipotent progenitors that give rise to distinct cell lineages (Livesey and Cepko, 2001; Malicki, 2004; Shen et al., 2006). The neural retina is an attractive model to investigate cell fate determination; it contains seven major cell types that derive from common pool(s) of multipotent progenitor cells (Turner et al., 1990; Wetts and Fraser, 1988). These retinal progenitors pass through sequential waves of competence, during which post-mitotic cells can be specified to only a subset of neuronal fates (Cepko et al., 1996; Livesey and Cepko, 2001). Birthdating studies in rodents indicate that ganglion cells, horizontal cells, cone photoreceptors, and amacrine cells are born prenatally, whereas most rod photoreceptors, bipolar cells and Muller glia are generated postnatally (Carter-Dawson and LaVail, 1979b; Rapaport et al., 2004; Young, 1985). The orderly sequence of cell birth and a considerable overlap in their generation suggest a sequential program of cell intrinsic mechanisms and extrinsic signals that control cell fate decisions (Dyer et al., 2003; Gan et al., 1996; Hatakeyama and Kageyama, 2004; Marquardt et al., 2001; Ng et al., 2001; Nishida et al., 2003; Roberts et al., 2006).

Each neuronal lineage is meticulously established by highly coordinated transcription factor network(s) in response to local micro-environmental cues

(Edlund and Jessell, 1999). Although extrinsic factors can promote differentiation (Levine et al., 2000; Watanabe and Raff, 1992), heterochronic mixing experiments demonstrate that progenitor cells at a particular time in development cannot be induced to generate temporally inappropriate cell types (Belliveau et al., 2000; Livesey and Cepko, 2001). Additionally, intrinsic priming of retinal progenitors appears to supersede the influence of environmental signals in specifying cell fate (Cayouette et al., 2003). Whether commitment of lineage-restricted precursors to a specific differentiation pathway is unidirectional has not been clearly elucidated.

Post-mitotic plasticity was first revealed in low-density retinal cell cultures derived from embryonic chicks; cells isolated on embryonic day (E) 6 became photoreceptors shortly after terminal mitosis, while those from E8 embryos gave rise to non-photoreceptors suggesting that the fate of a cell could be changed in response to the microenvironment (Adler and Hatlee, 1989). In another demonstration of cellular plasticity, treatment of retinal explants with ciliary neurotrophic factor (CNTF) was sufficient to block rhodopsin expression in post-mitotic rod precursors and resulted in the expression of bipolar interneuron markers (Ezzeddine et al., 1997). Interestingly, ETS transcription factors are selectively expressed during motor and sensory neuron development but only after their axons reach the periphery, suggesting that these proteins confer post-mitotic subtype identity during the establishment of selective connections (Arber et al., 2000; Lin et al., 1998). These findings indicate that neural identity can be

specified even after the terminal cell cycle exit; however, direct *in vivo* evidence of post-mitotic plasticity has not been reported.

The Maf-family transcription factor *Nrl* is expressed specifically in post-mitotic rod photoreceptors of the retina and in the pineal gland (Akimoto et al., 2006; Swain et al., 2001). NRL interacts with the homeodomain protein CRX (Mitton et al., 2000), orphan nuclear receptor NR2E3 (Cheng et al., 2004) and other retinal proteins (Lerner et al., 2001; Mitton et al., 2003; Wang et al., 2004) to regulate the expression of rod-specific genes (Akimoto et al., 2006; Yoshida et al., 2004). NRL is essential for rod differentiation, as rods are transformed to functional S-cones in the *Nrl*$^{-/-}$ mouse retina (Akimoto et al., 2006; Daniele et al., 2005; Mears et al., 2001). The apparent switch from rod to S-cone fate in the *Nrl*$^{-/-}$ mouse suggests that post-mitotic photoreceptor precursors retain some degree of plasticity. In this report, we generated a series of transgenic mice that express *Nrl* during early and late stages of photoreceptor differentiation in wild type or *Nrl*$^{-/-}$ background. We demonstrate that NRL is sufficient to guide post-mitotic photoreceptors towards rod lineage and that photoreceptor precursors are competent to make binary decisions of acquiring rod versus cone identity.

## Materials and Methods

**Plasmid constructs and generation of transgenic mice.** A 2.3 kb mouse *Crx* promoter DNA (from -2286 to +72, GenBank # AF335248 and AF301006) and the *Nrl* coding region (GenBank # NM008736) with an additional Kozak sequence were amplified and cloned into a modified promoter-less pCl (pClpl)

vector (Akimoto et al., 2004). The 3.7 kb *Crxp-Nrl* insert was purified and injected into fertilized *Nrl*$^{-/-}$ (mixed background of 129X1/SvJ and C57BL/6J) mouse oocytes (UM transgenic core facility). Transgenic founders were bred to the *Nrl*$^{-/-}$ mice to generate F1 progeny. The progeny was also mated to C57BL/6J to generate *Crxp-Nrl*/WT mice. The *BPp-Nrl* transgenic mice were generated in a similar manner, except that a 520 bp mouse *S-opsin* promoter DNA (Akimoto et al., 2004) was used. All studies involving mice were performed in accordance with institutional and federal guidelines and approved by the University Committee on Use and Care of Animals at the University of Michigan.

**Immunohistochemistry and confocal analysis.** Retinal sections and dissociated cells were prepared as described (Cheng et al., 2004; Strettoi et al., 2002). Sections were visualized under an Olympus FluoView 500 laser scanning confocal microscope or a Leica TSC NT confocal microscope, equipped with an argon-krypton laser. Images were digitized using FluoView software version 5.0 or Metamorph 3.2 software. Antibodies used for immunohistochemistry are as follows: rabbit anti S-opsin, M-opsin, and cone arrestin antibodies (generous gifts from C. Craft, University of Southern California, Los Angeles, CA), mouse anti-rhodopsin (1D4) (generous gift from R. Molday, University of British Columbia, Vancouver, BC, Canada), rabbit b-galactosidase (Cappel), rat anti-b-galactosidase (a generous gift from T. Glaser, University of Michigan, Ann Arbor, MI) rabbit anti-Cre (Covance), mouse anti-Cre (Chemicon), rabbit and mouse anti-Protein Kinase C a (Sigma); rabbit anti-mGluR6 (Neuromics); rabbit anti-

calbindin D-28k (Swant); mouse anti-$G_{0a}$ (Chemicon); mouse anti-Neurofilament 200 kDa (clone N52; Sigma); mouse anti-Glutamine Synthetase (Chemicon); mouse anti-NK3-receptor (Abcam, Novus Biologicals); mouse anti-bassoon (Stressgen); mouse anti-kinesin 2 (Covance); mouse anti-synaptophysin (Boehringer); mouse anti-PSD95 (Abcam); goat anti-Choline Acetyl Transferase (ChAT; Chemicon); and rabbit anti-Tyrosine Hydroxylase (Chemicon). Fluorescent detection was performed by using Alexa Fluor 488, 546, or 633 (Molecular Probes) conjugated secondary antibodies.

**Chromatin immunoprecipitation (ChIP).** Mouse retinas from different developmental stages were subjected to ChIP analysis using ChIP-IT™ kit (Active motif, Carlsbad, CA). IP was performed using anti-NRL or normal rabbit immunoglobulin (IgG). PCR primers, derived from the *Thrb* and *S-opsin* promoter region (GenBank # NT_039340.6 and NT_039595.6, respectively) spanning the putative NRE, were used for amplification (from nucleotides 26331250 to 26331458 and 13773280 to 13773502, respectively) using immunoprecipitated DNA as template. The albumin PCR primers were: 5' GGACACAAGACTTCTGAAAGTCCTC 3' and 5' TTCCTACCCCATTACAAAATCATA 3'.

**EMSA.** Oligonucleotides spanning the putative NRE were radiolabeled using [$\gamma$-$^{32}$]P-ATP (Amersham Biosciences) and incubated in binding buffer (20mM

HEPES pH 7.5, 60mM KCl, 0.5mM DTT, 1mM $MgCl_2$, 12% glycerol) with bovine retinal nuclear extract (RNE; (Mitton et al., 2003)) (20 µg) and 50µg/ml poly(dI-dC) for 30 min at room temperature, as described (Khanna et al., 2006) For competition experiments, non-radiolabeled oligonucleotides were used in molar excess of the labeled oligonucleotides. In some experiments, antibodies were added after the incubation of $^{32}$P-labeled oligonucleotides with RNE. Samples were analyzed by 7.5% non-denaturing PAGE.

**Electroretinography.** ERGs were recorded as described (Mears et al., 2001).

## Results

### Overexpression of *Nrl* in photoreceptor precursors drives rod differentiation at the expense of cones

We hypothesized that if cones develop from a unique pool of competent cells, early cone precursors would not be responsive to NRL. On the other hand, transformation of cone precursors to rods by NRL would indicate an intrinsic capacity to give rise to both rods and cones. To directly test this, we generated transgenic mouse lines (*Crxp-Nrl/WT*) expressing *Nrl* under the control of a previously-characterized 2.5 kb proximal promoter of the *Crx* gene (*Crxp-Nrl*), which is specifically expressed in post-mitotic cells that can develop into either cone or rod photoreceptors (Cheng et al., 2006; Furukawa et al., 2002).

Light micrographs of semi-thin (plastic) sections of *Crxp-Nrl/WT* mouse retina showed normal laminar organization (Figure II-1 A, B). Immuno-fluorescence studies demonstrated comparable rhodopsin expression relative to wild type and *Nrl*$^{-/-}$ mice (Figure II-1 E-G); however, staining of cone-specific markers (cone arrestin, peanut agglutinin (PNA), S-opsin and M-opsin) was undetectable in cryosections and flat-mount preparations from transgenic retinas (Figure II-1 I-K, and data not shown). Confocal examination of the outer nuclear layer revealed only the photoreceptor nuclei with dense chromatin (Figure II-1 A, B) that are characteristics of rods in the wild type retina (Carter-Dawson and LaVail, 1979a). Dark-adapted corneal flash electroretinograms (ERGs) from *Crxp-Nrl/WT* mice revealed normal rod function even at six months (Figure II-1 M, N), whereas the cone-derived photopic ERG response was absent at all ages (Figure II-1 O, P, and data not shown). These studies suggested a complete absence of cone functional pathway in the *Crxp-Nrl/WT* mice. Consistent with these, quantitative RT-PCR analysis demonstrated no expression of cone phototransduction genes in the *Crxp-Nrl/WT* retina, with little or no change in rod-specific genes (Figure II-6 C).

We then bred the *Crxp-Nrl* transgenic mice into the *Nrl*$^{-/-}$ background (*Crxp-Nrl/Nrl*$^{-/-}$) to test whether *Nrl* expression in a cone-only retina could convert a retina solely composed of cones to rods as seen in the *Crxp-Nrl/WT* mice. Analysis of retinal morphology uncovered a remarkable transformation of a dysmorphic retina with whorls and rosettes in the *Nrl*$^{-/-}$ mice (Mears et al., 2001) to a wild type-like appearance (Figure II-1 C, D). Images from toluidine blue-

stained retinal sections revealed clear extended outer segments and a highly organized laminar structure (Figure II-1 D). Similar to the wild type (Carter-Dawson and LaVail, 1979a), and unlike the all-cone retina in *Nrl*$^{-/-}$ mice (Mears et al., 2001), the outer nuclear layer of *Crxp-Nrl/Nrl*$^{-/-}$ retina had rod-like nuclei with dense chromatin. Immunolabeling of adult *Crxp-Nrl/Nrl*$^{-/-}$ retinal sections demonstrated a complete absence of cone proteins (cone arrestin data is shown in Figure II-1 L). In contrast to the *Nrl*$^{-/-}$ retinas (Figure II-1 G), *Crxp-Nrl/Nrl*$^{-/-}$ mice displayed normal levels of rhodopsin (Figure II-1 H). No photoreceptor degeneration was evident by histology or ERG at least up to 6 months (Figure II-1, data not shown).

**Retinal synaptic architecture is modified in the absence of cones**

Given that a complete rod-only retina did not reveal gross changes in retinal morphology, we contemplated whether cones are essential for proper development and lamination of cone-connected neurons. Cones are presynaptic to dendrites originating from the cell bodies of horizontal cells and to at least 9 different types of cone bipolar neurons (Ghosh et al., 2004; Pignatelli and Strettoi, 2004). Immunostaining of *Crxp-Nrl/WT* retinas with a panel of cell-type specific antibodies (Strettoi et al., 2002) did not reveal any major difference in the distribution of the marker proteins for horizontal, bipolar, amacrine and glial cells (Figure II-2). Despite the absence of cones, it was apparent that both the ON and OFF subtypes of cone bipolar cells were retained (Figure II-2 A, B, E). All ON bipolar neurons (both rod and cone bipolar cells) carried metabotropic

glutamate receptors on their dendritic tips (mGluR6) and thus, presumably, they were postsynaptic to rod spherules. It was unclear from these studies whether cone bipolar cells belonging to the OFF functional type received synapses from rod photoreceptors. The dendrites of one type of OFF cone bipolar cells, marked with Neurokinin receptor 3 (NK3-R), form basal (or flat) junctions with cone pedicles in the outer plexiform layer (Figure II-7). Although confocal microscopy does not reach the necessary resolution to detect such putative contacts, it is apparent from the preparations that not all the dendrites of NK3-R positive cone bipolar cells come in close apposition to the rod spherules, and that basal junctions are therefore unlikely (Figure II-2 E). It remains to be established if and how rod spherules make connections to OFF cone bipolar cells, and whether the OFF channel gains access to the scotopic pathway.

To study the morphology of horizontal cells, we stained *Crxp-Nrl/WT* retinas with a calbindin antibody (Figure II-2 F). While no gross changes were observed, we noticed rare ectopic sprouts emerging from the outer plexiform layer and extending into the outer nuclear layer. Other examined markers also revealed a normal distribution throughout the retina (see Figure II-2 G-I). AII amacrine neurons exhibited their peculiar bi-stratified morphology (Figure II-2 G). Cholinergic amacrine cells (Figure II-2 H, I) showed a typical distribution in two mirror-symmetric populations. Dopaminergic amacrines and Muller glial cells also showed normal organization (data not shown). Thus, besides the likely reconnections of ON cone bipolar and horizontal cells to rods, the retina from Crxp-Nrl/WT mice was indistinguishable from wild type.

**Ectopic expression of NRL can suppress cone function and induce rod characteristics in a subset of photoreceptors expressing S-opsin**

The onset of S-opsin expression begins at E16-E18 in rodents (Chiu and Nathans, 1994; Szel et al., 1993). To further delineate NRL's role in cell fate determination, we generated transgenic mouse lines (*BPp-Nrl/WT*) expressing NRL under the control of a previously-characterized *S-opsin* promoter (Akimoto et al., 2004). Immunostaining revealed a significant decrease of *S-opsin* positive cells in the inferior region of flat-mounted retinas (Figure II-3 A). Consistent with histological and immunohistochemical analysis, ERGs from the *BPp-Nrl/WT* mice showed a 50% reduction in the photopic b-wave amplitude compared to the wild type (Figure II-3 B); however, scotopic ERG a- and b- wave amplitudes were largely unaffected (data not shown).

We then transferred the *BPp-Nrl* transgene to the $Nrl^{-/-}$ background (*BPp-Nrl/*$Nrl^{-/-}$ mice). Ectopic expression of *Nrl* in the all-cone $Nrl^{-/-}$ retina even at this stage (i.e., under the control of S-opsin promoter) resulted in rhodopsin staining in the ONL; however, as in the $Nrl^{-/-}$ mice (Figure II-3 C-F) the outer and inner segments remained stunted (Figure II-3 G-N). The *BPp-Nrl/*$Nrl^{-/-}$ retina also revealed hybrid cells that expressed both S-opsin and rhodopsin in ONL, INL and ganglion cell layer (Figure II-3 G-N; Figure II-8 A). ERG data showed that while the phototopic b-wave (cone-derived) was somewhat reduced, the scotopic b-wave amplitude was still undetectable in *BPp-Nrl/*$Nrl^{-/-}$ mice (data not shown).

To examine the fate of S-opsin expressing cells, we mated the BP-Cre transgenic mice (that expresses Cre-recombinase under the control of the same *S-opsin* promoter) (Akimoto et al., 2004) with the R26R reporter line and the *BPp-Nrl/WT* line (Figure II-8 B-K). A large number of Cre-negative cells were labeled with ß-galactosidase in the *BP-Cre; R26R; BPp-Nrl/WT* background (Figure II-8 B-K). Approximately 40% of ß-galactosidase positive cells did not co-localize with S-opsin. Their position in the ONL and the lack of S-opsin staining indicate that these are rod photoreceptors, suggesting a possible fate switch in response to ectopic NRL expression. However, we could not validate their identity as rods because the rod marker – rhodopsin – does not clearly label the nuclear layer. TUNEL staining of sections from E18 retina did not detect obvious differences between wild type and *BPp-Nrl/WT* mice (data not shown).

**NRL can associate with cone-specific promoter elements**

NRL is established as a positive transcriptional regulator of rod-specific genes (Chen et al., 1997; Lerner et al., 2001; Mitton et al., 2000; Pittler et al., 2004; Rehemtulla et al., 1996; Yoshida et al., 2004). To examine whether NRL can directly modulate cone-specific promoters, we screened 3 kb of 5' upstream promoter regions of the two cone-expressed genes - *Thrb* (encoding TRβ2 that is involved in M-cone differentiation, (Ng et al., 2001)) and *S-opsin* - for the presence of *Nrl* or *Maf* response element (NRE / MARE) (Rehemtulla et al., 1996). Oligonucleotides spanning the single putative NRE sites, identified within the *Thrb* and *S-opsin* promoters, were used for electrophoretic mobility shift

assay (EMSA) with bovine retinal nuclear extracts. We detected a shifted band that could be specifically competed by the addition of 50-fold molar excess of unlabeled NRE-oligonucleotide, but not a random oligonucleotide (Figure II-4 A, B). The addition of anti-NRL antibody abolished the shifted band for the *Trβ2* oligonucleotide (Figure II-4 A), whereas S-opsin promoter-protein complex demonstrated an increased mobility in the native polyacrylamide gel (Figure II-4 B). Notably, disappearance of the shifted band may occur due to the dynamic nature of some DNA-protein interactions, whereas the net charge to mass (e/m) ratio of the ternary complex determines their rate of mobility in a native polyacrylamide gel (DW, 2001). Similar results were obtained when the radiolabeled oligonucleotides were incubated with anti-NRL antibody simultaneously with the retinal nuclear extract or with the nuclear extract pre-incubated with the anti-NRL antibody for 15 min. (data not shown). No effect on the gel-shift was observed in the presence of control rabbit IgG.

To further evaluate the association of NRL with *Thrb* and *S-opsin* promoter elements *in vivo*, we performed chromatin immunoprecipitation (ChIP) assays using wild type embryonic and adult mouse retinas. PCR primer sets spanning the *Thrb* and *S-opsin* NRE amplified specific products with DNA immunoprecipitated with the anti-NRL antibody but not with the rabbit IgG (Figure II-4 C). ChIP experiments using the *Nrl*$^{-/-}$ mouse retina (negative control) did not reveal specific amplified products (Figure II-4 C).

## DISCUSSION

Specification of neuronal cell fate involves changes in progenitor competence over time (Livesey and Cepko, 2001). In the developing retina, progenitor cells show heterogeneity in their developmental competence, and have the potential to produce many or all fates. Genesis of rod photoreceptors overlaps with the birth of all other retinal cell types. How regulatory factors orchestrate this decision-making has not been clearly delineated. As CRX is expressed in all post-mitotic photoreceptor precursors (Furukawa et al., 2002), we hypothesized that CRX-expressing cells are not committed to a specific fate and are plastic, and that NRL dictates the rod fate over a developmental time window. The transgenic data presented (see Figure II-1) strongly supports the hypothesis of post-mitotic plasticity in mammalian retina, as expression of NRL in even CRX-expressing cone precursors produces functional rods. It is therefore the timing of expression, availability and amount/activity of NRL that determine whether the post-mitotic precursor will acquire rod or a cone fate (Figure II-5; Figure II-9). Surprisingly, ectopic expression of NRL can still drive a subset of presumptive S-opsin expressing cone photoreceptors towards the rod lineage though not to a fully functional phenotype.

NR2E3 is a photoreceptor-specific orphan nuclear receptor, which is shown to suppress cone-specific gene expression in cultured cells (Chen et al., 2005; Peng et al., 2005) and in transgenic mice (Cheng et al., 2006). NRL is upstream of NR2E3 in regulatory hierarchy of photoreceptor differentiation (Cheng et al., 2004; Mears et al., 2001) and is an established activator of rod-

specific genes (Yoshida et al., 2004; Yu et al., 2004). It was therefore of interest to examine whether NRL can also directly suppress cone genes or all of its effects on cone differentiation are mediated via NR2E3. The finding of NRL binding to *Thrb* and *S-opsin* promoter sequences suggests that NRL functions, probably together with NR2E3, in suppressing cone differentiation *in vivo*. NRL's role as a transcriptional repressor is not surprising as key regulatory proteins can control transcription through context-dependent combinatorial mechanisms (Levine and Tjian, 2003). Notably, distinct phosphorylated isoforms of NRL are expressed during retinal development (Swain et al., 2001), and phosphorylation differences are suggested to modulate transcriptional activity of NRL by altering nuclear translocation, DNA binding, or protein interactions (Kanda et al., 2007). Additional studies are required to evaluate whether different NRL isoforms participate in transcriptional activation versus repression during early photoreceptor development.

The loss of cones produces an alteration in retinal synaptic connectivity such that ON cone bipolar cells are now connected to rods in the absence of their natural synaptic partner (i.e., cones). These results complement previous findings (Strettoi et al., 2004), demonstrating that neurons of the rod pathway are recruited by newly-generated cones in the *Nrl*$^{-/-}$ retina. In addition, the data provide support to the hypothesis that while intrinsic mechanisms may guide the formation of synaptic connections, the strength(s) of afferent input determine the final establishment of synaptic circuitry (Buffelli et al., 2003; Kasthuri and Lichtman, 2003; Katz and Shatz, 1996). In the cone-only *Nrl*$^{-/-}$ retina and the rod-

only transgenic mice reported here, a lack of input from the other competing afferent neurons leads to functional synaptic connections that are not usually observed in the wild type mouse retina.

These studies establish that NRL is not only essential (Mears et al., 2001) but is also sufficient for rod genesis. To what extent NRL can dictate rod specification in proliferating cells or other subsets of retinal precursors will require further investigations using transgenic mice expressing NRL under the control of early cell-type promoters. *In utero* infection of mouse embryonic retinas (Dejneka et al., 2004) with *Nrl* may also demonstrate whether we can stretch the developmental potential of retinal progenitors. These studies give rise to the prospect of exploiting the plastic nature of retinal precursors to replenish dying rods in degenerative retinal diseases by ectopic NRL expression in retinal stem cells (Banin et al., 2006; Coles et al., 2004) or by transplantation of NRL-expressing progenitors (MacLaren et al., 2006).

## Notes to Chapter II

**A modified version of this chapter has been previously published in: Proceedings of National Academy of Sciences of the United States of America. Oh, E.C., Khan, N., Novelli, E., Khanna, H., Strettoi, E., and Swaroop, A. (2007). Transformation of cone precursors to functional rod photoreceptors by bZIP transcription factor NRL.**

**<u>Outside contribution</u>: Naheed Khan conducted the ERG experiments. Elena Novelli and Enrica Strettoi performed the immunohistochemistry for bipolar cells. Hemant Khanna assisted with the EMSA and ChIP experiments.**

**Figure II-1. Expression of NRL in cone precursors.** Toluidine blue staining of WT (**A**), *Crxp-Nrl/WT* (**B**), *Nrl*$^{-/-}$ (**C**), and *Crxp-Nrl/Nrl*$^{-/-}$ (**D**) retinal sections demonstrate unique chromatin pattern in the photoreceptor layer for cones (indicated by arrowhead) and rods. Normal laminar structure is observed in both *Crxp-Nrl/WT* (**B**) and *Crxp-Nrl/Nrl*$^{-/-}$ (**D**) plastic sections. Immunohistochemical markers for rod photoreceptors (rhodopsin) can be detected in WT (**E**), *Crxp-Nrl/WT* (**F**) and *Crxp-Nrl/Nrl*$^{-/-}$ (**H**) retina but not in *Nrl*$^{-/-}$ (**G**). The pan cone photoreceptor marker, cone arrestin, is present only in WT (**I**) and *Nrl*$^{-/-}$ (**K**) retina, but is largely absent in the *Crxp-Nrl/WT* (**J**) and *Crxp-Nrl/Nrl*$^{-/-}$ (**L**). ERG intensity series and responses were recorded from 2-month-old WT, *Nrl*$^{-/-}$, *Crxp-Nrl/WT* and *Crxp-Nrl/Nrl*$^{-/-}$ mice under dark- (**M, N**) and light-adapted (**O, P**) conditions. The X axes for **M** and **O** indicate time lapsed after flash. Stimulus energy is indicated (log cd-s/m2). OS, outer segments; IS, inner segments, ONL, outer nuclear layer; INL, inner nuclear layer. Scale bar: 25 μm and 50 μm.

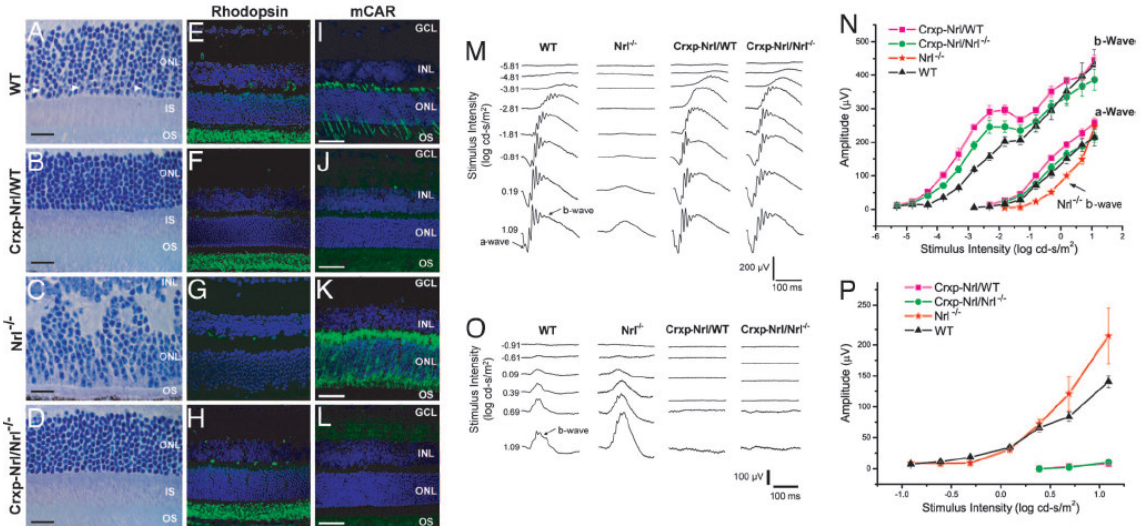


**Figure II-2. Synaptic organization of the inner retina in the absence of cones.** (**A**). The glutamatergic receptor mGluR6 is clustered selectively at puncta (Dejneka et al.) in the OPL, on the dendritic tips of ON bipolar cells, labeled by $G_{0\alpha}$ antibodies (green).

(**B**). $G_{0\alpha}$ antibody labels the whole population of ON bipolar cells (green signal), whereas PKCα labels rod bipolar cells only (RBC; red signal). Rod bipolar neurons are therefore double-labeled by both antibodies and appear yellow. Green cells are ON cone bipolar cells (indicated as CBC).

**(C)**. mGluR6 receptors are labelled as red puncta located at the dendritic tips of rod bipolar cells, labeled green by PKCα antibodies. In addition, clusters of mGluR6 are visible in the OPL, but not in association with rod biolar cell dendrites. These clusters are likely to be associated to the dendrites of ON cone bipolar cells.

(**D**). Rod bipolar cells (RBC), labeled by PKCα (red signal), are postsynaptic to photoreceptors in the OPL at ribbon synapses (indicated by R), as indicated by antibodies against kinesin, a synaptic ribbon marker (green signal).

(**E**). High magnification of one type of cone bipolar cell (CBC), labeled with NK3-R antibody (red signal). Rod spherules (RS) are labeled with anti-PSD95 antibody (green signal). Few dendrites of cone bipolar cells reach the basal aspect of some spherules (arrows); however, many spherules do not appear apposed to CBC dendrites, although these belong to one of the most abundant types of retinal cone bipolar cell.

(**F**). Calbindin staining (red signal) of the *Crxp-Nrl/WT* retina shows a normal distribution of intensely-labeled horizontal cells and weakly fluorescent amacrine cells with their processes in the IPL. Occasionally, horizontal cell sprouts are observed (arrow).

(**G**). AII amacrine cells (the most abundant population of mammalian amacrines) are specifically stained with DB3 antibodies (red signal). They exhibit a typical, bi-stratified morphology. Their innermost dendrites terminate in apposition to the axonal endings of rod bipolar cells, stained green by PKCα antibodies.

(**H**). Cholinergic amacrine cells are stained in the transgenic retina by ChAT antibodies (red signal). They form two, mirror symmetric populations of neurons. Axonal complexes of horizontal cells are labeled with neurofilament antibodies (green). Axonal fascicles of ganglion cells are also intensely stained in the optic fiber layer.

(**H**). Ethidium bromide nuclear staining (red signal) and ChAT immunostaining (green signal) demonstrate the normal layering and lamination of the transgenic retina.
OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer.

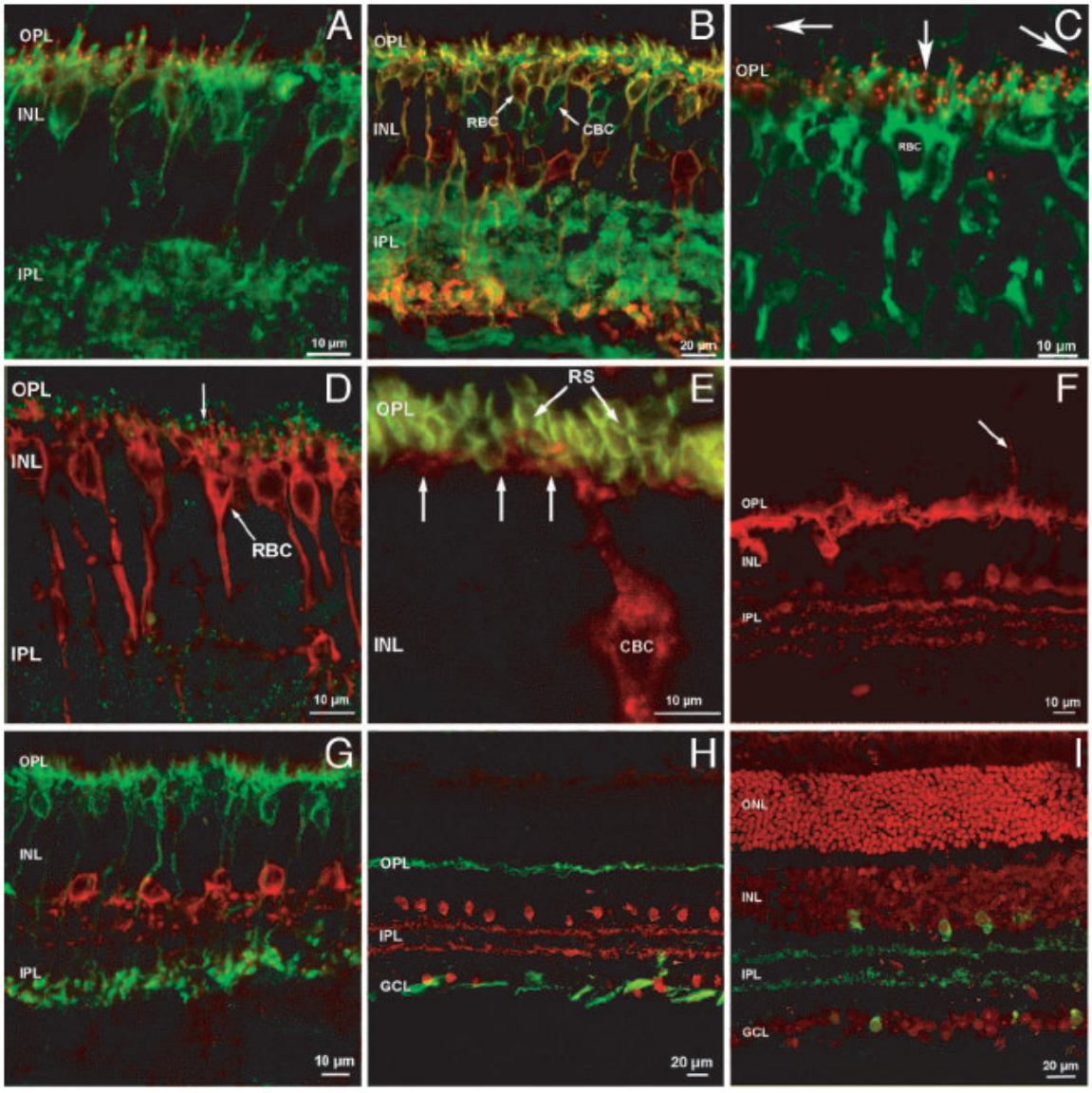


**Figure II-3. Ectopic expression of Nrl in S-opsin-expressing cone photoreceptors.** Quantification of S-cones in the inferior domain of flat-mounted retinas from WT and *BPp-Nrl/WT* mice with anti-S-opsin antibody (**A**) revealed a 40% decrease in S-cones. Light–adapted ERG photoresponses from WT and *BPp-Nrl/WT* mice are shown in (**B**). Immunostaining of cryosections from *Nrl*$^{-/-}$ retina (**C-F**). In the *BPp-Nrl/Nrl*$^{-/-}$ retina rhodopsin expression can be detected in the ONL and the OS (**G, K**). Hybrid photoreceptors expressing both S-opsin (**H, L**) and rhodopsin can be observed in the ONL, INL and the GCL (**G-N**). OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; BBZ, bisbenzamide. Scale bar: 25 μm and 50 μm.

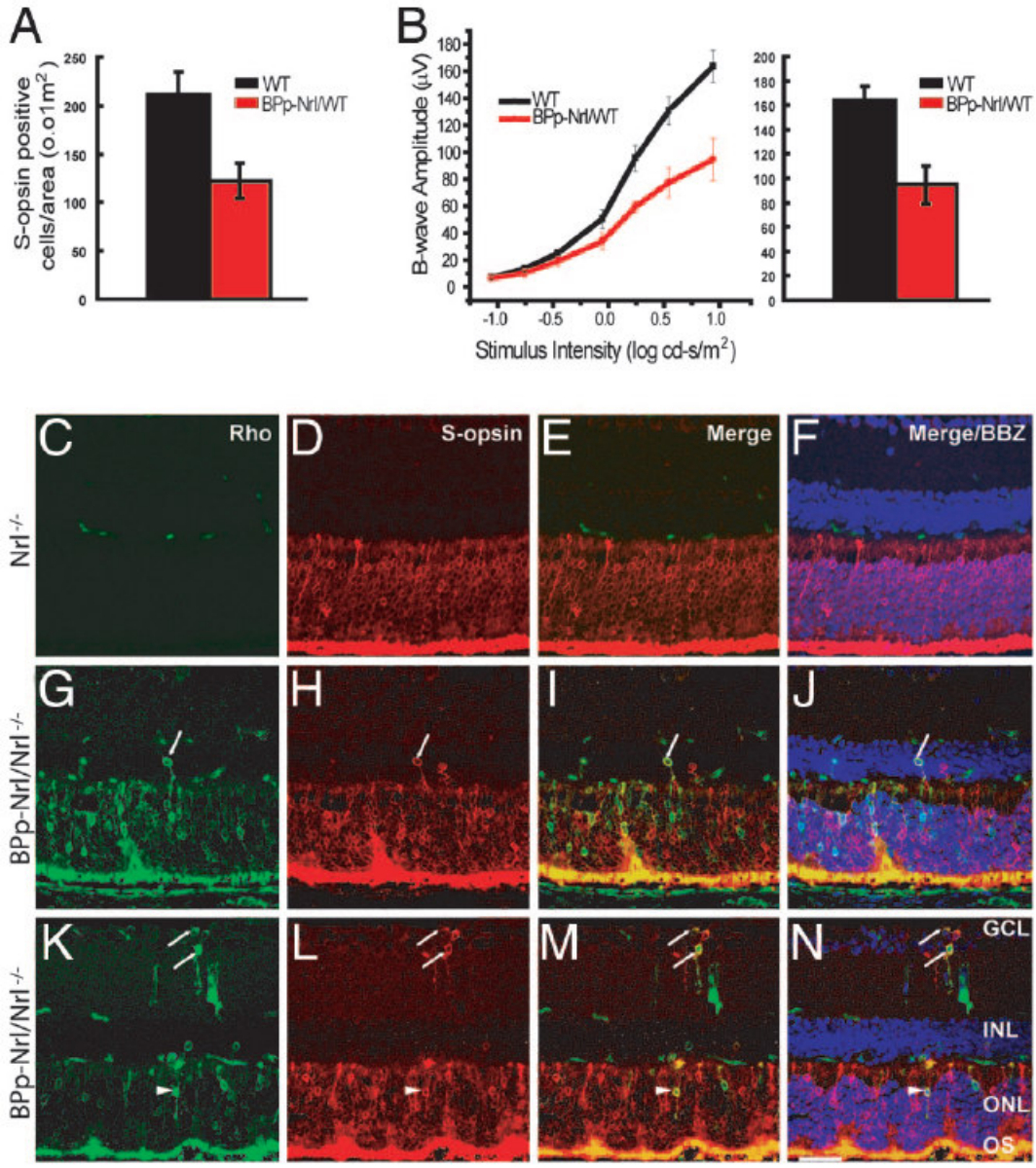


**Figure II-4. Association of Nrl to cone specific promoters.**
**A, B: EMSA.** Radiolabeled double-stranded oligonucleotides from *Thrb* and *S-opsin* promoters were incubated with RNE, followed by non-denaturing PAGE. Lanes are as indicated. Arrows represent specific shifted bands. Competition experiments were performed with increasing concentration (1, 5, or 50-fold molar excess, respectively) of unlabeled specific oligonucleotide or 50-fold higher concentration of non-specific (ns) oligonucleotide, to validate the specificity of band shift. Anti-NRL or normal rabbit IgG was added in some of the reactions, as indicated. Disappearance (see panel A) or increased mobility of the shifted band (B; shown by asterisk) was detected with anti-NRL antibody but not IgG. These experiments were performed three times and similar results were obtained. **C. ChIP assay.** WT or *Nrl*$^{-/-}$ mouse retina was used for ChIP with anti-NRL or rabbit IgG antibody. The positive and negative controls for ChIP assays are Pde6a and Albumin, respectively. Lanes are as indicated. Input DNA served as positive control for PCR. E: Embryonic day.

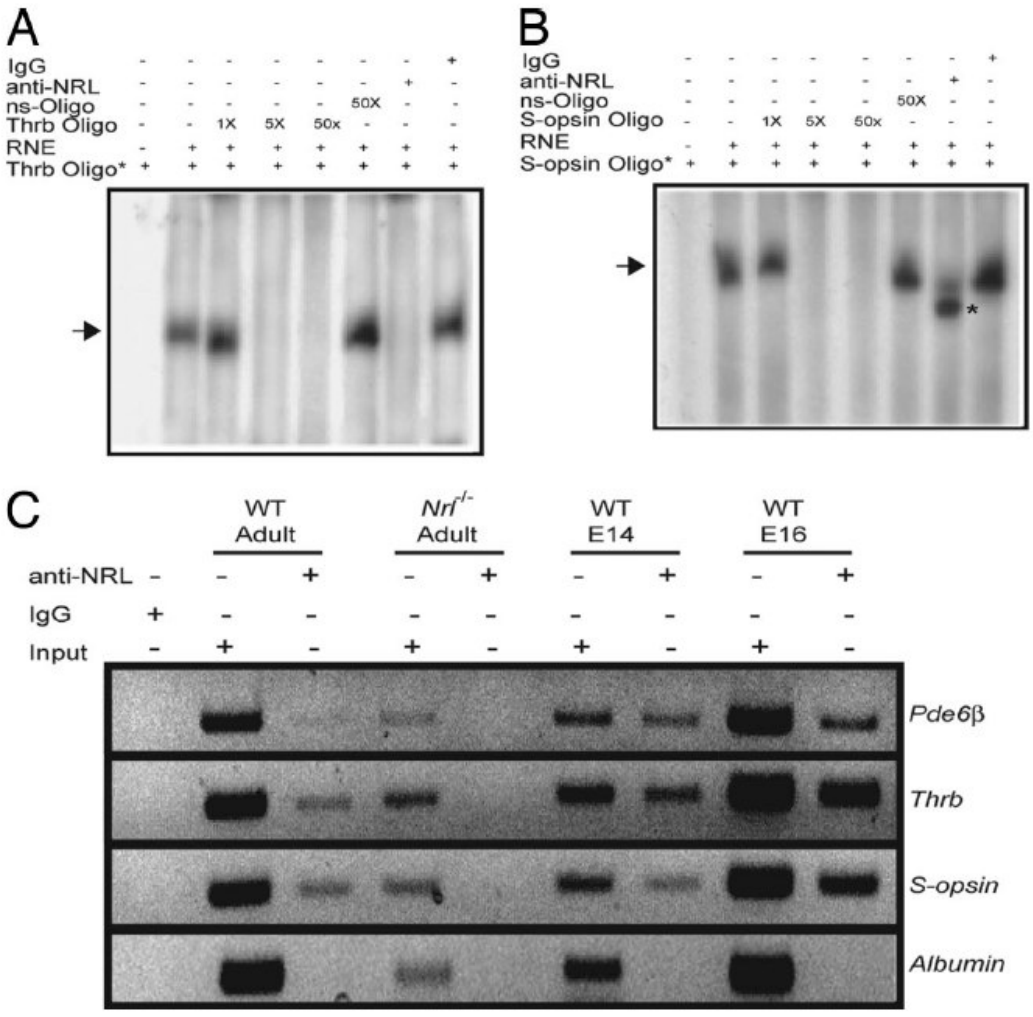


**Figure II-5. A model of photoreceptor specification.** Otx2 and Rb influence multipotent retinal neuroepithelial cells to exit cell cycle. We hypothesize that Crx is the competence factor in post-mitotic photoreceptor precursors. The cells that express Nrl are committed to rod photoreceptor fate, with subsequent expression of Nr2e3. The cells expressing only Crx are cone precursors. We propose that a degree of plasticity exists in early retinal development, such that changes in Nrl and/or Nr2e3 expression can lead to alterations in final ratio of rod and cone photoreceptors. Additional transcription factors (Ng et al., 2001; Srinivas et al., 2006) are required to guide the development to mature photoreceptors.

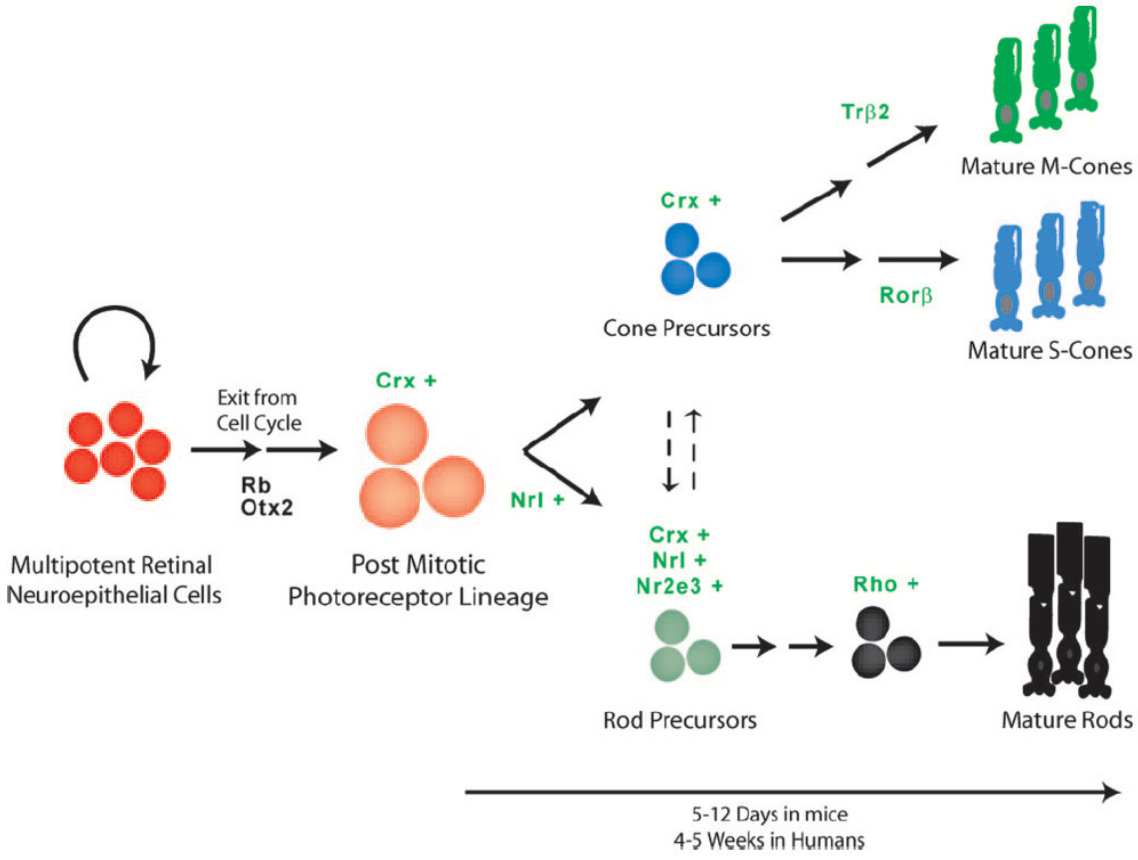


**Figure II-6. Nuclear morphology in the outer nuclear layer of WT and *Crxp-Nrl/WT* retina**. Flat-mounts of WT (A) and *Crxp-Nrl/WT* (B) retina were stained with the nuclear dye YOYO 1. The focal plane is set at the height of cone nuclei, illustrating their larger size and nonhomogeneous chromatin in the WT retina but not in the *Crxp-Nrl/WT* retina. (C) Gene expression analysis. Quantitative RT-PCR profiles show loss of cone-specific gene expression in both *Crxp-Nrl/WT* and *Crxp-Nrl/Nrl*$^{-/-}$ retinas, whereas rod-specific expression is largely unchanged. WT and *Nrl*$^{-/-}$ retinas show expected changes in gene expression. Expression levels are normalized to *Hprt*.

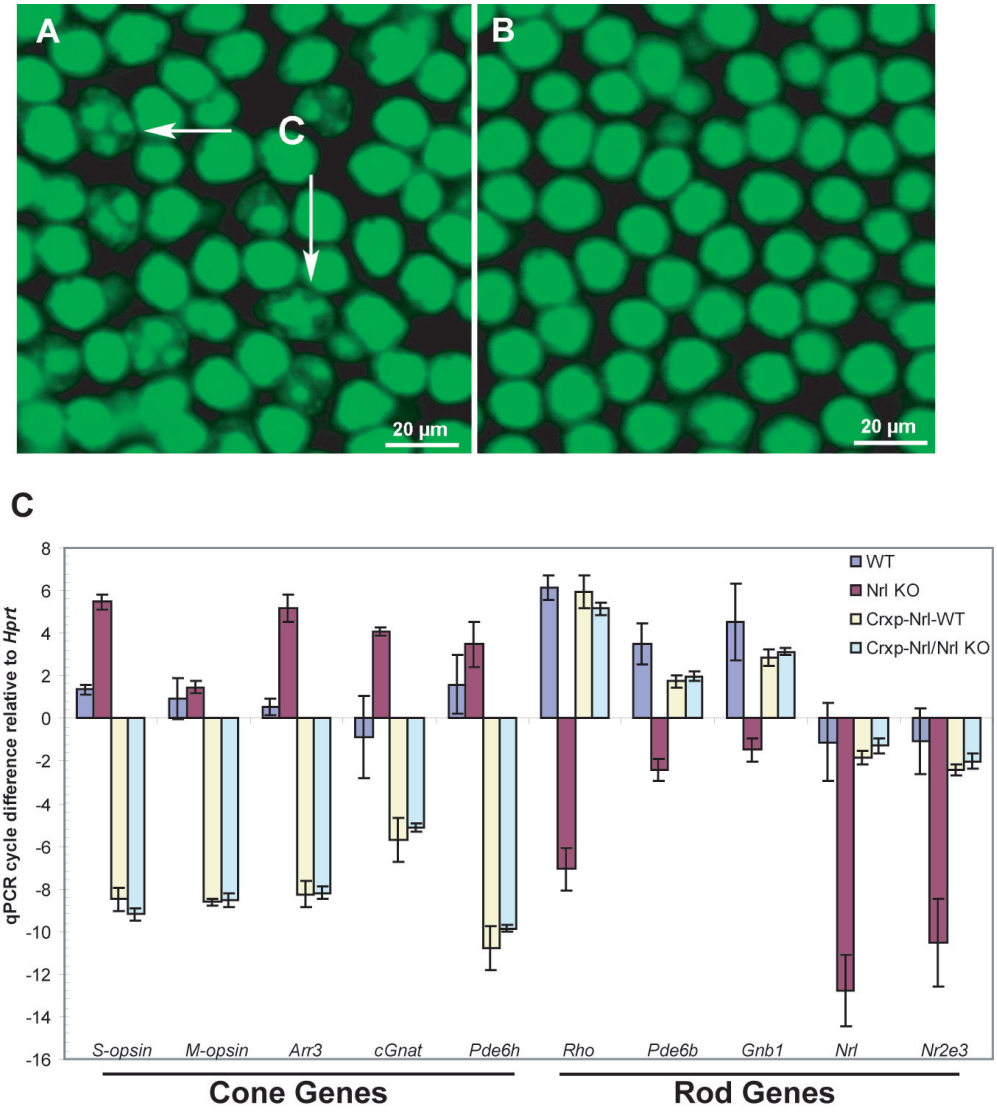


**Figure II-7. NK3-R immunostaining of OFF cone bipolar cells in the WT retina.** Using NK3-R antibody, the morphology and flat dendritic arbors of OFF cone bipolars are illustrated in WT P20 (A) and 7 month (B) retinas. PNA lectin and NK3-R staining (C) show the proximity of OFF cone bipolars to cone pedicles (*Inset*).

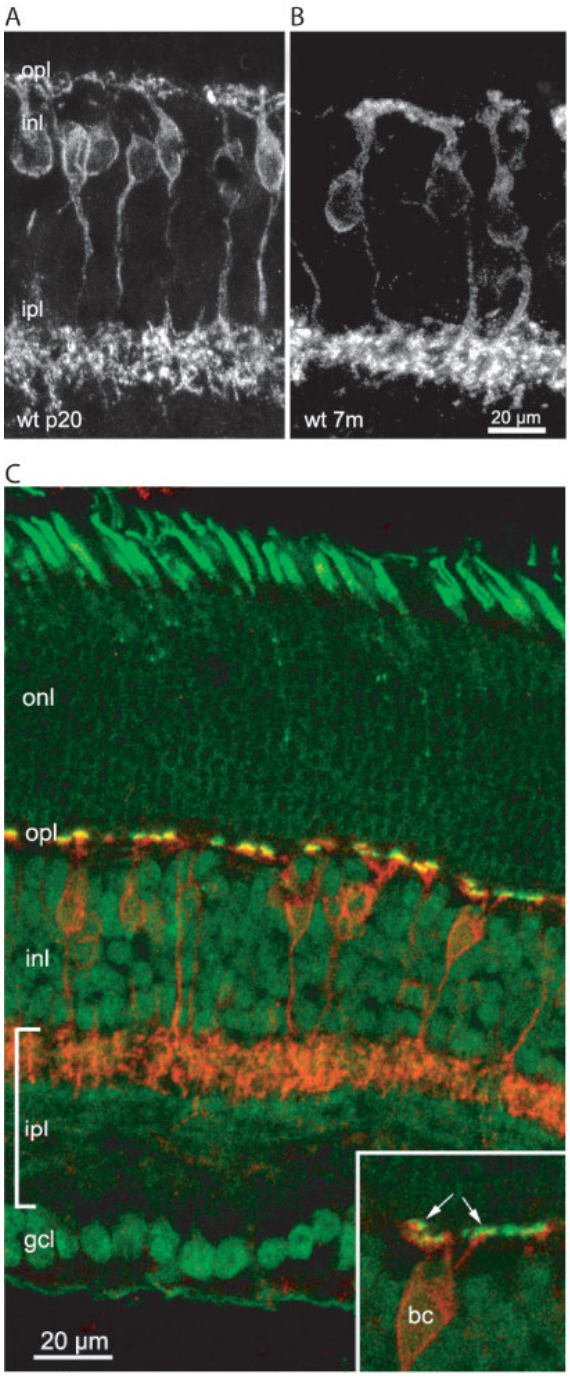


**Figure II-8. Quantification of photoreceptors and fate-mapping experiments.** Adult retinas were dissociated and assayed for rhodopsin and s-opsin expression (A) A total of 300 cells per sample in triplicate were scored. (B) A schematic illustration of transgenic constructs and breeding for the fate mapping is shown. (C-E) Presumptive cone precursors showing b-galactosidase immunoreactivity exhibit high degree of coexpression with Cre in the superior domain of the retina. (F-K) Central and inferior domains reveal an increase in b-galactosidase labeled cells that do not overlay with Cre and are presumably rods based on their position in the ONL.

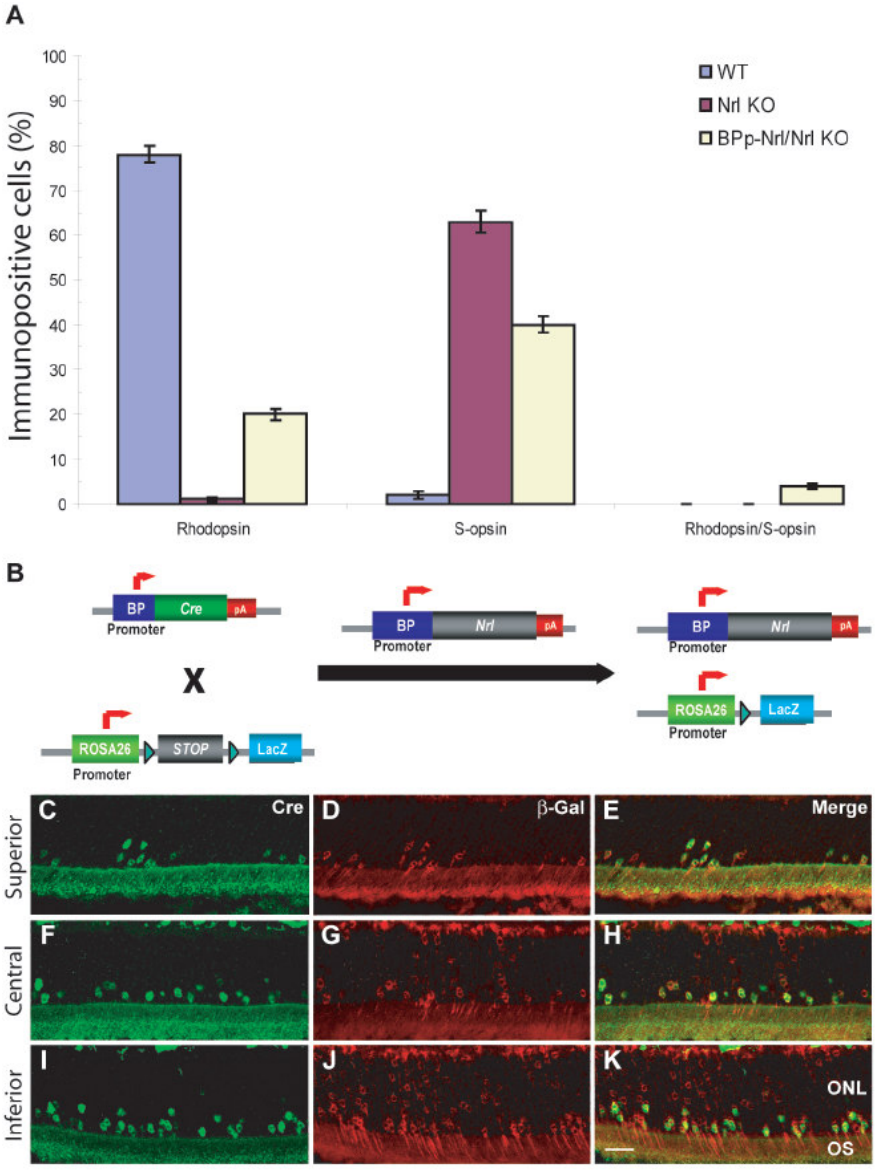
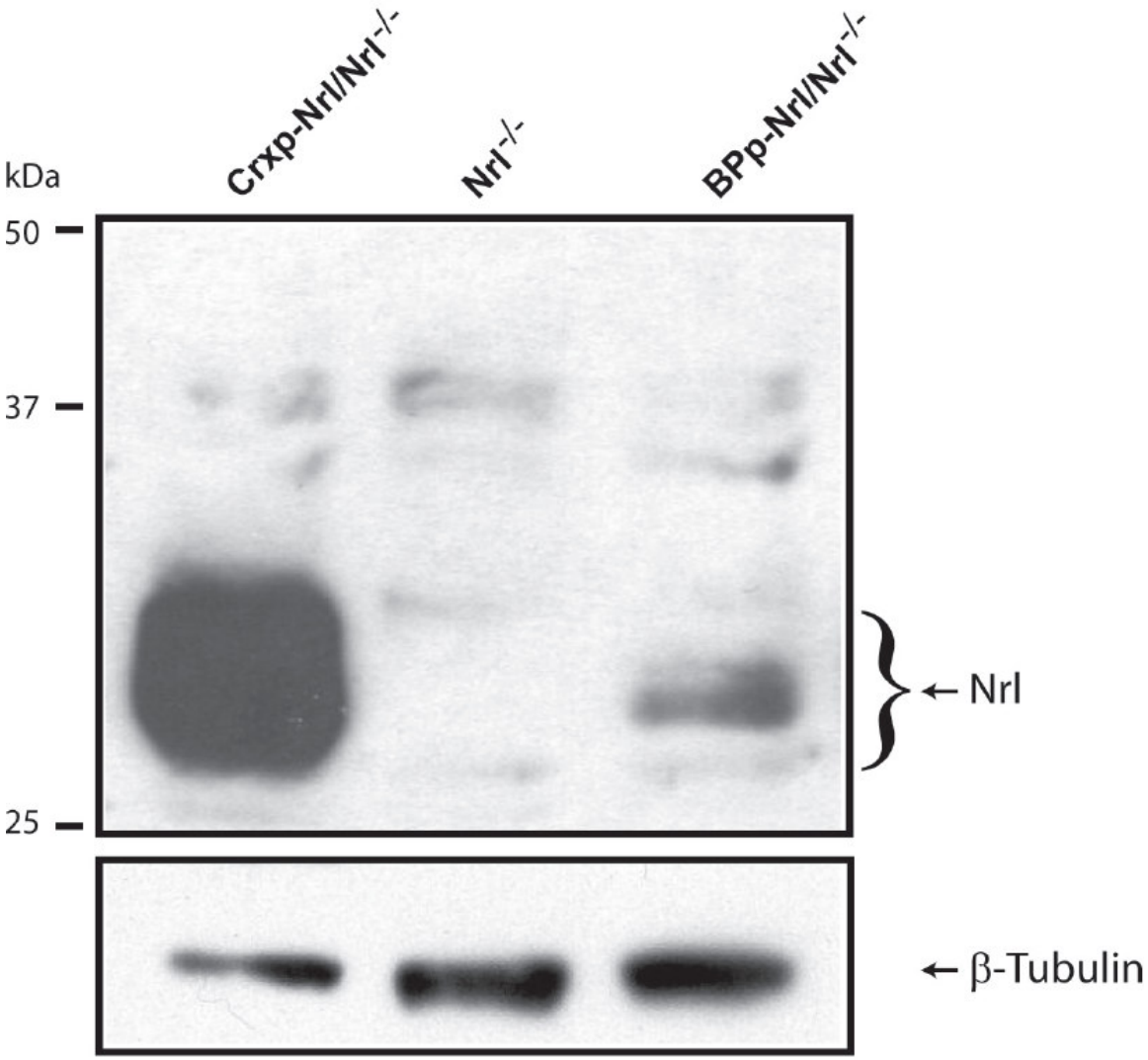


**Figure II-9. Immunoblot analysis to examine NRL expression in *Crxp-Nrl/Nrl*$^{-/-}$ and *BPp-Nrl/Nrl*$^{-/-}$ retinas.** Expression levels of the NRL protein were compared in retinas of transgenic mice. In contrast to *Crxp-Nrl/Nrl*$^{-/-}$, BP*p*-Nrl/Nrl$^{-/-}$ retinas contain > 5% of the NRL protein.



## References


Adler, R., and Hatlee, M. (1989). Plasticity and differentiation of embryonic retinal cells after terminal mitosis. Science *243*, 391-393.

Akimoto, M., Cheng, H., Zhu, D., Brzezinski, J. A., Khanna, R., Filippova, E., Oh, E. C., Jing, Y., Linares, J. L., Brooks, M.*, et al.* (2006). Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. Proc Natl Acad Sci U S A *103*, 3890-3895.

Akimoto, M., Filippova, E., Gage, P. J., Zhu, X., Craft, C. M., and Swaroop, A. (2004). Transgenic mice expressing Cre-recombinase specifically in M- or S-cone photoreceptors. Invest Ophthalmol Vis Sci *45*, 42-47.

Arber, S., Ladle, D. R., Lin, J. H., Frank, E., and Jessell, T. M. (2000). ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. Cell *101*, 485-498.

Banin, E., Obolensky, A., Idelson, M., Hemo, I., Reinhardtz, E., Pikarsky, E., Ben-Hur, T., and Reubinoff, B. (2006). Retinal incorporation and differentiation of neural precursors derived from human embryonic stem cells. Stem Cells *24*, 246-257.

Belliveau, M. J., Young, T. L., and Cepko, C. L. (2000). Late retinal progenitor cells show intrinsic limitations in the production of cell types and the kinetics of opsin synthesis. J Neurosci *20*, 2247-2254.

Buffelli, M., Burgess, R. W., Feng, G., Lobe, C. G., Lichtman, J. W., and Sanes, J. R. (2003). Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition. Nature *424*, 430-434.

Carter-Dawson, L. D., and LaVail, M. M. (1979a). Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. J Comp Neurol *188*, 245-262.

Carter-Dawson, L. D., and LaVail, M. M. (1979b). Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. J Comp Neurol *188*, 263-272.

Cayouette, M., Barres, B. A., and Raff, M. (2003). Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. Neuron *40*, 897-904.

Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M., and Ezzeddine, D. (1996). Cell fate determination in the vertebrate retina. Proc Natl Acad Sci U S A *93*, 589-595.

Chen, J., Rattner, A., and Nathans, J. (2005). The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes. J Neurosci *25*, 118-129.

Chen, S., Wang, Q. L., Nie, Z., Sun, H., Lennon, G., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Zack, D. J. (1997). Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron *19*, 1017-1030.

Cheng, H., Aleman, T. S., Cideciyan, A. V., Khanna, R., Jacobson, S. G., and Swaroop, A. (2006). In vivo function of the orphan nuclear receptor NR2E3 in establishing photoreceptor identity during mammalian retinal development. Hum Mol Genet *15*, 2588-2602.

Cheng, H., Khanna, H., Oh, E. C., Hicks, D., Mitton, K. P., and Swaroop, A. (2004). Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors. Hum Mol Genet *13*, 1563-1575.

Chiu, M. I., and Nathans, J. (1994). A sequence upstream of the mouse blue visual pigment gene directs blue cone-specific transgene expression in mouse retinas. Vis Neurosci *11*, 773-780.

Coles, B. L., Angenieux, B., Inoue, T., Del Rio-Tsonis, K., Spence, J. R., McInnes, R. R., Arsenijevic, Y., and van der Kooy, D. (2004). Facile isolation and the characterization of human retinal stem cells. Proc Natl Acad Sci U S A *101*, 15772-15777.

Daniele, L. L., Lillo, C., Lyubarsky, A. L., Nikonov, S. S., Philp, N., Mears, A. J., Swaroop, A., Williams, D. S., and Pugh, E. N., Jr. (2005). Cone-like morphological, molecular, and electrophysiological features of the photoreceptors of the Nrl knockout mouse. Invest Ophthalmol Vis Sci *46*, 2156-2167.

Dejneka, N. S., Surace, E. M., Aleman, T. S., Cideciyan, A. V., Lyubarsky, A., Savchenko, A., Redmond, T. M., Tang, W., Wei, Z., Rex, T. S.*, et al.* (2004). In utero gene therapy rescues vision in a murine model of congenital blindness. Mol Ther *9*, 182-188.

DW, S. J. a. R. (2001). Molecular Cloning, Vol 3 (Cold Spring Harbor, Cold Spring Harbor Laboratory Press).

Dyer, M. A., Livesey, F. J., Cepko, C. L., and Oliver, G. (2003). Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. Nat Genet *34*, 53-58.

Edlund, T., and Jessell, T. M. (1999). Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell *96*, 211-224.

Ezzeddine, Z. D., Yang, X., DeChiara, T., Yancopoulos, G., and Cepko, C. L. (1997). Postmitotic cells fated to become rod photoreceptors can be respecified by CNTF treatment of the retina. Development *124*, 1055-1067.

Furukawa, A., Koike, C., Lippincott, P., Cepko, C. L., and Furukawa, T. (2002). The mouse Crx 5'-upstream transgene sequence directs cell-specific and developmentally regulated expression in retinal photoreceptor cells. J Neurosci *22*, 1640-1647.

Gan, L., Xiang, M., Zhou, L., Wagner, D. S., Klein, W. H., and Nathans, J. (1996). POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells. Proc Natl Acad Sci U S A *93*, 3920-3925.

Ghosh, K. K., Bujan, S., Haverkamp, S., Feigenspan, A., and Wassle, H. (2004). Types of bipolar cells in the mouse retina. J Comp Neurol *469*, 70-82.

Hatakeyama, J., and Kageyama, R. (2004). Retinal cell fate determination and bHLH factors. Semin Cell Dev Biol *15*, 83-89.

Kanda, A., Friedman, J. S., Nishiguchi, K. M., and Swaroop, A. (2007). Retinopathy mutations in the bZIP protein NRL alter phosphorylation and transcriptional activity. Hum Mutat *28*, 589-598.

Kasthuri, N., and Lichtman, J. W. (2003). The role of neuronal identity in synaptic competition. Nature *424*, 426-430.

Katz, L. C., and Shatz, C. J. (1996). Synaptic activity and the construction of cortical circuits. Science *274*, 1133-1138.

Khanna, H., Akimoto, M., Siffroi-Fernandez, S., Friedman, J. S., Hicks, D., and Swaroop, A. (2006). Retinoic acid regulates the expression of photoreceptor transcription factor NRL. J Biol Chem *281*, 27327-27334.

Lerner, L. E., Gribanova, Y. E., Ji, M., Knox, B. E., and Farber, D. B. (2001). Nrl and Sp nuclear proteins mediate transcription of rod-specific cGMP-phosphodiesterase beta-subunit gene: involvement of multiple response elements. J Biol Chem *276*, 34999-35007.

Levine, E. M., Fuhrmann, S., and Reh, T. A. (2000). Soluble factors and the development of rod photoreceptors. Cell Mol Life Sci *57*, 224-234.

Levine, M., and Tjian, R. (2003). Transcription regulation and animal diversity. Nature *424*, 147-151.

Lin, J. H., Saito, T., Anderson, D. J., Lance-Jones, C., Jessell, T. M., and Arber, S. (1998). Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. Cell *95*, 393-407.

Livesey, F. J., and Cepko, C. L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci *2*, 109-118.

MacLaren, R. E., Pearson, R. A., MacNeil, A., Douglas, R. H., Salt, T. E., Akimoto, M., Swaroop, A., Sowden, J. C., and Ali, R. R. (2006). Retinal repair by transplantation of photoreceptor precursors. Nature *444*, 203-207.

Malicki, J. (2004). Cell fate decisions and patterning in the vertebrate retina: the importance of timing, asymmetry, polarity and waves. Curr Opin Neurobiol *14*, 15-21.

Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F., and Gruss, P. (2001). Pax6 is required for the multipotent state of retinal progenitor cells. Cell *105*, 43-55.

Mears, A. J., Kondo, M., Swain, P. K., Takada, Y., Bush, R. A., Saunders, T. L., Sieving, P. A., and Swaroop, A. (2001). Nrl is required for rod photoreceptor development. Nat Genet *29*, 447-452.

Mitton, K. P., Swain, P. K., Chen, S., Xu, S., Zack, D. J., and Swaroop, A. (2000). The leucine zipper of NRL interacts with the CRX homeodomain. A possible mechanism of transcriptional synergy in rhodopsin regulation. J Biol Chem *275*, 29794-29799.

Mitton, K. P., Swain, P. K., Khanna, H., Dowd, M., Apel, I. J., and Swaroop, A. (2003). Interaction of retinal bZIP transcription factor NRL with Flt3-interacting zinc-finger protein Fiz1: possible role of Fiz1 as a transcriptional repressor. Hum Mol Genet *12*, 365-373.

Ng, L., Hurley, J. B., Dierks, B., Srinivas, M., Salto, C., Vennstrom, B., Reh, T. A., and Forrest, D. (2001). A thyroid hormone receptor that is required for the development of green cone photoreceptors. Nat Genet *27*, 94-98.

Nishida, A., Furukawa, A., Koike, C., Tano, Y., Aizawa, S., Matsuo, I., and Furukawa, T. (2003). Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. Nat Neurosci *6*, 1255-1263.

Peng, G. H., Ahmad, O., Ahmad, F., Liu, J., and Chen, S. (2005). The photoreceptor-specific nuclear receptor Nr2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes. Hum Mol Genet *14*, 747-764.

Pignatelli, V., and Strettoi, E. (2004). Bipolar cells of the mouse retina: a gene gun, morphological study. J Comp Neurol *476*, 254-266.

Pittler, S. J., Zhang, Y., Chen, S., Mears, A. J., Zack, D. J., Ren, Z., Swain, P. K., Yao, S., Swaroop, A., and White, J. B. (2004). Functional analysis of the rod photoreceptor cGMP phosphodiesterase alpha-subunit gene promoter: Nrl and Crx are required for full transcriptional activity. J Biol Chem *279*, 19800-19807.

Rapaport, D. H., Wong, L. L., Wood, E. D., Yasumura, D., and LaVail, M. M. (2004). Timing and topography of cell genesis in the rat retina. J Comp Neurol *474*, 304-324.

Rehemtulla, A., Warwar, R., Kumar, R., Ji, X., Zack, D. J., and Swaroop, A. (1996). The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. Proc Natl Acad Sci U S A *93*, 191-195.

Roberts, M. R., Srinivas, M., Forrest, D., Morreale de Escobar, G., and Reh, T. A. (2006). Making the gradient: thyroid hormone regulates cone opsin expression in the developing mouse retina. Proc Natl Acad Sci U S A *103*, 6218-6223.

Shen, Q., Wang, Y., Dimos, J. T., Fasano, C. A., Phoenix, T. N., Lemischka, I. R., Ivanova, N. B., Stifani, S., Morrisey, E. E., and Temple, S. (2006). The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. Nat Neurosci *9*, 743-751.

Srinivas, M., Ng, L., Liu, H., Jia, L., and Forrest, D. (2006). Activation of the blue opsin gene in cone photoreceptor development by retinoid-related orphan receptor beta. Mol Endocrinol *20*, 1728-1741.

Strettoi, E., Mears, A. J., and Swaroop, A. (2004). Recruitment of the rod pathway by cones in the absence of rods. J Neurosci *24*, 7576-7582.

Strettoi, E., Porciatti, V., Falsini, B., Pignatelli, V., and Rossi, C. (2002). Morphological and functional abnormalities in the inner retina of the rd/rd mouse. J Neurosci *22*, 5492-5504.

Swain, P. K., Hicks, D., Mears, A. J., Apel, I. J., Smith, J. E., John, S. K., Hendrickson, A., Milam, A. H., and Swaroop, A. (2001). Multiple phosphorylated isoforms of NRL are expressed in rod photoreceptors. J Biol Chem *276*, 36824-36830.

Szel, A., Rohlich, P., Mieziewska, K., Aguirre, G., and van Veen, T. (1993). Spatial and temporal differences between the expression of short- and middle-wave sensitive cone pigments in the mouse retina: a developmental study. J Comp Neurol *331*, 564-577.

Turner, D. L., Snyder, E. Y., and Cepko, C. L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833-845.

Wang, Q. L., Chen, S., Esumi, N., Swain, P. K., Haines, H. S., Peng, G., Melia, B. M., McIntosh, I., Heckenlively, J. R., Jacobson, S. G.*, et al.* (2004). QRX, a novel homeobox gene, modulates photoreceptor gene expression. Hum Mol Genet *13*, 1025-1040.

Watanabe, T., and Raff, M. C. (1992). Diffusible rod-promoting signals in the developing rat retina. Development *114*, 899-906.

Wetts, R., and Fraser, S. E. (1988). Multipotent precursors can give rise to all major cell types of the frog retina. Science *239*, 1142-1145.

Yoshida, S., Mears, A. J., Friedman, J. S., Carter, T., He, S., Oh, E., Jing, Y., Farjo, R., Fleury, G., Barlow, C.*, et al.* (2004). Expression profiling of the developing and mature Nrl-/- mouse retina: identification of retinal disease candidates and transcriptional regulatory targets of Nrl. Hum Mol Genet *13*, 1487-1503.

Young, R. W. (1985). Cell differentiation in the retina of the mouse. Anat Rec *212*, 199-205.

Yu, J., He, S., Friedman, J. S., Akimoto, M., Ghosh, D., Mears, A. J., Hicks, D., and Swaroop, A. (2004). Altered expression of genes of the Bmp/Smad and Wnt/calcium signaling pathways in the cone-only Nrl-/- mouse retina, revealed by gene profiling using custom cDNA microarrays. J Biol Chem *279*, 42211-42220.

## CHAPTER III

## ROD DIFFERENTIATION FACTOR NRL ACTIVATES THE EXPRESSION OF NUCLEAR RECEPTOR NR2E3 TO SUPPRESS THE DEVELOPMENT OF CONE PHOTORECEPTORS

### Abstract

Neural developmental programs require a high level of coordination between the decision to exit cell cycle and acquisition of cell fate. The Maf-family transcription factor NRL is essential for rod photoreceptor specification in the mammalian retina as its loss of function converts rod precursors to functional cones. Ectopic expression of NRL or a photoreceptor-specific orphan nuclear receptor NR2E3 completely suppresses cone development while concurrently directing the post-mitotic photoreceptor precursors towards rod cell fate. Given that NRL and NR2E3 have overlapping functions and NR2E3 expression is abolished in the *Nrl*$^{-/-}$ retina, we wanted to clarify the distinct roles of NRL and NR2E3 during retinal differentiation. Here, we demonstrate that NRL binds to a sequence element in the *Nr2e3* promoter and enhances its activity synergistically with the homeodomain protein CRX. Using transgenic mice, we show that NRL can only partially suppress cone development in the absence of NR2E3. Gene profiling of retinas from transgenic mice that ectopically express NR2E3 or NRL in cone precursors reveals overlapping and unique targets of these two

transcription factors. Together with previous reports, the current findings establish the hierarchy of transcriptional regulators in determining rod versus cone cell fate in photoreceptor precursors during the development of mammalian retina.

## INTRODUCTION

The central nervous system is assembled from thousands of distinct cell types that must be produced in correct numbers and within appropriate spatial and temporal constraints (Donovan and Dyer, 2005, Dyer and Cepko, 2001 and Stevens, 1998). Unraveling the molecular mechanisms that control specification of diverse cell types during neurogenesis has been the subject of intense scrutiny. The vertebrate retina offers a convenient paradigm to examine cell fate decisions, as it is relatively less complex and more accessible to experimental manipulations. Birth-dating studies reveal that six neuronal cell types and one type of glia are produced from common pools of neuroepithelial progenitors in a conserved order of birth (Adler and Raymond, 2008, Carter-Dawson and LaVail, 1979, Livesey and Cepko, 2001, Marquardt and Gruss, 2002, Turner and Cepko, 1987, Turner et al., 1990 and Wetts and Fraser, 1988). Retinal ganglion cells are generated first, followed by cone, amacrine and horizontal cells, whereas rods, bipolar cells and Muller glia are generated later (Carter-Dawson and LaVail, 1979, Rapaport et al., 2004 and Young, 1985). A competence model has been proposed to explain the sequential birth order (Cepko et al., 1996 and Livesey and Cepko, 2001); according to this, multipotent pools of progenitor cells go

through discrete competence states during which they can only give rise to specific subsets of neurons. Restriction(s) in developmental potential can occur prior to or after exit from the final cell cycle through combinatorial regulatory mechanism(s) involving intrinsic factors and/or extrinsic signaling molecules (Cayouette et al., 2003, Edlund and Jessell, 1999 and Levine et al., 2000). As considerable overlap exists in the timings of cell birth, specific instructions and stringent controls are required to produce particular subtypes of neurons from a set of progenitor cells.

Rod and cone photoreceptors are specialized light-sensing neurons with highly structured membrane disks (called outer segments), which contain visual pigments and other phototransduction components. In most mammals (including humans and mice), rod photoreceptors greatly outnumber cones and generally account for over 70% of all cells in the retina (Carter-Dawson and LaVail, 1979). Although the morphology and physiology of photoreceptors are well documented, the developmental pathways from a multipotent retinal progenitor to a committed precursor and a terminally-differentiated photoreceptor are only beginning to be elucidated.

NRL belongs to the basic motif-leucine zipper (bZIP) family of transcription factors and is an essential regulator of early events leading to the birth and development of rod photoreceptors (Akimoto et al., 2006, Mears et al., 2001, Oh et al., 2007, Rehemtulla et al., 1996 and [waroop et al., 1992). It is preferentially expressed in rods (and not other retinal cells) and pineal gland (Akimoto et al., 2006 and Swain et al., 2001). In mice lacking NRL, rods are transformed to

cones that share morphological, molecular, and electrophysiological characteristics similar to wild type cones (Daniele et al., 2005, Mears et al., 2001 and Nikonov et al., 2005). More recently, gain of function studies reveal that NRL can influence all photoreceptor precursors to initiate a rod differentiation program at the expense of cones (Oh et al., 2007). Despite the absence of cones, cone bipolar and horizontal interneurons are present in the adult retina but do not attain appropriate neuronal morphology during synaptogenesis (Oh et al., 2007 and Raven et al., 2007). CRX is a photoreceptor-specific homeodomain protein that plays a critical role in the maturation of photoreceptors (Chen et al., 1997 and Furukawa et al., 1997), but it does not appear to be essential for initial specification events (Furukawa et al., 1999). Mis-expression studies in adult iris cells (Akagi et al., 2005) suggest that CRX acts as a photoreceptor competence factor before NRL defines rod identity. Control of photoreceptor cell fate also involves the participation of NR2E3, a photoreceptor-specific orphan nuclear receptor (Akhmedov et al., 2000, Chen et al., 2005, Cheng et al., 2004, Haider et al., 2000 and Peng et al., 2005). The *rd7* mice carrying an antisense L1 insertion into exon 5 of the *Nr2e3* gene exhibit a progressive photoreceptor degeneration accompanied by 1.5–2 fold increase in the number of S-cones (Akhmedov et al., 2000, Chen et al., 2006, Haider et al., 2001 and Ueno et al., 2005). Ectopic expression of NR2E3 or NRL (Cheng et al., 2006 and Oh et al., 2007) in the photoreceptor precursors of *Nrl*$^{-/-}$ mice results in the complete inhibition of cone developmental program (Cheng et al., 2006);

however, in contrast to NRL (Oh et al., 2007), functional rods are not generated by NR2E3 expression alone (Cheng et al., 2006).

Given that NRL and NR2E3 functions are overlapping and NR2E3 expression is undetectable in the *Nrl*$^{-/-}$ mice (Cheng et al., 2006, McIlvain and Knox, 2007, Mears et al., 2001 and Oh et al., 2007) it has been suggested that NR2E3 is downstream of NRL in transcriptional hierarchy controlling retinal development (Mears et al., 2001). In this report, we have examined whether NR2E3 is a direct target of NRL and evaluated the precise role NRL in cone specification in the absence of NR2E3. We also present expression profiles of retinas from transgenic mice that ectopically express either NRL and NR2E3 or NR2E3 alone in cone precursors, with a goal to identify cone-enriched genes in mature photoreceptors.

## Materials and Methods

**Transgenic mice.** The *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT* mice were generated previously (Cheng et al., 2006; Oh et al., 2007). We mated *Crxp-Nrl/WT* mice with the *rd7* mice (procured from Jackson Laboratory) to generate *Crxp-Nrl/rd7* mice. The mice, used for analysis reported here, were in a mixed background of 129X1/SvJ and C57BL/6J. PCR primers for genotyping the *Crxp-Nrl/WT* allele are: F: 5'-AGCCAATGTCACCTCCTGTT-3' and R: 5'-GGGCTCCCTGAATAGTAGCC-3'. PCR primers for genotyping the *rd7* allele are as reported (Haider et al., 2001). All studies involving mice were performed

in accordance with institutional and federal guidelines and approved by the University Committee on Use and Care of Animals at the University of Michigan.

**Gene Profiling.** The details of microarray analysis have been described earlier (Yoshida et al., 2004; Yu et al., 2004; Zhu et al., 2005). Briefly, total RNA (Trizol, Invitrogen) from P28 retinas was used to generate double-stranded cDNA for hybridization to mouse GeneChips MOE430.2.0, per guidelines (Affymetrix). Total retinal RNA from four independent samples was used for each evaluation. Normalized data were subjected to two-stage analysis based on false discovery rate with confidence interval (FDRCI) for identifying differentially expressed genes (Zhu et al., 2005).

**Immunohistochemistry.** Retinal whole mounts and 10 μm sections were probed with the following antibodies (Cheng et al., 2004; Strettoi et al., 2002): rabbit S-opsin, rabbit M-opsin, and rabbit cone-arrestin (generous gift from C. Craft, University of Southern California, Los Angeles, CA, and Chemicon), mouse anti-rhodopsin (1D4 and 4D2; generous gift from R. Molday, University of British Columbia, Vancouver, Canada). The secondary antibodies for fluorescent detection were AlexaFluor 488 and 546 (Molecular probes, Invitrogen). Sections were visualized using an Olympus FluoView 500 laser scanning confocal microscope. Images were subsequently digitized using FluoView software version 5.0.

**EMSA.** The electrophoretic mobility shift assays were performed using established methods (Hao et al., 2003), with minor modifications. Nuclear protein extracts from transfected COS-1 cells were prepared using a commercial kit (Active motif, Carlsbad, CA), and expression of NRL protein was confirmed by SDS-PAGE followed by immunoblotting. Nuclear extracts were incubated with 1 µg poly (dI-dC) at 4°C for 15 min in the binding buffer (12 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.9; 60 mM KCl; 4 mM $MgCl_2$; 1 mM EDTA [ethylenediaminetetra acetic acid]; 12% glycerol; 1 mM dithiothreitol; and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). Then, $^{32}$P-labeled double-stranded oligonucleotide (40,000 cpm) was added and the reaction was incubated at 4°C for 20 min. The DNA probe (-2820 nt to -2786 nt: NRE F5'-TGGCCTCTGGTGGCTTTGTCAGCAGTTCCAAGGCT-3', NRE R 5'-AGCCTTGGAACTGCTGACAAAGCCACCAGAGGCCA-3') contains a putative NRL-response element (NRE) (underlined) that is predicted by Genomatix. In competition studies, nuclear extracts were pre-incubated with 50 or 100X unlabeled oligonucleotide for 30 min at room temperature and incubated with labeled probe at room temperature for 20 min. A mutant oligonucleotide (F: 5'-TGGCCTCTGGTGGCTT TATTTGCAGTTCCAAGGCT-3', R: 5'-AGCCTTGGAACTGCAAATAAAGC CACCAGAGGCCA-3') with three nucleotide change in the NRE site was also used to compete for the protein binding to the probe. In order to immunologically identify the components in protein-DNA complexes, nuclear extracts were incubated with 2.0 µg of the anti-NRL antibody or normal rabbit IgG for 30 min at room temperature, followed by the addition of

labeled probe and a further incubation for 20 min at room temperature. The reaction mixtures were electrophoresed on 6% polyacrylamide gels at 175 volts for 2.5 hr and subjected to autoradiography.

**ChIP.** The chromatin immunoprecipitation assays were performed using a commercial kit (Active motif, Carlsbad, CA). Briefly, four snap-frozen retinas from wild type C57BL/6J mice were cross-linked for 15 min at room temperature with 1% formaldehyde in PBS containing protease inhibitors (Oh et al., 2007). The reaction was stopped by adding glycine (125 mM), followed by 5 min incubation at room temperature. The remaining steps were performed according to the manufacturer's instructions, using anti-NRL polyclonal antibody or normal rabbit IgG. ChIP DNAs were used for PCR amplification of a 248-bp fragment (-2989nt to -2742nt), containing a putative NRE (as determined by Genomatix), with primers 5'- GCATGCACTGTTCAAACACC-3' and 5'-GATAGGCTGTGCAGGGGTTA-3'. PCR with another pair of primers (5'-TGTCCTGAGTCTCC CTGCTT -3' and 5'- TAAGGCTGGCCAT AAAGTGG -3') that amplify a 209-bp fragment (1230 nt to 1438 nt) located about 4 kb downstream from the NRE site, served as a negative control.

**ERG.** Electroretinograms (ERGs) were recorded from 2–3 month old adult mice. Animals were dark-adapted for at least 12 h before intraperitonial administration of ketamine (93 mg/kg) and xylazine (8 mg/kg). After pupil dilation with topical 1% atropine and 0.5% tropicamide, corneal ERGs were recorded from both eyes

using gold wire loops with 0.5% tetracaine topical anesthesia and a drop of 2% methylcellulose for corneal hydration. A gold wire loop placed in the mouth was used as reference, and the ground electrode was attached to the tail. Body temperature was maintained at 37 ℃ with a heating pad. ERGs were recorded to single xenon white flashes (PS22 Photic Stimulator, Grass Telefactor, West Warwick, RI) presented in a Ganzfeld bowl. Responses were amplified at 10,000 gain at 1 to 1000 Hz (CP511 AC amplifier, Grass Telefactor), and digitized at a rate of 32 kHz. A notch filter was used to remove 60 Hz line noise. Stimulus intensity was attenuated with neutral density filters and ERGs were recorded to increasing intensity (– 6.0 to 1.09 log cd-s/$m^2$). Scotopic ERGs were recorded at 3 to 60 s interstimulus intervals depending on the stimulus intensity and responses were computer averaged with at least 20 averages at the lower intensities. Animals were then light adapted for 10 min by exposure to a white 32 cd/$m^2$ rod saturating background, and photopic ERGs were recorded for single flash white stimuli over a 2 log unit range. The a-wave was measured from the pre-stimulus baseline to the initial trough. B-waves were measured from the trough of the a-wave when present or from the baseline to the b-wave maximum. A second recording system was used to record S- and M-cone ERGs (Espion e2, Diagnosys LLC, Lowell, MA). Light-adapted ERGs were recorded on a 40 cd/$m^2$ background to a xenon flash and a UV filter (360 nm peak; Hoya U-360 filter, Edmund Optics, Barrington, NJ). M-cone ERGs were isolated using a green light-emitting diode (510 nm peak) on the Espion e2. The flash energy was adjusted to elicit responses of approximately equal amplitude for the two

wavelengths in WT mice. These stimuli were then used to record S- and M-cone ERGs in WT and in *Crxp-Nrl/rd7* mice.

## RESULTS

### NRL directly binds to the *Nr2e3* promoter

To determine whether NRL can modulate NR2E3 expression, we first analyzed the promoter of the *Nr2e3* gene and identified four sequence regions that are conserved in mammals (Figure III-1 A). *In silico* analysis revealed a putative NRL response element (NRE) in one of the conserved regions (see Figure III-1 A, grey box). Addition of nuclear extracts from COS-1 cells expressing the NRL protein, but not from mock-transfected cells, to $^{32}$P-labeled NRE oligonucleotide resulted in band-shift in electrophoretic mobility shift assays (EMSA) (Figure III-1 B; lanes 1–3), suggesting the binding of NRL to NRE sequence in the *Nr2e3* promoter region. The specificity of binding was substantiated by competition with an excess of unlabeled oligonucleotide spanning the NRE but not with a mutant sequence (lanes 4–6). The major shifted band (shown by the arrowhead) was clearly detectable upon the addition of rabbit IgG but not anti-NRL antibody (lanes 7, 8), providing further evidence in support of NRL's binding to *Nr2e3*-NRE. To determine whether NRL could bind the *Nr2e3* promoter *in vivo*, we performed chromatin immunoprecipitation (ChIP) experiments. Cross-linked protein–DNA complexes from wild-type adult retinas were immunoprecipitated with anti-NRL antibody, and purified ChIP DNA was used for PCR with primers flanking the *Nr2e3*-NRE site. A strong enrichment of

the *Nr2e3*-NRE promoter fragment was observed with anti-NRL antibody compared to rabbit IgG (Figure III-1C). Additionally, no significant enrichment was detected for another randomly-selected sequence in the *Nr2e3* gene (negative control) (Figure III-1 C).

**NRL induces the *Nr2e3* promoter activity in transfected cells**

We then examined the activity of a 4.5 kb *Nr2e3* promoter fragment (encompassing the conserved NRE sequence; see Figure III-1 A) in the presence of NRL. Transfection of HEK-293 cells with NRL, but not CRX, expression plasmid induced the luciferase reporter activity that was driven by the *Nr2e3* promoter (Figure III-1 D). Co-transfection of HEK-293 cells with both NRL and CRX plasmids resulted in further increase of the *Nr2e3* promoter activity (Figure III-1 D). This is consistent with previously-reported synergistic activation of several rod-specific genes by NRL and CRX (Chen et al., 1997, Cheng et al., 2004, Mitton et al., 2000 and Pittler et al., 2004).

**Overlapping yet distinct gene profiles are generated by NRL and NR2E3**

Recent investigations into the role of NRL and NR2E3 (Chen et al., 2005, Cheng et al., 2006, Hsiau et al., 2007, Oh et al., 2007 and Peng et al., 2005) and our findings reported here (Figure III-1) suggest that NRL suppresses cone differentiation by directly signaling through NR2E3. This level of regulation also implies that many molecular defects observed in mice lacking functional NR2E3 (e.g., the *rd7* mouse) are also present in the *Nrl*$^{-/-}$ mice (Corbo and Cepko, 2005

and Mears et al., 2001). To dissect the transcriptional activity of NRL versus NR2E3 in mature photoreceptors, we took advantage of two recently-generated transgenic mouse models — *Crxp-Nrl/WT* (Oh et al., 2007) and *Crxp-Nr2e3/WT* (Cheng et al., 2006). In these mice, a 2 kb *Crx* proximal promoter (Furukawa et al., 2002) leads to the expression of NRL or NR2E3 in photoreceptor precursors and transformation of cones to rod photoreceptors, without any obvious perturbation in retinal lamination or development of other cell types (Cheng et al., 2006 and Oh et al., 2007).

In the *Crxp-Nrl/WT* retinas, NRL and consequently NR2E3 ((Oh et al., 2007), see Figure III-1) are ectopically expressed in cone precursors; while only NR2E3 (and not NRL) is ectopically expressed in cone precursors of the *Crxp-Nr2e3/WT* retina. NRL and NR2E3 are also expressed in the developing rod precursors of both transgenic lines. Therefore, gene profiling of retinas from *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT* mice can reveal expression changes induced by NRL+NR2E3 or NR2E3 alone in cone precursors, respectively. Retinal RNA from P28 adult mice was hybridized to Affymetrix MOE430.2.0 GeneChips, which contain 45,101 probesets for mouse transcripts. A comparative analysis of gene clusters from *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT* retinas to WT samples revealed a number of genes involved in diverse signaling pathways and transcriptional regulation; Table III-1 shows the genes with FDRCI *P*-value of $< 0.1$ and a fold change $> 4$. In addition to established cone-specific genes, we also discovered several new genes down-regulated in the *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT* coneless groups which are potential cone-enriched target genes. We then

compared *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT* gene profiles to *Nrl*$^{-/-}$ (cone-only) and *rd7* (1.5–2 fold more S-cones) profiles. Many cone phototransduction genes that are up-regulated in the *Nrl*$^{-/-}$ (cone-only, Table III-2) and *rd7* (1.5–2 fold more S-cones, Table III-3) retinas are also significantly repressed in the *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT* coneless samples. Gene expression changes showing FDRCI *P*-value < 0.1 and a fold change > 10 are listed in Table III-2 and Table III-3.

**Expression of NRL can only suppress a subset of S-cones in the absence of NR2E3**

Similarities in gene profiles of *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT* retinas raise the question whether NRL can suppress cone gene expression and differentiation even in the absence of NR2E3. To evaluate this, we mated *Crxp-Nrl/WT* mice to *rd7* mice to generate a transgenic mouse line (*Crxp-Nrl/rd7*) that expresses NRL, but not NR2E3, in both cone and rod precursors. We first analyzed cone markers, such as S- and M-opsin, in retinal whole mounts. As previously demonstrated (Applebury et al., 2000), we observed an inferior to superior gradient of S-opsin expression (Figure III-2 A–C) and a superior to inferior gradient of M-opsin in the WT mice (data not shown). As predicted, S-opsin was detected throughout in the *Nrl*$^{-/-}$ retinal whole mounts (Figure III-2 D–F) and increased S-opsin staining was observed in the *rd7* retinas (Figure III-2 J–L); however, both S-opsin and M-opsin could not be detected in *Crxp-Nrl/WT* retinas (Figure III-2 G–I, and data not shown). In both *Nrl*$^{-/-}$ and *rd7* mice, whorls

are detected in the whole mount preparations (Figure III-2 D–F and J–L). In *Crxp-Nrl/rd7* retinal whole mounts, we observed a large absence of S-opsin staining in the superior domain (Figure III-2 M, N) yet detected a small population of S-opsin positive cells in the inferior retina (Figure III-2 M, O). The expression of M-opsin was unaltered (data not shown), and whorls could be detected throughout the retinas (Figure III-2 M–O).

As shown previously (Haider et al., 2001, Mears et al., 2001 and Oh et al., 2007), the number of cone arrestin (mCAR) and S-opsin positive cells in retinal cross-sections from *Nrl*$^{-/-}$ and *rd7* retinas were increased compared to WT, and there is an absence of cone-specific markers in *Crxp-Nrl/WT* mice (Figure III-3 A: a–o). In *Crxp-Nrl/rd7* sections, we observed normal cone arrestin and M-opsin staining but an absence of S-opsin in the superior domain (Figure III-3 A: m–o). In the inferior domain, we identified a few S-opsin positive cones and many S-opsin positive cell bodies at the inner portion of the ONL (Figure III-3 B: i, j). These findings were in contrast to S-opsin positive cell bodies distributed throughout the ONL and INL in *Nrl*$^{-/-}$ and *rd7* retinas (Figure III-3 B: c–d and g–h). The expression of cone arrestin and M-opsin in the *Crxp-Nrl/rd7* mice (harboring the *Crxp-Nrl* transgene in rd7 background with no NR2E3 function) but not in the *Crxp-Nrl/WT* mice (harboring the *Crxp-Nrl* transgene in wild-type background) demonstrates that NR2E3 is the primary suppressor of cone gene expression and cone differentiation.

**Cone function is detected but reduced in the *Crxp-Nrl/rd7* mice**

We performed electroretinography (ERG) recordings to measure the massed-field potential across the retina in the different transgenic lines. As reported previously (Oh et al., 2007), the ectopic expression of NRL in cone precursors (*Crxp-Nrl/WT*) resulted in an absence of cone-driven responses, whereas rod-driven components were preserved (Figure III-4 A–E). To characterize the functionality of cone-driven neurons in the absence of NR2E3, we analyzed the photopic response from *Crxp-Nrl/rd7* mice (Figure III-4 C, D). In response to brief flashes of white light, we first detected a cone-driven b-wave at 0.09 log cd-s/$m^2$. At the higher flash intensity of 1.09 log cd-s/$m^2$ the maximum b-wave amplitude was about 40% of the WT and *rd7* response amplitude (Figure III-4 C, D).

We further examined the photopic ERG in the *Crxp-Nrl/rd7* transgenic mice by recording light-adapted cone-mediated responses at 360 nm and 510 nm to isolate S-cone and M-cone function, respectively (Figure III-4 F). As predicted, the *Nrl*$^{-/-}$ mice showed an enhanced S-cone response when compared with WT mice (Daniele et al., 2005 and Mears et al., 2001). There was no significant difference in the M-cone response amplitude between *Nrl*$^{-/-}$ and WT mice. We then recorded from *Crxp-Nrl/rd7* mice and found that while the M-cone response was reduced by 40% from WT mice, S-cone responses were undetectable (Figure III-4 F).

## DISCUSSION

Regulatory networks defining rod versus cone identity are under the direct control of bZIP transcription factor NRL (Mears et al., 2001 and Oh et al., 2007). In this report, we demonstrate that NR2E3 is a direct transcriptional target of NRL and that specification of rod cell fate over cone differentiation is dictated by the activation of NR2E3 in response to NRL. Restricted expression of these two key transcriptional regulators in photoreceptor precursors is essential for proper development of rods. Ectopic expression of either protein in cone precursors can reprogram the cone development pathway to generate rod photoreceptors (Cheng et al., 2006 and Oh et al., 2007). We had shown previously that ectopic NR2E3 expression can inhibit the development of functional S and M-cones in the *Nrl*$^{-/-}$ retina (Cheng et al., 2006). The current data suggest that NR2E3 is necessary to completely repress the development of M and some S-cones, and NRL alone can only repress a subset of S-cones. These genetic models therefore raise the possibility of heterogeneity within S-cones.

Several studies have indicated the association of NRL and NR2E3 with promoter elements of cone-specific genes (Oh et al., 2007, Peng et al., 2005 and Peng and Chen, 2005). In this report, we analyzed the relationship of NRL and NR2E3 in modulating the cone developmental program. Data from immunohistochemical and physiological studies presented herein suggest that NRL modulates the development of S-cones, and its gain or loss of function primarily results in alterations of the S-cone pathway. One possibility is that S-cones represent the “default fate” for early-born photoreceptors in mice ([Cepko,

2000] and [Szel et al., 2000]) and that the expression of NRL controls an important node for this process.

The presence of ectopic S-opsin cells in the INL of *rd7* and *Nrl*$^{-/-}$ retinas is reminiscent of previous findings showing opsin-like immunoreactive cells in the developing retina (Gunhan et al., 2003). Our study reveals the presence of ectopic S-opsin positive cells that persist and survive in the adult retinas from *Nrl*$^{-/-}$ and *rd7* mice. What can account for the existence and survival of these neurons outside of their normal retinal photoreceptor layer? It is possible that NRL and NR2E3 dictate the expression of specific guidance cues that facilitate photoreceptor pathfinding to the vicinity of their appropriate target regions in a highly stereotyped and directed manner. Several candidate proteins that show an altered expression profile in the *Nrl*$^{-/-}$ retina appear to match the role of an axonal guidance cue (Yoshida et al., 2004 and Yu et al., 2004). These include members of families of secreted signaling molecules, such as Wingless/Wnt and Decapentaplegic/Bone Morphogenic Protein/Transforming Growth Factor B (Dpp/BMP/TGFb) (Charron and Tessier-Lavigne, 2005), which appear to have important functions during retinal development (Belecky-Adams and Adler, 2001, Liu et al., 2006, Liu et al., 2003, Osakada et al., 2007, Van Raay and Vetter, 2004, Xu et al., 2004 and Yu et al., 2004). We hypothesize that in the absence of NRL, and consequently NR2E3, changes in Wnt and BMP pathway may create noise in a homing signal that is required to (i) bring all photoreceptors to the ONL, and/or (ii) promote the appropriate wiring of rods and cones to bipolar and horizontal neurons. Although our current microarray experiments of P28

retinas did not reveal significant changes in classical pathfinding genes, future efforts will focus on profiling early postnatal stages of retinal development.

The absence of cones in the *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT* retinas resulted in normal architecture and lamination features, similar to the WT. A lack of structural abnormalities has allowed us to profile expression changes that may be specifically due to the absence of one class of neurons (i.e., cones). While the retinal profiles of *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT* mice had many common genes, the *Crxp-Nrl/WT* profile contained more unique changes in gene expression, consistent with NRL being upstream of NR2E3 in transcriptional hierarchy. One interesting novel gene revealed from the gene profiling experiments is PTTG1, which is down-regulated in the coneless retinas. The inhibitory chaperone PTTG1 has been implicated as a mitotic checkpoint gene involved at the metaphase-anaphase interface (Zou et al., 1999). Elucidation of specific roles of PTTG1 and other cone-enriched genes will require further investigation.

In conclusion, our work refines the roles of NRL and NR2E3 during photoreceptor differentiation. We show, for the first time, that NR2E3 is a direct downstream target of NRL and that the correct sequential expression of these transcriptional regulators may be required for appropriate expression of rod-specific opsin and suppression of cone phototransduction genes during normal retinal development. Additional studies are needed to precisely define how specific down-stream targets of NRL and NR2E3 fine-tune the differentiation of functional photoreceptors from post-mitotic committed precursors.

## Notes to Chapter III

**A modified version of this chapter has been previously published in: Brain Research. Oh, E.C., Cheng, H., Hao, H., Jia, L., Khan, N.W., and Swaroop, A. (2007). Rod differentiation factor NRL activates the expression of nuclear receptor NR2E3 to suppress the development of cone photoreceptors.**

**<u>Outside contribution</u>: Hong Cheng performed luciferase experiments. Hong Hao performed ChIP and EMSA experiments. Lin Jia performed perfusions and some immunohistochemistry. Naheed Khan conducted the ERG experiments. We also thank Matthew Brooks and Ritu Khanna for microarray chip experiments and analyses.**

**Figure III-1. NRL directly binds to and activates the *Nr2e3* promoter.** (**A**) Schematic of approximately 4.5 kb genomic DNA upstream of the *Nr2e3* transcription start site (denoted as +1). The four boxes indicate sequence regions conserved in mammals. A comparison of sequences in the second conserved region including a putative NRE (highlighted in grey) is shown. (**B**) EMSA. NRL containing COS-1 nuclear extract and $^{32}$P-labeled NRE probe (-2820 nt to -2786 nt) were used in EMSA. Lanes 1 to 8, 40 000 cpm $^{32}$P-labeled probe; lane 2, 10 µg nuclear extract (NE) from untransfected COS-1 cells; lanes 3 to 8, 10 µg nuclear extract from COS-1 cells transfected with *Nrl* cDNA expression plasmid (NRL NE); lane 4, 50-fold excess wild-type unlabeled NRE probe; lane 5, 100-fold excess wild-type unlabeled NRE probe; lane 6, 100-fold unlabeled mutant NRE probe; lane 7, 2.0 µg anti-NRL antibody; and lane 8, 2.0 µg normal rabbit IgG. (**C**) ChIP assays with chromatin from adult C57BL/6J retinas. Lane1, NRL antibody used for IP; lane 2, normal rabbit IgG used for IP, a negative control; and lane 3, input DNA used as template for PCR. Top panel: primers amplifying the NRE containing region (-2989 nt to -2742 nt) in the *Nr2e3* promoter region were used for PCR. Bottom panel: primers amplifying an irrelevant region (1230 nt to 1438 nt) in the *Nr2e3* gene were used for PCR. (**D**) Luciferase transactivation assays showing the activation of *Nr2e3* promoter by NRL and CRX.

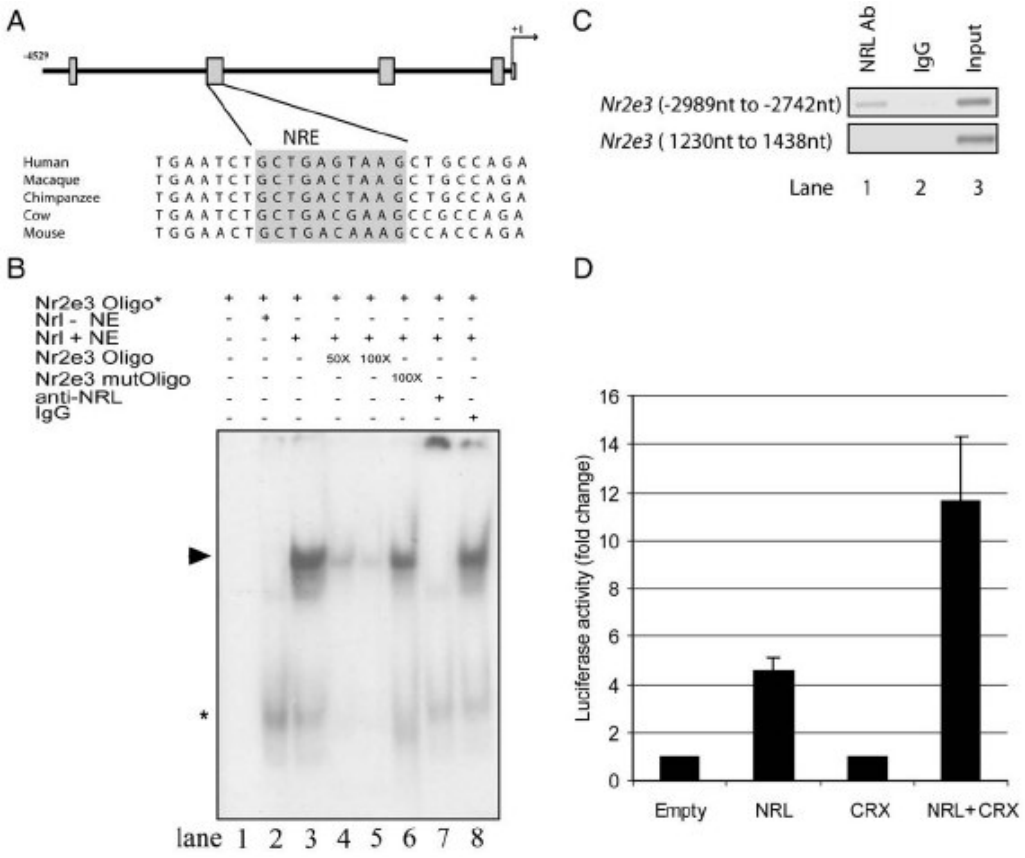


**Figure III-2. NRL does not completely suppress S-opsin expression in the absence of NR2E3.** (A–C) WT retina, showing superior to inferior gradient of S-opsin expression. (D–F) *Nrl*$^{-/-}$ retina. In the absence of NRL and NR2E3, whorls (arrows) and S-opsin can be detected throughout the retina. (G–I) *Crxp-Nrl/WT* retina. Ectopic expression of NRL in early cone precursors results in the complete absence of S-opsin. (J–L) *rd7* retina. In the absence of functional NR2E3, enhanced S-opsin expression and whorls (arrows) are observed in both superior and inferior domain. (M–O) *Crxp-Nrl/rd7* retina. In the presence of NRL but absence of NR2E3, expression of S-opsin is reduced but detectable in the inferior domain. Asterisks are positioned at 3-o'clock relative to the whorls (M). Arrows indicate the irregular S-opsin staining of whorls (O). Scale bar: 200 μm (A, D, G, J, and M), and 50 μm (B, C, E, F, H, I, K, L, N, and O).

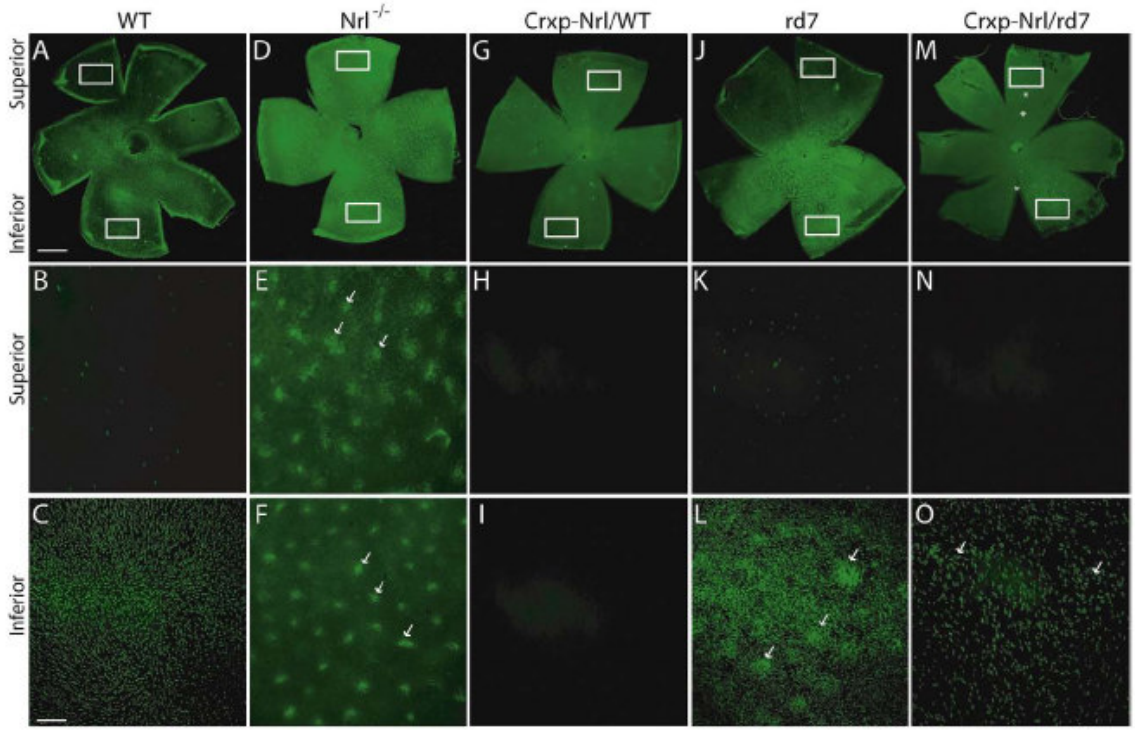


**Figure III-3. Expression of cone-specific markers and targeting of some photoreceptors to the ONL is perturbed in the absence of NRL and NR2E3.** Immunostaining with mCAR, S-opsin, and M-opsin from WT (**A: a-c**), *Nrl*$^{-/-}$ (**A: d-f**), *Crxp-Nrl/WT* (**A: g-i**), *rd7* (**A: j-l**) and *Crxp-Nrl/rd7* (**A: m-o**) retinal cryosections. Compared to WT (**B: a-b**) and *Crxp-Nrl/WT* (**B: e-f**), targeting of S-cones (arrows) to the ONL is perturbed in *Nrl*$^{-/-}$ (**B: c-d**) and rd7 retinas (**B: g-h**), and S-opsin positive nuclei are present in the INL. S-cone staining (arrowheads) in the *Crxp-Nrl/rd7* retinas (**B: i-j**) is observed in cells closest to the outer plexiform layer. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; BBZ, bisbenzamide. Scale bar: 25 μm.

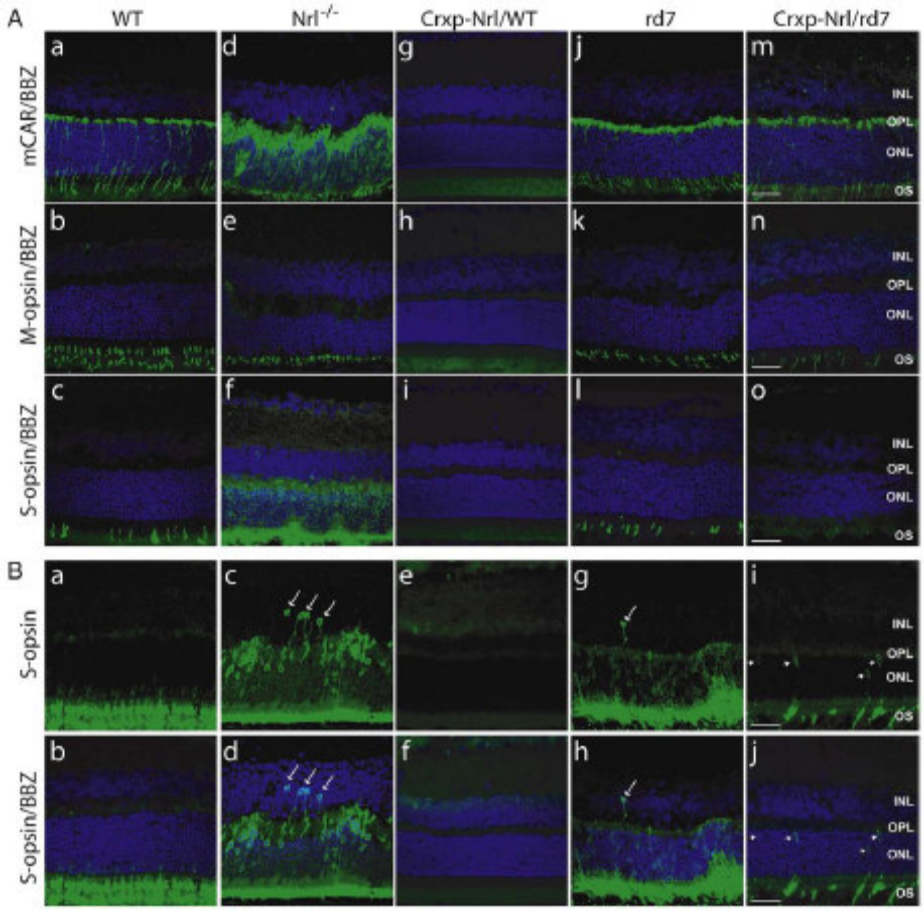
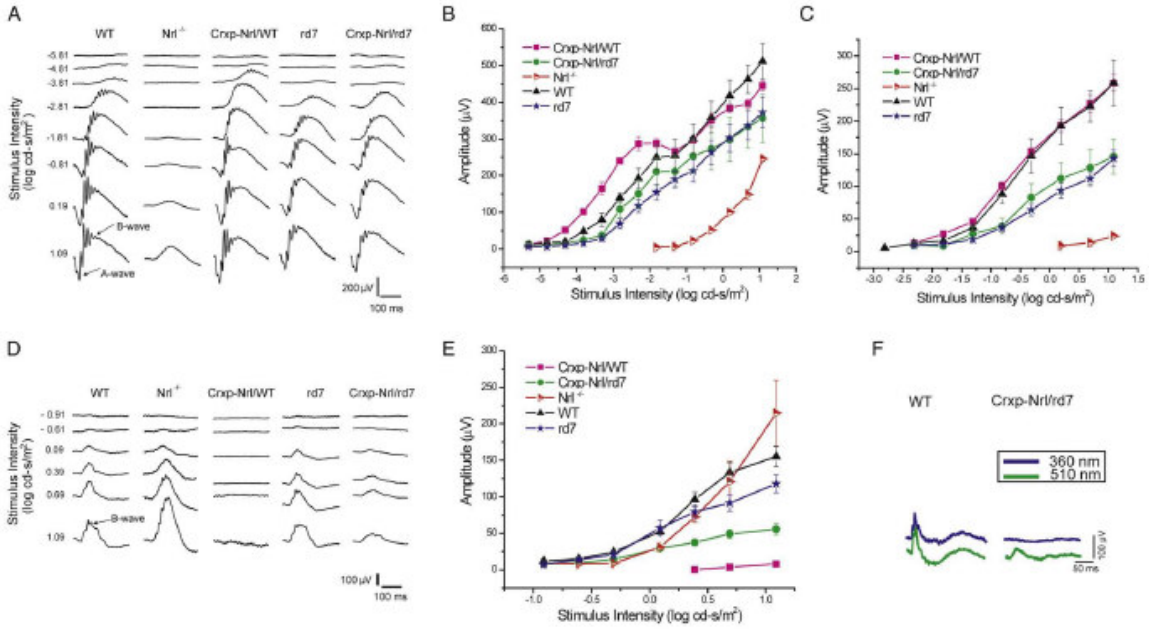


**Figure III-4. Absence of normal cone function in cone photoreceptors expressing NRL but not NR2E3.** (A) Representative dark-adapted ERGs for increasing stimulus intensities are shown for WT, *Nrl*$^{-/-}$, *Crxp-Nrl/WT*, *rd7* and *Crxp-Nrl/rd7* mice at two months age. Intensity-response functions for the (B) a-wave and (C) b-wave amplitude were plotted on log-linear coordinates. (D) Representative light-adapted ERGs waveforms with increasing stimulus intensity for WT, *Nrl*$^{-/-}$, *Crxp-Nrl/WT*, *rd7* and *Crxp-Nrl/rd7* mice, as indicated. (E) Plots of the b-wave amplitude as a function of stimulus intensity for light-adapted conditions. At 2 months of age, there was no significant difference in the photopic response between WT and *rd7* mice. (D–E) B-wave amplitude at the maximum intensity for the *Crxp-Nrl/rd7* mice. A reduction of about 40% is observed from the WT and *rd7* mice. (F) Representative light-adapted S- (360 nm) and M- (510 nm) cone ERGs showing a smaller M-cone response and undetectable S-cone response in *Crxp-Nrl/rd7* mice compared to WT mice. Bars indicate ± standard error.



## Table III-1. Non-redundant differentially expressed genes in *Crxp-Nrl/WT* or *Crxp-Nr2e3/WT* samples compared to WT retinas.

**Table 1 – Non-redundant differentially expressed genes in *Crxp-Nrl/WT* or *Crxp-Nr2e3/WT* samples compared to WT retinas**

| Gene symbol | Gene title | AFC *Crxp-Nrl/WT* versus WT | AFC *Crxp-Nr2e3/WT* versus WT | GO biological process description |
|---|---|---|---|---|
| *Overlapping genes in Crxp-Nrl/WT and Crxp-Nr2e3/WT versus WT group* | | | | |
| *Gdpd3* | Glycerophosphodiester phosphodiesterase domain containing 3 | 21.4 | 4.6 | Alcohol metabolic process |
| *Sgcg* | Sarcoglycan, gamma (dystrophin-associated glycoprotein) | 14.8 | 12.1 | Cytoskeleton organization and biogenesis |
| *Cap1* | CAP, adenylate cyclase-associated protein 1 (yeast) | 10.2 | 2.1 | Actin cytoskeleton organization and biogenesis |
| *Plekhk1* | Pleckstrin homology domain containing, family K member 1 | 6.3 | 4.7 | Regulation of cell proliferation |
| *Tsc22d1* | TSC22 domain family, member 1 | 6.0 | 6.5 | Regulation of transcription |
| *Paip1* | Polyadenylate binding protein-interacting protein 1 | 4.4 | 2.6 | Regulation of translation |
| *Huwe1* | HECT, UBA and WWE domain containing 1 | 4.1 | 3.0 | DNA packaging |
| *Abca13* | ATP-binding cassette, sub-family A (ABC1), member 13 | −4.0 | −6.5 | Intracellular protein transport |
| *Clca3* | Chloride channel calcium activated 3 | −4.5 | −4.9 | Chloride transport |
| *Camk2b* | Calcium/calmodulin-dependent protein kinase II, beta | −5.1 | −20.0 | Regulation of phosphorylation |
| *Gnat2* | Guanine nucleotide binding protein, alpha transducing 2 | −8.3 | −10.1 | Phototransduction |
| *Pttg1* | Pituitary tumor-transforming 1 | −10.3 | −4.2 | Meiotic chromosome segregation |
| *Pde6c* | Phosphodiesterase 6C, cGMP specific, cone, alpha prime | −15.9 | −13.8 | Activation of MAPK activity/ visual perception |
| *Opn1mw* | Opsin 1 (cone pigments), medium-wave-sensitive (color blindness, deutan) | −38.7 | −37.6 | Phototransduction |
| *Arr3* | Arrestin 3, retinal | −56.3 | −31.1 | Regulation of phosphorylation |
| *Pde6h* | Phosphodiesterase 6H, cGMP-specific, cone, gamma | −75.6 | −70.9 | Activation of MAPK activity/ visual perception |
| *Opn1sw* | Opsin 1 (cone pigments), short-wave-sensitive (color blindness, tritan) | −124.0 | −98.4 | Phototransduction |
| *Unique genes in Crxp-Nrl/WT versus WT group* | | | | |
| *Rds* | Retinal degeneration, slow (retinitis pigmentosa 7) | 56.5 | 1.5 | Visual perception |
| *Nudt21* | Nudix (nucleoside diphosphate linked moiety X)-type motif 21 | 10.3 | 1.1 | |
| *Deadc1* | Deaminase domain containing 1 | 6.6 | 1.9 | tRNA processing |
| *Atp2b2* | ATPase, Ca++ transporting, plasma membrane 2 | 5.6 | −1.1 | Inner ear development |
| *Scd2* | Stearoyl-Coenzyme A desaturase 2 | 5.5 | −1.2 | Fatty acid biosynthetic process |
| *Atp6v0a1* | ATPase, H+ transporting, lysosomal V0 subunit A1 | 5.2 | −1.1 | Transcription initiation |
| *Stk35* | Serine/threonine kinase 35 | 5.2 | 1.2 | Protein amino acid phosphorylation |
| *Uhmk1* | U2AF homology motif (UHM) kinase 1 | 4.3 | −1.3 | Protein amino acid phosphorylation |
| *Gucy2e* | Guanylate cyclase 2e | 4.3 | 1.4 | Visual perception |
| *Thbs1* | Thrombospondin 1 | 4.2 | 1.1 | Blood vessel morphogenesis |
| *Fabp4* | Fatty acid binding protein 4, adipocyte | −4.0 | 1.2 | Regulation of protein kinase activity |
| 5730410E15Rik | RIKEN cDNA 5730410E15 gene | −4.1 | −1.2 | |
| 5330426P16Rik | RIKEN cDNA 5330426P16 gene | −4.1 | −1.4 | |
| *Ssbp2* | Single-stranded DNA binding protein 2 | −4.3 | −1.0 | Regulation of transcription |
| *Ing3* | Inhibitor of growth family, member 3 | −4.4 | 1.0 | Regulation of transcription |
| *Dmd* | Dystrophin, muscular dystrophy | −4.4 | 1.1 | Peptide biosynthetic process |
| A930033H14Rik | RIKEN cDNA A930033H14 gene | −4.7 | 1.4 | |
| *Aff1* | AF4/FMR2 family, member 1 | −5.0 | −1.3 | Regulation of transcription |
| *Vapb* | Vesicle-associated membrane protein, associated protein B and C | −5.1 | −1.2 | |
| *Scn2b* | Sodium channel, voltage-gated, type II, beta | −8.2 | −1.1 | Sodium ion transport |

*(continued on next page)*

**Table 1** *(continued)*

| Gene symbol | Gene title | AFC *Crxp-Nrl*/WT versus WT | AFC *Crxp-Nr2e3*/WT versus WT | GO biological process description |
|---|---|---|---|---|
| *Unique genes in Crxp-Nr2e3/WT versus WT group* | | | | |
| *Sox30* | SRY-box containing gene 30 | 1.8 | 6.8 | Regulation of transcription |
| *2900056M20Rik* | RIKEN cDNA 2900056M20 gene | −1.9 | −6.6 | |
| *LOC552908* | Hypothetical LOC552908 | −1.7 | −10.1 | |

Gene profiles of P28 retinal samples from Crxp-Nrl/WT or Crxp-Nr2e3/WT mice were compared to those from the WT retina. Common genes in *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT*, or unique genes from *Crxp-Nrl/WT* or *Crxp-Nr2e3/WT* with a minimum fold change of 4 and FDRCI P-value of <0.1 are shown. AFC, average fold change.

**Table III-2. Non-redundant differentially expressed genes in Crxp-Nrl/WT or Crxp-Nr2e3/WT samples compared to *Nrl*$^{-/-}$ retinas.**

**Table 2 – Non-redundant differentially expressed genes in *Crxp-Nrl/WT* or *Crxp-Nr2e3/WT* samples compared to *Nrl*$^{-/-}$ retinas**

| Gene symbol | Gene title | AFC Crxp-Nrl/ WT versus Nrl$^{-/-}$ | AFC Crxp-Nr2e3/ WT versus Nrl$^{-/-}$ | GO biological process description |
|---|---|---|---|---|
| *Overlapping genes in Crxp-Nrl/WT and Crxp-Nr2e3/WT versus Nrl$^{-/-}$ group* | | | | |
| Nrl | Neural retina leucine zipper gene | 386.9 | 381.8 | Photoreceptor cell development |
| Rho | Rhodopsin | 347.1 | 332.5 | Phototransduction |
| Nr2e3 | Nuclear receptor subfamily 2, group E, member 3 | 115.8 | 83.9 | Photoreceptor cell development |
| Gnb1 | Guanine nucleotide binding protein, beta 1 | 69.7 | 46.1 | Phototransduction |
| Slc24a1 | Solute carrier family 24 (sodium/potassium/calcium exchanger), member 1 | 50.9 | 43.3 | Calcium ion transport/visual perception |
| A930036K24Rik | RIKEN cDNA A930036K24 gene | 50.1 | 88.7 | |
| BC016201 | cDNA sequence BC016201 | 40.8 | 33.8 | |
| Esrrb | Estrogen related receptor, beta | 32.4 | 26.9 | Intracellular protein transport |
| Susd3 | Sushi domain containing 3 | 23.4 | 21.5 | |
| Aqp1 | Aquaporin 1 | 21.3 | 31.4 | Water transport |
| BC038479 | cDNA sequence BC038479 | 20.4 | 17.5 | |
| Reep6 | Receptor accessory protein 6 | 17.9 | 20.9 | Protein binding |
| Mef2c | Myocyte enhancer factor 2C | 16.7 | 10.8 | Regulation of transcription |
| Pde6b | Phosphodiesterase 6B, cGMP, rod receptor, beta polypeptide | 16.7 | 19.4 | Activation of MAPK activity/ visual perception |
| Wisp1 | WNT1 inducible signaling pathway protein 1 | 16.1 | 20.3 | Regulation of cell growth |
| Sh2d1a | SH2 domain protein 1A | 15.8 | 18.7 | Intracellular signaling cascade |
| Sgcg | Sarcoglycan, gamma (dystrophin-associated glycoprotein) | 14.6 | 10.6 | Cytoskeleton organization and biogenesis |
| Samd11 | Sterile alpha motif domain containing 11 | 13.9 | 16.5 | Negative regulation of transcription |
| Plekha2 | Pleckstrin homology domain-containing, family A (phosphoinositide binding specific) member 2 | 11.4 | 14.7 | Phosphatidylinositol binding |
| Vax2os1 | Vax2 opposite strand transcript 1 | 10.3 | 15.6 | |
| Gucа1b | Guanylate cyclase activator 1B | 10.2 | 10.6 | Phototransduction |
| Gulo | Gulonolactone (L-) oxidase | −10.0 | −31.3 | L-ascorbic acid biosynthetic process |
| Pik3ap1 | Phosphoinositide-3-kinase adaptor protein 1 | −11.1 | −11.3 | Transmembrane receptor protein tyrosine kinase signaling protein activity |
| Gngt2 | Guanine nucleotide binding protein (G-protein), gamma transducing activity polypeptide 2 | −11.7 | −11.9 | G-protein coupled receptor protein signaling pathway |
| En2 | Engrailed 2 | −12.0 | −10.4 | Regulation of transcription |
| Myocd | Myocardin | −12.5 | −12.7 | Regulation of cell growth |
| Kcne2 | Potassium voltage-gated channel, Isk-related subfamily, gene 2 | −12.7 | −12.4 | Potassium ion transport |
| Arhgdib | Rho, GDP dissociation inhibitor (GDI) beta | −13.8 | −12.8 | Rho GDP-dissociation inhibitor activity |
| Parvb | Parvin, beta | −13.9 | −14.0 | Actin binding |
| Cckbr | Cholecystokinin B receptor | −17.4 | −33.3 | G-protein coupled receptor protein signaling pathway |
| Klhl4 | Kelch-like 4 (*Drosophila*) | −19.8 | −17.4 | |
| A930009A15Rik | RIKEN cDNA A930009A15 gene | −24.4 | −28.9 | |
| Otop3 | Otopetrin 3 | −39.2 | −39.3 | |
| Cngb3 | Cyclic nucleotide gated channel beta 3 | −40.8 | −49.4 | Potassium ion transport |
| Gnat2 | Guanine nucleotide binding protein, alpha transducing 2 | −46.0 | −56.2 | Phototransduction |
| Fabp7 | Fatty acid binding protein 7, brain | −60.1 | −48.8 | Lipid binding |
| Opn1mw | Opsin 1 (cone pigments), medium-wave-sensitive (color blindness, deutan) | −77.9 | −76.2 | Phototransduction |
| Clca3 | Chloride channel calcium activated 3 | −91.6 | −93.5 | Chloride transport |
| Pde6c | Phosphodiesterase 6C, cGMP specific, cone, alpha prime | −158.3 | −136.8 | Activation of MAPK activity/ visual perception |
| Arr3 | Arrestin 3, retinal | −271.2 | −166.0 | Regulation of phosphorylation |

*(continued on next page)*

**Table 2 (continued)**

| Gene symbol | Gene title | AFC Crxp-Nrl/ WT versus $Nrl^{-/-}$ | AFC Crxp-Nr2e3/ WT versus $Nrl^{-/-}$ | GO biological process description |
|---|---|---|---|---|
| *Overlapping genes in Crxp-Nrl/WT and Crxp-Nr2e3/WT versus $Nrl^{-/-}$ group* | | | | |
| *Pde6h* | Phosphodiesterase 6H, cGMP-specific, cone, gamma | −429.1 | −409.3 | Activation of MAPK activity/ visual perception |
| *Opn1sw* | Opsin 1 (cone pigments), short-wave-sensitive (color blindness, tritan) | −559.2 | −546.8 | Phototransduction |
| *Unique genes in Crxp-Nrl/WT versus $Nrl^{-/-}$ group* | | | | |
| *Rds* | Retinal degeneration, slow (retinitis pigmentosa 7) | 149.7 | 3.3 | Sensory perception of light stimulus |
| *Stk35* | Serine/threonine kinase 35 | 11.7 | 2.3 | Protein amino acid phosphorylation |
| *Mtmr7* | Myotubularin related protein 7 | −11.8 | 1.8 | Phospholipid dephosphorylation |
| *Pcdh15* | Protocadherin 15 | −12.6 | −3.3 | Sensory perception of light stimulus |
| *Pip5k2b* | Phosphatidylinositol-4-phosphate 5-kinase, type II, beta | −12.6 | 1.3 | Glycerophospholipid metabolic process |
| *Unique genes in Crxp-Nr2e3/WT versus $Nrl^{-/-}$ group* | | | | |
| *A930003C13Rik* | RIKEN cDNA A930003C13 gene | 3.5 | 11.5 | |
| *Skiv2l2* | Superkiller viralicidic activity 2-like 2 (S. cerevisiae) | −4.4 | −17.0 | RNA splicing |

Gene profiles of P28 retinal samples from *Crxp-Nrl/WT* or *Crxp-Nr2e3/WT* were compared to the profiles from the $Nrl^{-/-}$ retina. We show common differentially expressed genes in *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT* retina, or unique genes from *Crxp-Nrl/WT* or *Crxp-Nr2e3/WT*, with a minimum fold change of 10 and FDRCI P-value of <0.1. AFC, average fold change.

## Table III-3. Non-redundant differentially expressed genes in Crxp-Nrl/WT or Crxp-Nr2e3/WT samples compared to *rd7* retinas.

**Table 3 – Non-redundant differentially expressed genes in *Crxp-Nrl/WT* or *Crxp-Nr2e3/WT* samples compared to *rd7* retinas**

| Gene symbol | Gene title | AFC *Crxp-Nrl/WT* versus *rd7* | AFC *Crxp-Nr2e3/WT* versus *rd7* | GO biological process description |
|---|---|---|---|---|
| *Overlapping genes in Crxp-Nrl/WT and Crxp-Nr2e3/WT versus rd7 group* | | | | |
| *Eif2s3y* | Eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked | 65.3 | 79.1 | Macromolecule biosynthetic process |
| *Ddx3y* | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked | 62.1 | 74.0 | |
| *Sgcg* | Sarcoglycan, gamma (dystrophin-associated glycoprotein) | 14.8 | 10.7 | Cytoskeleton organization and biogenesis |
| *Jarid1d* | Jumonji, AT rich interactive domain 1D (Rbp2 like) | 11.8 | 11.2 | Regulation of transcription |
| *LOC640072 /// LOC677194* | Hypothetical protein LOC640072 /// Hypothetical protein LOC677194 | −11.3 | −12.6 | |
| *A230097K15Rik* | RIKEN cDNA A230097K15 gene | −12.7 | −11.9 | |
| *Arhgdib* | Rho, GDP dissociation inhibitor (GDI) beta | −12.8 | −11.9 | Rho GDP-dissociation inhibitor activity |
| *Gulo* | Gulonolactone (L-) oxidase | −13.6 | −42.6 | L-ascorbic acid biosynthetic process |
| *Socs3* | Suppressor of cytokine signaling 3 | −15.8 | −11.6 | Regulation of phosphorylation |
| *Bub1b* | Budding uninhibited by benzimidazoles 1 homolog, beta (*S. cerevisiae*) | −20.1 | −14.7 | Regulation of phosphorylation |
| *Edn2* | Endothelin 2 | −20.1 | −11.1 | Regulation of vasoconstriction |
| *Otop3* | Otopetrin 3 | −28.1 | −28.1 | |
| *Fabp7* | Fatty acid binding protein 7, brain | −37.7 | −30.6 | Lipid binding |
| *A930009A15Rik* | RIKEN cDNA A930009A15 gene | −38.0 | −45.1 | |
| *Gnat2* | Guanine nucleotide binding protein, alpha transducing 2 | −39.7 | −48.5 | Phototransduction |
| *Opn1mw* | Opsin 1 (cone pigments), medium-wave-sensitive (color blindness, deutan) | −40.5 | −39.6 | Phototransduction |
| *Arr3* | Arrestin 3, retinal | −50.1 | −25.3 | Regulation of phosphorylation |
| *Clca3* | Chloride channel calcium activated 3 | −78.5 | −80.1 | Chloride transport |
| *Pde6c* | Phosphodiesterase 6C, cGMP specific, cone, alpha prime | −127.0 | −109.7 | Activation of MAPK activity/ visual perception |
| *Opn1sw* | Opsin 1 (cone pigments), short-wave-sensitive (color blindness, tritan) | −223.2 | −218.3 | Phototransduction |
| *Pde6h* | Phosphodiesterase 6H, cGMP-specific, cone, gamma | −365.1 | −348.2 | Activation of MAPK activity/ visual perception |
| *Unique genes in Crxp-Nrl/WT versus rd7 group* | | | | |
| *Rds* | Retinal degeneration, slow (retinitis pigmentosa 7) | 74.7 | 1.6 | Sensory perception of light stimulus |
| *Cap1* | CAP, adenylate cyclase-associated protein 1 (yeast) | 11.3 | 2.3 | Actin cytoskeleton organization and biogenesis |
| *Scn2b* | Sodium channel, voltage-gated, type II, beta | −10.2 | −1.3 | Sodium ion transport |
| *Fabp4* | Fatty acid binding protein 4, adipocyte | −11.3 | −2.4 | Regulation of protein kinase activity |
| *Mtmr7* | Myotubularin related protein 7 | −27.7 | −1.3 | Phospholipid dephosphorylation |
| *Unique genes in Crxp-Nr2e3/WT versus rd7 group* | | | | |
| *Camk2b* | Calcium/calmodulin-dependent protein kinase II, beta | −3.1 | −12.2 | Regulation of phosphorylation |
| *LOC552908* | Hypothetical LOC552908 | −2.4 | −14.0 | |

Gene profiles of P28 retinal samples from *Crxp-Nrl/WT* or *Crxp-Nr2e3/WT* were compared to those of *rd7* retina. Common genes in *Crxp-Nrl/WT* and *Crxp-Nr2e3/WT*, or unique genes from *Crxp-Nrl/WT* or *Crxp-Nr2e3/WT* with a minimum fold change of 10 and FDR(1) P-value of <0.1 are shown. AFC, average fold change.

## References


Adler, R., and Raymond, P. A. (2007). Have we achieved a unified model of photoreceptor cell fate specification in vertebrates? Brain Res.

Akagi, T., Akita, J., Haruta, M., Suzuki, T., Honda, Y., Inoue, T., Yoshiura, S., Kageyama, R., Yatsu, T., Yamada, M., and Takahashi, M. (2005). Iris-derived cells from adult rodents and primates adopt photoreceptor-specific phenotypes. Invest Ophthalmol Vis Sci *46*, 3411-3419.

Akhmedov, N. B., Piriev, N. I., Chang, B., Rapoport, A. L., Hawes, N. L., Nishina, P. M., Nusinowitz, S., Heckenlively, J. R., Roderick, T. H., Kozak, C. A.*, et al.* (2000). A deletion in a photoreceptor-specific nuclear receptor mRNA causes retinal degeneration in the rd7 mouse. Proc Natl Acad Sci U S A *97*, 5551-5556.

Akimoto, M., Cheng, H., Zhu, D., Brzezinski, J. A., Khanna, R., Filippova, E., Oh, E. C., Jing, Y., Linares, J. L., Brooks, M.*, et al.* (2006). Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. Proc Natl Acad Sci U S A *103*, 3890-3895.

Applebury, M. L., Antoch, M. P., Baxter, L. C., Chun, L. L., Falk, J. D., Farhangfar, F., Kage, K., Krzystolik, M. G., Lyass, L. A., and Robbins, J. T. (2000). The murine cone photoreceptor: a single cone type expresses both S and M opsins with retinal spatial patterning. Neuron *27*, 513-523.

Belecky-Adams, T., and Adler, R. (2001). Developmental expression patterns of bone morphogenetic proteins, receptors, and binding proteins in the chick retina. J Comp Neurol *430*, 562-572.

Carter-Dawson, L. D., and LaVail, M. M. (1979). Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. J Comp Neurol *188*, 263-272.

Cayouette, M., Barres, B. A., and Raff, M. (2003). Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. Neuron *40*, 897-904.

Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M., and Ezzeddine, D. (1996). Cell fate determination in the vertebrate retina. Proc Natl Acad Sci U S A *93*, 589-595.

Charron, F., and Tessier-Lavigne, M. (2005). Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance. Development *132*, 2251-2262.

Chen, J., Rattner, A., and Nathans, J. (2005). The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes. J Neurosci *25*, 118-129.

Chen, J., Rattner, A., and Nathans, J. (2006). Effects of L1 retrotransposon insertion on transcript processing, localization and accumulation: lessons from the retinal degeneration 7 mouse and implications for the genomic ecology of L1 elements. Hum Mol Genet *15*, 2146-2156.

Chen, S., Wang, Q. L., Nie, Z., Sun, H., Lennon, G., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Zack, D. J. (1997). Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron *19*, 1017-1030.

Cheng, H., Aleman, T. S., Cideciyan, A. V., Khanna, R., Jacobson, S. G., and Swaroop, A. (2006). In vivo function of the orphan nuclear receptor NR2E3 in establishing photoreceptor identity during mammalian retinal development. Hum Mol Genet *15*, 2588-2602.

Cheng, H., Khanna, H., Oh, E. C., Hicks, D., Mitton, K. P., and Swaroop, A. (2004). Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors. Hum Mol Genet *13*, 1563-1575.

Corbo, J. C., and Cepko, C. L. (2005). A hybrid photoreceptor expressing both rod and cone genes in a mouse model of enhanced S-cone syndrome. PLoS Genet *1*, e11.

Daniele, L. L., Lillo, C., Lyubarsky, A. L., Nikonov, S. S., Philp, N., Mears, A. J., Swaroop, A., Williams, D. S., and Pugh, E. N., Jr. (2005). Cone-like morphological, molecular, and electrophysiological features of the photoreceptors of the Nrl knockout mouse. Invest Ophthalmol Vis Sci *46*, 2156-2167.

Donovan, S. L., and Dyer, M. A. (2005). Regulation of proliferation during central nervous system development. Semin Cell Dev Biol *16*, 407-421.

Dyer, M. A., and Cepko, C. L. (2001). Regulating proliferation during retinal development. Nat Rev Neurosci *2*, 333-342.

Edlund, T., and Jessell, T. M. (1999). Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell *96*, 211-224.

Furukawa, T., Morrow, E. M., and Cepko, C. L. (1997). Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell *91*, 531-541.

Furukawa, T., Morrow, E. M., Li, T., Davis, F. C., and Cepko, C. L. (1999). Retinopathy and attenuated circadian entrainment in Crx-deficient mice. Nat Genet *23*, 466-470.

Gunhan, E., van der List, D., and Chalupa, L. M. (2003). Ectopic photoreceptors and cone bipolar cells in the developing and mature retina. J Neurosci *23*, 1383-1389.

Haider, N. B., Jacobson, S. G., Cideciyan, A. V., Swiderski, R., Streb, L. M., Searby, C., Beck, G., Hockey, R., Hanna, D. B., Gorman, S.*, et al.* (2000). Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. Nat Genet *24*, 127-131.

Haider, N. B., Naggert, J. K., and Nishina, P. M. (2001). Excess cone cell proliferation due to lack of a functional NR2E3 causes retinal dysplasia and degeneration in rd7/rd7 mice. Hum Mol Genet *10*, 1619-1626.

Hao, H., Qi, H., and Ratnam, M. (2003). Modulation of the folate receptor type beta gene by coordinate actions of retinoic acid receptors at activator Sp1/ets and repressor AP-1 sites. Blood *101*, 4551-4560.

Levine, E. M., Fuhrmann, S., and Reh, T. A. (2000). Soluble factors and the development of rod photoreceptors. Cell Mol Life Sci *57*, 224-234.

Liu, H., Thurig, S., Mohamed, O., Dufort, D., and Wallace, V. A. (2006). Mapping canonical Wnt signaling in the developing and adult retina. Invest Ophthalmol Vis Sci *47*, 5088-5097.

Liu, J., Wilson, S., and Reh, T. (2003). BMP receptor 1b is required for axon guidance and cell survival in the developing retina. Dev Biol *256*, 34-48.

Livesey, F. J., and Cepko, C. L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci *2*, 109-118.

Marquardt, T., and Gruss, P. (2002). Generating neuronal diversity in the retina: one for nearly all. Trends Neurosci *25*, 32-38.

McIlvain, V. A., and Knox, B. E. (2007). Nr2e3 and Nrl can reprogram retinal precursors to the rod fate in Xenopus retina. Dev Dyn *236*, 1970-1979.

Mears, A. J., Kondo, M., Swain, P. K., Takada, Y., Bush, R. A., Saunders, T. L., Sieving, P. A., and Swaroop, A. (2001). Nrl is required for rod photoreceptor development. Nat Genet *29*, 447-452.

Mitton, K. P., Swain, P. K., Chen, S., Xu, S., Zack, D. J., and Swaroop, A. (2000). The leucine zipper of NRL interacts with the CRX homeodomain. A possible mechanism of transcriptional synergy in rhodopsin regulation. J Biol Chem *275*, 29794-29799.

Nikonov, S. S., Daniele, L. L., Zhu, X., Craft, C. M., Swaroop, A., and Pugh, E. N., Jr. (2005). Photoreceptors of Nrl -/- mice coexpress functional S- and M-cone opsins having distinct inactivation mechanisms. J Gen Physiol *125*, 287-304.

Oh, E. C., Khan, N., Novelli, E., Khanna, H., Strettoi, E., and Swaroop, A. (2007). Transformation of cone precursors to functional rod photoreceptors by bZIP transcription factor NRL. Proc Natl Acad Sci U S A *104*, 1679-1684.

Osakada, F., Ooto, S., Akagi, T., Mandai, M., Akaike, A., and Takahashi, M. (2007). Wnt signaling promotes regeneration in the retina of adult mammals. J Neurosci *27*, 4210-4219.

Peng, G. H., Ahmad, O., Ahmad, F., Liu, J., and Chen, S. (2005). The photoreceptor-specific nuclear receptor Nr2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes. Hum Mol Genet *14*, 747-764.

Pittler, S. J., Zhang, Y., Chen, S., Mears, A. J., Zack, D. J., Ren, Z., Swain, P. K., Yao, S., Swaroop, A., and White, J. B. (2004). Functional analysis of the rod photoreceptor cGMP phosphodiesterase alpha-subunit gene promoter: Nrl and Crx are required for full transcriptional activity. J Biol Chem *279*, 19800-19807.

Rapaport, D. H., Wong, L. L., Wood, E. D., Yasumura, D., and LaVail, M. M. (2004). Timing and topography of cell genesis in the rat retina. J Comp Neurol *474*, 304-324.

Raven, M. A., Oh, E. C., Swaroop, A., and Reese, B. E. (2007). Afferent control of horizontal cell morphology revealed by genetic respecification of rods and cones. J Neurosci *27*, 3540-3547.

Rehemtulla, A., Warwar, R., Kumar, R., Ji, X., Zack, D. J., and Swaroop, A. (1996). The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. Proc Natl Acad Sci U S A *93*, 191-195.

Stevens, C. F. (1998). Neuronal diversity: too many cell types for comfort? Curr Biol *8*, R708-710.

Strettoi, E., Porciatti, V., Falsini, B., Pignatelli, V., and Rossi, C. (2002). Morphological and functional abnormalities in the inner retina of the rd/rd mouse. J Neurosci *22*, 5492-5504.

Swain, P. K., Hicks, D., Mears, A. J., Apel, I. J., Smith, J. E., John, S. K., Hendrickson, A., Milam, A. H., and Swaroop, A. (2001). Multiple phosphorylated isoforms of NRL are expressed in rod photoreceptors. J Biol Chem *276*, 36824-36830.

Swaroop, A., Xu, J. Z., Pawar, H., Jackson, A., Skolnick, C., and Agarwal, N. (1992). A conserved retina-specific gene encodes a basic motif/leucine zipper domain. Proc Natl Acad Sci U S A *89*, 266-270.

Turner, D. L., and Cepko, C. L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature *328*, 131-136.

Turner, D. L., Snyder, E. Y., and Cepko, C. L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833-845.

Van Raay, T. J., and Vetter, M. L. (2004). Wnt/frizzled signaling during vertebrate retinal development. Dev Neurosci *26*, 352-358.

Wetts, R., and Fraser, S. E. (1988). Multipotent precursors can give rise to all major cell types of the frog retina. Science *239*, 1142-1145.

Xu, Q., Wang, Y., Dabdoub, A., Smallwood, P. M., Williams, J., Woods, C., Kelley, M. W., Jiang, L., Tasman, W., Zhang, K., and Nathans, J. (2004). Vascular development in the retina and inner ear: control by Norrin and Frizzled-4, a high-affinity ligand-receptor pair. Cell *116*, 883-895.

Yoshida, S., Mears, A. J., Friedman, J. S., Carter, T., He, S., Oh, E., Jing, Y., Farjo, R., Fleury, G., Barlow, C*., et al.* (2004). Expression profiling of the developing and mature Nrl-/- mouse retina: identification of retinal disease candidates and transcriptional regulatory targets of Nrl. Hum Mol Genet *13*, 1487-1503.

Young, R. W. (1985). Cell differentiation in the retina of the mouse. Anat Rec *212*, 199-205.

Yu, J., He, S., Friedman, J. S., Akimoto, M., Ghosh, D., Mears, A. J., Hicks, D., and Swaroop, A. (2004). Altered expression of genes of the Bmp/Smad and Wnt/calcium signaling pathways in the cone-only Nrl-/- mouse retina, revealed by gene profiling using custom cDNA microarrays. J Biol Chem *279*, 42211-42220.

Zhu, D., Hero, A. O., Qin, Z. S., and Swaroop, A. (2005). High throughput screening of co-expressed gene pairs with controlled false discovery rate (FDR) and minimum acceptable strength (MAS). J Comput Biol *12*, 1029-1045.

Zou, H., McGarry, T. J., Bernal, T., and Kirschner, M. W. (1999). Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science *285*, 418-422.

# CHAPTER IV

# AFFERENT CONTROL OF HORIZONTAL CELL MORPHOLOGY REVEALED BY GENETIC RE-SPECIFICATION OF RODS AND CONES

## Abstract

The first inhibitory interneurons of the retina, the horizontal cells, stratify within the outer plexiform layer, extending dendritic terminals that connect to the pedicles of cone photoreceptors, and an axon terminal system contacting the spherules of rod photoreceptors. How the horizontal cells acquire this morphology is unknown, but instructive interactions with afferents are suggested to play a role in the development of synaptic circuits. Here we show that the morphology of the axon terminal system and the dendritic field are selectively regulated by innervation from their respective afferents: Genetic re-specification of all cones to become rods, in *Crxp-Nrl* transgenic mice, produces an atrophic dendritic field, yet leaves the axon terminal system largely intact. By contrast, in the retinas of $Nrl^{-/-}$ mice, where the population of rod photoreceptors is re-specified to adopt a cone fate, the dendritic field is hypertrophic while the axon terminal system is underdeveloped. Our studies reveal that, while cell-intrinsic mechanisms drive the formation of independent dendritic versus axonal domains, the afferents play a selectively instructive role in defining their respective morphologies.

## Introduction

Horizontal cells in the mammalian retina give rise to dendrites that ramify in a radiate pattern within the outer plexiform layer (OPL), where their sole source of innervation is from the terminals of cone photoreceptors, the pedicles (Kolb, 1974). One form of horizontal cell, being the only type present in the murine retina (Peichl et al., 1998), also extends an axonal-like process that courses for a short distance away from the dendritic field before elaborating a terminal arbor. Despite the similarity to axons elsewhere in the nervous system, the horizontal cell axon does not conduct nerve impulses but rather provides metabolic support for its terminal arbor, which is selectively innervated by the spherules of rod photoreceptors and serves as an independent functional entity comparable to the dendritic domain (Peichl et al., 1998). The dendritic and the axonal domains are not only post-synaptic to the cones and the rods, respectively, but also provide inhibitory feedback onto those photoreceptors through a currently debated mechanism (Kamermans et al., 2001; Hirasawa and Kaneko, 2003; Hirano et al., 2005). The molecular mechanisms generating this polarization of the cell into a dendritic and an axonal domain are unknown, but cell-intrinsic specification seems likely (Horton and Ehlers, 2003; Jan and Jan, 2003; Horton et al., 2005). Afferent innervation may still, however, shape the differentiation of these cells, through secreted factors or by way of membrane-bound or activity-dependent signaling (McAllister, 2000; Cline, 2001; Lom et al.,

2002; Wong and Ghosh, 2002; Hua and Smith, 2004; Sorensen and Rubel, 2006).

In the mouse retina, the afferent population of photoreceptors is composed of 97% rods and 3% cones (Carter-Dawson and LaVail, 1979b). We sought to define the role of the afferents by taking advantage of two different mouse models that genetically re-specify the cones to adopt a rod fate, or the rods to adopt a cone fate, by manipulating the expression of the transcription factor gene *Nrl* (Swaroop et al., 1992). In *Crxp-Nrl* transgenic mice, (referred to herein as rod-*full*), the *Crx* promoter drives *Nrl* expression, instructing the post-mitotic cone precursors to become rods; hence, cones are never formed (Oh et al., 2007). This transgenic mouse is distinct from a previously described cone-*less* mouse (Reese et al., 2005), in which the expression of an attenuated diphtheria toxin gene driven by a cone opsin promoter obliterates the cones during the first postnatal week (Soucy et al., 1998). The loss of *Nrl* in *Nrl*$^{-/-}$ mice (referred to herein as cone-*full*), in contrast, causes all post-mitotic rod precursors to become cones (Mears et al., 2001; Daniele et al., 2005). In this report, we first describe the general condition of the outer retina in these two mouse models, using immunofluorescence techniques to identify the organization of the outer plexiform layer (OPL). We subsequently examine the morphology of individually labeled horizontal cell axons and dendrites to determine the extent to which their properties are dependent upon the afferents.

## Materials and Methods

**Tissue preparation.** All experiments were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals, and under authorization by respective Institutional Animal Care and Use Committees at UCSB and at the University of Michigan. Wild-type C57BL/6 mice, rod-full (*Crxp-Nrl*) mice (Oh et al., 2007) and cone-full ($Nrl^{-/-}$) mice (Mears et al., 2001) were given a lethal dose of sodium pentobarbital (120 mg/kg, i.p.) and perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.2 at 20$^{o}$C). Whole retinas were either dissected from the eyes, or the entire eye was embedded in agarose and sectioned through the optic nerve head on a Vibratome at a thickness of 100 µm. Retinal sections were prepared for immunofluorescence, while whole retinas were prepared for DiI labeling.

**Immunofluorescence**. Retinal sections from one month old mice were triple-labeled using antibodies to calbindin (a mouse monoclonal antibody at 1:10,000 from Sigma, C8666; St. Louis, MO), neurofilaments (a rabbit polyclonal antiserum to the 150kDa neurofilament subunit at 1:500 from Millipore, AB1981; Temecula, CA), cytochrome oxidase (a mouse monoclonal antibody at 1:100 from Invitrogen, AB6403; Eugene OR), piccolo (an affinity-purified guinea pig antibody at 1:1000; Dick et al., 2001; a generous gift from W. Altrock, Leibniz Institute for Neurobiology) and mouse cone arrestin (an affinity-purified rabbit antibody (LUMIj) at 1:1000; Zhu et al., 2002; a generous gift from C. Craft,

University of Southern California). Primary antibodies were detected with a cocktail of appropriate donkey secondary IgGs conjugated to Cy2 or Alexa 488, Cy3 and Cy5 (at 1:200 from Jackson ImmunoResearch Labs; West Grove, PA, or from Invitrogen; Eugene, OR). Specimens were examined using an Olympus Fluoview laser scanning confocal microscope with a 60× objective, in which image stacks were collected at 1 µm intervals across 10 µm.

**DiI labeling.** Crystals of the lipophilic dye, DiI (Invitrogen, D3911; Eugene, OR) were inserted into fixed whole retinas from adult (2-4 month old) mice, and in P-5 and P-10 mice, as previously described (Reese et al., 2005). Individual horizontal cell dendritic fields or axon terminals, labeled by diffusion through their axons, were subsequently imaged at 0.5 µm intervals using confocal microscopy, and reconstructed through their full three-dimensional extent using Metamorph (Downington, PA). For adult labeled cells, the dendritic field area, the number of primary dendrites, and the depth of dendritic processes were determined using Metamorph, as were the area and the depth of the axonal terminal fields. One-way ANOVAs were conducted, followed by post-hoc Scheffe tests to determine if any of these morphological features differed between the adult wild-type, rod-full and cone-full retinas. Additional details are provided elsewhere (Reese et al., 2005).

## Results

### Re-organization of the outer retina in rod-full and cone-full mice

The characteristic features of the outer retina in the wild-type adult mouse are shown in Figure IV-1 a-d: an antibody to mouse cone arrestin, in blue, shows the cone outer segments at the top (Figure IV-1 a); beneath these, the cone inner segments and somata are labeled less intensely. The somata are restricted to this outermost portion of the outer nuclear layer (ONL), largely adjacent to the mitochondria-rich inner segments of the rods and cones, detected using an antibody to cytochrome oxidase, shown in green in Figure IV-1 c. Each cone soma has a prominent mitochondrion situated basally (Carter-Dawson and LaVail, 1979a), at the location where the axon emerges (Figure IV-1 c). Those axons extend through the full thickness of ONL, expanding to produce a stratum of pedicles within the OPL (blue profiles in the OPL in Figure IV-1 a, c). The presynaptic cytomatrix protein, piccolo, present along the tip of synaptic ribbons in both pedicles and spherules (Dick et al., 2001), has also been labeled with an antibody, shown in red (Figure IV-1 a-d). Figure 1b and d are higher magnification views of the OPL, showing the localization of piccolo along the base of the pedicles, revealing the clustered distribution of the synaptic ribbons associated with each pedicle; the remaining piccolo labeling, largely outside or overlapping the apical aspect of the pedicles, identifies the distribution of the ribbon synapses associated with the rod spherules (unlabeled). The pedicles and spherules are also rich in mitochondria (Figure IV-1 d), though the

mitochondria are more apically positioned relative to the ribbons, never co-localizing with the piccolo labeling (Carter-Dawson and LaVail, 1979a). Shown as well, in green in Figure IV-1 a, is labeling for the calcium binding protein, calbindin, a marker for horizontal cells and their processes, particularly their proximal dendrites. Because the plexus of horizontal cell dendrites is so dense and overlapping (Reese et al., 2005), the processes from individual horizontal cells cannot be resolved using immunostaining techniques. Notice the sites of contact between the calbindin-positive processes and the pedicles (Figure IV-1 b).

The architecture of the adult rod-full retina (*Crxp-Nrl*) is comparable to that of the wild-type retina, except that no cones are present (Figure IV-1 g-j). Labeled cone outer segments and somata are entirely absent (note the lack of blue profiles in Figure IV-1 g, i), as is the stratum of perinuclear mitochondria in the outer parts of the ONL (Figure IV-1 i; compare with green basal labeling in the blue somata in Figure IV-1 c), along with the stratum of pedicles within the OPL (Figure IV-1 g-j). Consistent with this absence of stratified cone pedicles, the pattern of piccolo-immunostaining in the OPL is now devoid of an isolated stratum of clustered ribbons adjacent to the horizontal cell plexus (compare Figure IV-1 h, j, l with b, d, f). The calbindin-positive plexus of dendrites is still labeled in the rod-full retina, showing no overt changes relative to the wild-type retina (green label in Figure IV-1 h). The architecture of these rod-full retinas is similar to that of a cone-less transgenic mouse model in which nearly all (97%) of the cone photoreceptors die during the early postnatal period following activation

of an attenuated diphtheria toxin transgene driven by a cone opsin upstream regulatory sequence (Soucy et al., 1998; M.A.R. and B.E.R. unpublished observations).

The cone-full (*Nrl*$^{-/-}$) retina, by contrast, shows cone somata labeled for mouse cone arrestin throughout the full thickness of the ONL (Figure IV-1 m, n), and large cytochrome oxidase-positive puncta throughout the ONL (Figure IV-1 o, p), consistent with other evidence indicating that the entire population of cells in the ONL has now adopted a cone fate (Mears et al., 2001; Daniele et al., 2005; Nikonov et al., 2005). Within the OPL, a single stratum of large cone pedicles is no longer detectable; cone arrestin labeling now fills the entire depth of the OPL, but discrete profiles are less readily discriminated (Figure IV-1 m-p). The piccolo labeling pattern within the OPL also lacks an isolated stratum of basally located, clustered, ribbons adjacent to the horizontal cell plexus (Figure IV-1 n, p; compare with 1 b, d). These morphological changes are consistent with EM data showing a reorganization of the OPL, in which pedicle-like structures with multiple synaptic ribbons are present throughout the depth of the OPL, but that the ribbons within a pedicle are not stratified along the basal surface (Strettoi et al., 2004).

The axon of the horizontal cell is rich in neurofilaments (Peichl and Gonzalez-Soriano, 1993), and antibodies to the medium neurofilament subunit label processes within the OPL that do not, for the most part, colocalize with calbindin-labeled dendrites (Figure IV-1 e, f). Both are distributed within the OPL of the wild-type retina; however, the distal tips of the dendrites and the axon

terminal system, where these contact the pedicles and spherules, respectively, are less readily labeled (Figure IV-1 e, f). In the rod-full retina, the organization of the OPL in such immunolabeled specimens is not appreciably different, but for the presence of occasional sprouting by labeled processes into the ONL (Figure IV-1 k, l). The sprouting is most clearly revealed using antibodies to NF, but these processes are also occasionally calbindin-positive, and cannot be unambiguously associated with either a dendritic or axonal component on the basis of their immunolabeling alone (Figure IV-1 k, l). Such sprouting is not detected in the cone-full (*Nrl*$^{-/-}$) retina, where the immunolabeling pattern for NF is qualitatively like that seen in the wild-type retina (Figure IV-1 q, r).

**Dendritic morphology is regulated by the cones**

Horizontal cells give rise to, on average, six primary dendrites that extend from the soma to course within the OPL, branching to establish a dendritic field with an average diameter of 79 µm (Figure IV-2 a). Higher order branches give rise to periodic terminal clusters that contact individual pedicles within the dendritic field, and because of their overlapping dendritic coverage, about six neighboring horizontal cells contact each pedicle (Reese et al., 2005).

Horizontal cells in the rod-full retina, by contrast, show an atrophic dendritic morphology when compared with wild-type retina (Figure IV-2 b), yet dendritic field size is largely unchanged, being on average 78 µm in diameter. The periodic terminal clusters are absent, although small clusters are infrequently found within the field. The atrophic morphology of these cells is comparable to

that observed in the retinas of cone-less transgenic mice, but the latter show conspicuously larger and dystrophic, if comparably rare, terminal clusters (Reese et al., 2005). The overall similarity in phenotype between these models demonstrates that the atrophy cannot reflect putative degenerative changes following denervation, because the horizontal cells in the rod-full retina are never exposed to cone pedicles during development.

Horizontal cells in the cone-full retina have an average of eight primary dendrites, and establish a dendritic field that is slightly smaller than that in the wild-type or cone-less retinas, averaging 71 μm in diameter (Figure IV-2 d, e). More conspicuously, the density of higher order branching within the dendritic field is far greater in the cone-full retina (Figure IV-2 c). Within that dense dendritic field, individual terminal clusters associated with single pedicles are not discriminable when compared with wild-type horizontal cells, due to the density of pedicles within the OPL. Consistent with this, labeled dendritic fields, when viewed in rotation, now occupy the full depth of the OPL rather than arborizing only within the innermost portion of this synapse-rich region, where their synaptic contacts with pedicles are normally found (compare with Figures IV-1 b and n). Dendritic arborization depth was significantly greater in these cone-full retinas (Figure IV-2 f).

These features discriminating the dendritic field of horizontal cells in wild-type, rod-full and cone-full retinas emerge after the normal period of synaptogenesis with the cone pedicles, beginning at P-5. Prior to this stage, the dendrites of horizontal cells radiate in multiple directions from the soma. On P-5,

they begin to stratify in the nascent OPL, though periodic terminal clusters have yet to form (Reese et al., 2005). Horizontal cells in the wild-type, rod-full and cone-full retinas are barely discriminable at this initial stage of stratification (Figure IV-3 a-c). By P-10, however, the wild-type cells display periodic clusters studded across the stratified dendritic field, associated with the sites of pedicle contact (Figure IV-3 d), while those in the rod-full retina show no evidence of terminal clustering (Figure IV-3 e), well after they have stratified within a cone-free environment. Rather, in the absence of pedicles, the dendritic fields of horizontal cells in the rod-full retina exhibit hypertrophic branching (Figure IV-3 e), as they do in the cone-less transgenic retina at this stage (Reese et al., 2005). The dendritic fields of P-10 horizontal cells in the cone-full retina are also hypertrophic (Figure IV-3 f), maintaining this excessive branching into maturity (Figure IV-2 c), whereas the horizontal cells deprived of any cone afferents subsequently atrophy (Figure IV-2 b).

**Axonal morphology is regulated by the rods**

The axon terminal and its appendages, forming contacts exclusively with rod spherules, has a more variable size, shape and orientation (Figure IV-4 a). The axon broadens to form the thicker body of the terminal, giving rise to multiple branches that in turn yield individual branchlets extending into the spherules, with 2-4 per spherule in association with the 1-2 ribbons made by each spherule (Migdale et al., 2003). In the rod-full retina, the morphology of single axon terminals appears expanded in size, although not significantly (Figure IV-4 b, d).

Cone-full retinas, by contrast, having no rod photoreceptors, display an axon terminal arbor that is comparably atrophic: while the primary branch of the axon terminal is present, the higher-order branchlets that characterize the wild-type and rod-full terminal field are often reduced in density in the cone-full retina (Figure IV-4 c). Associated with this, the overall area of the axon terminal field is smaller (Figure IV-4 d), though the depth of its distribution is greater (Figure IV-4 e), spanning the thickness of the expanded OPL (Figure IV-1 n, p, r).

The rod spherules normally establish synaptic contacts after the cone pedicles initiate synaptogenesis (Olney, 1968; Blanks et al., 1974; Sherry et al., 2003). Curiously, changes in the axon terminals can be detected as early as P-5, when both the developing arbor, as well as the girth of the axonal process itself, are reduced relative to those axonal arbors for wild-type and rod-full retinas (Figure IV-5). In the absence of any rods, their growth would appear to be stunted even at this early stage, but they go on to develop a morphology similar to that of a wild-type axon terminal arbor, albeit reduced in area and expanded in depth (Figure IV-4).

## Discussion

The establishment of neuronal architecture within the central nervous system is a complex developmental process that is as yet poorly elucidated. The lesser complexity and ready access to the neural retina render it an ideal system for dissecting the underlying mechanisms that generate synaptic connectivity and

cellular architecture using genetically-manipulated mouse models. In this report, we show that the distinct properties of the dendritic and the axonal arbors of horizontal cells are independently controlled by their afferents during development. The periodic distribution of cone pedicles instructs a comparably periodic formation of terminal clusters in the wild-type retina. In strains of mice with a 50% reduction in cone afferents, the frequency of such terminal clustering is coincidently reduced (Reese et al., 2005), while a complete elimination of cone afferents in the rod-full retina leads to a failure of these clustered terminals to form. Conversely, the large increase in cone afferents in the cone-full retina induces a blanket of dendritic terminals to form, no longer showing any periodicity nor stratification in their formation, extending to fill the depth of the OPL. Together, these results make clear that the cone afferents play an instructive, rather than merely a permissive, role in defining the dendritic morphology of a horizontal cell.

The dense distribution of rod spherules present in the wild-type retina, differentiating slightly later than the cone pedicles (Olney, 1968; Blanks et al., 1974), acts independently upon the axon terminal system, inducing a comparably dense branching and terminal sprouting to form. Re-specification of the cones to become rods appears to yield a larger axonal arbor, while the entire loss of the rods in the cone-full retina yields an underdeveloped axon terminal field, although those changes do not appear to be proportional to the size of the afferent population. The stunted development of the axon terminal system in the cone-full retina is apparent even before synaptogenesis, suggesting that differentiating

rods are already supplying a signal for axon terminal outgrowth during normal development. Together, these results demonstrate how different afferent populations regulate the morphology of a nerve cell. While horizontal cell morphology is unique in having discrete compartments as synaptic targets, nerve cells throughout the central nervous system exhibit regional specificity in the distribution of their afferents upon the cell surface, and may in turn prove to differentiate those select portions of the post-synaptic cell under the instructive effects of those afferents.

The selective responsiveness of these two compartments of the horizontal cell cannot be explained by the effects of regionally distinct signals, because the axon terminals and the dendritic arbors of neighboring horizontal cells overlap one another within the OPL. Despite their overlapping distributions, they must recognize one another as distinct entities since each forms gap junctional contacts exclusively with their homotypic partners (He et al., 2000). While there is a slight temporal delay between the maturation of cone pedicles and rod spherules, both the dendritic arbor and a discriminable axon are already present by the time of synaptogenesis (Raven et al., 2005), and so each must be exposed to the same local environment within the developing OPL. Instead, our results suggest that cell-intrinsic mechanisms establish a polarization of the neuron, trafficking proteins to each compartment that constrain their subsequent development and render them sensitive to distinct instructive signals. The fact that horizontal cell dendrites become hypertrophic at P-10 in the rod-full retina, much like they do in the cone-less retina (Reese et al., 2005), suggests that,

while this cellular compartment is instructively regulated by afferent density, there is an intrinsic component to this developmental process as well, as though the horizontal cells are programmed to seek out afferent innervation. Failing to find it, they undergo regression, while finding normal or excessive densities of cone afferents, they branch accordingly to innervate all of the pedicles within their dendritic fields.

Our data, showing how the local retinal environment regulates neuronal morphology, run counter to recent publications favoring a cell-autonomous specification of morphological differentiation. For example, *Math5*$^{-/-}$ and *Brn3b*$^{-/-}$ mice contain reduced numbers of retinal ganglion cells that still develop a normal dendritic morphology (Lin et al., 2004), even in the presence of excessive amacrine cells (Wang et al., 2001). Cholinergic amacrine cells likewise develop a normal dendritic arbor despite reductions in the local density of homotypic cells as well as other cell types (Farajian et al., 2004). In comparison, the horizontal cells would appear to be sensitive to both their afferents (present data) as well as their homotypic neighbors, given the reciprocal relationship between dendritic field size and horizontal cell density across different strains of mice (Reese et al., 2005). Somewhat surprisingly, the horizontal cells of the cone-full retinas had smaller dendritic fields, indicating that field size is not exclusively controlled by proximity to homotypic neighbors. The expansion of cellular volume associated with this degree of dendritic branching may come at a cost to the overall dendritic field extent, reducing slightly dendritic coverage.

The present results are also unexpected given the recent demonstration that the primary target of the rod spherules, the rod bipolar cells, do *not* fail to differentiate their dendrites in the absence of rods in the *Nrl*$^{-/-}$ retina. Rather, they become innervated by the re-specified population of cone photoreceptors (Strettoi et al., 2004). Rod bipolar cells do not differentiate normally in the *rd1* retina, in which the rod and cone photoreceptor populations die postnatally, indicating that they do require an afferent population to form (Strettoi and Pignatelli, 2000). Our data suggest that, while the central post-synaptic elements at the ribbons of such re-specified cones may now include rod bipolar dendrites (Strettoi et al., 2004), many of the lateral post-synaptic elements should be the dendritic terminals of horizontal cells, indicating that the recruitment of the rod pathway in this mouse model may be only a partial one. That the axon terminal arbor is not entirely atrophic in these cone-full retinas reveals some intrinsic capacity to differentiate; as these terminals extend throughout a thicker OPL in such retinas, but do not exhibit conspicuous sprouting into the ONL (present results and Oh et al., 2007), they are also likely to form synaptic contacts with the re-specified cones.

The developmental plasticity associated with horizontal cell morphology described in the present study is distinct from recent descriptions of horizontal cell sprouting in rodent models of retinal degeneration. Disrupted gene function, affecting either phototransduction in the outer segment (Park et al., 2001; Strettoi et al., 2002; Cuenca et al., 2004; Claes et al., 2004) or neurotransmission within the outer plexiform layer (Mansergh et al., 2005; Chang et al., 2006) can lead to

an outgrowth of ectopic horizontal cell processes into the inner and/or outer nuclear layers. This sprouting appears to be relatively slow, progressive and in some cases reactive, indicating the cellular response to maintain normal morphology or function in degenerating retina (Lewis et al., 1998; Fariss et al., 2000; Jones et al., 2003). In this report, the present mouse models primarily reveal the cellular determinants for attaining appropriate neuronal morphology during synaptogenesis.

## Notes to Chapter IV

**A modified version of this chapter has been accepted for publication in: Journal of Neuroscience. Raven, M.A., Oh, E.C., Swaroop, A., and Reese, B.E. (2007). Afferent Control of Horizontal Cell Morphology Revealed by Genetic Re-specification of Rods and Cones.**

**<u>Outside contribution</u>: Edwin Oh generated the coneless mice for the analysis and prepared samples for immunohistochemistry and DiI labeling (performed by Mary Raven). He also generated gene profiles from P5 and P10 coneless and rodless mice.**

**Figure IV-1. A comparison of retinal markers in WT, rod-full, and cone-full retinas.** Sections were immunolabeled for piccolo to identify the ribbon synapses, and to reveal the plexiform layers as a landmark. Sections in the top two-thirds of the figure (**a-d; g-j; m-p**) also show labeling for cone arrestin (blue), to identify the cone photoreceptor cells and their inner and outer segments, axons and pedicles. Sections in the top (**a, b; g, h; m, n**) and bottom (**e, f; k, l; q, r**) thirds were also labeled for calbindin (green) to identify the horizontal cells and their dendritic plexus, while those in the middle third (**c, d; i, j; o, p**) were labeled for mitochondria using an antibody to cytochrome oxidase (green). Sections in the bottom third (**e, f; k, l; q, r**) were also labeled for neurofilaments (blue), to identify the axonal plexus. Calibration bar in q = 50 μm for the low magnification images, and = 14 μm for the high magnification images.

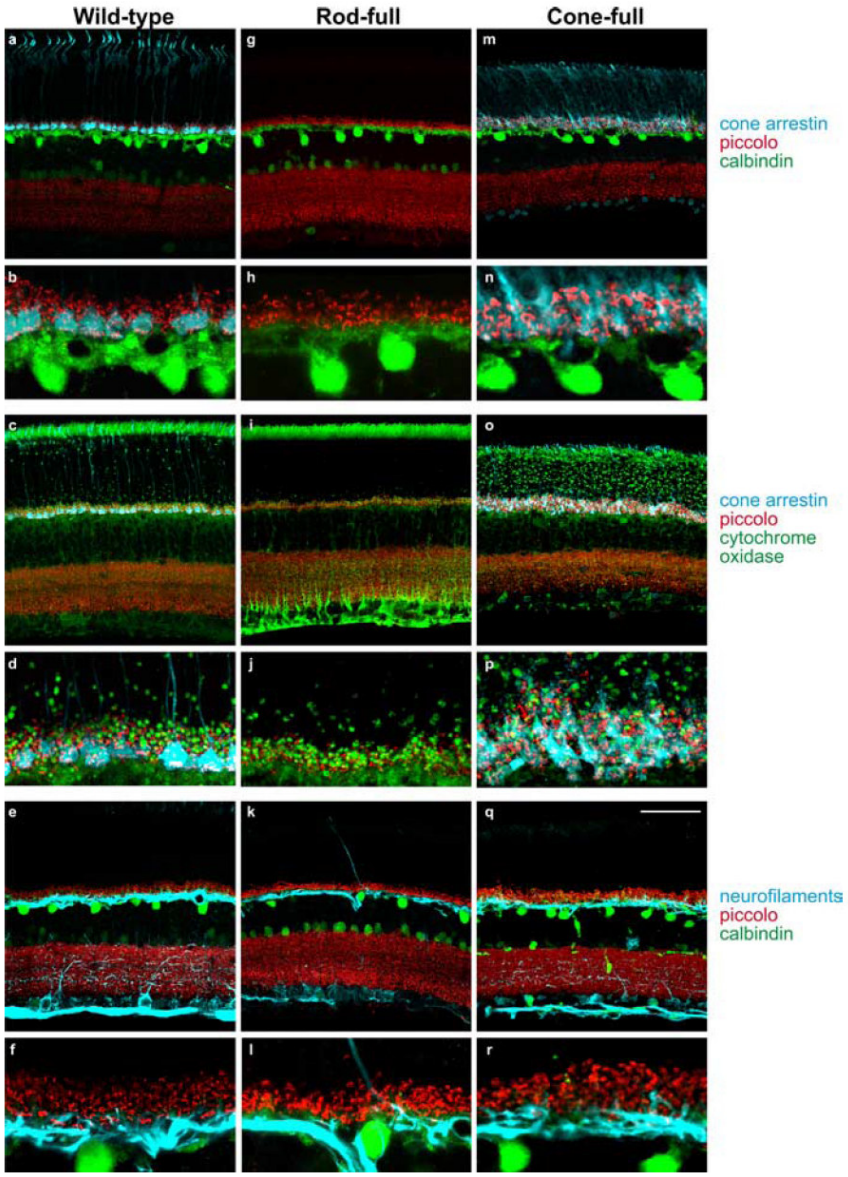


**Figure IV-2. Examples of individual horizontal cells and their dendritic fields labeled with DiI.** Staining can be observed in the mature wild-type (**a**), rod-full (**b**) and cone-full (**c**) retinas. The dendritic field is sparse and atrophic in the rod-full retina, while it is hypertrophic in the cone-full retina. Calibration bar = 50 µm. The dendritic field area is slightly smaller in the cone-full retina (**d**), and the number of primary dendrites is slightly greater (**e**), while the thickness of the dendritic arbor is larger (**f**).

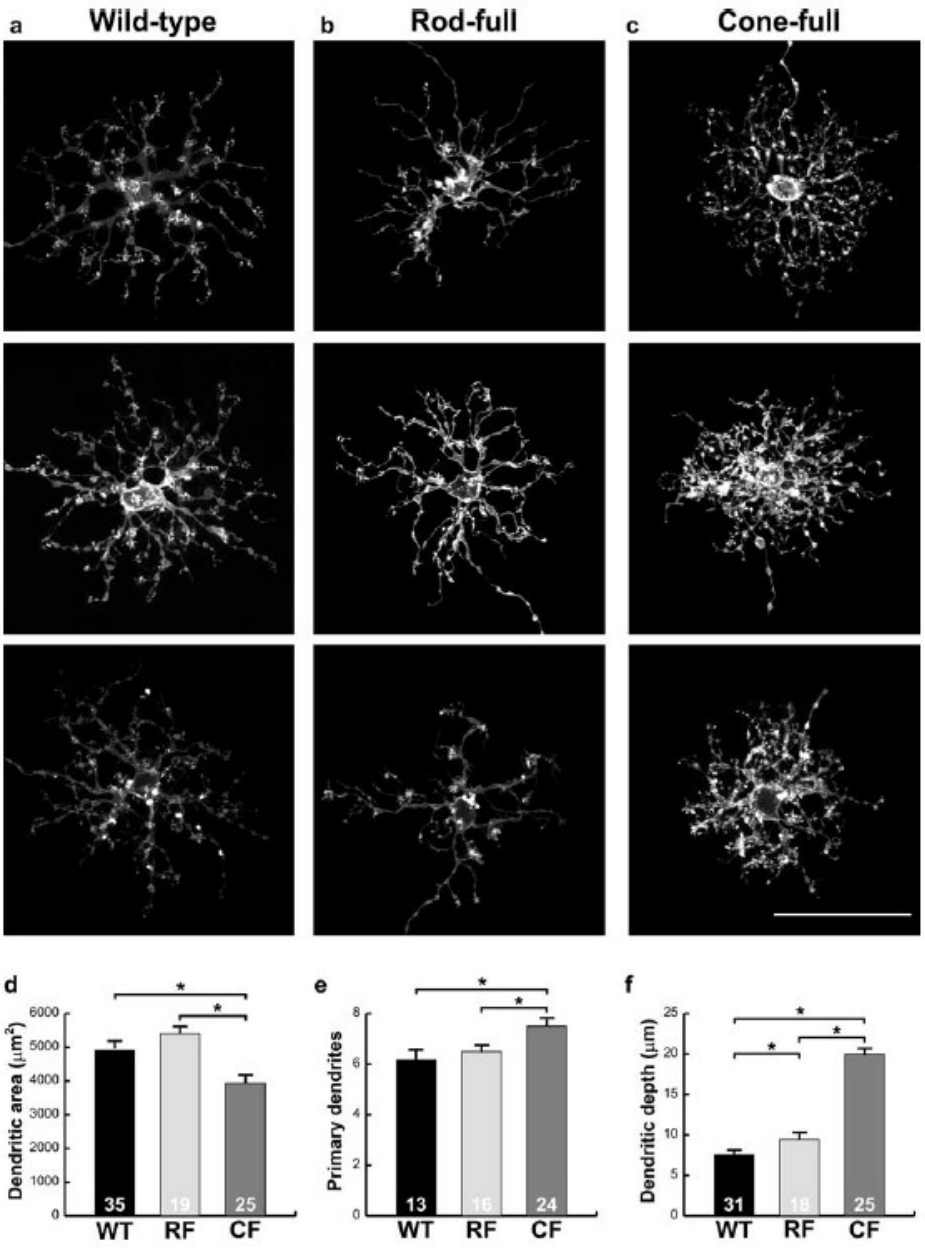


**Figure IV-3. Developing dendritic fields in the transgenic animals.** Wild-type (**a, d**), rod-full (**b, e**) and cone-full (**c, f**) retinas were labeled with DiI at P-5 (**a-c**) and P-10 (**d-f**). Dendritic fields become discriminable only after P-5, when synaptogenesis between the pedicles and horizontal cells takes place. Calibration bar = 50 μm.

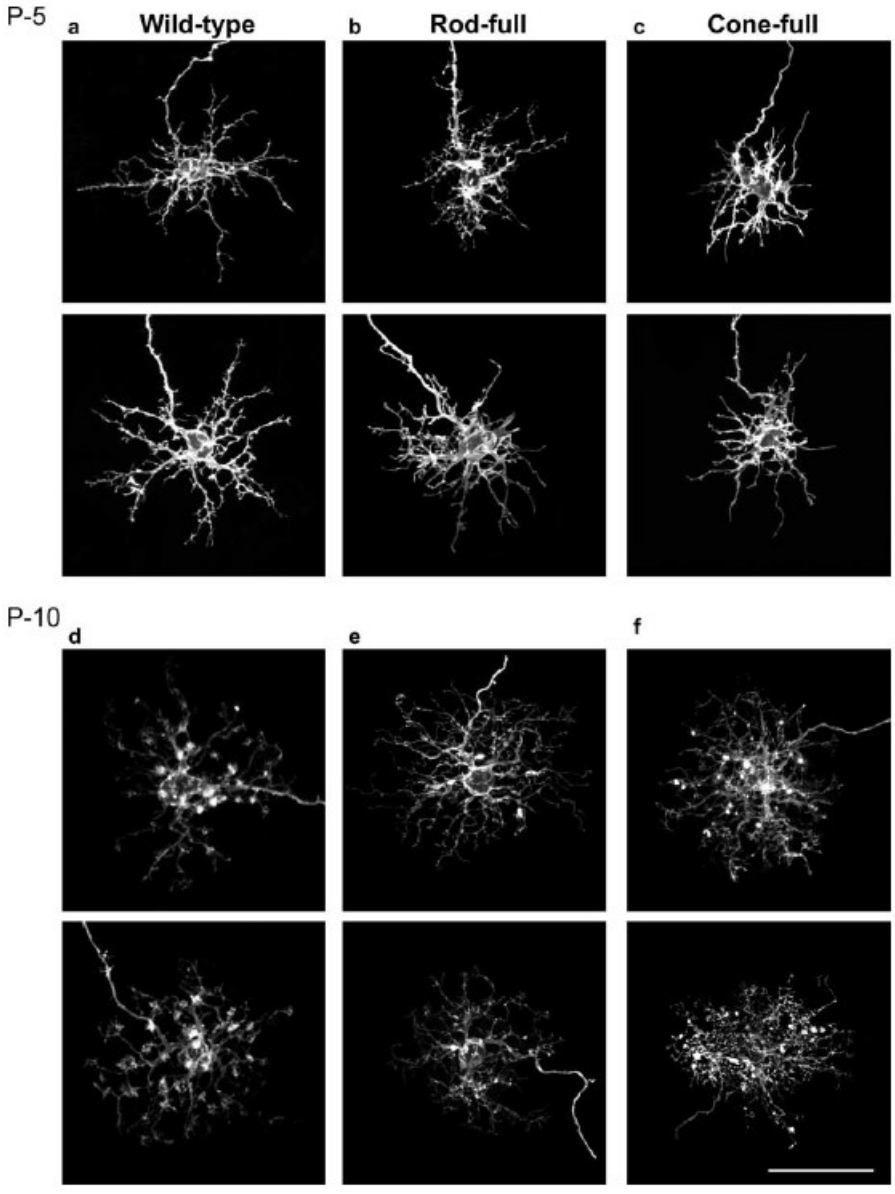


**Figure IV-4. Individual horizontal cell axon terminals labeled with DiI.** Staining is shown for wild-type (**a**), rod-full (**b**) and cone-full (**c**) retinas. The terminal field is dense in the wild-type and rod-full retinas, while it is relatively atrophic in the cone-full retina. Calibration bar = 50 µm. The size of the axon terminal field is reduced in the cone-full retina, while its depth of termination is greater (**d, e**).

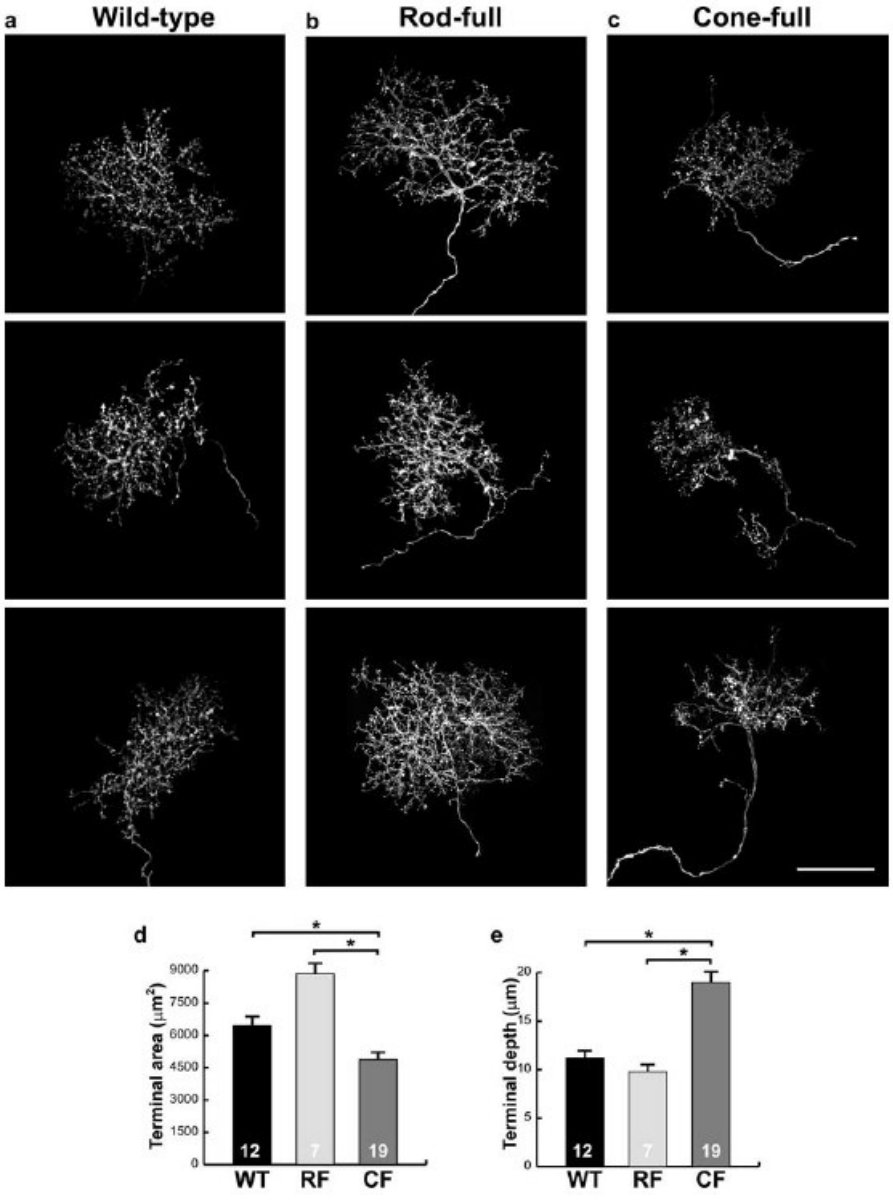
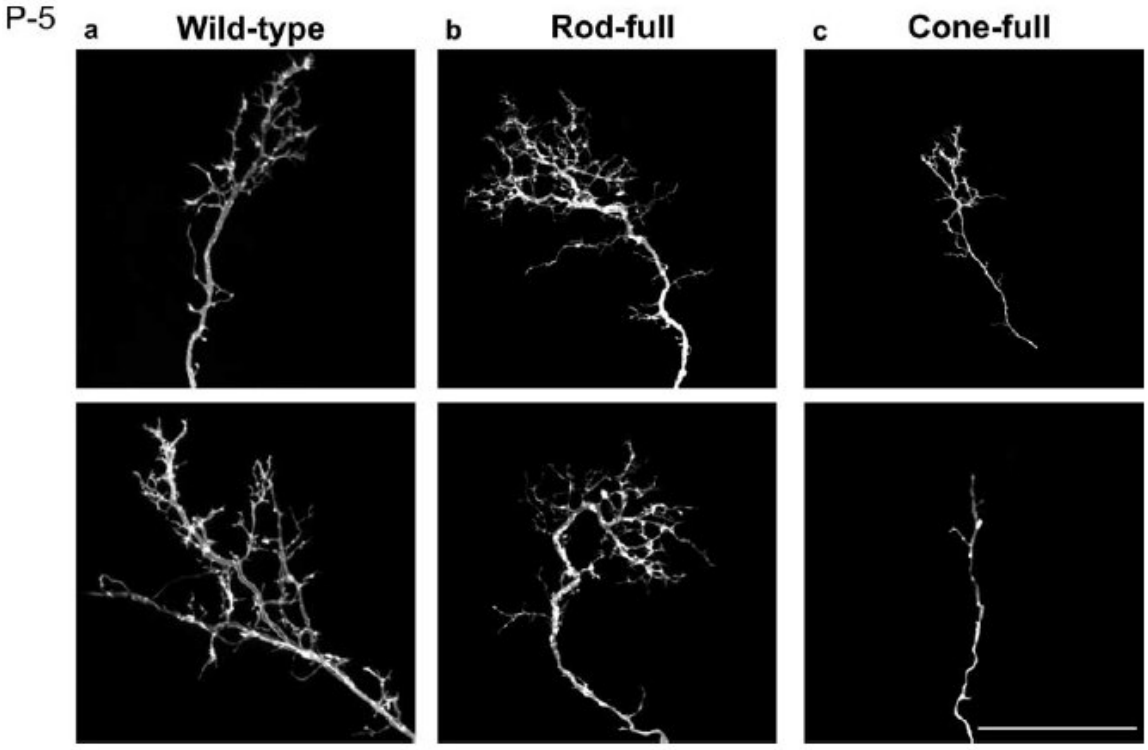


**Figure IV-5. Developing axonal arbors in the transgenic animals.** Wild-type (**a**), rod-full (**b**) and cone-full (**c**) retinas are labeled with DiI at P-5. Even at this early stage, before synaptogenesis between the spherules and axonal arbor, the girth of the axon and the size of the developing terminal are reduced in the cone-full retina. Calibration bar = 50 µm.



## References


Blanks JC, Adinolfi AM, Lolley RN (1974) Synaptogenesis in the photoreceptor terminal of the mouse retina. J Comp Neurol 156:81-93.

Carter-Dawson LD, LaVail MM (1979a) Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. J Comp Neurol 188:245-262.

Carter-Dawson LD, LaVail MM (1979b) Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. J Comp Neurol 188:263-272.

Chang B, Heckenlively JR, Bayley PR, Brecha NC, Davisson MT, Hawes NL, Hirano AA, Hurd RE, Ikeda A, Johnson BA, McCall MA, Morgans CW, Nusinowitz S, Peachey NS, Rice DS, Vessey KA, Gregg RG (2006) The nob2 mouse, a null mutation in *Cacna1f*: anatomical and functional abnormalities in the outer retina and their consequences on ganglion cell visual responses. Vis Neurosci 23:11-24.

Claes E, Seeliger M, Michalakis S, Biel M, Humphries P, Haverkamp S. (2004) Morphological characterization of the retina of the CNGA3(-/-) Rho(-/-) mutant mouse lacking functional cones and rods. Invest Ophthalmol Vis Sci 45:2039-2048.

Cline HT (2001) Dendritic arbor development and synaptogenesis. Curr Opin Neurobiol 11:118-126.

Cuenca N, Pinilla I, Sauvé Y, Lu B, Wang S, Lund RD (2004) Regressive and reactive changes in the connectivity patterns of rod and cone pathways of P23H transgenic rat retina. Neuroscience 127:301-317.

Cuenca N, Pinilla I, Sauve Y, Lu B, Wang S, Lund RD. Daniele LL, Lillo C, Lyubarsky AL, Nikonov SS, Philp N, Mears AJ, Swaroop A, Williams DS, Pugh EN (2005) Cone-like morphological, molecular, and electrophysiological features of the photoreceptors of the *Nrl* knockout mouse. Invest Ophthalmol Vis Sci 46:2156-2167.

Dick O, Hack I, Altrock WD, Garner CC, Gundelfinger ED, Brandstätter JH (2001) Localization of the presynaptic cytomatrix protein Piccolo at ribbon and conventional synapses in the rat retina: Comparison with Bassoon. J Comp Neurol 439:224-234.

Farajian R, Raven MA, Cusato K, Reese BE (2004) Cellular positioning and dendritic field size of cholinergic amacrine cells are impervious to early ablation of neighboring cells in the mouse retina. Vis Neurosci 21:13-22.

Fariss RN, Li Z-Y, Milam AH (2000) Abnormalities in rod photoreceptors, amacrine cells, and horizontal cells in human retinas with retinitis pigmentosa. Am J Ophthalmol 129:215-223.

He S, Weiler R, Vaney DI (2000) Endogenous dopaminergic regulation of horizontal cell coupling in the mammalian retina. J Comp Neurol 418:33-40.

Hirano AA, Brandstätter JH, Brecha NC (2005) Cellular distribution and subcellular localization of molecular components of vesicular transmitter release in horizontal cells of rabbit retina. J Comp Neurol 448:70-81.

Hirasawa H, Kaneko A (2003) pH changes in the invaginating synaptic cleft mediate feedback from horizontal cells to cone photoreceptors by modulating $Ca^{2+}$ channels. J Gen Physiol 122:657–671.

Horton AC, Ehlers MD (2003) Neuronal polarity and trafficking. Neuron 40:277-295.

Horton AC, Rácz B, Monson EE, Lin AL, Weinberg RJ, Ehlers MD (2005) Polarized secretory trafficking directs cargo for asymmetric dendrite growth and morphogenesis. Neuron 48:757-771.

Hua JY, Smith SJ (2004) Neural activity and the dynamics of central nervous system development. Nat Neurosci 7:327-332.

Jan Y-N, Jan LY (2003) The control of dendrite development. Neuron 40:229-242.

Jones BW, Watt CB, Frederick JM, Baehr W, Chen CK, Levine EM, Milam AH, Lavail MM, Marc RE. (2003) Retinal remodeling triggered by photoreceptor degenerations. J Comp Neurol 464:1-16.

Kamermans M, Fahrenfort I, Schultz K, Janssen-Bienhold U, Sjoerdsma T, Weiler R (2001) Hemichannel-mediated inhibition in the outer retina. Science 292:1178–1180.

Kolb H (1974) The connexions between horizontal cells and photoreceptors in the retina of the cat: electron microscopy of Golgi-preparations. J Comp Neurol 155:1-14.

Lewis GP, Linberg KA, Fisher SK (1998) Neurite outgrowth from bipolar and horizontal cells after experimental retinal detachment. Invest Ophthalmol Vis Sci 39:424-434.

Lin B, Wang SW, Masland RH (2004) Retinal ganglion cell type, size, and spacing can be specified independent of homotypic dendritic contacts. Neuron 43:475-485.

Lom B, Cogen J, Sanchez AL, Vu T, Cohen-Cory S (2002) Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells *in vivo*. J Neurosci 22:7639-7649.

Mansergh F, Orton NC, Vessey JP, Lalonde MR, Stell WK, Tremblay F, Barnes S, Rancourt DE, Bech-Hansen NT (2005) Mutation of the calcium channel gene *Cacna1f* disrupts calcium signaling, synaptic transmission and cellular organization in mouse retina. Human Mol Gen 14:3035-3046.

McAllister AK (2000) Cellular and molecular mechanisms of dendrite growth. Cereb Cortex 10:963-973.

Mears AJ, Kondo M, Swain PK, Takada Y, Bush RA, Saunders TL, Sieving PA, Swaroop A (2001) Nrl is required for rod photoreceptor development. Nat Gen 29:447-452.

Migdale K, Herr S, Klug K, Ahmad K, Linberg K, Sterling P, Schein S (2003) Two ribbon synaptic units in rod photoreceptors of macaque, human, and cat. J Comp Neurol 455:100-112.

Nikonov SS, Daniele LL, Zhu X, Craft CM, Swaroop A, Pugh EN (2005) Photoreceptors of *Nrl-/-* mice coexpress functional S- and M-cone opsins having distinct inactivation mechanisms. J Gen Physiol 125:287-304.

Oh ECT, Khan N, Novelli E, Khanna H, Strettoi E, Swaroop A (2007) Transformation of cone precursors to functional rod photoreceptors by bZIP transcription factor NRL. Proc Nat'l Acad Sci in press.

Olney JW (1968) An electron microscopic study of synapse formation, receptor outer segment development, and other aspects of developing mouse retina. Invest Ophthalmol 7:250-268.

Park S-J, Kim I-B, Choi K-R, MoonJ-I, Oh S-J, Chung J-W, Chun M-H (2001) Reorganization of horizontal cell processes in the developing FVB/N mouse retina. Cell Tissue Res 306:341-346.

Peichl L, Gonzalez-Soriano J (1993) Unexpected presence of neurofilaments in axon-bearing horizontal cells of the mammalian retina. J Neurosci 13:4091-4100.

Peichl L, Sandmann D, Boycott BB (1998) Comparative anatomy and function of mammalian horizontal cells. In: Development and Organization of the Retina. (Chalupa LM, Finlay BL, eds). New York: Plenum Press.

Raven MA, Stagg SB, Nassar H, Reese BE (2005) Developmental improvement in the regularity and packing of mouse horizontal cells: Implications for mechanisms underlying mosaic pattern formation. Vis Neurosci in press.

Reese BE, Raven MA, Stagg SB (2005) Afferents and homotypic neighbors regulate horizontal cell morphology, connectivity and retinal coverage. J Neurosci 25:2167-2175.

Sherry DM, Wang MM, Bates J, Frishman LJ (2003) Expression of vesicular glutamate transporter 1 in the mouse retina reveals temporal ordering in development of rod vs. cone and ON vs. OFF circuits. J Comp Neurol 465:480-498.

Sorensen SA, Rubel EW (2006) The level and integrity of synaptic input regulates dendritic structure. J Neurosci 26:1539-1550.

Soucy E, Wang Y, Nirenberg S, Nathans J, Meister M (1998) A novel signaling pathway from rod photoreceptors to ganglion cells in mammalian retina. Neuron 21:481-493.

Strettoi E, Pignatelli V (2000) Modifications of retinal neurons in a mouse model of retinitis pigmentosa. Proc Nat'l Acad Sci USA 97:11021-11025.

Strettoi E, Porciatti V, Falsini B, Pignatelli V, Rossi C (2002) Morphological and functional abnormalities in the inner retina of the rd/rd mouse. J Comp Neurol 22:5492-5504.

Strettoi E, Mears AJ, Swaroop A (2004) Recruitment of the rod pathway by cones in the absence of rods. J Neurosci 24:7576-7582.

Swaroop A, Xu JZ, Pawar H, Jackson A, Skolnick C, Agarwal N (1992) A conserved retina-specific gene encodes a basic motif/leucine zipper domain. Proc Nat'l Acad Sci 89:266-270.

Wang SW, Kim BS, Ding K, Wang H, Sun D, Johnson RL, Klein WH, Gan L (2001) Requirement for math5 in the development of retinal ganglion cells. Genes Dev 15:24-29.

Wong ROL, Ghosh A (2002) Activity-dependent regulation of dendritic growth and patterning. Nat Rev Neurosci 3:803-812.

Zhu X, Li A, Brown B, Weiss ER, Osawa S, Craft CM (2002) Mouse cone arrestin expression pattern: light induced translocation in cone photoreceptors. Mol Vis 11:462-471.

# CHAPTER V

# FUNCTIONAL CONSERVATION OF LARGE MAF TRANSCRIPTION FACTORS

## Abstract

The family of large Maf transcription factors is involved in controlling cell fate and coordinating various biological processes. During retinal development in mammals, expression of the large Maf protein NRL is sufficient and necessary for the differentiation of rod photoreceptors. A complete loss of rod function and super-normal cone function, mediated by S cones is observed in *Nrl*$^{-/-}$ mice. We have used genetic rescue experiments to investigate the functional equivalence of large Maf proteins in the differentiation of rod precursors. Using transgenic mice, we first demonstrate that the *Nrl*$^{-/-}$ phenotype can be rescued by targeted expression of the NRL protein under the control of a rod-specific *Nrl* proximal promoter. We then show that under the control of these rod-specific regulatory elements, three other large Maf gene products are able to substitute NRL in activating the expression of a key rod-specific marker, rhodopsin. However, only the expression of NRL can suppress S-opsin activity. Using transient transfection studies, we provide biochemical evidence that large Maf proteins together with CRX can activate a rhodopsin-promoter reporter, while only NRL can suppress Crx-mediated activation of the S-opsin promoter. Our results indicate that despite considerably diverged sequences, large Maf proteins can

partially promote rod photoreceptor identity. We propose that differences in large Maf function rely in part on their spatiotemporal expression during development.

## Introduction

In the vertebrate retina, a common proliferating progenitor gives rise to amacrine, ganglion, horizontal, cone, rod, and bipolar neurons and Muller glia (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988). The earliest postmitotic neuron in mice is born around embryonic day (E) 11 and cells are added to the retina up to postnatal day (P) 21 (Carter-Dawson and LaVail, 1979b; Young, 1985). While the inner retina is mainly composed of interneurons (horizontal, amacrine, and bipolar cells) and ganglion cells, the outer retina consists of photoreceptors (rods and cones) that are responsible for converting light information into an electrical signal. In rodents, rhodopsin is the visual pigment in rods while cones utilize either S or M-opsin (reviewed in Fu and Yau, 2007). The retina is therefore particularly unique in that it is the only neuronal tissue directly exposed to light. This anatomical feature has permitted facile genetic manipulation (Matsuda and Cepko, 2004; Matsuda and Cepko, 2007) of specific genes in the central nervous system.

Maf proteins constitute a family of diverse and well-conserved transcription factors implicated in the control of cell fate and coordinating various developmental processes (Artner et al., 2007; Cordes and Barsh, 1994; Ho et al., 1996; Kim et al., 1999b; Matsuoka et al., 2003; Mears et al., 2001; Ogino and Yasuda, 1998; Tsuchiya et al., 2006; Zhang et al., 2005). The Maf family shares

homology to the v-Maf oncoprotein, which was originally identified in the genome of the AS42 chicken musculoaponeurotic sarcoma retrovirus (Nishizawa et al., 1989). Sequence analysis of these transcription factors reveal that they contain a highly conserved basic region and a leucine zipper domain that allows them to bind to target DNA elements and dimerize with the same or other proteins possessing a bZIP domain (Kataoka et al., 1994; Kerppola and Curran, 1994). All Maf proteins are subdivided into a large or small group of transcription factors depending on the presence of a putative activation domain (Kataoka, 2007). Collectively, Maf proteins dictate the specification of cellular identity and function through tightly regulated spatiotemporal expression patterns, interactions with co-factors and regulation of specific downstream genes.

In mammals, four large Maf proteins have emerged as important regulators of insulin gene expression (Artner et al., 2007; Matsuoka et al., 2003; Zhang et al., 2005), erythroid differentiation (Ho et al., 1996), segmentation (Cordes and Barsh, 1994) and retinal (Mears et al., 2001) and lens development (Kim et al., 1999b). Structural studies of MafA (L-Maf), MafB (Kreisler), c-Maf (v-Maf), and NRL reveal that in addition to the bZIP domain, they share a conserved Maf N-terminal domain related to transactivator function. Recent findings suggest that this domain is not only important for the activation of downstream targets, but also for its biological activity (Benkhelifa et al., 2001; Bessant et al., 1999; Ochi et al., 2003). Maf protein function has been elucidated through loss of function studies. Mice lacking either MafA or MafB show a defect in the regulation of insulin transcription. While the loss of MafB resulted in a 50%

decrease of insulin and glucagon producing cells (Artner et al., 2007), MafA$^{-/-}$ mice revealed a decrease in insulin secretion with no change in cell numbers (Zhang et al., 2005). Disruption of the c-Maf gene confirmed a role for the cellular counterpart of v-Maf in Th2 differentiation and interleukin-4 production from CD4$^{+}$ T lymphocytes (Kim et al., 1999a). Interestingly, *c-maf*$^{-/-}$ mice are also microphthalmic and display a defect in the elongation of the posterior lens fiber cells (Kim et al., 1999b). Expression analyses of the neural leucine zipper (NRL) protein reveal that it is the only Maf protein expressed in the retina (Akimoto et al., 2006). NRL is both necessary (Mears et al., 2001) and sufficient (Oh et al., 2007) for rod photoreceptor development, and recent studies demonstrate that Nrl determines whether photoreceptor precursors differentiate into a rod or cone photoreceptor (Oh et al., 2007).

Given their differences in expression patterns and proposed roles in the differentiation of diverse tissues, it seemed possible that individual Maf proteins might perform some common activity in the retina. However, since not all Maf proteins are expressed in the retina, we queried whether factors with conserved activity mediate different functional outcomes when expressed in the appropriate tissue. To gain insight into whether all large Maf proteins display functional conservation in the retina we first generated transgenic mice that expressed Nrl under the control of a 2.5 kb proximal promoter (Akimoto et al., 2006) in the *Nrl*$^{-/-}$ background. Our analyses revealed that the expression of the transgene resulted in various levels of histological and functional rescue of the *Nrl*$^{-/-}$ phenotype. With the knowledge that the *Nrl* promoter could facilitate the proper

spatiotemporal expression of NRL, we then utilized an *in vivo* electroporation technique (Matsuda and Cepko, 2004; Matsuda and Cepko, 2007) to express all large Maf proteins under the control of the *Nrl* promoter in the *Nrl*$^{-/-}$ background. Our results revealed that while rhodopsin expression could be activated *in vivo*, high levels of S-opsin expression in the photoreceptor layer was unchanged. These findings suggest that while all large Maf proteins could promote rod-specific identity, each factor has evolved to direct specific developmental activities and cannot completely compensate for each other.

## Materials and Methods

**Transgene constructs and generation of transgenic mice.** A 2.5 kb mouse *Nrl* promoter DNA (GenBank # AY526079) and the *Nrl* coding region (GenBank # NM008736) with an additional Kozak sequence were amplified and cloned into a pEGFP-1 vector (Clontech). The 4 kb *Nrlp-Nrl* insert was purified and injected into fertilized *Nrl*$^{-/-}$ (mixed background of 129X1/SvJ and C57BL/6J) mouse oocytes (UM transgenic core facility). Transgenic founders were bred to the *Nrl*$^{-/-}$ mice to generate F1 progeny. All studies involving mice were performed in accordance with institutional and federal guidelines and approved by the University Committee on Use and Care of Animals at the University of Michigan.

**In vivo electroporation.** P2-P4 mice were anesthetized on ice before receiving subretinal DNA injections (1–2 µl). A homemade copper probe was then placed

on both eyes and 5 pulses of 75 V were delivered using an electroporation pulse generator.

**Immunohistochemistry and confocal analysis.** Mice were sacrificed at P9-11 and eyes were removed and immersed in 4% paraformaldehyde (pH 7.2) for 1 h at room temperature. After the lens was removed, we used an epifluorescent dissecting microscope to identify the retinal region that revealed GFP fluorescence. Eyecups were then equilibrated in 30% sucrose and frozen in embedding medium for cryosectioning. Sections (10 µm) were used for immunostaining. At least three retinas from three different injections were used for analysis. Retinal sections were probed with rhodopsin (1D4 and 4D2; generous gift from R. Molday, University of British Columbia, Vancouver, Canada) and S-opsin (generous gift from C. Craft, University of Southern California, Los Angeles, CA, and Chemicon). Sections were visualized under an Olympus FluoView 500 laser scanning confocal microscope or a Leica TSC NT confocal microscope, equipped with an argon-krypton laser. Images were digitized using FluoView software version 5.0 or Metamorph 3.2 software.

**Electroretinography.** Six-week old animals were anesthetized with intraperitoneal ketamine 86 mg/kg and xylazine 13 mg/kg. Pupils were fully dilated with topical0.5% tropicamide and 0.5% phenylepherine HCl. Body temperature was maintained by placing the animal on a heating pad before and during recordings. Gold wire loops placed on the cornea as active and reference electrodes. Mice were dark-adapted for overnight (16 hrs) and recordings were

made from both eyes simultaneously beginning at dark-adapted threshold. A 30 µs full-field stimulus was used with maximum intensity of 0.6 log cd-s/m2, and attenuated with neutral density filters. Photopic responses were recorded on a 34 cd/m2 full-field background. Amplified and filtered responses (5,000 gain, 0.1-1000 Hz) from each eye were used as separate data points since variability within a group depended more on the ERG setup than phenotype of individual animals.

**Transient transfection and luciferase assays.** HEK 293 cells (ATCC CRL-1573) were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, penicillin G (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified incubator with 5% $CO_2$. Cells were transfected in 24-well plates ($1x10^5$ cells/well) using Fugene 6 (Roche, Indianapolis, IN, USA) according to manufacturer's instructions. Expression constructs for CRX, NR2E3, NRL (Cheng et al., 2004), MafA, MafB, and c-Maf were used for transfection . Luciferase assays were performed as described earlier (Mitton et al., 2003). All transfections included the reporter construct pGL2-BRP-130 or pGL3-S-opsin as well as pCMV-ß-gal to normalize for transfection efficiency. Luciferase expression was measured using luminescence-based assay system (Promega, Madison, WI, USA). The luciferase activity was calculated as fold change on the basis of the control transfections using empty vector and pGL2-BRP-130 or pGL3- S-opsin.

## RESULTS AND DISCUSSION

**The expression of NRL under the control of the *Nrl* promoter results in the rescue of rhodopsin expression and suppression of S-opsin expression**

To first establish an *in vivo* system to examine the function of large Maf proteins, we took advantage of the *Nrl*$^{-/-}$ mice. This allowed us to analyze whether the exogenous expression of NRL could: (1) decrease the expression of cone-specific markers; (2) increase the expression of rhodopsin expression in the photoreceptor layer; (3) convert the morphology of an all-cone retina back to a predominantly rod-full retina; and (4) lead to a rescue of rod function. An analysis of a previously characterized mouse *Nrl* promoter revealed that the 2.5 kb proximal promoter shared four conserved domains in mammals (Figure V-1 a) (Akimoto et al., 2006). We therefore generated a construct (*Nrlp-Nrl*) that permitted the expression of mouse *Nrl* under the control of this promoter (Figure V-1 b). Injection of the transgene into *Nrl*$^{-/-}$ eggs yielded 9 founder lines of transgenic mice in a mixed 129X1/SvJ and C57BL/6J background (*Nrlp-Nrl/Nrl*$^{-/-}$). We determined through PCR and Southern blot analysis of *Nrlp-Nrl/Nrl*$^{-/-}$ mouse tail DNA that only seven lines transmitted the transgene to their offspring (Figure V-1 d). By immunoblot assays, the NRL protein was detected in only four founders (Figure 1 D).

We then characterized the expression of rod and cone specific markers in the retina of adult transgenic mice. As can be observed in wild-type mice, both S-opsin and rhodopsin are expressed at low levels in the cytoplasm, but highly expressed in the outer segments of photoreceptors (Figure V-2 A-D). Since wild-

type mice have rod-dominant retinas and *Nrl*$^{-/-}$ mice have cone-only retinas, obvious differences in rod and cone opsin expression can be easily distinguished (Figure V-2 E-H). Of the seven founder lines, we determined that there were at least three groups of mice showing rhodopsin expression (low, medium, and high) when compared to retinas from the *Nrl*$^{-/-}$ mice. Three of the seven founders expressed high levels of rhodopsin, one founder expressed medium levels, and three founders expressed low levels of rhodopsin. The difference in the expression level is probably due to the integration site of the transgene. Retinal confocal images taken from a high rhodopsin-expressing founder, *Nrlp-Nrl/Nrl*$^{-/-}$ 37, resembled staining observed from WT retinas (Figure V-2 A-D, Q-T). A normal level of both S-opsin and rhodopsin expression indicated that this founder expressed NRL in most rod photoreceptors (Figure V-2 Q-T). Medium rhodopsin expression was detected in an unusual line, *Nrlp-Nrl/Nrl*$^{-/-}$ 32. Interestingly, rhodopsin staining was detected at high levels in both the outer segments and in the innermost rod cell bodies (Figure V-2 M-P). Since these cell bodies typically belong to early born rods, it is possible that the *Nrlp-Nrl* transgene integrated in a genomic locus that corresponds to a novel gene expressed in firstborn rods. Low or scattered rhodopsin expression is illustrated in retinal immunohistochemical images from founder *Nrlp-Nrl/Nrl*$^{-/-}$ 910 (Figure V-2 I-J). Here, we noticed that cells expressing rhodopsin no longer expressed S-opsin (Figure V-2 I-J). These transgenic lines demonstrate that when NRL is expressed under the control of the *Nrl* promoter, rhodopsin expression is activated while S-opsin expression is repressed.

**NRL can rescue rod morphology and function in the *Nrl*$^{-/-}$ retina**

In the wild-type retina, rods and cones can be distinguished by several anatomical features. Rods have small nuclei (4–5 μm diameter) with a characteristic polyhedral shape and contain a single, large clump of heterochromatin (Figure V-3 A), while cones have large nuclei (8-10 μm diameter) with several large clumps of heterochromatin (Figure V-3 A) (Carter-Dawson and LaVail, 1979a). Cone nuclei also typically occupy the outermost nuclei layer in the outer nuclear layer (ONL), while rod nuclei are distributed throughout the ONL (Figure V-3 A). In the *Nrl*$^{-/-}$ retina, only cone nuclei can be detected in plastic sections and whorls and rossettes are frequently observed (Figure V-3 B) (Mears et al., 2001). In agreement with immunohostochemical data on rhodopsin and S-opsin expression (Figure V-2), we discovered that the retinal histology in the *Nrlp-Nrl/Nrl*$^{-/-}$ transgenic mice varied according to the level of rhodopsin expression. The high rhodospin-expressing line *Nrlp-Nrl/Nrl*$^{-/-}$ 37 showed no signs of whorls and rossettes, and cone nuclei could only be detected in the outermost nuclei of the ONL (Figure V-3 E). The *Nrlp-Nrl/Nrl*$^{-/-}$ 32 line again displayed an interesting phenotype where only the innermost nuclei of the ONL appeared rod-like (Figure V-3 D). Finally, as predicted, the *Nrlp-Nrl/Nrl*$^{-/-}$ 910 founder line revealed few characteristic rod nuclei, and appeared similar to *Nrl*$^{-/-}$ retinas (Figure V-3 C).

In order to determine whether the presence of various histological features translated into a functional rescue of the *Nrl*$^{-/-}$ phenotype, we recorded

electroretinograms (ERGs) from the different founder lines. We first established the response profiles from wild-type and *Nrl*$^{-/-}$ mice (Figure V-3 F-H). As previously established, the *Nrl*$^{-/-}$ mice exhibit no dark-adapted ERG responses at or below -2.4 log cd-s/m$^2$ (Figure V-3 F) but have super-normal photopic responses (Figure V-3 G). Their dark-adapted thresholds are slightly below that of the rhodopsin knockout mouse, which has only cone function (Toda et al., 1999). This lower threshold and larger scotopic responses compared to the rhodopsin knockout mouse at and above -1.4 log cd-s/m2 is expected for *Nrl*$^{-/-}$ retinas that have a much greater number of cones. The transgenic founder lines revealed dark-adapted responses at -2.4 log cd-s/m$^2$ and below arising from rod activity. These transgenic lines segregate by rod b-wave amplitude at -2.4 log cd-s/m2 in the order of *Nrlp-Nrl/Nrl*$^{-/-}$ 37 having the largest response and *Nrlp-Nrl/Nrl*$^{-/-}$ 32 having the smallest. The *Nrlp-Nrl/Nrl*$^{-/-}$ 32 rod response is considerably smaller than *Nrlp-Nrl/Nrl*$^{-/-}$37, indicating fewer rods or rod responsivity (Figure V-3 F). This functional data is consistent with our immunohistochemical and morphological analyses. Since little rod function could be detected in *Nrlp-Nrl/Nrl*$^{-/-}$ 32, we did not record from the *Nrlp-Nrl/Nrl*$^{-/-}$ 910 line. Photopic amplitudes of the transgenic lines were inversely related to the order of the rod scotopic responses, indicating that the presence of more functional cones resulted in fewer functional rods (Figure V-3 G). One can note that for the highest stimulus intensity in the dark-adapted state there is intermixing of response amplitudes for these two categories, but this is of no consequence as

this condition evokes a complex rod and cone response that is difficult to interpret.

### *In vivo* electroporation of large Maf proteins in the *Nrl*$^{-/-}$ retina results in the expression of rhodopsin in the photoreceptor layer

Having established that a 2.5 kb proximal promoter could effectively drive the expression of NRL in the photoreceptor layer (Akimoto et al., 2006), we were curious whether other large family members could substitute for NRL function. We first examined the level of sequence identity and homology between the DNA binding domain from the four large Maf proteins. Interestingly, the bZIP domain from MafA, MafB, and c-Maf was 70-80% identical and almost 90% similar to NRL (Figure V-4 A).

To examine whether the sequence conservation translated to functional conservation, we then wanted to express the Maf proteins in *Nrl*$^{-/-}$ retinas. As a first step to elucidate this function, we first generated transgenes in which the *Nrl* promoter was cloned upstream of each *Maf* gene (*Nrlp-Nrl*, *Nrlp-MafA*, *Nrlp-MafB*, and *Nrlp-c-Maf*). Using a modified version of a recently reported electroporation technique (Matsuda and Cepko, 2004), we injected each construct in the subretinal space of P2-4 newborn mice and electroporated the DNA in the direction of the retina. A GFP vector was also co-electroporated into the retina to track the presence of the transgene. Retinas were then harvested 7-9 days later, and sections that revealed the expression of GFP were collected for further immunohistochemical analyses. Though GFP could be detected

throughout the retina, expression of all large Maf proteins revealed rhodopsin expression only in the ONL (Figure V-4). Interestingly, high rhodopsin expression could be detected both in the cell bodies and the outer segments. In a parallel experiment, we also co-electroporated a GFP control vector, the large Maf transgenes, and a rhodopsin-DsRedII reporter. Confocal analysis of retinal sections from this experiment also confirmed the activation of the rhodopsin-DsRedII construct (data not shown).

**The expression of large Maf proteins drives the transactivation of the bovine rhodopsin promoter**

Using HEK 293 cells, we have previously established that NRL can bind and activate the *rhodopsin* promoter in transient transfection assays (Rehemtulla et al., 1996). Expression of NRL with the homeobox transcription factor, CRX (Chen et al., 1997; Mitton et al., 2000) or nuclear receptor, NR2E3 (Cheng et al., 2004; Peng et al., 2005) also demonstrated a synergistic or additive activation of the rhodopsin promoter construct, respectively. Due to the high level of amino acid conservation in the bZIP domain and that large Maf proteins can activate rhodopsin expression *in vivo*, we then used the same rhodopsin promoter-luciferase activity assay to test whether MafA, MafB, or c-maf could substitute for NRL. While NRL alone resulted in a seven-fold activation of the reporter (Figure 5-4 A), expression of all Maf proteins resulted in only a two to four-fold activation (Figure V-5 B-D). We then transfected NRL together with CRX in our assay system and determined that the *rhodopsin* promoter was activated twenty-fold

(Figure V-5 A). Co-expression of the three Maf family members with CRX did not result in synergistic activation of the *rhodopsin* promoter. Instead, an additive effect of three to seven-fold activation could be observed (Figure V-5 B-D).

**Not all large Maf proteins can inhibit S-opsin expression in the *Nrl*$^{-/-}$ retina**

Given that *Nrlp-Nrl/Nrl*$^{-/-}$ transgenic mice reveal rhodopsin expression in the ONL and an absence of S-opsin expression, we wanted to verify whether the expression of the large Maf proteins, using *the in vivo* electroporation technique, could show a change in S-opsin expression. We first tested the Nrlp-Nrl construct, and discovered that like the *Nrlp-Nrl/Nrl*$^{-/-}$ transgenic mice, S-opsin was not expressed in any rhodopsin-positive cell (Figure V-6 A-D). We then analyzed retinal samples that had been electroporated with each Maf construct. Though rhodopsin could be observed in response to the expression of the Maf protein, high levels of S-opsin was also expressed in the same cell (Figure V-6 E-P).

**NRL is the only large Maf protein that can inhibit Crx-mediated transactivation of the S-opsin promoter *in vitro***

The *in vivo* expression of NRL in photoreceptor precursors resulted in the inactivation of S-opsin expression (Figure V-2). Although we have previously demonstrated that NRL can bind to elements in the S-opsin promoter (Oh et al., 2007), our recent studies suggest NR2E3 is the major repressor of M and S-opsin expression. These findings demonstrate that NRL activates NR2E3 transcription, thereby suppressing cone opsin expression. To test whether we

could phenocopy this result *in vitro*, we utilized similar transient transfection assays as earlier using a *S-cone opsin* promoter (Srinivas et al., 2006). Consistent with previous reports, we found that the expression of CRX alone could activate the *S-cone* promoter reporter six-fold (Peng et al., 2005; Srinivas et al., 2006). We then expressed NRL with CRX and determined that NRL reduced the activation of the *S-cone* promoter to three-fold (Figure V-7 A). We then tested whether the other large Maf proteins could substitute for NRL in suppressing Crx-mediated transactivation of the S-opsin promoter. As can be observed in Figure V-7 B-D, no significant effect on the reporter could be recorded when MafA, MafB, or c-Maf was co-expressed with CRX.

**CONCLUSION**

During the development of diverse tissues, proper spatiotemporal control of large Maf proteins is observed in several cell-types. How lymphocytes, photoreceptors, lens fiber cells or insulin producing cells induce the expression of the correct large Maf is still unknown. In this report, we test a hypothesis that large Maf proteins can function outside of the tissue that they are normally expressed. Using transgenic mice and *in vivo* electroporation, we show for the first time that large Maf proteins can partially function in photoreceptors. Our selective use of genetic tools represents the first study in the mammalian retina of the functional equivalence of a well-conserved gene family.

**Figure V-1. Injection of the *Nrlp-Nrl* transgene results in several transgenic founders.** Schematic of approximately 2.5 kb genomic DNA upstream of the *Nrl* transcription start site (denoted as +1) (A). The four boxes indicate sequence regions conserved in mammals. The mouse *Nrl* promoter was used to drive the expression of mouse *Nrl* (B). A probe corresponding to *Nrl* (B) was employed to assay 4 transgenic founders using mouse genomic tail DNA. Southern blot analysis revealed a ~ 4 kb transgene band (C). Retinal tissue from these founders was used to test for NRL expression (D).

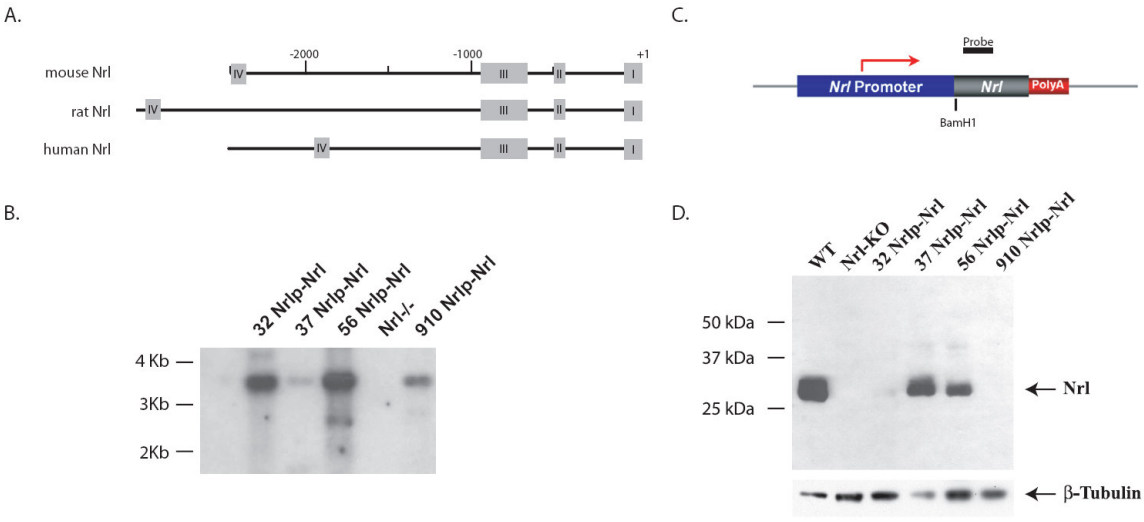


**Figure V-2. Expression of NRL rescues the *Nrl*$^{-/-}$ phenotype.** High levels of both rhodopsin and S-opsin staining could be observed in the outer segments from wild-type retinas (A-D). In *Nrl*$^{-/-}$ retinas, rhodopsin could not be detected while S-opsin staining was distributed throughout the photoreceptors (E-H). The *Nrlp-Nrl/Nrl*$^{-/-}$ 910 revealed high levels of S-opsin staining (I), similar to *Nrl*$^{-/-}$ retinas. However, rhodopsin staining was detected in areas where S-opsin was not expressed (Arrows; J-L). The *Nrlp-Nrl/Nrl*$^{-/-}$ 32 also revealed high levels of S-opsin staining (M), similar to Nrl retinas. In this line, we observed a row of nuclei closest to the INL that expressed rhodopsin (Arrows; N). In these cells, no S-opsin could be detected (M-P). The *Nrlp-Nrl/Nrl*$^{-/-}$ 37closely resembled the wild-type phenotype and showed normal levels of S-opsin (Bessant et al.) and rhodopsin (R-T). OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; BBZ, bisbenzamide. Scale bar: 25 μm.

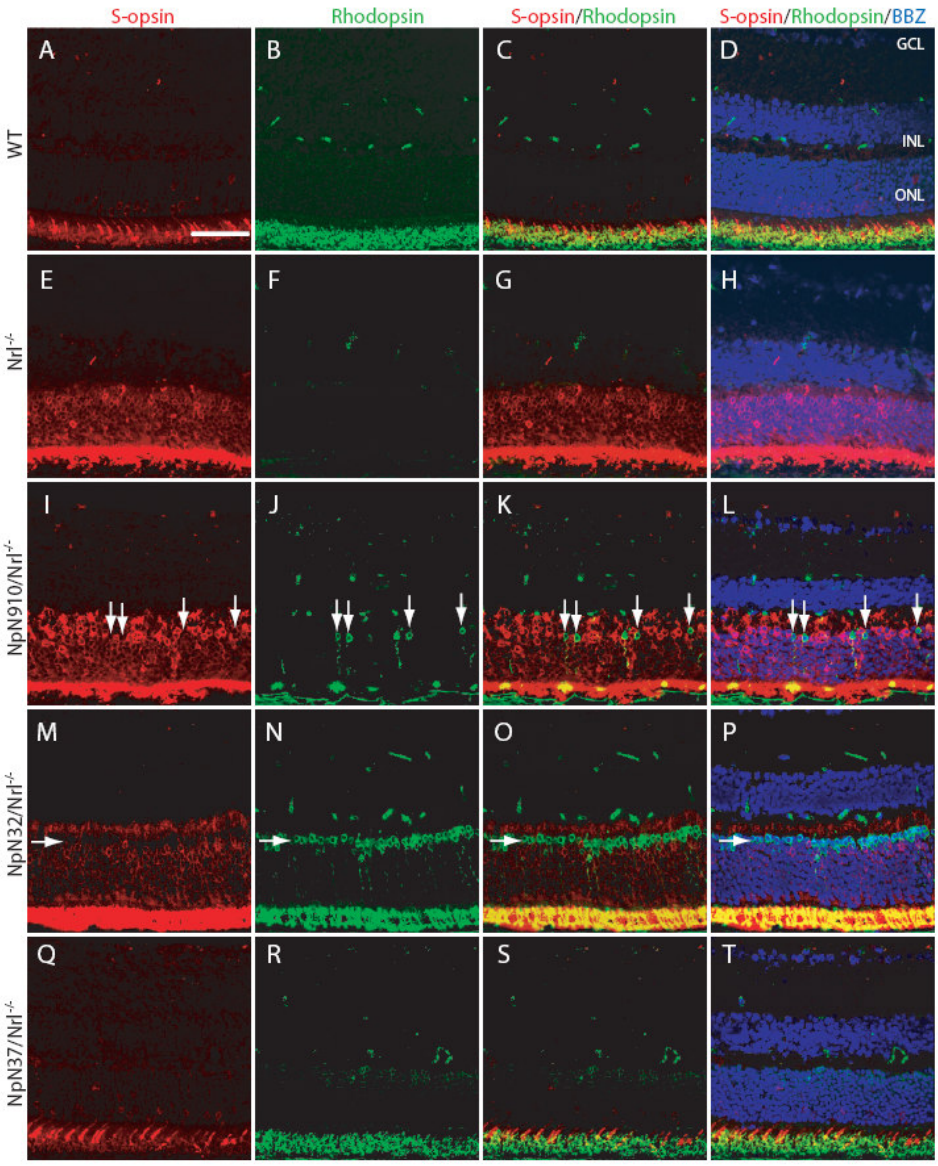


**Figure V-3. Rod morphology and function results from the expression of the *Nrlp-Nrl* transgene.** Semi-thin plastic sections from WT (A), *Nrl*$^{-/-}$ (B), *Nrlp-Nrl/Nrl*$^{-/-}$ 910 (C), *Nrlp-Nrl/Nrl*$^{-/-}$ 32 (D), and *Nrlp-Nrl/Nrl*$^{-/-}$ 37 (E) were stained with toluidine blue. A major difference between rods and cones is most obvious in the chromatin organization in the ONL (Arrows; A, E). Whorls and rosettes are present in retinal sections from *Nrl*$^{-/-}$ and the *Nrl/Nrl*$^{-/-}$ 910 line (B-C). Figures F-G show the average b-wave amplitude (± SEM) of three mice (6 eyes) at each point. *Nrlp-Nrl/Nrl*$^{-/-}$ 37 mice have a normal intensity vs. response relationship for both the scotopic (F) and photopic (G) ERG. The appearance of a dark-adapted b-wave (F) below cone threshold in *Nrlp-Nrl/Nrl*$^{-/-}$ 32 and a reduction in the photopic (G) response compared to the *Nrl*$^{-/-}$ suggests a partial shift from all cone to cone plus rod function. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 25 µm.

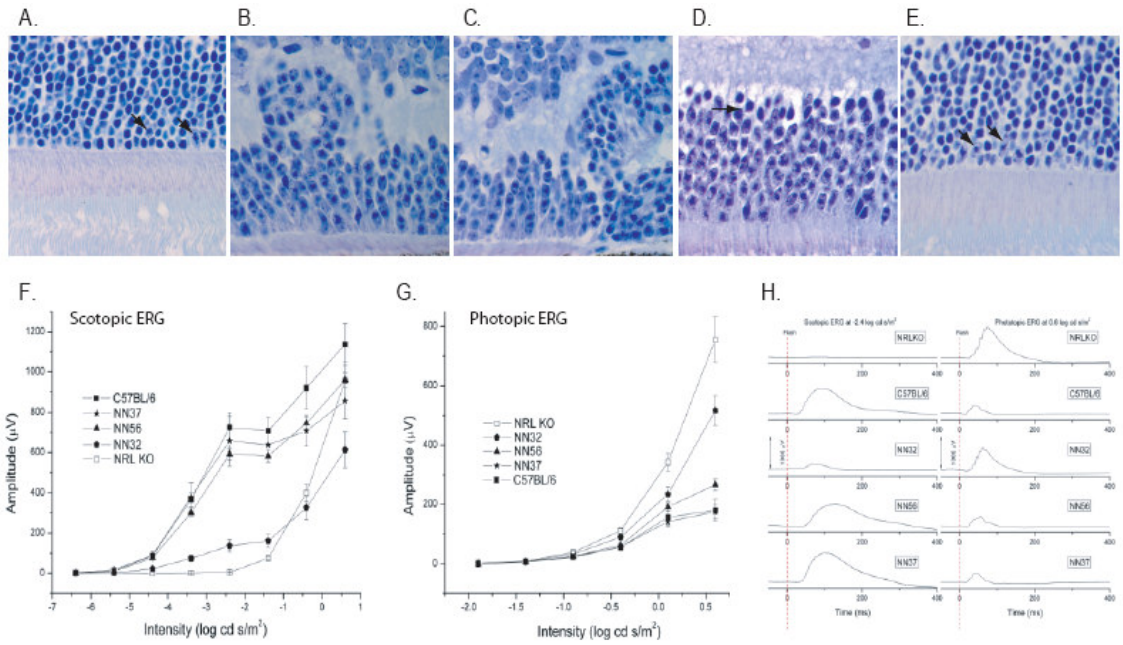


**Figure V-4. Expression of large Maf proteins in the retina results in rhodopsin expression.** The sequence identity and similarity of bZIP domain from the large Maf proteins to NRL was computed using an alignment program (A). Co-electroporation of a GFP control vector and the *Nrlp-Nrl* transgene resulted in high levels of rhodopsin staining in the rod end feet, through the cell body and into the outer segments (B-E). Substituting the *Nrlp-Nrl* transgene with the *Nrlp-MafA* (F-I), *Nrlp-MafB* (J-M), and *Nrlp-c-Maf* (N-Q) transgenes also resulted in rhodopsin staining in the ONL. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 25 µm.

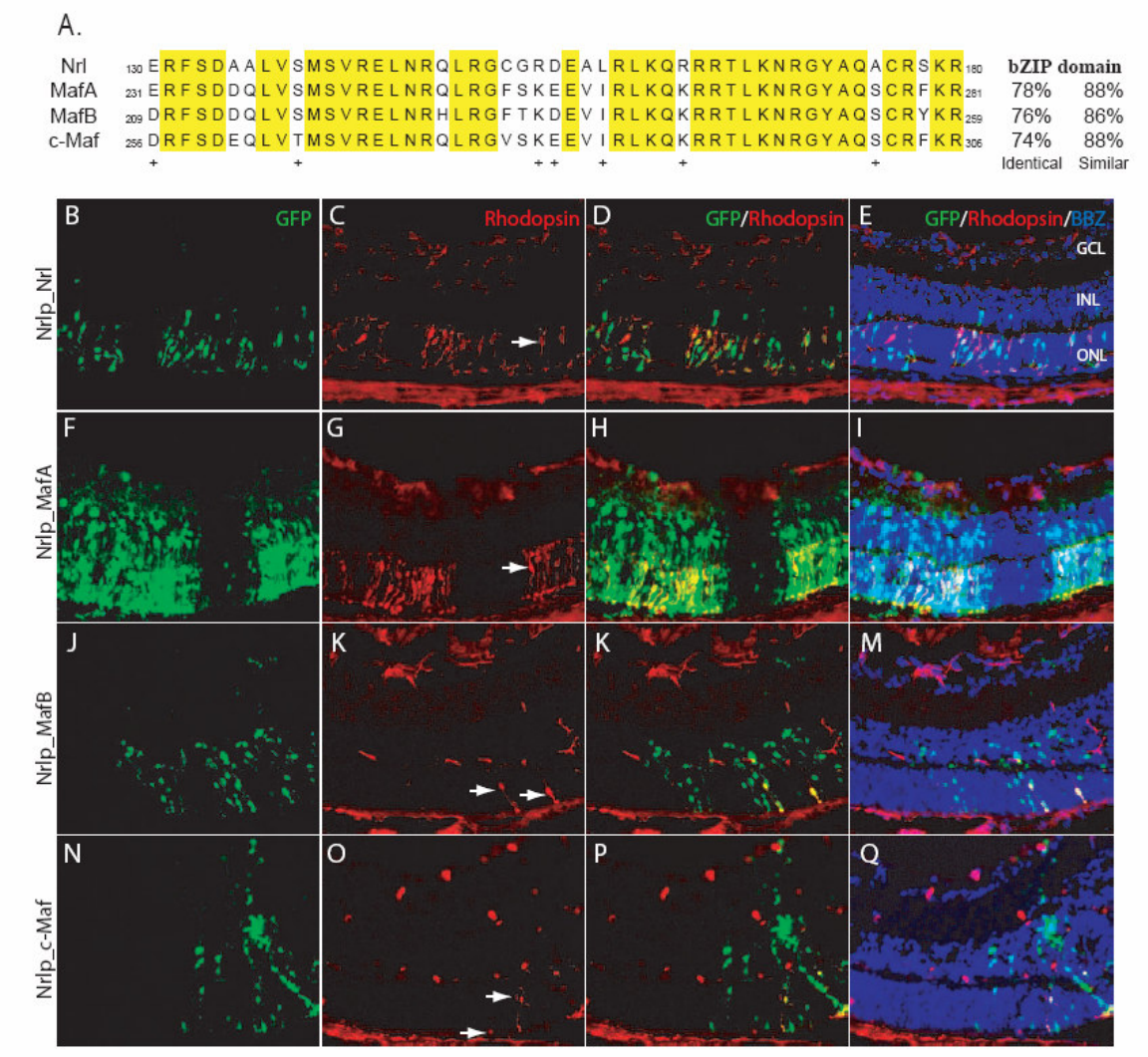


**Figure V-5. Large Maf proteins transactivate the bovine rhodopsin reporter construct.** HEK 293 cells were co-transfected with 0.3 µg of bovine rhodopsin –130 to +72/luciferase fusion construct (pGL2-BRP-130) together with the indicated amount of CRX, NR2E3 and NRL (A), or CRX, NR2E3 and MafA (B), or CRX, NR2E3 and MafB (C), or CRX, NR2E3 and c-Maf (D) expression plasmids. Luciferase activity was corrected for transfection efficiency using a ß-galactosidase internal control (pCMV-ß-gal) (0.3 µg) and shown as fold change, which was calculated as the ratio of each combination to the reporter construct with empty vector (lane 1). Error bars show the standard deviation, *n*=9.

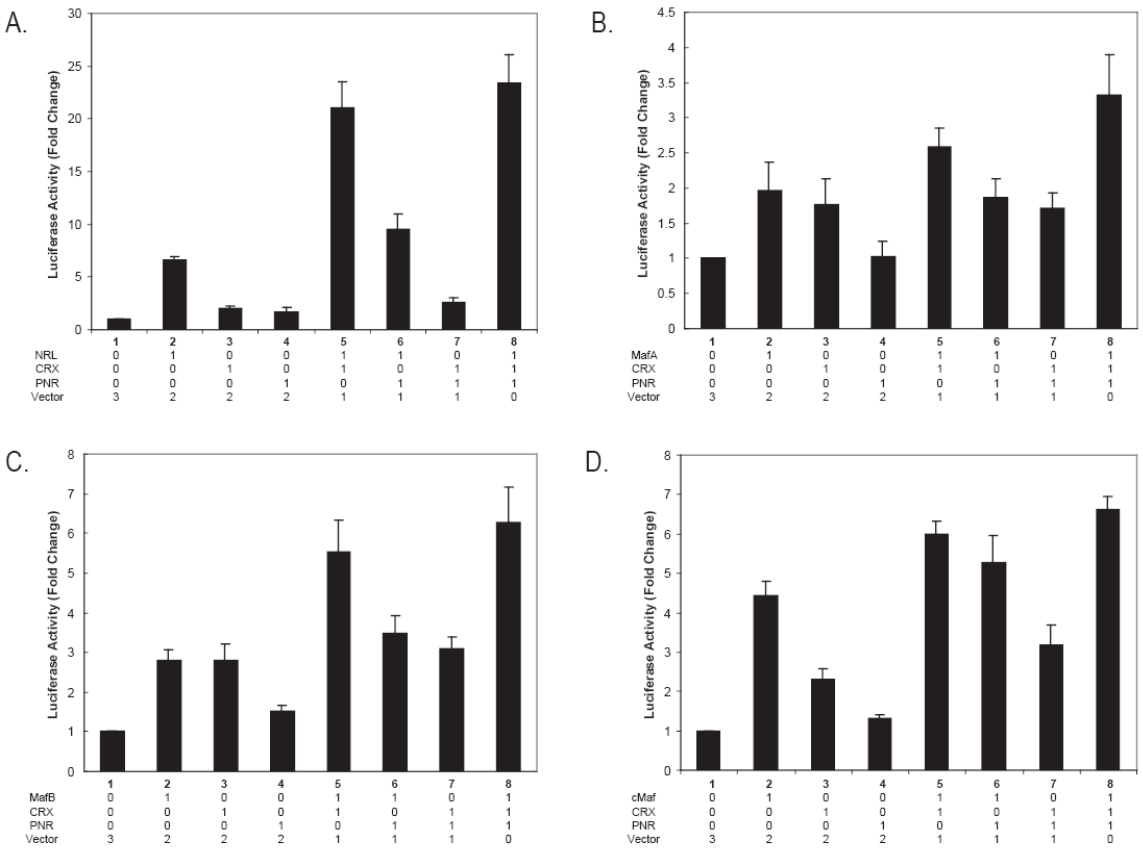


**Figure V-6. Not all large Mafs can block S-opsin expression in the retina.** Co-electroporation of a GFP control vector and the *Nrlp-Nrl* transgene revealed specific areas of S-opsin staining that were absent in the ONL (A-D). In contrast, substituting the *Nrlp-Nrl* transgene with the *Nrlp-MafA* (E-F), *Nrlp-MafB* (I-L), and *Nrlp-c-Maf* (M-P) transgenes did not result in changes in S-opsin expression in the ONL. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 25 μm.

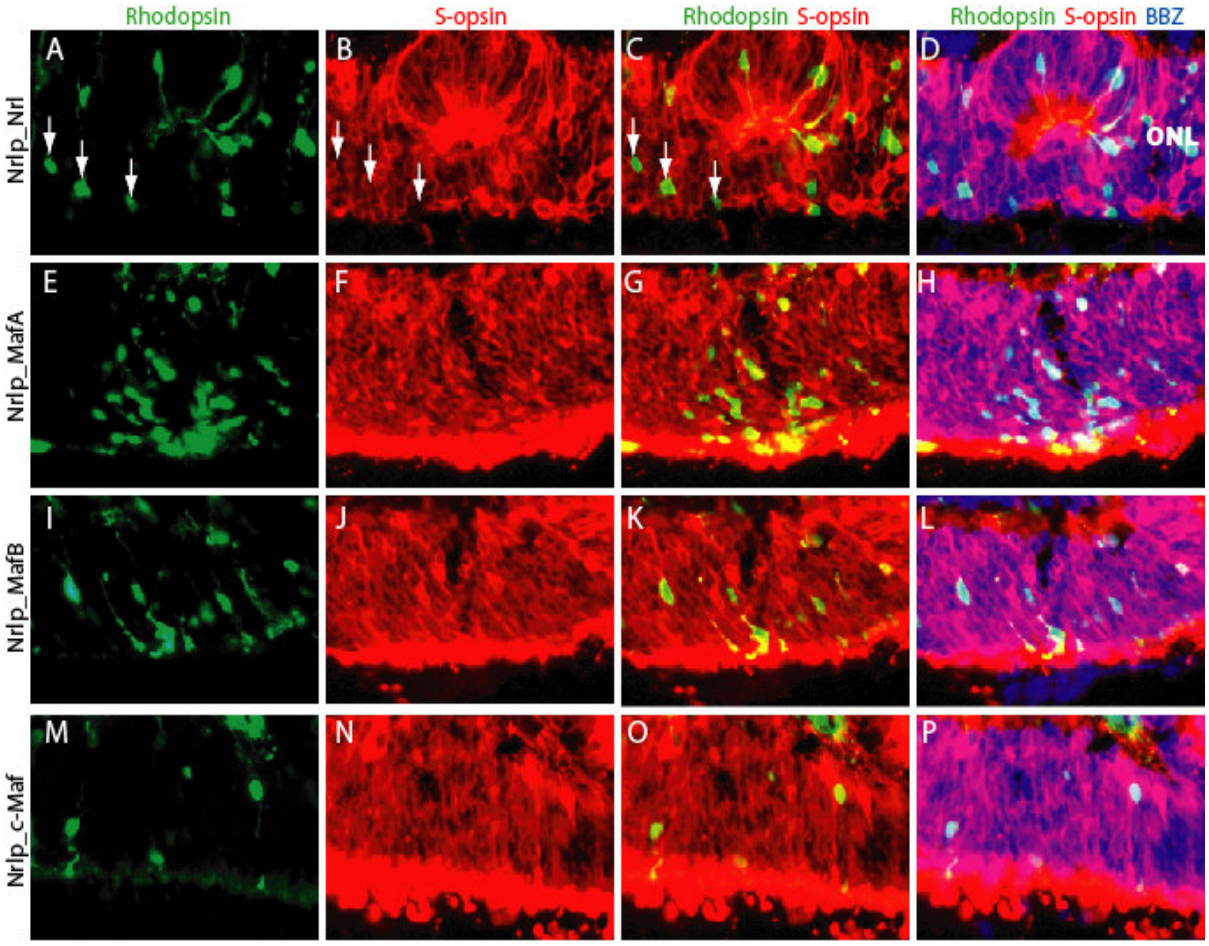
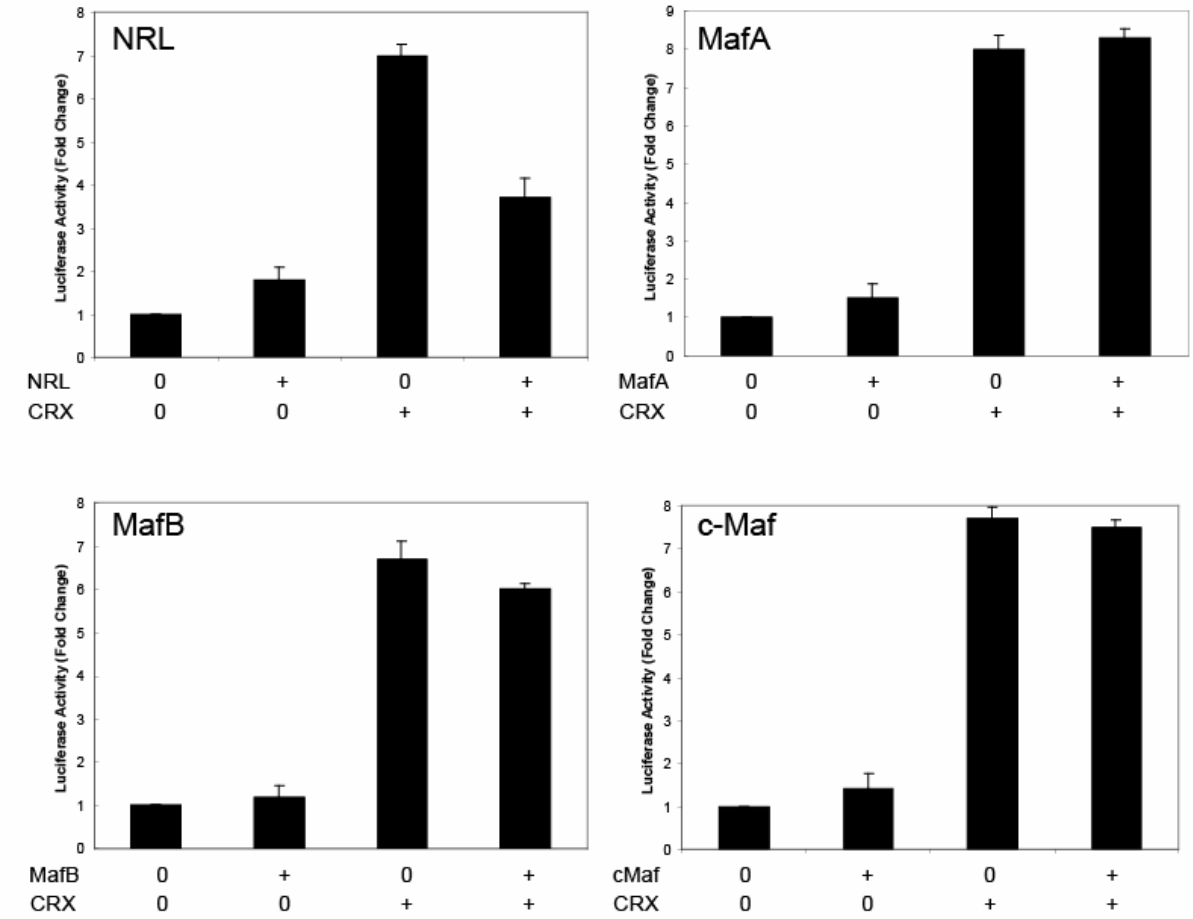


**Figure V-7. NRL suppresses Crx-mediated transactivation of the S-opsin reporter construct.** Similar to earlier experiments in Figure 4, HEK 293 cells were co-transfected with 0.3 µg of S-opsin luciferase fusion construct together with the indicated amount of CRX, NR2E3 and NRL (A), or CRX, NR2E3 and MafA (B), or CRX, NR2E3 and MafB (C), or CRX, NR2E3 and c-Maf (D) expression plasmids.



## References


Akimoto, M., Cheng, H., Zhu, D., Brzezinski, J. A., Khanna, R., Filippova, E., Oh, E. C., Jing, Y., Linares, J. L., Brooks, M.*, et al.* (2006). Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. Proc Natl Acad Sci U S A *103*, 3890-3895.

Artner, I., Blanchi, B., Raum, J. C., Guo, M., Kaneko, T., Cordes, S., Sieweke, M., and Stein, R. (2007). MafB is required for islet beta cell maturation. Proc Natl Acad Sci U S A *104*, 3853-3858.

Benkhelifa, S., Provot, S., Nabais, E., Eychene, A., Calothy, G., and Felder-Schmittbuhl, M. P. (2001). Phosphorylation of MafA is essential for its transcriptional and biological properties. Mol Cell Biol *21*, 4441-4452.

Bessant, D. A., Payne, A. M., Mitton, K. P., Wang, Q. L., Swain, P. K., Plant, C., Bird, A. C., Zack, D. J., Swaroop, A., and Bhattacharya, S. S. (1999). A mutation in NRL is associated with autosomal dominant retinitis pigmentosa. Nat Genet *21*, 355-356.

Carter-Dawson, L. D., and LaVail, M. M. (1979a). Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. J Comp Neurol *188*, 245-262.

Carter-Dawson, L. D., and LaVail, M. M. (1979b). Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. J Comp Neurol *188*, 263-272.

Cheng, H., Khanna, H., Oh, E. C., Hicks, D., Mitton, K. P., and Swaroop, A. (2004). Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors. Hum Mol Genet *13*, 1563-1575.

Cordes, S. P., and Barsh, G. S. (1994). The mouse segmentation gene kr encodes a novel basic domain-leucine zipper transcription factor. Cell *79*, 1025-1034.

Ho, I. C., Hodge, M. R., Rooney, J. W., and Glimcher, L. H. (1996). The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. Cell *85*, 973-983.

Holt, C. E., Bertsch, T. W., Ellis, H. M., and Harris, W. A. (1988). Cellular determination in the Xenopus retina is independent of lineage and birth date. Neuron *1*, 15-26.

Kataoka, K. (2007). Multiple mechanisms and functions of maf transcription factors in the regulation of tissue-specific genes. J Biochem (Tokyo) *141*, 775-781.

Kataoka, K., Noda, M., and Nishizawa, M. (1994). Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. Mol Cell Biol *14*, 700-712.

Kerppola, T. K., and Curran, T. (1994). A conserved region adjacent to the basic domain is required for recognition of an extended DNA binding site by Maf/Nrl family proteins. Oncogene *9*, 3149-3158.

Kim, J. I., Ho, I. C., Grusby, M. J., and Glimcher, L. H. (1999a). The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. Immunity *10*, 745-751.

Kim, J. I., Li, T., Ho, I. C., Grusby, M. J., and Glimcher, L. H. (1999b). Requirement for the c-Maf transcription factor in crystallin gene regulation and lens development. Proc Natl Acad Sci U S A *96*, 3781-3785.

Matsuda, T., and Cepko, C. L. (2004). Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc Natl Acad Sci U S A *101*, 16-22.

Matsuda, T., and Cepko, C. L. (2007). Controlled expression of transgenes introduced by in vivo electroporation. Proc Natl Acad Sci U S A *104*, 1027-1032.

Matsuoka, T. A., Zhao, L., Artner, I., Jarrett, H. W., Friedman, D., Means, A., and Stein, R. (2003). Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells. Mol Cell Biol *23*, 6049-6062.

Mears, A. J., Kondo, M., Swain, P. K., Takada, Y., Bush, R. A., Saunders, T. L., Sieving, P. A., and Swaroop, A. (2001). Nrl is required for rod photoreceptor development. Nat Genet *29*, 447-452.

Mitton, K. P., Swain, P. K., Chen, S., Xu, S., Zack, D. J., and Swaroop, A. (2000). The leucine zipper of NRL interacts with the CRX homeodomain. A possible mechanism of transcriptional synergy in rhodopsin regulation. J Biol Chem *275*, 29794-29799.

Nathans, J., and Hogness, D. S. (1983). Isolation, sequence analysis, and intron-exon arrangement of the gene encoding bovine rhodopsin. Cell *34*, 807-814.

Nathans, J., Thomas, D., and Hogness, D. S. (1986). Molecular genetics of human color vision: the genes encoding blue, green, and red pigments. Science *232*, 193-202.

Nishizawa, M., Kataoka, K., Goto, N., Fujiwara, K. T., and Kawai, S. (1989). v-maf, a viral oncogene that encodes a "leucine zipper" motif. Proc Natl Acad Sci U S A *86*, 7711-7715.

Ochi, H., Ogino, H., Kageyama, Y., and Yasuda, K. (2003). The stability of the lens-specific Maf protein is regulated by fibroblast growth factor (FGF)/ERK signaling in lens fiber differentiation. J Biol Chem *278*, 537-544.

Ogino, H., and Yasuda, K. (1998). Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. Science *280*, 115-118.

Oh, E. C., Khan, N., Novelli, E., Khanna, H., Strettoi, E., and Swaroop, A. (2007). Transformation of cone precursors to functional rod photoreceptors by bZIP transcription factor NRL. Proc Natl Acad Sci U S A *104*, 1679-1684.

Peng, G. H., Ahmad, O., Ahmad, F., Liu, J., and Chen, S. (2005). The photoreceptor-specific nuclear receptor Nr2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes. Hum Mol Genet *14*, 747-764.

Rehemtulla, A., Warwar, R., Kumar, R., Ji, X., Zack, D. J., and Swaroop, A. (1996). The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. Proc Natl Acad Sci U S A *93*, 191-195.

Srinivas, M., Ng, L., Liu, H., Jia, L., and Forrest, D. (2006). Activation of the blue opsin gene in cone photoreceptor development by retinoid-related orphan receptor beta. Mol Endocrinol *20*, 1728-1741.

Toda, K., Bush, R. A., Humphries, P., and Sieving, P. A. (1999). The electroretinogram of the rhodopsin knockout mouse. Vis Neurosci *16*, 391-398.

Tsuchiya, M., Taniguchi, S., Yasuda, K., Nitta, K., Maeda, A., Shigemoto, M., and Tsuchiya, K. (2006). Potential roles of large mafs in cell lineages and developing pancreas. Pancreas *32*, 408-416.

Turner, D. L., and Cepko, C. L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature *328*, 131-136.

Turner, D. L., Snyder, E. Y., and Cepko, C. L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833-845.

Wetts, R., and Fraser, S. E. (1988). Multipotent precursors can give rise to all major cell types of the frog retina. Science *239*, 1142-1145.

Young, R. W. (1985). Cell differentiation in the retina of the mouse. Anat Rec *212*, 199-205.

Zhang, C., Moriguchi, T., Kajihara, M., Esaki, R., Harada, A., Shimohata, H., Oishi, H., Hamada, M., Morito, N., Hasegawa, K.*, et al.* (2005). MafA is a key regulator of glucose-stimulated insulin secretion. Mol Cell Biol *25*, 4969-4976.

# CHAPTER VI

# CONCLUSIONS AND UNRESOLVED ISSUES

## Summary of Findings

This thesis presents studies on the role of NRL in photoreceptor cell fate determination. When I first approached this project in 2004, the specific aim was to determine whether NRL was sufficient to induce rod-specific gene expression and generate functional rods *in vivo*. At that time, we were aware that NRL activated rod-specific genes *in vitro* and was necessary for rod photoreceptor specification *in vivo* (Chen et al., 1997; Lerner et al., 2001; Mears et al., 2001; Mitton et al., 2000; Mitton et al., 2003; Pittler et al., 2004; Rehemtulla et al., 1996; Swain et al., 2001; Wang et al., 2004; Yoshida et al., 2004; Yu et al., 2004). Based on these important observations, we hypothesized that expression of NRL in post-mitotic cone precursors would drive rod differentiation at the expense of cone development.

We began these investigations by obtaining genomic DNA that corresponded to the proximal promoter elements of the *Crx* gene. In 2003, Furukawa and colleagues reported that a 12 kb or a 2 kb promoter fragment could drive Cre-recombinase in the photoreceptor layer (Furukawa et al., 2002). Upon closer examination, we noticed that the promoter was indeed active in both rods and cones, but not bipolar cells. I then communicated with

Dr Furukawa and discovered that both the 2 kb and the 12 kb promoters worked equivalently. This information set in motion a number of experiments with the 2 kb promoter that led to the generation of several transgenic lines used in this thesis.

My findings in Chapter II relate to the potency of NRL in the retina during photoreceptor development. Although this body of work is summarized in Figure II-5, I will reiterate a number of salient points. As reviewed in Chapter I, a previously proposed model of retinal cell fate determination predicts that retinal progenitors pass through a series of competence states, during each of which the progenitors are competent to produce only a subset of retinal cell types (Cepko et al., 1996; Livesey and Cepko, 2001). This may imply that cone precursors would not be responsive to NRL (a putative rod determining factor) since cones develop from an early competence state. To test this hypothesis, we expressed NRL under the control of the *Crx* promoter and examined the retina of our transgenic mice (Oh et al., 2007). Through ultrastructural, histochemical, molecular, and physiological analyses of transgenic mouse lines, I discovered that the expression of NRL in cone precursors resulted in an all rod retina. Due to the lack of any obvious increase in TUNEL stained cells and that only cone photoreceptor cell numbers were perturbed, the data was consistent with cone precursors transforming into rod photoreceptors. Although we could not directly show that Crx positive cone precursors transformed to rods, I genetically tagged S-opsin positive cells and demonstrated that they had

converted to rods. These findings highlight the robust activity of NRL as an activator of rod genes and more importantly as a potential suppressor of cone specification (as shown in ChIP and EMSA data, Figure II-4). In addition, we were able to conclude that a bipotent photoreceptor precursor exists during early retinal development and is competent to develop into either a rod or cone photoreceptor (Figure II-5 and VI-1).

While this work in Chapter II was being conducted, another study from our laboratory addressed the role of ectopic expression of NR2E3 in cone precursors (using the same *Crx* promoter) (Cheng et al., 2006). Interestingly, the expression of NR2E3 — a putative downstream transcriptional target of NRL (Akimoto et al., 2006; Mears et al., 2001; Yoshida et al., 2004) — was sufficient to suppress the specification of cone photoreceptors. A key difference in these mice from the *Crxp-Nrl* transgenic mice was that in the absence of NRL, cone photoreceptors were converted to rhodopsin-expressing neurons that lacked rod-specific photoresponse (Figure VI-2). This implied that a switch from cone to rod cell fate had commenced but was incomplete. Cheng and colleagues then determined through quantitative PCR analyses that at least one of the phototransduction proteins rod transducin *Gnat1,* was not expressed in the absence of NRL. This information provided at least one of the missing links to why cones could not be converted to functional rods and was consistent with my earlier experiments demonstrating that *Gnat1* is a transcriptional target of NRL, CRX and NR2E3 (Cheng et al., 2004). More importantly, these findings provided a

reason to cross the *Crxp-Nr2e3* mice into a wild-type background and test whether the expression of NRL could facilitate the expression of rod specific genes. As anticipated, the *Crxp-Nr2e3* mice in a wild-type background revealed a full functional transformation from cones to rods.

The published studies on NR2E3 by our lab (Cheng and colleagues) and other laboratories have provided key insights into mechanisms that regulate photoreceptor differentiation (Chen et al., 2005; Chen et al., 2006; Cheng et al., 2006; Haider et al., 2000; Milam et al., 2002; Peng et al., 2005). As a result, we can now ask questions about genetic relationships between various transcription factors in the retina. In particular, we wanted to figure out how NRL regulates NR2E3 and vice versa. Although we had microarray data from mouse knockout models that suggested NR2E3 was a downstream transcriptional target of NRL (Akimoto et al., 2006; Yoshida et al., 2004), we lacked a direct evidence. Therefore, I started the experiments that are covered in Chapter III. We hypothesized that NR2E3 was a downstream target of NRL and that NR2E3 facilitates cone photoreceptor suppression in the *Crxp-Nrl* transgenic mice. To test the initial part of the hypothesis, my first step involved using a bioinformatics program to establish the presence of NRL-response element (NRE) in the *Nr2e3* promoter. I then used these sites for EMSA and found NRL could bind to these DNA sequences. In addition, NRL in concert with CRX could activate the *Nr2e3* promoter in transient activation assays. These findings suggested that the *Nr2e3* promoter is in fact a direct transcriptional target of NRL. In the second part of hypothesis, I

then asked whether ectopic expression of NR2E3 in cones, in the *Crxp-Nrl* mice, results in the suppression of cone specification? To approach this, I needed to remove NR2E3 from the mice and test for cone-specific markers and function. I crossed the *Crxp-Nrl* mice to *rd7* mice (which lacks functional NR2E3) (Chang et al., 2002; Haider et al., 2001) and analyzed retinal histology. Both retinal whole-mounts and cryosections revealed that M-opsin was expressed at levels similar to wild-type mice and S-opsin was partially rescued. ERG recordings then suggested that cone function was present though at lower levels than wild-type mice. These results provided additional evidence that NRL is a strong activator of rod differentiation but a weak suppressor of cone development, while NR2E3 is a weak activator of rod differentiation and a strong suppressor of cone development. In order to identify cone-specific genes, we also carried out microarray studies on whole retinal tissue from various transgenic and knockout backgrounds. The new targets listed in this report may potentially lead to new projects on cone biology.

The *Crxp-Nrl* mice are now being used in many (at least 8) laboratories around the world to characterize various aspects of photoreceptor biology ranging from rod morphogenesis to physiological questions. In addition to these studies, our lab — in collaboration with Dr Enrica Strettoi at CNR, Italy and Dr. Benjamin Reese at UCSB — was interested in understanding how retinal interneurons develop in the absence of photoreceptors (Raven et al., 2007; Strettoi et al., 2004). Photoreceptors directly connect to one type of rod

bipolar cell, 9 different types of cone bipolar cells, and horizontal cells (Ghosh et al., 2004; Kolb, 1974; Pignatelli and Strettoi, 2004; Reese et al., 2005). In Chapter II, we were able to show that in the absence of cones, cone bipolar cells were still born. Since bipolar cells develop from a later born retinal progenitors, it appeared that they do not need extracellular signals from cones to develop in the retina (Oh et al., 2007). We next wanted to understand how horizontal cells differentiate in response to a pure cone or a pure rod retina and much of this work constitutes Chapter IV. Horizontal cells connect to rods and cones through their axon and dendrites, respectively (Kolb, 1974). One fascinating question that arises from this systematic synaptic partnership is what happens when either the rods or cones are depleted? To answer this question, we examined horizontal cells from P5, P10 and adult retinal preparations from *Nrl*$^{-/-}$ (rodless) and *Crxp-Nrl* (coneless) animals. Our analyses revealed that although horizontal cells are born, the absence of either rods or cones resulted in the progressive atrophy of the axon terminal and dendritic field, respectively (Raven et al., 2007).

In Chapter V, I made use of the *Nrl* promoter to ask questions about the functional conservation of Maf protein function and photoreceptor differentiation. I first tested a hypothesis that the expression of NRL under the control of the *Nrl* promoter (*Nrlp-Nrl*) could rescue the *Nrl*$^{-/-}$ phenotype (Mears et al., 2001). After generating and characterizing several *Nrlp-Nrl* lines, I discovered that at least three lines could completely rescue the *Nrl*$^{-/-}$ background. I also observed a range of rescue effects that could be

categorized by variable amounts of rhodopsin expression in the photoreceptor layer (Figure V-2). Based on the immunohistochemical analyses, it appeared the re-expression of NRL in the *Nrl*$^{-/-}$ background could promote rhodopsin expression levels similar to wild-type levels and a reduction in S-opsin expression. To test the rod function, ERGs were carried out and both photopic and scotopic ERGs from two transgenic mouse lines looked identical to wild-type mice. These experiments allowed us to conclude that a 2.5 kb *Nrl* proximal promoter was sufficient to express the NRL protein in the correct temporospatial manner. Since the expression of NRL in transgenic mice could rescue the *Nrl*$^{-/-}$ phenotype, we were curious whether other large Maf proteins could play the same role. Therefore, we generated new constructs in which MafA, MafB or c-Maf was expressed under the control of the *Nrl* promoter. We then proceeded to optimize a new protocol (Figure VI-4) to inject these constructs into the subretinal space of P2 *Nrl*$^{-/-}$ pups and electroporate the DNA transgenes. This technique led to our discovery that the expression of large Maf proteins resulted in the activation of rhodopsin. Although we do not know if these rhodopsin-expressing photoreceptors behave like functional rods, this study demonstrates the utility of the *Nrl* promoter and *in vivo* electroporation into neonatal pups. We then explored whether the large Maf proteins could directly transactivate the *rhodopsin* promoter in cultured cells using transient transfection studies. We found that the expression of each Maf member either alone or in concert with CRX positively influenced the *rhodopsin* reporter construct. We then asked

whether the Maf family members could down-regulate the S-opsin promoter in transient transfection assays. Although the expression of NRL could lead to the inhibition of CRX-mediated activation of the S-opsin promoter activity, the other three Maf proteins could not.

## Unresolved Issues

### Can NRL instruct all retinal progenitors to become rods?

Ectopic expression of NRL in photoreceptor precursors drives the expansion of the rod photoreceptor population at the expense of cones (Chapter I) (Oh et al., 2007). This observation suggests that NRL acts like a master regulator in post-mitotic neurons where it plays an instructive role during differentiation. These results provide a new platform to ask whether NRL can instruct all cells to become rods, or whether this effect is limited to post-mitotic photoreceptor precursors in the retina (Akimoto et al., 2006; Mears et al., 2001; Oh et al., 2007).

Several recent studies have described the expression of specific promoters in the retina. More specifically, work on SIX3 (Furuta et al., 2000), Pax6 (Zhang et al., 2003), and CHX10 (Rowan and Cepko, 2004; Rowan et al., 2004) reveal their promoters can be used to drive Cre recombinase in both early retinal progenitors and subsequently in a number of different post-mitotic cells (Figure VI-5). These promoters present an interesting resource to ask whether the expression of NRL in retinal precursors can drive the

expansion of rod photoreceptors. Since cell intrinsic signals are essential in dictating the fate of early born cells (Cepko et al., 1996; Livesey and Cepko, 2001), I predict that the generation and characterization of such transgenic mice will result in a number of different possibilities: First, the expression of NRL may not affect retinal differentiation. This may indicate the limits of NRL function in post-mitotic neurons. Secondly, we may find that late-born cells (such as bipolar cells and Muller glia) are transformed to rods. This outcome will not be surprising as ectopic expression of several master control genes at specific times during development is shown to result in the exclusion of specific cell fates (Dyer, 2003; Dyer et al., 2003; Li et al., 2004; Livne-Bar et al., 2006; Ma et al., 2004). Finally, another possibility is that NRL reprograms all retinal progenitors to rods. In light of recent findings that the removal of Notch in the retina can reprogram retinal precursors to cones (Jadhav et al., 2006; Yaron et al., 2006), it is possible that NRL could have the same effect for rods. Although Notch is normally expressed in mitotic cells and NRL is not, it will be interesting to fully explore the dominant function of NRL.

**How do human mutations in NRL result in retinitis pigmentosa?**

The characterization of the *Nrlp-Nrl* transgenic mice and the efficiency at which the *Nrl* proximal promoter can be used to express genes of interest (Akimoto et al., 2006) allowed us to investigate new issues about rod biology. Several published studies clearly define an association of mutations in NRL with retinal degeneration (Acar et al., 2003; Bessant et al., 2000; Martinez-

Gimeno et al., 2001; Nishiguchi et al., 2004; Sullivan et al., 2006; Wright et al., 2004). These genetic studies provide a reason to use transgenic mouse tools to investigate the direct correlation between the mutation and the disease. I propose that the generation of transgenic lines of NRL phosphorylation mutants under the control of the *Nrl* promoter will help determine a direct link between mutation and disease.

A direct association of the S50T allele and adRP has been clearly documented in a number of different human patients (Bessant et al., 2000; Nishiguchi et al., 2004). In addition, our lab has established an *in vitro* assay to demonstrate that lower protein levels of $Nrl^{S50T}$ can transactivate the *rhodopsin* promoter to much higher levels than wild-type NRL (Nishiguchi et al., 2004). I hypothesize that the generation of an $Nrl^{S50T}$ mouse will lead to higher rhodopsin levels *in vivo* and retinal degeneration over time (Figure VI-6). A first test before generating these transgenic mice would be to utilize the *in vivo* electroporation technique to test whether the presence of the *Nrlp-*$Nrl^{S50T}$ transgene can lead to expression of rhodopsin. Since we have shown that wild-type NRL — when expressed at P2 in the $Nrl^{-/-}$ background — can activate rhodopsin expression, we can use this time-point as a control. If rhodopsin expression is observed, several transgenic lines should be generated, aged, and analyzed for retinal histology and function. If adRP can be modeled in mice, it will be important to examine whether a single copy of the transgene in an $Nrl^{+/-}$ heterozygote mouse will best phenocopy what is observed in humans.

**What are the downstream targets of NRL?**

One satisfying outcome of microarray results is observing dramatic changes in the expression of novel genes. Our analysis of the retinas from *Nrl*$^{-/-}$ (rodless) and *Crxp-Nrl* (coneless) mice revealed a number of potential cone- and rod-specific genes. An example of these findings is that the *Pituitary Tumor-Transforming 1* gene (Chapter III) appears to be enriched in cones. This gene has been previously demonstrated to orchestrate sister-chromatid separation (Stemmann et al., 2001; Zou et al., 1999), and it would be interesting if it regulates chromatin remodeling in cones versus rods. Gene expression profiling from rods has also revealed many exciting novel genes that are yet to be characterized. For example, we determined that REEP6 was down regulated in *Nrl*$^{-/-}$ retinal profiles (Chapter III). REEP6 (receptor enhancing expression protein 6) is also known as DP1L1 (Deleted in Polyposis 1 like 1) (Behrens et al., 2006; Sato et al., 2005) and belongs to a family of proteins that play a role in transporting G-protein coupled receptors (GPCRs) such as olfactory receptors in neurons (Behrens et al., 2006). Although REEP6/DP1L1 was initially characterized as a novel gene enriched in ganglion cells, many experiments in that report lacked appropriate controls (Sato et al., 2005).

I have recently generated antibodies to REEP6. REEP6 protein expression can be observed in the retina, liver, kidney and testis. In the retina, a rod-enriched pattern of expression can be observed when I stain

wild-type and *Nrl*$^{-/-}$ retinal cryosections (Figure VI-7). In addition, the initiation of REEP6 expression coincides with the birth of rod photoreceptors (Figure VI-7). The protein expression data is supported by quantitative PCR and in situ analyses of retinal tissue. By confocal analysis, REEP6 appears to be enriched in the inner segments and synaptic terminals of rods. The ribbon synapses of the vertebrate retina are unique chemical synapses characterized by presynaptic ribbons, sheet-like organelles with a lamellar organization (Dowling, 1987; Sterling, 1988). In contrast to the detailed knowledge about the morphology, physiology, and overall protein complement of the terminals of ribbon synapses, little is known about the composition of the ribbons themselves. It has been suggested that the primary function of the ribbons is to speed up vesicle traffic by serving as a conduit for synaptic vesicles (Schmitz et al., 2000). The expression of REEP6 in the ribbons could therefore enhance the transport of synaptic proteins. Additional studies will be needed to purify ribbon synapses and verify the presence of REEP6 in this compartment. In addition, knockdown studies will assist in demonstrating the role of REEP6 in ribbon formation.

In conclusion, my studies provide significant new insights into the role of NRL in photoreceptor differentiation. The studies presented here show that NRL is not only essential but is sufficient for rod differentiation. Our data provide direct evidence of post-mitotic plasticity in the developing mammalian retina by the expression a single transcription factor *in vivo*. Finally, my

research gives rise to the prospect of exploiting the plastic nature of retinal precursors to replenish dying rods in degenerative retinal diseases.

**Figure VI-1: New models of retinal differentiation.** (A) Retinal progenitors comprise a dynamic mixture of distinct mitotic cell types that interact with the environment to make the different postmitotic cell types. A complex of transcription factors can define the competence of progenitors to make a particular retinal cell type, or small set of cell types. Retinal progenitors are modeled to progress from one state of competence another in only one direction. Early progenitor cells appear to be unable to jump ahead to later stages of competence. There are most likely many more states of competence than shown here, and there may be many branch points along this progression. The environment is shown to be changing over time, in part due to the production of postmitotic, differentiating cells. The postmitotic cell types can regulate, via feedback inhibition, the production of more neurons of the same type, and possibly regulate cell fate choices through other types of interactions. (B) Our research suggests that cone precursors can jump ahead and make rod photoreceptors. In turn, our model reveals that there are exceptions to what is commonly observed in the vertebrate retina (Livesey and Cepko, 2001).

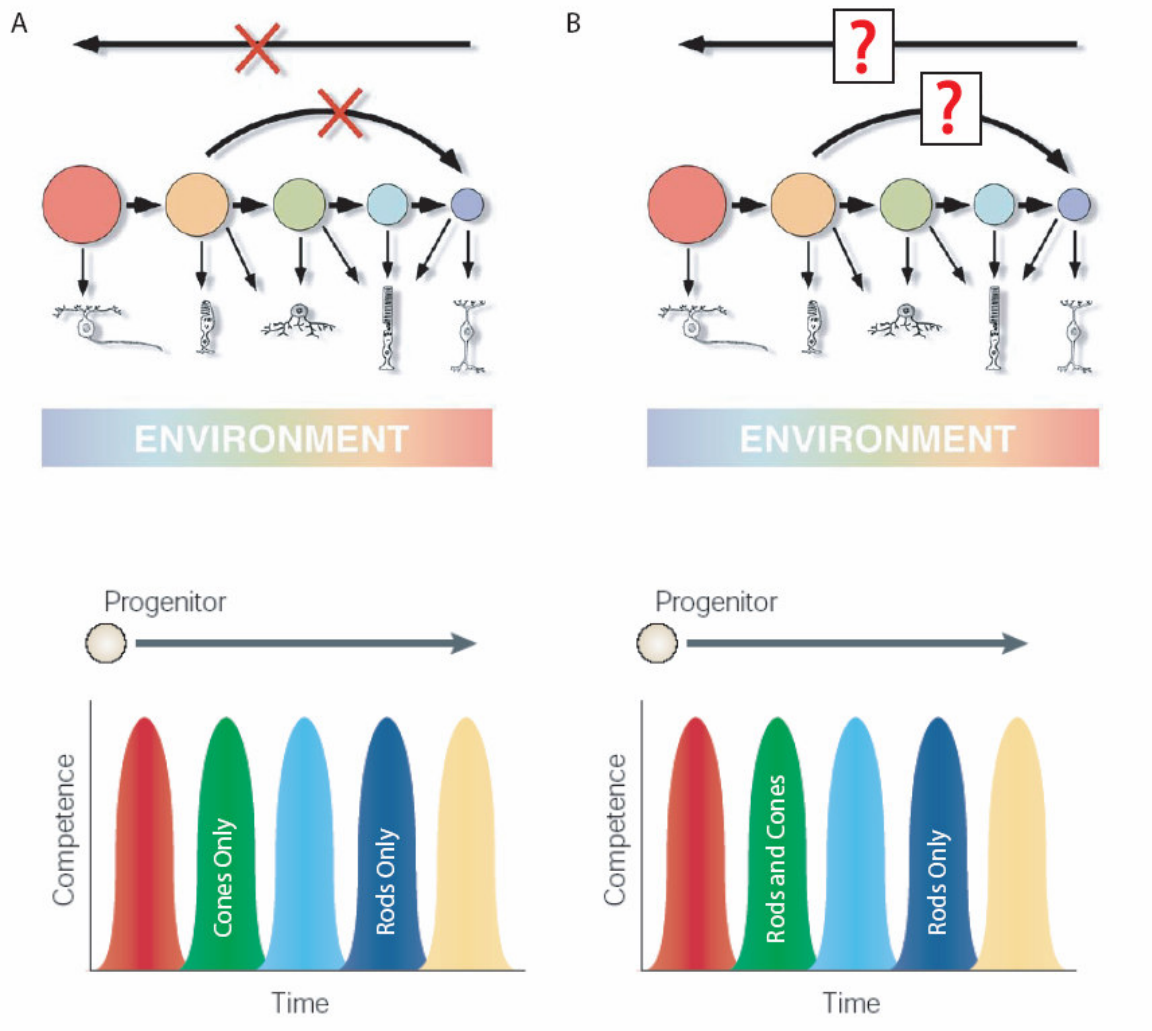


**Figure VI-2: Ectopic expression of NR2E3 in cone precursors suppresses cone differentiation.** (A) Rhodopsin is detected in the ONL and OS of the WT and *CrxpNr2e3/Nrl*$^{-/-}$ retina. S-opsin and cone arrestin are enriched in the *Nrl*$^{-/-}$ retina but are undetectable in the *CrxpNr2e3/Nrl*$^{-/-}$ retina. (B) Light-adapted, spectral ERG amplitudes demonstrate the enrichment of S-cone activity (360 nm) in *Nrl*$^{-/-}$ mice compared with WT. *CrxpNr2e3/Nrl*$^{-/-}$ mice (gray symbols) show responses indistinguishable from noise. (C) Dark-adapted ERGs in WT mice show rods dominating the first flash photoresponse (dark line). In *Nrl*$^{-/-}$ mice, dark-adapted photoresponses are smaller and slower than WT; the paired-flash response closely tracks the first flash response. ERG photoresponses are not detectable in the *CrxpNr2e3/Nrl*$^{-/-}$ mice (Cheng et al., 2006).

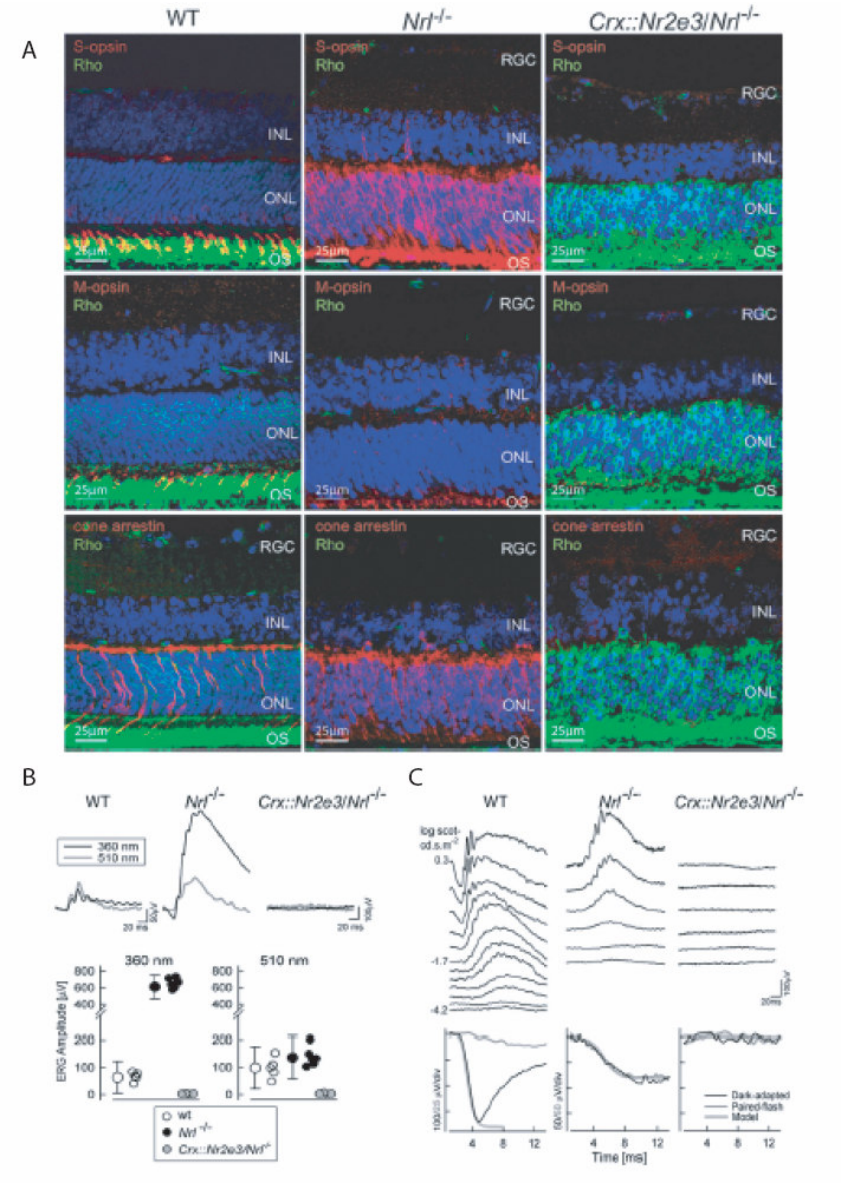


**Figure VI-3: Schematic of in vivo electroporation of the mouse and rat retina.** (*A*) Illustration showing the subretinal injection and *in vivo* electroporation method. (*B*) The electrodes used in this study are shown. Tweezer-type electrodes (*a*) are placed to hold the head of newborn (P0) rat or mouse (*b*) (Matsuda and Cepko, 2004).

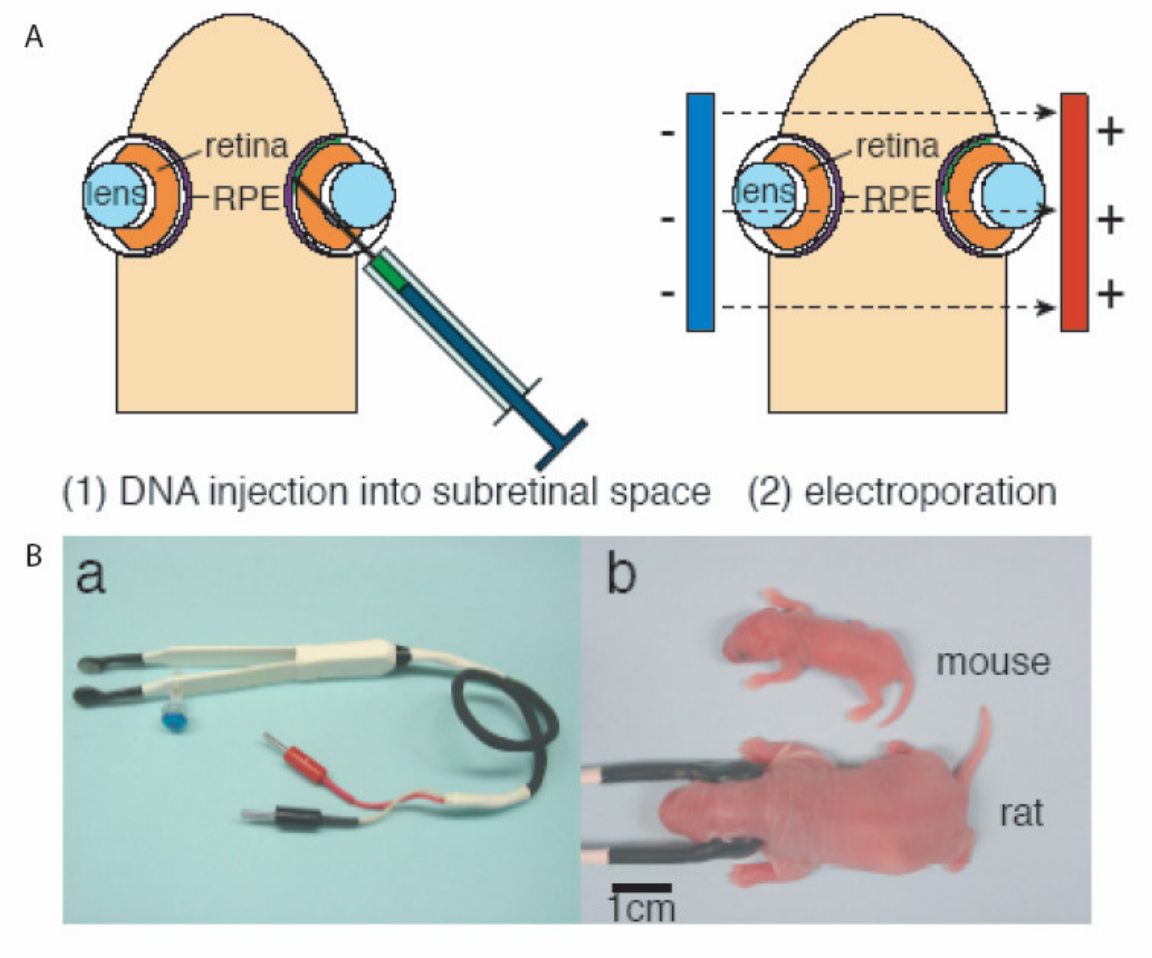


**Figure VI-4: The CHX10, PAX6, and SIX3 promoters can be used to drive NRL expression in retinal precursors.** (A) Schematic of a modified BAC that was used for integration of the GFP Cre-IRES-AP cassette into the first exon of Chx10. Here, we would remove this cassette and insert Nrl. (Rowan and Cepko, 2004). (B) Schematic diagram of the *Pax6* P0 enhancer region and transgenic constructs. The number of independent transgenic (denominators) and β-galactosidase positive embryos (numerators) analyzed transiently from E10.5 to E14.5 are shown (Zhang et al., 2003). (C) Schematic diagram of a 9-kb genomic clone of the mouse *Six3* locus. HD in the shaded box in the exon represents the homeodomain-coding region that would be replaced by NRL (Furuta et al., 2000).

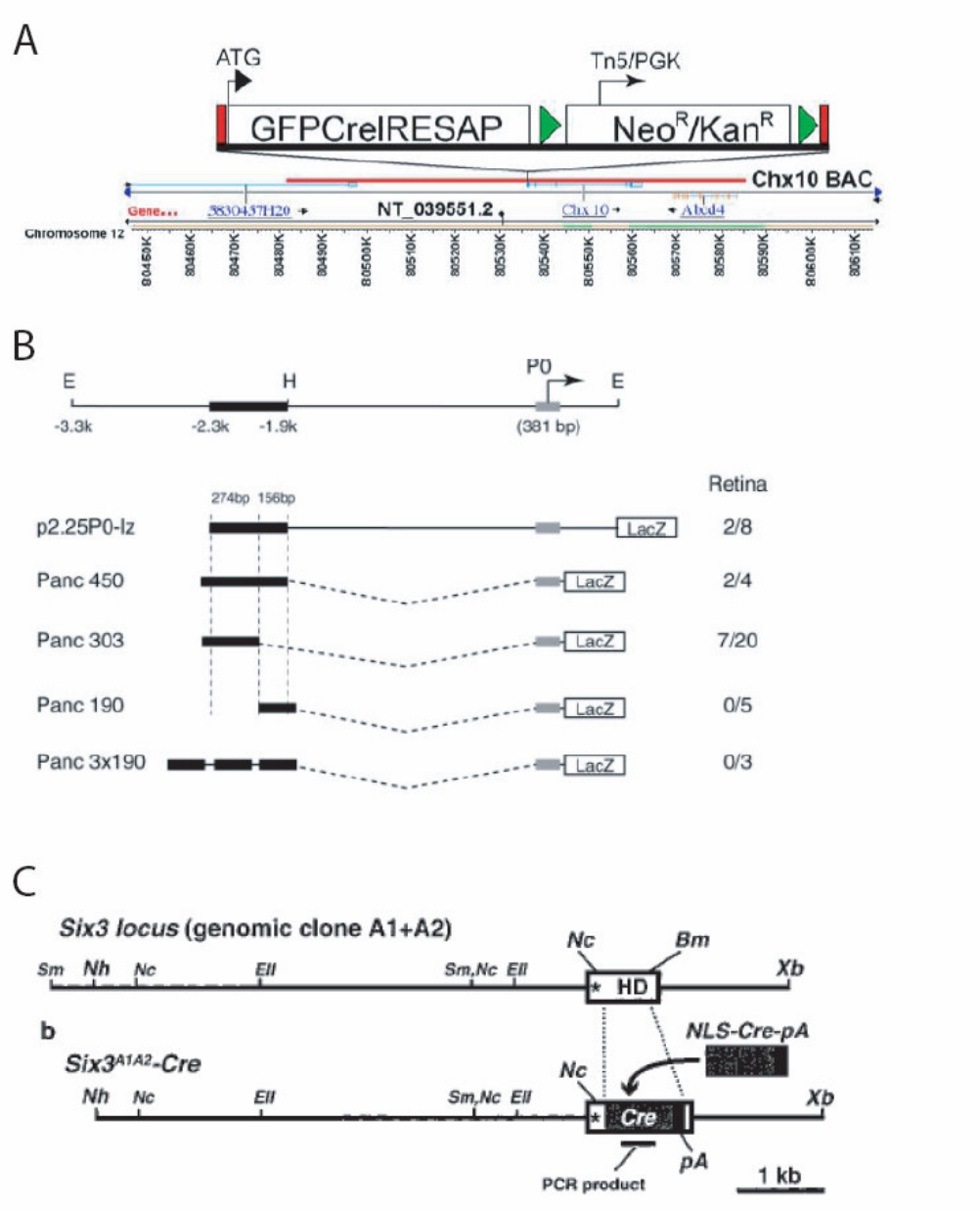


**Figure VI-5: Generation and characterization of NRL S50T mice.** (A) Schematic of a construct that can be used to generate transgenic mouse lines for NRL S50T. (B) Schematic of mouse breeding and the different generations that can be aged and analyzed for the retinal degeneration.

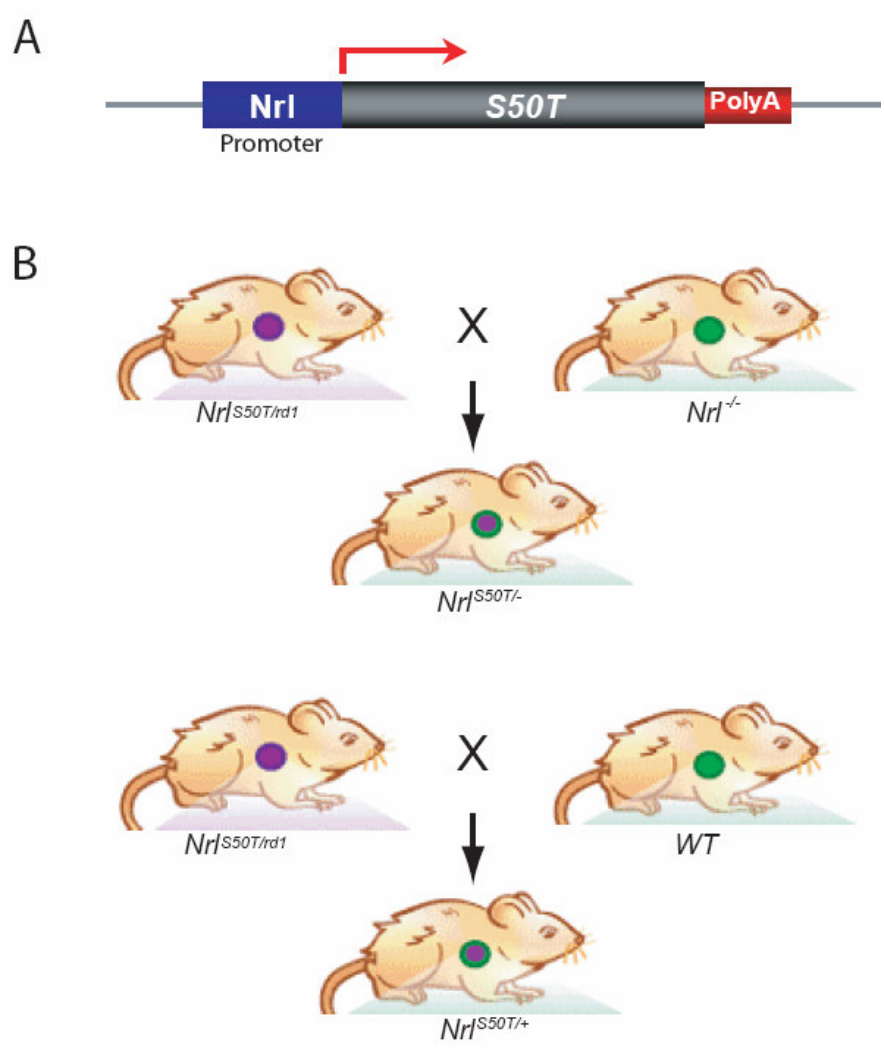
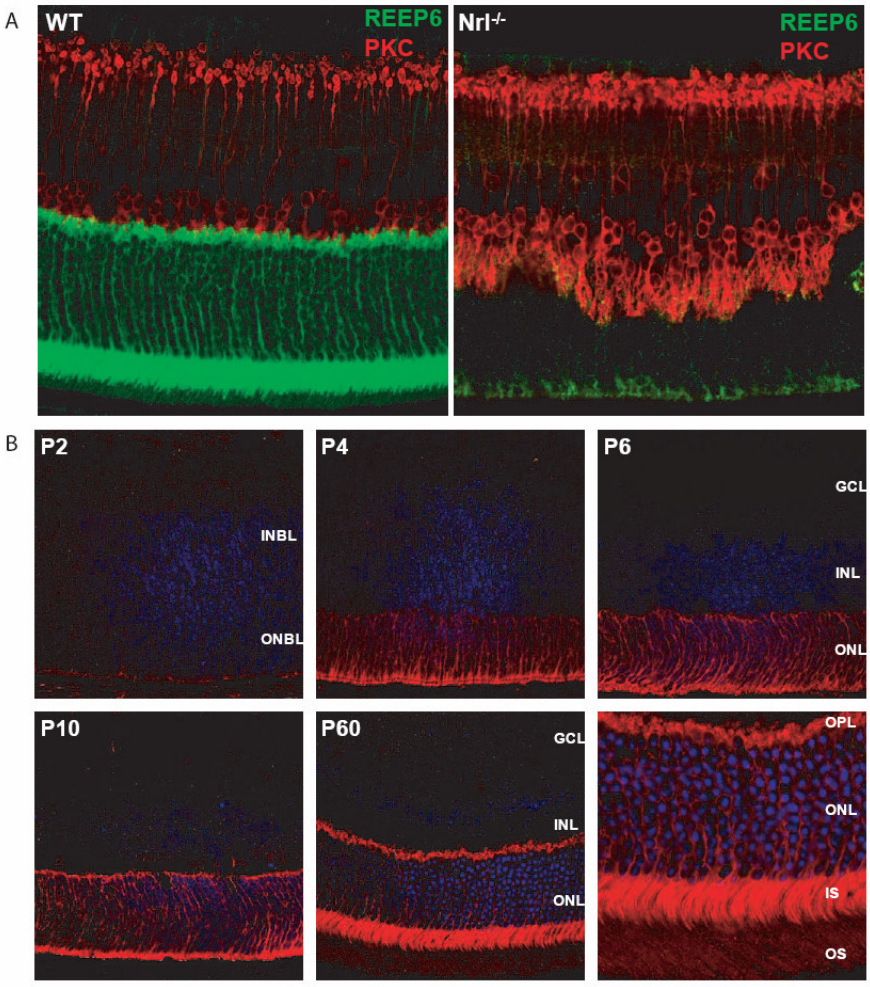


**Figure VI-6: Characterization of REEP6, a novel rod-enriched protein.** (A) WT and Nrl$^{-/-}$ mouse retinal cryosections stained for REEP6 (green) and PKCa (Wright et al.). The REEP6 signal is predominantly in the photoreceptor layer. In the absence of NRL, REEP6 signal is dramatically reduced. (B) P2 to adult WT mouse retinal sections stained for REEP6. The staining can be seen again localized to the photoreceptor layer and starts to peak around P4-6.



## References


Acar, C., Mears, A. J., Yashar, B. M., Maheshwary, A. S., Andreasson, S., Baldi, A., Sieving, P. A., Iannaccone, A., Musarella, M. A., Jacobson, S. G., and Swaroop, A. (2003). Mutation screening of patients with Leber Congenital Amaurosis or the enhanced S-Cone Syndrome reveals a lack of sequence variations in the NRL gene. Mol Vis *9*, 14-17.

Akimoto, M., Cheng, H., Zhu, D., Brzezinski, J. A., Khanna, R., Filippova, E., Oh, E. C., Jing, Y., Linares, J. L., Brooks, M.*, et al.* (2006). Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. Proc Natl Acad Sci U S A *103*, 3890-3895.

Behrens, M., Bartelt, J., Reichling, C., Winnig, M., Kuhn, C., and Meyerhof, W. (2006). Members of RTP and REEP gene families influence functional bitter taste receptor expression. J Biol Chem *281*, 20650-20659.

Bessant, D. A., Payne, A. M., Plant, C., Bird, A. C., Swaroop, A., and Bhattacharya, S. S. (2000). NRL S50T mutation and the importance of 'founder effects' in inherited retinal dystrophies. Eur J Hum Genet *8*, 783-787.

Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M., and Ezzeddine, D. (1996). Cell fate determination in the vertebrate retina. Proc Natl Acad Sci U S A *93*, 589-595.

Chang, B., Hawes, N. L., Hurd, R. E., Davisson, M. T., Nusinowitz, S., and Heckenlively, J. R. (2002). Retinal degeneration mutants in the mouse. Vision Res *42*, 517-525.

Chen, J., Rattner, A., and Nathans, J. (2005). The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes. J Neurosci *25*, 118-129.

Chen, J., Rattner, A., and Nathans, J. (2006). Effects of L1 retrotransposon insertion on transcript processing, localization and accumulation: lessons from the retinal degeneration 7 mouse and implications for the genomic ecology of L1 elements. Hum Mol Genet *15*, 2146-2156.

Chen, S., Wang, Q. L., Nie, Z., Sun, H., Lennon, G., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Zack, D. J. (1997). Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron *19*, 1017-1030.

Cheng, H., Aleman, T. S., Cideciyan, A. V., Khanna, R., Jacobson, S. G., and Swaroop, A. (2006). In vivo function of the orphan nuclear receptor NR2E3 in

establishing photoreceptor identity during mammalian retinal development. Hum Mol Genet *15*, 2588-2602.

Cheng, H., Khanna, H., Oh, E. C., Hicks, D., Mitton, K. P., and Swaroop, A. (2004). Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors. Hum Mol Genet *13*, 1563-1575.

Dyer, M. A. (2003). Regulation of proliferation, cell fate specification and differentiation by the homeodomain proteins Prox1, Six3, and Chx10 in the developing retina. Cell Cycle *2*, 350-357.

Dyer, M. A., Livesey, F. J., Cepko, C. L., and Oliver, G. (2003). Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. Nat Genet *34*, 53-58.

Furukawa, A., Koike, C., Lippincott, P., Cepko, C. L., and Furukawa, T. (2002). The mouse Crx 5'-upstream transgene sequence directs cell-specific and developmentally regulated expression in retinal photoreceptor cells. J Neurosci *22*, 1640-1647.

Furuta, Y., Lagutin, O., Hogan, B. L., and Oliver, G. C. (2000). Retina- and ventral forebrain-specific Cre recombinase activity in transgenic mice. Genesis *26*, 130-132.

Ghosh, K. K., Bujan, S., Haverkamp, S., Feigenspan, A., and Wassle, H. (2004). Types of bipolar cells in the mouse retina. J Comp Neurol *469*, 70-82.
Haider, N. B., Jacobson, S. G., Cideciyan, A. V., Swiderski, R., Streb, L. M., Searby, C., Beck, G., Hockey, R., Hanna, D. B., Gorman, S.*, et al.* (2000). Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. Nat Genet *24*, 127-131.

Haider, N. B., Naggert, J. K., and Nishina, P. M. (2001). Excess cone cell proliferation due to lack of a functional NR2E3 causes retinal dysplasia and degeneration in rd7/rd7 mice. Hum Mol Genet *10*, 1619-1626.

Jadhav, A. P., Mason, H. A., and Cepko, C. L. (2006). Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development *133*, 913-923.

Kolb, H. (1974). The connections between horizontal cells and photoreceptors in the retina of the cat: electron microscopy of Golgi preparations. J Comp Neurol *155*, 1-14.

Lerner, L. E., Gribanova, Y. E., Ji, M., Knox, B. E., and Farber, D. B. (2001). Nrl and Sp nuclear proteins mediate transcription of rod-specific cGMP-

phosphodiesterase beta-subunit gene: involvement of multiple response elements. J Biol Chem *276*, 34999-35007.

Li, S., Mo, Z., Yang, X., Price, S. M., Shen, M. M., and Xiang, M. (2004). Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. Neuron *43*, 795-807.

Livesey, F. J., and Cepko, C. L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci *2*, 109-118.

Livne-Bar, I., Pacal, M., Cheung, M. C., Hankin, M., Trogadis, J., Chen, D., Dorval, K. M., and Bremner, R. (2006). Chx10 is required to block photoreceptor differentiation but is dispensable for progenitor proliferation in the postnatal retina. Proc Natl Acad Sci U S A *103*, 4988-4993.

Ma, W., Yan, R. T., Xie, W., and Wang, S. Z. (2004). bHLH genes cath5 and cNSCL1 promote bFGF-stimulated RPE cells to transdifferentiate toward retinal ganglion cells. Dev Biol *265*, 320-328.

Martinez-Gimeno, M., Maseras, M., Baiget, M., Beneito, M., Antinolo, G., Ayuso, C., and Carballo, M. (2001). Mutations P51U and G122E in retinal transcription factor NRL associated with autosomal dominant and sporadic retinitis pigmentosa. Hum Mutat *17*, 520.

Mears, A. J., Kondo, M., Swain, P. K., Takada, Y., Bush, R. A., Saunders, T. L., Sieving, P. A., and Swaroop, A. (2001). Nrl is required for rod photoreceptor development. Nat Genet *29*, 447-452.

Milam, A. H., Rose, L., Cideciyan, A. V., Barakat, M. R., Tang, W. X., Gupta, N., Aleman, T. S., Wright, A. F., Stone, E. M., Sheffield, V. C., and Jacobson, S. G. (2002). The nuclear receptor NR2E3 plays a role in human retinal photoreceptor differentiation and degeneration. Proc Natl Acad Sci U S A *99*, 473-478.

Mitton, K. P., Swain, P. K., Chen, S., Xu, S., Zack, D. J., and Swaroop, A. (2000). The leucine zipper of NRL interacts with the CRX homeodomain. A possible mechanism of transcriptional synergy in rhodopsin regulation. J Biol Chem *275*, 29794-29799.

Mitton, K. P., Swain, P. K., Khanna, H., Dowd, M., Apel, I. J., and Swaroop, A. (2003). Interaction of retinal bZIP transcription factor NRL with Flt3-interacting zinc-finger protein Fiz1: possible role of Fiz1 as a transcriptional repressor. Hum Mol Genet *12*, 365-373.

Nishiguchi, K. M., Friedman, J. S., Sandberg, M. A., Swaroop, A., Berson, E. L., and Dryja, T. P. (2004). Recessive NRL mutations in patients with clumped

pigmentary retinal degeneration and relative preservation of blue cone function. Proc Natl Acad Sci U S A *101*, 17819-17824.

Oh, E. C., Khan, N., Novelli, E., Khanna, H., Strettoi, E., and Swaroop, A. (2007). Transformation of cone precursors to functional rod photoreceptors by bZIP transcription factor NRL. Proc Natl Acad Sci U S A *104*, 1679-1684.

Peng, G. H., Ahmad, O., Ahmad, F., Liu, J., and Chen, S. (2005). The photoreceptor-specific nuclear receptor Nr2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes. Hum Mol Genet *14*, 747-764.

Pignatelli, V., and Strettoi, E. (2004). Bipolar cells of the mouse retina: a gene gun, morphological study. J Comp Neurol *476*, 254-266.

Pittler, S. J., Zhang, Y., Chen, S., Mears, A. J., Zack, D. J., Ren, Z., Swain, P. K., Yao, S., Swaroop, A., and White, J. B. (2004). Functional analysis of the rod photoreceptor cGMP phosphodiesterase alpha-subunit gene promoter: Nrl and Crx are required for full transcriptional activity. J Biol Chem *279*, 19800-19807.

Raven, M. A., Oh, E. C., Swaroop, A., and Reese, B. E. (2007). Afferent control of horizontal cell morphology revealed by genetic respecification of rods and cones. J Neurosci *27*, 3540-3547.

Reese, B. E., Raven, M. A., and Stagg, S. B. (2005). Afferents and homotypic neighbors regulate horizontal cell morphology, connectivity, and retinal coverage. J Neurosci *25*, 2167-2175.

Rehemtulla, A., Warwar, R., Kumar, R., Ji, X., Zack, D. J., and Swaroop, A. (1996). The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. Proc Natl Acad Sci U S A *93*, 191-195.

Rowan, S., and Cepko, C. L. (2004). Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. Dev Biol *271*, 388-402.

Rowan, S., Chen, C. M., Young, T. L., Fisher, D. E., and Cepko, C. L. (2004). Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene Chx10. Development *131*, 5139-5152.

Sato, H., Tomita, H., Nakazawa, T., Wakana, S., and Tamai, M. (2005). Deleted in polyposis 1-like 1 gene (Dp1l1): a novel gene richly expressed in retinal ganglion cells. Invest Ophthalmol Vis Sci *46*, 791-796.

Schmitz, F., Konigstorfer, A., and Sudhof, T. C. (2000). RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. Neuron *28*, 857-872.

Stemmann, O., Zou, H., Gerber, S. A., Gygi, S. P., and Kirschner, M. W. (2001). Dual inhibition of sister chromatid separation at metaphase. Cell *107*, 715-726.

Strettoi, E., Mears, A. J., and Swaroop, A. (2004). Recruitment of the rod pathway by cones in the absence of rods. J Neurosci *24*, 7576-7582.

Sullivan, L. S., Bowne, S. J., Birch, D. G., Hughbanks-Wheaton, D., Heckenlively, J. R., Lewis, R. A., Garcia, C. A., Ruiz, R. S., Blanton, S. H., Northrup, H.*, et al.* (2006). Prevalence of disease-causing mutations in families with autosomal dominant retinitis pigmentosa: a screen of known genes in 200 families. Invest Ophthalmol Vis Sci *47*, 3052-3064.

Swain, P. K., Hicks, D., Mears, A. J., Apel, I. J., Smith, J. E., John, S. K., Hendrickson, A., Milam, A. H., and Swaroop, A. (2001). Multiple phosphorylated isoforms of NRL are expressed in rod photoreceptors. J Biol Chem *276*, 36824-36830.

Wang, Q. L., Chen, S., Esumi, N., Swain, P. K., Haines, H. S., Peng, G., Melia, B. M., McIntosh, I., Heckenlively, J. R., Jacobson, S. G.*, et al.* (2004). QRX, a novel homeobox gene, modulates photoreceptor gene expression. Hum Mol Genet *13*, 1025-1040.

Wright, A. F., Reddick, A. C., Schwartz, S. B., Ferguson, J. S., Aleman, T. S., Kellner, U., Jurklies, B., Schuster, A., Zrenner, E., Wissinger, B.*, et al.* (2004). Mutation analysis of NR2E3 and NRL genes in Enhanced S Cone Syndrome. Hum Mutat *24*, 439.

Yaron, O., Farhy, C., Marquardt, T., Applebury, M., and Ashery-Padan, R. (2006). Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. Development *133*, 1367-1378.

Yoshida, S., Mears, A. J., Friedman, J. S., Carter, T., He, S., Oh, E., Jing, Y., Farjo, R., Fleury, G., Barlow, C.*, et al.* (2004). Expression profiling of the developing and mature Nrl-/- mouse retina: identification of retinal disease candidates and transcriptional regulatory targets of Nrl. Hum Mol Genet *13*, 1487-1503.

Yu, J., He, S., Friedman, J. S., Akimoto, M., Ghosh, D., Mears, A. J., Hicks, D., and Swaroop, A. (2004). Altered expression of genes of the Bmp/Smad and Wnt/calcium signaling pathways in the cone-only Nrl-/- mouse retina, revealed by gene profiling using custom cDNA microarrays. J Biol Chem *279*, 42211-42220.

Zhang, X., Heaney, S., and Maas, R. L. (2003). Cre-loxp fate-mapping of Pax6 enhancer active retinal and pancreatic progenitors. Genesis *35*, 22-30.

Zou, H., McGarry, T. J., Bernal, T., and Kirschner, M. W. (1999). Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science *285*, 418-422.