# TRANSCRIPTIONAL REGULATION OF THE DEVELOPING RETINA

By

NOAH BRANCH GOODSON

B.S., Appalachian State University, 2011

M.Cert., Nova Southeastern University, 2015

A thesis submitted to the

Faculty of the Graduate School the

University of Colorado in partial fulfillment

Of the requirements for the degree of

Doctor of Philosophy

Neuroscience

2020

This thesis for the Doctor of Philosophy degree by

Noah Branch Goodson

Has been approved for the

Neuroscience Program

By

Julie Siegenthaler, Chair

Joseph Brzezinski, Advisor

Kristen Artinger

Santos Franco

Kenneth Jones

Alan Palestine

Date: May 15th, 2020

Goodson, Noah Branch (PhD, Neuroscience)

Transcriptional Regulation of the Developing Retina

Thesis directed by Associate Professor Joseph Brzezinski

**ABSTRACT**

The retina is a complex stratified neural tissue required for all mammalian vision. During development, tightly controlled transcriptional regulation drives diversification from a homogenous population of retinal progenitor cells into seven major types and dozens of subtypes of cells. Here, we explored the role of the transcription factors *Otx2, Prdm1, Prdm13,* and *Vsx2* in driving the formation of specific neurons from a homogenous pool of retinal progenitors. We first showed that PRDM13 is expressed in a subset of developing amacrine interneurons. Loss of *Prdm13* leads to a 25% reduction in amacrine cells and disruption of retinal sublamina formation. Next, we explored the role of *Prdm1* and *Vsx2* in driving the correct formation of rod photoreceptors and bipolar interneurons. In OTX2+ cells late in retinal development, *Prdm1* and *Vsx2* work in opposition, such that *Prdm1* promotes photoreceptor cell fate and *Vsx2* bipolar cell fate. We deleted *Prdm1* and *Vsx2* or their cell type-specific enhancers simultaneously using a CRISPR/Cas9 *in vivo* retina electroporation strategy. Double enhancer targeting favored bipolar outcomes whereas double gene targeting favored photoreceptor fates. Both conditions generated excess amacrine cells. We show photoreceptors are a default fate outcome in OTX2+ cells and VSX2 is required during a narrow temporal window to drive bipolar cell formation. *Prdm1* and *Vsx2* appear to redundantly restrict the competence of OTX2+ cells, preventing amacrine cell formation. Next, we explored the stability of VSX2+ bipolar fates. To do this, we created a system to conditionally misexpress *Prdm1* during bipolar cell development. We found that *Prdm1* blocks bipolar formation if expressed before the fate choice decision occurred. Constitutive PRDM1 expression in nascent bipolar cells can cause a fate shift to rods but has no effect on mature bipolar cells. PRDM1 was selectively toxic to mature rods. Our data show that bipolar fate is malleable, but only for a short period following fate specification. OTX2+ cells use *Prdm1* and *Vsx2* to stabilize photoreceptor and bipolar cell identities during development and

maturation of proper amacrine subtypes requires *Prdm13*. Our work provides novel insights into the complex regulatory mechanisms that control cell fate choice in the developing murine retina.

The form and content of this abstract are approved. I recommend its publication.

Approved by: Joseph Brzezinski

## DEDICATION

There are dozens of people who made this possible, but there are only two who are irreplaceable.

To Mom, for teaching me to read, write, and do math. You showed me every day that problems can be solved. You empowered my young mind with the tools to understand the world and set me loose with bright-eyed enthusiasm.

To Bethany, for your unending love, support, and sacrifice. You stuck with me through the darkest days and the longest nights. We did it.

"Your eye is the lamp of your body. When your eyes are healthy, your whole body also is full of light. But when they are unhealthy, your body also is full of darkness." Luke 11:34

**ACKNOWLEDGEMENTS**

The pursuit of a PhD is a monumental undertaking. There are the challenges of learning a discipline, pursuing rigorous research, and solving problems with no easy answers. Added to these obstacles are the vicissitudes of life that the doctoral candidate passes through, developing their identity, discovering purpose, and forging a career path. To accomplish this work, I have been supported by many who are not listed here but deserve to be praised and thanked.

I am thankful to Dr. Joseph Brzezinski for his unerring support. Over the last four years he provided mentorship, guidance, and generous assistance in my extensive and variegated endeavors. He put enormous effort into encouraging my work, empowered me to pursue diverse interests, and pushed my thinking as a person and scientist.

I am grateful to my thesis committee. Dr. Santos Franco, Dr. Kristen Artinger, and Dr. Kenneth Jones provided significant guidance and support. Dr. Alan Palestine not only helped shape my intellectual experience but afforded me the chance to spend many days in the Ophthalmology clinic, offered critical guidance to the trajectory of my career, and aided me in obtaining funding. Finally, I am particularly thankful for Dr. Julie Siegenthaler who was an outstanding thesis committee chair.

In addition, I am thankful to the past and present members of the Brzezinski lab, particularly Ko Park for logistical support, and Michael Kaufman, who paved the way for much of this thesis by his pioneering of novel techniques. I would also like to acknowledge the predoctoral training grants I have received, including T32-NS099042, NIH/NCATS Colorado CTSA Grant Number TL1 TR002533, and the Kitty Hirs Research Travel Award. Thanks to the University of Colorado Anschutz Medical Campus, the Neuroscience Graduate Program, and the Department of Ophthalmology for welcoming me and empowering this research.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| Abbreviation | Meaning |
|---|---|
| AP2a | Transcription Factor AP-2 Alpha |
| AP2b | Transcription Factor AP-2 Beta |
| ASCL1 | Achaete-Scute Family BHLH Transcription Factor 1 |
| ATOH7 | (MATH5) Atonal Homolog BHLH Transcription Factor 7 |
| bHLH | basic Helix-Loop-Helix transcription factor |
| BHLHB5 | Basic Helix-Loop-Helix family member B5 |
| BP | Base Pair |
| CCTSI | Colorado Clinical and Translational Sciences Institute |
| cDNA | Complimentary Deoxyribonucleic Acid |
| ChAT | Choline O-Acetyltransferase |
| CNS | Central Nervous System |
| CRE | Cre Recombinase |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| DAPT | γ-secretase inhibitor; (2S)-N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine 1,1-dimethylethyl ester |
| DLX1/2 | Distal-Less Homeobox 1 |
| DNA | Deoxyribonucleic Acid |
| E | Embryonic Day |
| EBF3 | EBF Transcription Factor 3 |
| EGF | Epidermal Growth Factor |
| EIU | Endotoxin Induced Uveitis |
| ERG | Electroretinogram |
| FDA | Food Drug Administration |
| FGF | Fibroblast Growth Factor |
| FOXN4 | Forkhead Box N4 |
| GAD65 | Glutamate Decarboxylase 65 |
| GAD67 | Glutamate Decarboxylase 67 |
| GCL | Ganglion Cell Layer |
| GLYT1 | Glycine Transporter Type 1 |
| HES1 | Hes Family BHLH Transcription Factor 1 |
| HES5 | Hes Family BHLH Transcription Factor 2 |
| IHC | Immunohistochemistry |
| ILM | Inner Limiting Membrane |
| INL | Inner Nuclear Layer |
| IPL | Inner Plexiform layer |
| iPSC | Induced Pluripotent Stem Cells |
| ISLET1/2 | Insulin Gene Enhancer Protein ISL-1 |
| KB | Kilobase |
| LHX2 | LIM Homeobox 2 |
| nGnG | Non-Glycinergic Non-GABAergic |

| | |
|---|---|
| NRL | Neural Retina Leucine Zipper |
| OLM | Outer Limiting Membrane |
| ONL | Outer Nuclear Layer |
| OPL | Outer Plexiform Layer |
| OTC | Optical Coherence Tomography |
| OTX2 | Orthodenticle Homeobox 2 |
| P | Postnatal Day |
| PAX6 | Paired Box 6 |
| PEA3 | (ETV4) ETS Variant Transcription Factor 4 |
| PRDM | PR/Set Domain Protein |
| PRDM1 | PR Domain Zinc Finger Protein 1 |
| PRDM13 | PR Domain Zinc Finger Protein 13 |
| PROX1 | Prospero Homeobox 1 |
| PTF1a | Pancreas Associated Transcription Factor 1a |
| RNA | Ribonucleic Acid |
| RORB | RAR Related Orphan Receptor B |
| RPE | Retinal Pigmented Epithelium |
| RPE65 | Retinal Pigmented Epithelium-specific 65-kDa protein |
| SAC | Starburst Amacrine Cell |
| scRNAseq | Single-Cell RNA Sequencing |
| SHH | Sonic Hedgehog |
| SOX2 | SRY-Box Transcription Factor 2 |
| SVA | Scalar Visual Assessment |
| TH | Tyrosine Hydroxylase |
| vGLUT3 | Vesicular glutamate transporter 3 |
| VSX2 | Visual Systems Homeobox 2 |
| WNT | Wingless Int-1 |
| WT | Wild-Type |

# CHAPTER I

# INTRODUCTION

## Retinal Disease and Neural Degeneration Cause Permanent Blindness

The retina is a thin layer of neural tissue at the back of the eye and is the foundational sensory processing center for visual information. This tissue is less than 0.5mm thick in humans and is composed of multiple layers of neurons that perform a variety of functions. Rod and cone photoreceptors convert photons into neural signals. Various interneurons including bipolar, horizontal, and amacrine cells conduct complex calculations on the raw data from the photoreceptors. Ganglion cells compute the processed inputs and pass their signals out through the optic nerve to the brain where further signal decoding and encoding is performed.

The retina is supported by a group of cells called Müller glia, which provide support to the high-functioning neurons by maintaining structure and mediating neurotransmitter maintenance. Underlying the retina is the retinal pigmented epithelium (RPE). This supporting structure plays a critical role in the visual cycle by retinoid recycling, providing ion buffering, light absorption, controlling oxidative stress, and mediating immune privilege within the retina.

Dysfunction in any part of the retinal or associated RPE tissue can cause loss of visual function. This can result from genetic disorders, like retinitis pigmentosa, diseases of aging like macular degeneration, other disease states like diabetic retinopathy, various forms of cancer, autoimmune diseases like uveitis, or traumatic events like retinal detachment. Untreated, nearly all forms of retinal degeneration will lead to permanent blindness. Indeed, nearly 90% of legal blindness in the United States is caused by degradation of the retina or its supporting tissue[1].

Blindness due to loss of retinal neurons is permanent and affects more than 10 million Americans[2]. This is because once retinal neurons begin to die, they are not regenerated within

mammalians systems. This has led to significant work attempting to understand the foundations of developmental and degenerative diseases of the retina. A critical prerequisite to producing therapies that alleviate human disease is answering questions about the underlying biological mechanisms. For example, what are the mechanisms of retinal degradation in various disease states and how can they be stopped? How do we created regenerative therapies that restore vision to those who have already suffered significant neural loss? Pursuing either of these lines of inquiry requires significant advancement in our understanding of the mechanisms governing how neurons of the retina differentiate into unique cell types, laminate appropriately, and develop into functional neural circuits.

Here I explore the role of a variety of genes in retinal development, several of which have been associated with disorders of the developing eye or congenital degenerative diseases. Throughout this introduction I will introduce the broad evolutionary patterns of visual development, the structure and diversity within a retina, and the major forces that contribute to the development of retinal neuron formation in vertebrates including various transcription factors and DNA enhancer regions. I then specifically explore how three key transcription factors *Prdm1*, *Prdm13,* and *Vsx2*, contribute to the development of the murine retina.

**Evolution of the Vertebrate Eye**

The detection of photons and their conversion into behaviorally relevant information may be one of the oldest sensory systems in biology. There are numerous examples of single celled organisms responding to light to migrate, change their metabolic functions, or modify cell cycle processes[3,4]. The first proto-eye likely evolved around 600 million years ago during the Cambrian explosion[4]. Visual systems across the animal kingdom are incredibly diverse and some

of the most complex eyes in existence are found in non-vertebrate species. The eyes of the mantis shrimp have twelve or more unique kinds of photoreceptors that see from deep ultraviolet to far-red with incredible spatiotemporal clarity[5].

The eye is a functional visual organ that is not merely a light detector. All eyes involve some form of lens that focuses light onto one or more photoreceptors which then pass light onto the rest of the central nervous system for further processing[6]. They can be divided into compound eyes and non-compound eyes[7]. Compound eyes, like those found in the mantis shrimp, have thousands of individual photo-detection units called ommatidia. Each one functions as a small eye with a visual focusing lens and photoreceptors. Compound eyes are further divided into apposition eyes, in which each lens focuses onto a photoreceptor with no gap between, and superposition eyes in which there is a gap between the lens and the rhabdom, where photoreception takes place. Superposition eyes create poor visual acuity, but provide incredible light sensitivity[7].

Non-compound eyes, sometimes called simple eyes, have evolved numerous times throughout evolution through unique developmental pathways (Fig 1.1)[8,9]. The humblest version are called pit eyes, which are a simple sheet of photoreceptors set in a tissue pit, and are considered by many a proto-eye or visual detector rather than a camera eye[6]. Spherical lens eyes possess a lens with some form of refractive abilities to focus light onto the visual tissue. This allows for improved visual clarity, as well as increased aperture diameter, improving light sensitivity. Simple spherical lens eyes exist across the animal kingdom from insects and arachnids to fish and mammals. In rare cases, animals have evolved multi-lens eyes, that function more like a camera lens where a series of unique lens tissues perform separate functions to focus the light properly[6].

Vertebrate eyes themselves have enormous diversity, however many share a common structure and I will focus on these species for the remainder of this work. Broadly, light enters the eye through an aperture called an iris which controls light access. Light is then passed through a clear crystalline structure called a lens which focuses light onto the back of the eye (Fig 1.2A). At the back of the eye the light is encoded into neural information by the thin multilayered neural tissue called a retina. The retina itself sits on top of a supporting retinal pigment epithelium (RPE). Between the lens and the retina is a space filled with a clear gelatinous liquid called the vitreous. Surrounding the eye is the sclera at the back and sides and cornea on the outward facing portion of the tissue. Small muscles in the ciliary body may be used to stretch the lens and change focus. There are additional supporting structures in many systems, like muscles that move the entire eye, tear ducts that provide lubricant and protectant to the sclera, and systems for correctly maintaining eye pressure by draining excess fluid[10].

In mammals, the retina may be the highest energy demanding tissue in the body when scaled to size[11]. To maintain this demand, the retina is an incredibly vascularized tissue. But the vasculature itself is not sufficient to control the energy needs and waste flow of the retina[10]. The underlying RPE plays a critical role in the cycling of visual metabolites and breakdown in the structure, function, or metabolic function of the RPE results in retinal dysfunction[12]. Each portion of the eye is essential to processing information effectively. However, the single most functionally, structurally, and cellularly complex tissue of the eye is the retina.

**Structure and Function of the Retina**

The retina is a laminated tissue and is positioned “upside-down” to the flow of light. That is to say, photons pass through the layers of tissue and impact the photoreceptors located at the

back of the eye. The encoded neural signal is then transduced back through the tissue toward the front of the eye, out the optic nerve, and back to the brain (Fig 1.2A-C). The basic signal path is photoreceptors to bipolar cell to ganglion cell. Horizontal and amacrine cells are involved in modifying signals for circuit-specific functions. Ganglion cells are projection neurons that carry signals to the brain. Müller glia and the RPE are supporting cells[13]. There are ten major layers of the retina passing from the vitreous toward the back of the eye. First is an inner limiting membrane (ILM) which separates the vitreous from the retina and then the nerve fiber layer (NFL) where ganglion cell axons pass on their way to the optic nerve (Fig 1.2B). Next is the ganglion cell layer (GCL) which contains ganglion cell nuclei and some amacrine cells. These project dendrites into the inner plexiform layer (IPL), which contains bipolar cell axons, amacrine dendritic arbors, and ganglion cell dendrites in a laminated structure. The IPL itself is divided into at least five major sub-lamina in mammals and plays a critical role in signal processing[14]. The majority of amacrine cells, all bipolar cells, and horizontal cells have their nuclei in the inner nuclear layer (INL)[14,15]. Bipolar cells and horizontals have projections into the outer plexiform layer (OPL) where signals from photoreceptors are passed to bipolar cells[16]. The photoreceptor nuclei are located in the outer nuclear layer (ONL) and they project their outer segments (OS) toward the back of the eye where they are surrounded by cells from the retinal pigmented epithelium (RPE)[17]. Between photoreceptors' outer segment and nuclei is a tissue barrier called the outer limiting membrane (OLM). The ten lamina of the retina are composed of seven major cell types, each of which plays a unique role in processing visual signals.

Photoreceptors are divided into two classes: rods and cones. Rods are sensitive to low light signals and their ability to detect visual input is driven by the protein Rhodopsin. Rods are so sensitive that a single photon of light can generate a cascade leading to a neural signal (Fig

1.2C)[18]. Cones have two primary functions: First, they detect color information and second, they are involved in high acuity vision in many species. Color vision is generated by cones expressing unique opsins that are sensitive to different wavelengths of light. A given cone will only express one kind of opsin in higher vertebrates, and thus represents a single color-channel. In humans, there are three primary opsins that are most sensitive to red, green, or blue light respectively[19]. Cones are also involved in high acuity vision in many species, including humans. They require two to three orders of magnitude more photons to activate their photo cascade than rods[20]. This means that they are only functional in high-light settings which explains their role in diurnal vision. The acuity itself is a function of the circuitry. Rods, particularly within the peripheral retina, tend to cluster multiple inputs onto a single bipolar cell. Functionally, this means the spatial input of this signal is imprecise but highly photosensitive. In general, fewer cones synapse onto a single bipolar cell compared to rods. But in the central retina at the macula, and particularly in the fovea of humans, cones and bipolars can form circuits as tightly coupled as 1:1[20]. While not all mammals have the level of central acuity of the human fovea, the general pattern from peripheral to central retina is consistent. This allows the output from the circuit to provide high spatial-temporal precision.

Bipolar interneurons play a critical role within the retina. They pass all visual signals from sensory neurons to projection neurons. However, they are not simply a non-processing intermediate. As mentioned above, they may take inputs from 15-50 rods all the way down to a single cone. In mice, there are up to 15 unique bipolar cell subtypes depending on how you quantify morphology and molecular markers (Fig 1.2C, E)[21–24]. Some have proposed that only one kind of rod bipolar exists, called rod bipolar cells. Others have suggested based on molecular markers that there are two rod bipolar cells subtypes, rod bipolar 1 and rod bipolar 2 cells[23,24].

Whether there are one or two kinds of rod bipolar cells, the remainder of the bipolar diversity in mammals exists within cone bipolars. There are two broad classifications of cone bipolars: ON or OFF. ON-bipolar cells send signals when their associated cones respond to light. OFF-bipolars send their signals when the cones do not respond to light. This ability of cone bipolars to send signals through a positive as well as negative information channel has significant implications for downstream processing of information and the sorting of visual information into circuits. ON or OFF signals are sent through unique information channels that are spatially separated within the retina. I previously mentioned that the IPL is a laminated structure. It has distinct layers where dendrites of amacrines and ganglion cells synapse with the axons of bipolar cells. These layers are numbered one through five with layer one being closest to INL and five closests to the GLC. This lamination is integral to signal processing in ON and OFF bipolar cells. ON bipolar cells project their axons to layers four and five of the IPL, whereas OFF bipolars project to layers one and two. Rod bipolars also generally project their axons to layers four and five of the IPL. Some bipolar subtypes project to multiple layers as well as layer three where ON and OFF projections are both processed[24–26].

Integrating the signal passed from photoreceptors to bipolar cells are a group of interneurons called horizontal cells. These cells have their nuclei in the INL, but project dendritic processes into the OPL (Fig 1.2B-C). Horizontal cells are networked together through connexin57 gap junctions that allow signals to be transported and diffused across a network. They form unique triad synapses at the junction between photoreceptors and bipolar cells[27–29]. This network has been shown to feed back onto photoreceptors as well as feed forward onto bipolar cells depending on the nature of the signal. The signal transduction between photoreceptors, bipolars, and horizontals is reciprocal. Horizontal cells receive glutamatergic

input from photoreceptors and can then send negative or positive signals back onto the photoreceptors or onto their targeted bipolar cells[30–34]. The complexity of this network is poorly understood. However, if horizontal cells are deleted during development, the outer nuclear layer fails to pattern perfectly, and the loss of so-called triad synapses effects retinal function[35–37]. When horizontal cells were knocked out in a mature retina, the signals to ON-bipolar cells were impaired while OFF-bipolar signal were less effected, but the net retinal output appeared to largely compensate for this loss[37]. It is likely that horizontal cells play roles in specific neural networks within the retina, though their function and subtypes are not well understood at this time.

Amacrine cells primarily function as inhibitory interneurons that alter and control the signal transduction between bipolar cells and ganglion cells (Fig 1.2B-C, E). They make up approximately 8% of the mouse retina but there are over 30 unique subtypes as defined by morphology alone[38,39]. There are three primary categories of amacrines by molecular markers: GABAergic amacrines that express GAD65/67 and are about 43% of amacrines, glycinergic amacrines that express GlyT1 but make up about 43%, and finally non-glycinergic, non-GABAergic (nGnG) make up the remaining 15%[40]. However, each of these broad categories can project to all five laminae of the IPL. Additionally, there are numerous other molecular markers that span multiple morphological subtypes, including transcription factors like AP2a, EBF3, and BHLHB5, calcium binding proteins like calretinin and calbindin, and neurotransmission proteins including ChAT, TH, and vGlut3[30,40–45]. The morphological and molecular diversity does not always align, with some types of morphological amacrines expressing divergent molecular expression patterns[30,40–45]. Taken together, the identification and classification of amacrine cellular subtypes within the retina is a critical undertaking and may be considered a preamble to

electrophysiological studies of circuit function. I discuss our efforts exploring amacrine subtype development and identification in Chapter II of this work.

Retinal ganglion cells are the projection neurons of the retina (Fig 1.2B-C, E). They receive inputs from bipolar cells and target their axons to the brain, but they represent a functionally diverse group of neurons that are tiled across the retina. There are ganglion cells that send signals based on local motion, directional motion, as well as direct illumination[46–49]. Within the mouse, ganglion cells have been classified into at least 12 unique functional groups based on responses to light, but morphological studies show up to 20 unique subtypes (Fig 1.2F)[50–54]. It is unclear if morphology perfectly overlaps with function and how retinal ganglion cell subtypes are best identified[55]. While the responses to light and the morphology are one consideration, axon targeting is another. In mice, ganglion cells are known to target over 10 major areas of the brain and over 40 unique cellular population targets (Fig 1.2F)[56,57]. Further studies based physiological cell signals have shown there are up to 49 subtypes of ganglion cells including fast and slow firing ON and OFF signals as well as intermediate signals, and immense diversity within each subcategory[55]. Taken together, this suggests that information conveyed from the retina to the brain is massively sorted into unique processing channels. It also highlights the fact that the processing of visual information that occurs via retinal interneurons is likely integral to the proper function of the mammalian visual system.

The final major cell type within the retina is the Müller glia. These supporting cells extend the width of the retina and are laid out in a mosaic pattern with extended radiating processes touching the surrounding cells. Müller glia are physically integrated into retinal structure, surrounding blood vessels and the extracellular clefts and touching every neuron in the retina[58]. Müller glia play a significant role in regulating metabolism, controlling neural functions,

and are involved in every form of retinal degeneration[58–60]. Additionally, Müller glia are important to light scattering in the retinal tissue, maintenance of structural integrity, and are involved in nearly every biochemical cycle of the retina[58]. Not only are Müller glia involved in almost every function of the retina, in non-mammalian species, they are the source of neural regeneration in response to damage[61–63]. Unlike other types of retinal cells, there do not appear to be subtypes of Müller glia. Each cell is arranged in a mosaic columnar fashion across the retina and does not overlap much, if at all, with the field of other Müller glia. They arrange themselves during development so that all retinal neurons, including photoreceptors, interneurons, and project neurons, are contacted by a Müller glia[59].

**Functional Neural Circuits of the Retina**

The seven major cell types and dozens of subtypes of neurons within the retina work in circuits to transform information about the presence of photons into meaningful computational visual information. As discussed, this results in ganglion cells projecting to 10 areas of the brain with over 40 targets receiving unique computationally relevant information (Fig 1.2F). Many of the circuits driving this output are poorly understood at this time because of barriers to studying them, like limited unique markers of interneuron subtypes. A thorough review of known retinal circuits is beyond the scope of this work (see Baden et al., 2018 to start)[64]. However, there are well studied circuits that are relevant to our understanding of retinal development, patterning, and fate specification.

*Physiological Patterns of Retinal Neural Circuits*

A central feature of retinal physiology is that two sets of parallel neurons often respond in an opposite fashion to the same input signal. This creates parallel lanes of opposing signaling which broadly functions to reduce signaling noise and refine net circuit computational output[64]. Photoreceptors hyperpolarize in response to the presence of photons meaning that their glutamatergic output decreases when light is present, however, only one in three bipolar cells respond similarly[64]. Bipolar cells can process the same glutamatergic input with various levels or even an opposite response[65–67]. Mammalian OFF bipolar cells have ionotropic glutamate receptors and fire most when photoreceptors are *not* responding to light. ON bipolar cells express metabotropic glutamate receptors and their signaling increases in response to *decreased* photoreceptor output, when light is highest[64,66,68].

These ON and OFF or dark and light signaling pathways are integrated in a complex fashion in the IPL based on the specific layer as previously mentioned. Amacrine cells may further mediate the signal from ON or OFF bipolar cells and incorporate the information into numerous circuits with ganglion cells. Some amacrines provide glycinergic inhibition to retinal ganglion cells while other provide glutamatergic excitation. By segregating dendritic arbors to different regions of the IPL, some amacrines perform both of these functions simultaneously[69]. In the context of a neural circuit, an excitatory signal may at times be inhibitory to the function of the circuit by creating excess noise or increasing a post-depolarization refractory period[70]. An inhibitory signal may actually provide increased probability of signaling by providing input synchrony[69,71,72]. The combination of ON and OFF currents with complex excitatory and inhibitory interactions create a multitude of potential retinal circuits. I previously mentioned the massive morphological and histological variance among amacrine cells and ganglion cell output.

It is likely that this emmense diversity integrates into fascinating, yet to be studied, neural circuits.

*Center-Surround Receptive Fields*

One of the challenges in identifying a specific retinal circuit is there needs to be a testable visual input that results in consistent output from a specific set of neurons. Once you have identified a physiologically testable circuit, it is much easier to begin dissecting the roles of various interneurons on regulating the flow of information. One way the retina is known to leverage the ON and OFF currents is through the use of center-surround receptive fields (Fig 1.2D).

There are two kinds of center-surround receptive fields. Imagine a light spot, surrounded by dark, or a dark spot surrounded by light. These two overlapping dots are center (dark or light) and surround (the opposite). There are bipolar cells that respond specifically to center-surround dots within their field of the retina. A given center-surround bipolar cell may hyperpolarize in response to a 1000µm laser dot with a dark circle enclosed, but depolarize in response to a 200µm laser dot surrounded by a dark sphere in primate retinas (Fig 1.2D)[73]. Extensive additional studies have shown this mechanism to be critical to retinal ganglion cell processing across the retina and subtypes of ganglion cells have been identified that respond better as the center grows larger or grows smaller suggesting that one role for these circuits may be identifying when an object is approaching or receeding[73–75]. This fundamental ability for neurons within a region of the retina to process ON and OFF patterns can be combined into even more complex calculations, for example, directionally selective circuits.

*Starburst Amacrine Cells and Directional Selectivity*

Directional selective neurons were first recorded in the visual cortex of cats and the Nobel Prize winning work was published in 1959[76]. This work showed that certain neurons responded positively to an object moving in one direction across visual field and did not respond to the same object moving in other directions. Unique neurons are tuned to different spatial orientations. While these recordings were originally made in the cortex, evidence, starting with work in rabbits, showed that certain retinal ganglion cells exhibited this same directionally selective property[77]. Years of evidence eventually revealed that there are direct lines of communication from directionally selective ganglion cells, through the lateral geniculate nucleus, where information was further refined and sent directly on to the directionally selective neurons in the superficial layers of the visual cortex[78].

For directionally selective information to be computed, both spatial and temporal information needs to be encoded and computed simultaneously within a neuron. A bright/dark difference will appear, passing over hundreds to millions of photoreceptors that send signals in sequence in response to the photons. Yet only a subset of photoreceptors are involved in directional selectivity and within a given region of the retina. There are directionally selective circuits tuned to each direction. How then are these complex computations computed within the retina?

At the heart of the directionally selective circuit is the Starburst Amacrine Cell, commonly called SACs. These unique amacrines are both cholinergic and GABAergic. SACs have their nuclei in either the INL or the GCL. They have large diffuse arbors of dendrites with processes in either ON or OFF layers of the IPL[79]. The function of SACs is incredibly complex and not fully understood (for a thorough review see Mauss et al 2017)[79]. However, the basic

mechanism is that when stimulated SACs produce inhibitory signals in their null direction and excitatory signals in the positive direction. Significant debate about the species-specific mechanisms exists. However, what is clear is that precise radial and laminar wiring of their dendrites within the IPL is absolutely critical to the function of directionally selective neurons[79].

*Neural Circuits and the Developing Retina*

These two circuits encapsulate many of the challenges faced when attempting to understand retinal development. For directionally selective ganglion cells of center-surround circuits to function properly, the retina must give rise to all the major cell types. These neurons then need to migrate to the correct layer and laminate properly. But more than just types are needed, specific subtypes of bipolars, amacrines, and ganglion cells must form meaningful circuits by interconnecting their axons and dendritic arbors across the retina in a mosaic pattern, accurately express a suite a cell-cell signaling molecules and finally adjust and tune the circuit so that there is directional selectivity in every direction across the entire retina that functions in both high contrast and low contrast situations. In the case of directionally selective circuits, the ganglion cells must correctly target directionally selective interneurons in the lateral geniculate nucleus and convey information in a precise spatiotemporal way. This involves a subset of photoreceptors, one subtype of amacrine cell, a bipolar population, and one type of ganglion cell and thus represents only a fraction of information the retina is processing and encoding. Understanding the complex cascade of intrinsic developmental changes and extrinsic signaling mechanisms involved in developing such a complex circuit represents and monumental undertaking, yet it is required to uncover a complete picture of retinal development. In chapter II, I attempt to elucidate molecular markers for a subset of amacrine interneurons which likely

function in similarly complex yet to be studied retinal circuits. Loss of these neurons dysregulates the proper lamination of the IPL and likely results in meaningful changes in the function of specific retinal circuits.

**Patterns and Mechanisms of Neural Development**

Development of the retina involves exit from the cell cycle (*e.g.* birthdate) of seven major cell types, specification into subtypes, maturation, correct migration of nuclei, lamination of dendrites and axons, and ultimately the formation of neural circuits (Fig 1.4)[80]. These processes take variable lengths of time depending on the species. The retina itself forms a mosaic tissue with circuits spread out in predictable yet stochastic patterns, like those observed in directionally selective circuits[80]. The general pattern in vertebrates is that there is more high acuity cone-rich tissue in the central retina, and more low light sensitive rod-rich tissue at the periphery, although across the whole retina rods outnumber cones 35:1 in mice[80,81]. The specifics of this can vary significantly by species from nocturnal creatures like mice and rats, to diurnal ones like humans. Yet, for cells to develop in any of these systems they must exit the cell cycle, make a fate choice, and mature into a functional neural circuit. Two dominant, non-exclusive models are used to describe the broad patterns of retinal development.

*Intrinsic and Stochastic Development Models*

Foundational studies in retinal development showed that all the seven major cell types descend from a common progenitor pool[82,83]. A key finding of these and many other studies was that a given progenitor could, but does not always, give rise to each of the seven major cell types[84]. All cell types are not born at the same time. Certain types tend to be born early and other

cell types tend to be born late. At any given time in retinal development, progenitors appear to give rise to only a few types at a time[81,83,85,86]. These discoveries, along with many others, led to the *competence model* of retinal development. In this model, retinal progenitors pass through progressive states in which they are competent (able) to give rise to certain fates, but not others (Fig 1.3A-B)[84]. An early progenitor may decide between a ganglion cell and a cone, while a later progenitor may decide between a bipolar cell and a rod photoreceptor. The model is considered progressive, because late progenitors will not give rise to early cell types, and early progenitors generally do not give rise to late cell types, even when transplanted into different molecular contexts[82–84]. However, a given progenitor when fate tracked may first give rise to early cell fates, then middle cell fates, and finally late cell fates[82–84]. Indeed, numerous studies showed that cell fates appear to arise from specific competence windows, which change across developmental time (Fig 1.3A-B)[87–89]. Additionally, these windows of development do not appear to be regulated by extrinsic signaling but by changes in the internal environment of the progenitor[90–94].

The competence model states that retinal progenitors pass through progressive phases regulated by their intrinsic environment where they are competent to give rise to certain cells types but not others (Fig 1.3A-B). This model successfully defines the many of the phenomenon observed in lower vertebrates retinal development, like *Xenopus laevis*[95]. However, it does not fully explain retinal development in higher vertebrates like mice, rats, and humans. Why do some progenitors produce large lineages while others do not? Why do some progenitors give rise to predominantly early or predominantly late fates while others produce fates across all of developmental time? Why do some lineages occasionally produce late cell types before early ones[96]? A *stochastic model* of retinal development implies that there is a degree of

unpredictability at each decision point for a given progenitor cell. If a stochastic model of development were true, then patterns like whether a progenitor gives rise to two progenitors, a progenitor and a neuron, or two neurons would be unpredictable at each specific choice point, but on average fates would match the total cell number needed in the retina (Fig 1.3C-F). An additional aspect of a stochastic model is that it would predict the probability of a progenitor giving rise to a specific type of cell as directly correlated with the final percentage of that cell in the retina (Fig 1.3C-F). For example, rods make up ~78% of the mouse retina, and thus a dividing progenitor would have an 78% chance of generating a rod and this probability would increase toward the end of retinal development as other early born cell types, like ganglion cells and cones, were no longer generated[96,97]. The stochastic model of development is far better at explaining specific phenomenon in higher vertebrate retinal develop but may not be enough to explain all developmental patterns.

Evidence suggests that both the intrinsic model of development and the stochastic model of development may be at play within mammalian systems and these two ways of approaching retinal development are non-exclusive (Fig 1.3). However, mechanisms by which either of these models' function are not fully understood at this time. Indeed, what defines a progenitor competence state and how a decision is made from that competence window or why a given progeny will permanently exit the cell cycle while another remains in progenitor form are not well understood. Within this work I specifically explore the regulation of amacrine, bipolar, and rod cell fate determination at multiple competence windows. I am also able to disrupt a competence state and dysregulate cell fate leading to an increased understanding of the role of transcription factors in regulating retinal fates and competence.

*Mechanisms Underlying Retinal Developmental Models*

The choice for a cell to remain a progenitor or exit the cell cycle and become a specific neuron is actively regulated by a variety of transcription factors (Fig 1.4C)[98]. During development, all cells go through a series of changes in gene expression and thus pass through different intrinsic cellular environments. The fate and maturation of the cell depends on transcription factors binding to specific regions of DNA and activating or repressing genes. The ability of an individual transcription factor to bind a given region of DNA depends on the ultrastructure of the chromatin. Chromatin can be open with reading frames readily accessible for transcription, or tightly wound around nucleosomes that prohibit access from the machinery necessary to transcribe it into mRNA. Regulators, like transcription factors, can cause the DNA to be more tightly wound and block transcription or less tightly wound allowing for transcription. However, unwound DNA where a specific gene exists within an open reading frame does not guarantee that the gene will be transcribed. Transcriptional regulators also affect the ability of transcriptional machinery to bind a specific gene. Thus, transcription factors are involved transcription of individual genes as well as epigenetic control of a cell's state.

These developmental paradigms are iterative. Tightly wound or open chromatin responds to the presences of specific transcriptional regulators, which are also able to drive production of different transcription factors. These new factors will then further alter the genes that are being transcribed, which in turn may wind specific regions of chromatin tight while other previously closed regions are now accessible for transcription. Understanding the state of a given cell, especially a developing one, is not as simple as knowing what mRNA or proteins are present. There may be robust production of an mRNA at the same time the gene is never translated into

protein because of post-translational modification. These complex processes make unpacking the specific means by which a cell develops extremely challenging.

In this section I attempt to provide a broad picture of the identified roles of some transcriptional regulators of retinal development. The complexity of cell fate regulation and the multifaceted intrinsic landscape of a developing cell make the generation of a complete picture challenging. However, to identify the role of a given transcription factor in development there are some steps that can develop clarity. First, we can ask what happens to cell fate when a transcription factor is lost? Second, we can ask how cell fate changes if there is excess of a specific transcription factor during different developmental time frames? We can look for places that the factor binds and attempt to understand if it is acting as a driver of cell fate or through inhibition of gene expression. We may also ask questions about what regulates the expression of a given transcription factor as well as what genes that factor may be acting on. Putting these pieces together into a holistic picture of cell fate regulation, even within a limited temporal window, remains an incredible challenge. The dominant evidence in the field suggests that the majority of retinal cell fates and the shift in cell competence is largely regulated by intrinsic gene regulation. Thus, to advance our understanding of retinal development, we must refine our understanding of intrinsic transcriptional regulation of cell fate.

*The Roles of Transcription Factors in Developing Neurons*

During development, a common pool of retinal progenitor cells give rise to all major subtypes, including rod and cone photoreceptors, bipolar, amacrine, and horizontal interneurons, ganglion projection neurons, and Müller glia[82]. As previously stated, a single retinal progenitor is capable of generating all the major cell types. However, the generation of this diversity does not

occur simultaneously, rather cell types are born in a stereotyped overlapping fashion with some cells like cones and ganglion cells born early, and others like bipolars are born late[99]. If the intrinsic progressive competence model is correct, how does it function[84,99,100]?

Transcription factors are proteins that are involved in gene expression. There are a wide variety of transcription factors, but most function by binding a specific consensus sequence of DNA that is often 4-8 base pairs (bp) long, though this length can vary. Once bound to a consensus sequence, transcription factors will either work as an activator, promoting the binding of RNA polymerase to drive expression, or as a repressor, blocking the same suite of proteins[84]. Many transcription factors can act as activators or repressors, depending on the specific cellular context and even the site they are binding. They can do this by directly stabilizing or blocking the action of RNA polymerase, recruiting coactivators or corepressors, or through changing the chromatin accessibility by driving acetylation or deacetylation of histones[101,102]. Often a given transcription factor may be playing multiple roles in a cell at the same time and distinguishing their function(s) can be challenging. Added to this complexity is the fact that not all transcription factors function in the same way across vertebrate development. To limit the background to the model species I use, throughout the remainder of this section, unless specified, all studies refer to transcriptionally regulated developmental paradigms in mice. Next, I discuss several major classes of transcription factor and highlight the diverse functions in murine development.

*bHLH Transcription Factors in the Developing Retina*

Basic Helix-Loop-Helix (bHLH) transcription factors function throughout retinal development and play critical roles in patterning of the eye and driving specific cell fates[103]. The name basic helix-loop-helix refers to an approximately 60 amino acid protein structural motif

where two alpha-helices are connected via a loop. They are formed from dimeric helix domains that may not be the same size and diversity in region gives bHLH proteins a massive range of functions. However, they all bind to a specific consensus sequence referred to as an E-Box, though the nature of this sequence can vary depending on the specific protein[104]. Not only can bHLHs form homodimers, but they have been shown to also form heterodimers, leading to an immense array of functions that a given bHLH can perform, even within a single cellular context[104]. The dimer controls where the bHLH binds and the strength of the bond, however, the remainder of the protein can have an array of functions in transcriptional regulation including driving and inhibiting transcription.

BHLHs have been implicated in numerous cell-fate determining roles in the retina. *Mash1* (*Ascl1*) deletion causes a delay in bipolar cell differentiation and *Math3* (*Neurod4*) seems to promote rod fates, but simultaneous deletion of both genes disrupts bipolar cell formation[105,106]. Almost all amacrine and horizontal interneurons require the expression of the bHLH *Ptf1a* and I discuss this gene in more detail later[107,108]. These genes are critical to controlling cell type formation, but bHLHs may also govern the development of specific subtypes, for example *Bhlhb5*, mentioned above regulating bipolar cells, is also required for GABAergic amacrines, and in its absence they fate shift to glycinergic amacrines[42]. While photoreceptor fates are predominately controlled by homeobox domain and zinc-finger transcription factors, some bHLHs including *NeuroD, Mash1* have been implicated in contributing to their correct formation[98,109].

*PRDM Zinc-Finger Transcription Factors in the Developing Retina*

The *Prdm* family of genes are the source of significant interest across multiple disciplines. The proteins are characterized by a PR domain at the N-terminus and a variable number of so-called zinc-finger binding motifs in the middle[110–112]. PRDMs may function in unique ways depending on the cofactors present at the time[113–116]. While there is some evolution, the genes have been shown to be fairly conserved across invertebrate and vertebrate species[117].

PRDMs were independently discovered in various systems and have been implicated in extremely diverse roles including involvement in B cell lymphoma, tumor suppression, leukemia, breast cancer, and many other cancers[112,118–120]. PRDMs work through a variety of mechanisms to control DNA transcription. When bound to a consensus sequence they may recruit histone acetylases, acetyltransferases, or methyltransferases as well as factors associated with direct transcriptional activation[121]. These actions may be brought about by direction of from the PR domain or through recruiting associated epigenetic enzymes[122,123].

Within developing systems, *Prdm1* has been implicated in numerous roles including, but not limited to enterocytes of developing milk-dependent mice, B and T cell function, primordial germ cell development, patterning of the heart, and proper skin development[112,124–126]. Of significant interest to us is the critical role PRDM1 plays in stabilizing photoreceptor identity within the retina though its mode of action is not fully understood[127,128]. *Prdm13* is known to function in the human retina in North Carolina Macular Dystrophy[129]. I explore the roles of both PRDM1 and PRDM13 in more detail later.

In summary, PRDMs are capable of playing an enormous variety of roles within a developing system, possibly executing multiple actions at the same time. A single PRDM could theoretically inhibit one segment of DNA, drive histone acetylation on another portion of the

genome, and attract cofactors that perform deacetylation on still another set of histones all at the same time in the same cell. The ability of these transcription factors to interact with numerous co-factors to perform various complex tasks creates a significant challenge in understanding their role in the developing retina. However, when broadly generalized, PRDMs act to inhibit a specific cellular function or cell fate, not necessarily to instruct a fate choice in many cellular contexts[97,112,127,130,131].

*Homeobox Domain Transcription Factors in the Developing Retina*

Homeobox transcription factors contain a common 60 amino acid DNA binding sequence called a homeobox which canonically binds TAAT/ATTA motifs[132]. However, homeobox domain transcription factors can bind a variety of sequences and the presence of various additional domains within the protein may have a significant impact on the precise targeting[133]. Of all transcription factor families, homeobox domains may play the single largest role in patterning, shifting progenitor competence, and driving specific cell fates in the retina. Numerous homeobox domain transcription factors have been implemented in so many functions that I will not review them all here (see Holland 2013 and Zagozewski et al, 2014) (Fig 1.4)[134].

*Otx2* is at the center of a major gene regulatory network studied within this work and discussed in significant detail elsewhere. To summarize, early in mouse development OTX2 is a critical regulator of early neural development and patterning of head structures[135]. It is turned on again around embryonic day (E) 11.5 in the RPE and developing neural progenitor cells and its expression increases across neural development and is necessary for proper formation of the RPE[136–138]. Loss of *Otx2* results in a complete loss of bipolar and photoreceptor fates and a

switch to amacrine fates, making *Otx2* a critical regulator of cell competence in retinal progenitor cells[138,139].

Like *Otx2*, *Pax6* is expressed in the RPE and the neural epithelium of the developing retina[140]. Interestingly, in the absence of *Pax6*, retinal progenitors lose their competence and produce only amacrines, yet *Pax6* is required in mature amacrine cells and remains on in them into adulthood[141]. Not only is *Pax6* involved in progenitors and amacrines, but it plays divergent roles within the central and peripheral retina. Central retinal progenitors do not require PAX6 to specify their fates, but peripheral progenitors do[142]. The reasons for this differential regulation of cell fate are not understood.

*Vsx2* is a complex homeodomain protein involved in numerous functions across several systems[143–145]. Like *Pax6* and *Otx2*, *Vsx2* appears to have multiple roles across retinal development. It is turned on as early as E9.5 and in its absence only a small neural retina forms[146–148]. Late in retinal development, progenitor VSX2 expression decreases, however as I discuss in more detail later, it is necessary for bipolar formation and in its absence bipolar cells are not formed[148–150].

Many transcription factors function like *Pax6, Otx2*, and *Vsx2,* with one role early and another role late, and at times even a third or fourth temporally linked role in retinal development and maturation. Since transcription factors have a limited and specific binding sequence there are two plausible explanations for why they drive different cell responses at different times. The associated binding proteins may change within the retina. In this scenario, a transcription factor that used to bind and activator now binds a repressor and thus reverses its function. Perhaps more common is that the epigenetic state shifts as cells progress through competence states. These changes in accessible chromatin allow transcription factors to bind to different enhancers or

promoters and thereby the same transcriptional regulator can have a novel or even contradictory function as a cell changes its fate. A perfect example of this mentioned above is that deletion of *Pax6* early results in excess amacrines, yet *Pax6* is on in all mature amacrine cells[134]. This suggests that PAX6 prevents amacrine formation early yet contributes to it later. How transcription factors play contradictory roles like this in development is not completely understood. What is clear is that the presence of a specific transcription factor alone is not sufficient to explain cell fate in the developing retina.

*Cis-regulatory Elements are Vital to Retinal Function*

Cis-regulatory elements are non-coding DNA regions that control the transcription of DNA[151]. There are a wide variety of cis-regulatory elements including promotors, enhancers, silencers, and operators. Each plays a unique role by recruiting a set of transcription factors which may then drive or inhibit expression of a given gene. Nearly all cis-regulatory elements are acted upon by more than one transcriptional regulator and many may be hundreds or even thousands of base pairs away from the gene they are acting on[152]. The distance does not inhibit activity, because when bound by the appropriate transcription factors, the DNA may be looped to bring long distance regions close together, thus aiding in the initiation or inhibition of transcription[153,154]. Cis-regulatory elements are thought to be one of the primary mechanisms of species evolution[155–158]. However, they can also play a major role in human disease pathogenesis[159]. For example, a 6,523-bp deletion of a cis-regulatory region, 20-kb upstream of the gene *ATOH7 (MATH5)*, causes congenital retinal nonattachment and blindness from birth[160]. This loss of a single distal enhancer bit is sufficient to cause a major developmental disorder

specific to the retina, highlighting the critical nature of cis-regulatory elements in controlling development.

*Promotors Are Highly Conserved*

Every known gene that is expressed in eukaryotes has at least one, and occasionally more than one promotor which is located proximal to the site of transcriptional initiation but may be located before or in the middle of a gene[153]. Compared to the diversity of elements like enhancers, promotor sequences, like TATA boxes, are relatively conserved throughout the genome[153–155]. This makes them excellent at their job which is to coordinate with the conserved initiation machinery involved in transcription. Indeed, it may be that they are evolutionarily conserved because of the need to properly bind to limited transcriptional machinery[153–155]. Since thousands of genes within an organism may contain similar promotor sequences, these make poor regulatory elements. Additionally, significant evidence suggests that in most cases, sustained promotor activity requires the aid of additional transcription factors that bind DNA distally to maintain transcription[153–155].

*Cell Type-Specific Enhancers*

Unlike promotors which are relatively consistent in their location near a gene and their sequence, enhancers are a complex group of cis-regulatory elements that may be upstream, downstream, or in the introns of a given gene. Enhancers may have a broad function and act on a gene in several cellular contexts, or narrow function and drive gene activity in a cell type-specific way[151,161,162].

Enhancers may be tens-of-thousands of base pairs away from the gene they act on. This makes identification challenging. However, the general method is to utilize computational approaches to identify conserved regions of open chromatin, and significant datasets already exist with predicted enhancer regions[163–165]. After a region is identified, further computational work can predict candidate binding sites. These can then be tested *in vivo* using a reporter assay[166,167]. Reporter assays can only show the sufficiency of an enhancer, but not its necessity. To demonstrate necessity, the enhancer must be removed, and the expression of the gene changed as a result.

An additional challenge exists in predicting which transcription factors bind to a specific enhancer. A given enhancer region may have several to dozens of consensus binding sites. Each one may be capable of binding a wide range of transcription factors. Additionally, it is likely that many enhancers work by the combinatorial action of several transcription factors simultaneously[155]. Immunoprecipitation of a given enhancer, followed by protein extraction and mass spectrometry or quantitative assessment on a microarray may provide insights into the bound transcription factors, but these assays are complex, time consuming, and have numerous technical limitations[168–170].

Despite the technical constraints, understanding the interactions between enhancer regions and the transcription factors that bind them is critical to understanding developmental process. Indeed, the action of specific transcription factors on cell type-specific enhancers is one of the single most important elements in understanding intrinsic mechanisms of cell fate in developing tissue.

*Extrinsic Signaling Mechanisms in the Developing Retina*

Extensive evidence compiled over years suggests that the development of the neural retina is dominated by intrinsic mechanisms of cell fate[84]. However, these mechanisms are insufficient to explain all the behaviors of cells within the tissue. For example, the formation of directionally selective circuits has been shown to involve SAC arbors specifically dispersing without interfering with proximal SACs[79]. A second example is the mosaic dispersion of Müller glia across the retina where each separate cell integrates columnarly within the tissue and does not interfere with adjacent cells[58]. These patterns indicate that cells are able to communicate during development. Indeed, the mosaicism that is a dominate characteristic of the retina itself suggest that cells are communicating in some fashion to arrange themselves appropriately throughout the developmental paradigm. Additionally, some external signaling mechanism must be present for cells to appropriately laminate and stratify.

It is possible that most of development is driven by intrinsic mechanisms of cell fate, but once fate is determined, cells utilize cell-cell signaling for alignment and circuit formation. Several extrinsic signaling mechanisms are known to play roles in early patterning of the retina, including WNT, Notch, and SHH.

Notch signaling plays a critical role in retinal development by maintaining progenitor like identity[171–173]. Suppression of notch signaling by the γ-secretase inhibitor DAPT has been shown to force cells out of their progenitor state and adopt neural fates within the retina[173]. As previously mentioned, Notch is required to properly maintain expression of HES1 and HES5 which in turn maintain progenitor identity, though the mechanisms are not fully understood.

WNT and SHH signaling have been implicated in early formation of the eye cup[174,175]. In the absence of WNT the eye cup of zebrafish fails to form properly, but ectopic expression of

WNT11 results in abnormally large eyes[174,176]. WNT signaling also plays critical roles across vertebrate species in defining the lens, separating RPE from neural tissue, maintaining dorso-ventral patterning, and proper retinal vascularization[177,178].

One role of SHH signaling is in the formation, propagation, and axon guidance of ganglion cells. Newborn ganglion cells will produce SHH early in retinal development and are the major stable source of SHH in the early retina[179]. In a complex feedback loop, mammalian SHH, produced by retinal ganglion cells, prevents progenitors from exiting the cell cycle, and blocking SHH early results in excess ganglion cells, the primary source of SHH in the retina[180]. Added to the multifaceted role in ganglion cells is how SHH interacts with axon guidance. Axons of the ganglion cells that are producing SHH proceed away from the high source and towards a low level of SHH located through the optic disc and at the ventral midline of the diencephalon[181,182]. Misregulation of SHH levels in the retina results in disorganized ganglion cell axon guidance and viral mediated overexpression of SHH at the optic chiasm restricts ganglion cell axon guidence[183–186]. How these cells are capable of creating a gradient that they also respond to is not well understood.

While WNT and SHH play roles in the early patterning and Notch maintains progenitor identity, few other mechanisms have been well elucidated within the field. The predominant theory remains that once the neural retina is established, intrinsic mechanisms of fate specification are the driving force[84].

*Putting the Retina Together*

One simile for retinal development patterns is to think of transcription factors as tools. Each can perform a variety of functions, like a hammer, but they also perform specific functions.

You cannot use a hammer to drill a hole, but there are a variety of tasks you can use a hammer for at different stages of a construction project. Each stage of a developing cell may be somewhat like a construction site. It is roughly the same cell, but after a week of progress the nails you could hit last week are no longer accessible while a whole new set of nails are extant. In this analogy, cell type-specific enhancers represent the ability to bring out the correct tool in the correct context. Like a building that is under construction, cells are in a constant state of flux during development. Genes are upregulated and inhibited, this results in remapping of the chromatin structure, opening of certain regions, closing and tightly winding other regions, all while the cell may be migrating, dividing, or communicating to adjacent cells. These complex waves of interaction mean that the development of any tissue may pass through hundreds or even thousands of checkpoints in which the loss of a single transcription factor could disrupt the process. Because transcription factors are so multi-functional and because their expression is also driven by transcription factors, it is critical that developing tissue be able to precisely regulate expression of the regulatory proteins. To assemble a retina, each cell must properly manage the expression patterns of unique genes, assemble the cellular structure, organize and reorganize chromatin, signal associated neurons, and integrate into a fully formed visual tissue.

**Early Patterning of the Developing Retina**

Before developing the complexity of the final retina, the developing embryo sets the rough pattern and gives rise to the progenitors which will eventually generate the ocular tissue. The inner layer of the optic cup transitions into the group of neural retinal progenitor cells that will give rise to all seven major types of retina cells by E10.5 in mice. The first visible formation of the eye occurs around E8 in mice when optic pits begin to form[187]. Over the course of the next

couple of days the expression of critical transcription factors pass through a series of steps to establish an eye field and ultimately an optic vesicle (Fig 1.4)[187–189]. This process involves both WNT and SHH signaling, as well as an array of transcription factors, including OTX2, PAX6, and VSX2, all critical regulators in early and late retinal development (Fig 1.4A). Loss of any of these three early will disrupt proper retinal formation[187]. The optic vesicle will expand outward till it impacts the surface ectoderm. Once there the vesicle will begin to invaginate, bringing with it the surface ectoderm and forming two nested cups. One of which will become the neural retina and the other the RPE. The surface ectoderm will form the lens, as well as other ocular tissue including the cornea. Significant crosstalk likely exists between each portion of this tissue and loss of any layer of this tissue results in a failure to properly form an eye (Fig 1.4B)[187,190,191].

The remainder of this work will explore events occurring after the patterning of the eye and development of the neural retina has occurred. I investigate the processes that drive this homogenous group of retinal progenitors to generate the complex tissue of a fully developed retina.

**OTX2, VSX2, and PRDM13 in Developmental and Degenerative Retinal Diseases**

I have briefly discussed the evolution of vision, explored the structure and function of the retina, and highlighted the major processes that govern retinal development, including the role of transcription factors in driving specific cell fates as well as neural patterning. Before delving into the regulation and function of specific transcription factors in a particular developmental context, I wanted to underscore the known roles of several genes studied here in human-specific disease

states. *Otx2, Vsx2,* and *Prdm13* have been specifically implicated in human developmental disorders or retinal degenerative diseases.

*The Transcription Factor OTX2 can Cause Developmental and Degenerative Retinal Diseases*

The homeobox domain transcription factor *OTX2* is critical for retinal development[138]. *Otx2* is involved throughout the process of initial patterning of the retina, through retinal neurogenesis, and remains on in mature photoreceptors and bipolar interneurons[127,192,193]. Loss-of-function mutations in the *OTX2* gene cause developmental alterations including anophthalmia, microphthalmia, and degradation of the optic nerve[194–196]. Certain *OTX2* mutations have been associated with Leber congenital amaurosis, a progressive photoreceptor degenerative disease[197].

*The Transcription Factor VSX2 is Associated with Microphthalmia*

*VSX2* is a transcription factor that plays a central role in the development of retinal progenitor cells, which ultimately give rise to all neural tissue within the retina. Loss of function in *VSX2* can result in mild to severe microphthalmia as well as severe coloboma (holes in ocular tissues, like the retina and iris)[149,198–202]. At this time, there are no known retinal degenerative diseases associated with *VSX2*, but all published phenotypes for mutations in this gene in humans have severe degradation of the ocular tissue and generally little to no visual function.

*PRDM13 is Implicated in North Carolina Macular Dystrophy*

The central portion of the human retina is called the macula. There is a high density of photoreceptors, and especially cones within this region, which is responsible for human high acuity vision[203]. Since the primary source of functional visual information in humans arises from

the macular region of the retina macular dystrophy or degeneration is considered a debilitating disease state. In macular dystrophy the central portion of the retina begins to degrade, photoreceptors die, and visual function is permanently lost[204].

North Carolina Macular Dystrophy (OMIM-136550) is a rare form of the disease, named for the location where it was first characterized[205]. The disease is an autosomal dominant disorder and has variable phenotypes. Unlike many other forms of macular dystrophy, North Carolina Macular Dystrophy is generally not progressive and is thus likely a developmental disorder.

The zinc-finger transcription factor *PR Domain Zinc Finger Protein 13* (*Prdm13*) has been implicated in a large subset of North Carolina Macular dystrophy patients[129]. Within these patients, 14 distinct variants along a single allele resulting in five distinct mutations of the *Prdm13* gene[129]. These mutations resulted in failure to properly maintain the macular region of the retina and cause lifelong visual deficits.

### ***Prdm13*** **and Amacrine Cell Formation**

Cellular diversity within the retina, and indeed all neural tissues, can be approached from three broad categories of classification: 1) cellular morphology, 2) molecular markers, and 3) neural circuits. In most cases, these three categories will broadly harmonize to identify specific subtypes of neurons. However, it is not always possible to collate techniques in these disciplines together. If a cell subtype cannot be clearly labeled by a molecular marker that corresponds to a specific morphology, identifying that cell's role within neural circuits is challenging. Indeed, one of the major advances required to improve our understanding of retinal development and function is clearly identifiable subtypes of retinal interneurons neurons.

*Challenges in Defining Amacrine Subtypes*

Amacrine interneurons are one of the most diverse and least defined groups of cells within the retina. As mentioned previously, amacrine nuclei are located in the INL or GCL and they project dendritic arbors in the IPL where they are involved in mitigating the signals passing from bipolar cells to ganglion cells. There are numerous morphological and histological markers of amacrines but identifying the specific molecular markers and developmental history of subtypes has remained challenging[38–40,206,207]. Here I define a subtype of amacrine cells as a group that are characterized by a specific morphology including dendritic arborization and function within an explicit neural circuit[30,40–42,44,45,208,209].

All mature amacrines are marked by the transcription factor PAX6, but many share a variety of other markers. A given amacrine might express both GLYT1 and AP2a, while the next cell is only marked by AP2a[210]. Further, just because a cell expresses common markers, say AP2a/GLYT1, does not mean it is morphologically or functionally homogenous with other similarly labeled cells. As a consequence of this incredible cellular diversity, studying specific amacrine subtypes is problematic. To advance our understanding of retinal circuits, the unique subtypes of amacrine cells need to be identified by a specific group of molecular markers that allow the cells to be labeled, tracked, and their population segregated for physiological studies.

*Patterns of Amacrine Cell Formation and Transcriptional Regulation*

Amacrine cells in mice are born from E12.5 through P2 with the majority being born embryonically, peaking around E16.5[85,211,212]. The transcription factors FOXN4 and RORB work in concert to turn on PTF1a, and these cells are then lineage committed to amacrine or horizontal

cell fates[107,108,213]. PTF1a+ cells may then turn on *Prox1* and go on to become horizontal cells[214]. The mechanisms by which they remain amacrine cells are not fully understood, though all mature amacrines express PAX6.

There is temporal patterning to amacrine subtype formation. Extensive birthdating experiments have shown that GABAergic amacrines tend to birthdate early, while glycinergic amacrines are late-born[212]. Even when amacrine cells were disassociated from their tissue context and raised in culture, they tended to express transcription factors that matched their birthdate, suggesting that there were intrinsic mechanisms of subtype-specification present in cells upon cell-cycle exit[212]. This led us to ask, how do PTF1a+ cells choose between becoming an amacrine or horizontal cell? Additionally, what intrinsic forces cause early born cells to form some amacrine subtypes while late born amacrines choose other subtypes? If it is driven by cell birthdate, how is that tied to the intrinsic environment of a neuron and what is it about that internal transcriptional environment that drives an amacrine toward a specific subtype? Finally, can we utilize an improved understanding of amacrine subtype formation to generate identifiable subpopulations of amacrine cells and thereby study their role in retinal circuits?

While it has been shown that amacrine subtypes correlate with their birthdate, there are a number of transcription factors that are implicated in playing diverse roles in type and subtype formation[42,44,212]. There are factors that set primary type-fate, like *Ptf1a*. Loss of these results in a failure to form any amacrine cells. Others are intermediate, like *Islet1,* loss of these factors causes a subset of amacrines to not form or die during development, decreasing total amacrine numbers[215]. Still other factors appear to promote specific subtypes; loss of *Neurod6* causes a subtype fate-shift, but there is no change in the total number of amacrines[40]. This argues that fate choice is progressive, where PTF1a+ cells first adopt amacrine/horizontal identity before

becoming further restricted to a type and then particular subtype identity. The finding that cells will go through these processes in culture, suggests that amacrine neurons may be predisposed at cell-cycle exit to follow a particular specification pathway. The means by which such mechanisms of intrinsic fate specification work are not well understood.

*Ptf1a and Prdm13 in the Developing Retina*

PTF1a is the prerequisite transcriptional regulator of horizontal and amacrine cells[108]. Mice that lack *Ptf1a* die at birth and essentially lack horizontal and amacrine cells[108]. During development, *Ptf1a* will come on in nascent horizontals and amacrines and then turn off after a short period of time. This suggests that it may play some role in activating particular cellular machinery and that once active these mechanisms repress *Ptf1a.* To better understand the temporal and spatial mechanisms that diversify the PTF1a+ precursor population, I looked for factors that act downstream of PTF1a.

The zinc finger transcription factor *Prdm13* is genetically downstream of *Ptf1a* in the mouse spinal cord and retina[216]. PTF1a directly activates *Prdm13,* which acts as a transcriptional repressor to promote inhibitory interneuron identity at the expense of excitatory fates[216]. In the developing spinal cord, the primary function of PRDM13 appears to be inhibitory. Additionally, PRDM13 acts in a co-repressive manner, collaborating with associated transcriptional regulators to control cell fate[217]. In particular, PRDM13 interacts with bHLH factors like PTF1a in the developing spinal cord to convert transcriptional activators into repressors, helping to silence competing gene expression programs in bistable precursors[217]. Evidence suggests that PRDM13 is involved in retinal amacrine cells during development[218]. Taken together with data from the

spinal cord, I suspected that PRDM13 may be acting downstream of PTF1a within the retina to set interneuron subtypes and potentially label a subpopulation.

Here, I explored the expression pattern and developmental role of PRDM13 in the retina utilizing a combination of antibodies, *Prdm13-GFP* knock-in mice, and a hypomorphic version of *Prdm13* (*Prdm13-Δ115*)[217]. I set out to ask a variety of questions, first I wanted to explore the expression pattern of PRDM13 during development and if it remains on into adulthood. Second, I asked what role PRDM13 plays in developing and mature interneurons of the mouse retina. What population of cells does PRDM13 effect and is it a necessary cell type-specific enhancer? Without PRDM13, do these cells fail to form, do they die off, or do they fate shift to alternative identities? Ultimately, I attempted to further unpack the processes of interneuron fate specification in the developing murine retina (Chapter II).

### *Otx2*, *Prdm1*, and *Vsx2* Control Bipolar and Rod Fates in the Developing Retina

One of the fundamental questions in development is how progenitor cells give rise to the incredible variety of cell types found in the central nervous system. A pool of retinal progenitor cells generate all of the primary cell fates within the retina (Fig 1.4C). However, as I have discussed, these fates do not arise at the same time. Certain cell types like ganglion cells are born early, and others are born late. In the first portion of this work I focus on the role of *Prdm13* in amacrine subtype formation. Next, I explore the roles a gene regulatory network that occurs late in retinal development during the postnatal period when progenitors primarily give rise to rod photoreceptors or bipolar interneurons[81–83,85,219,220].

There are three cell types that are predominantly born postnatally in mice; rods, bipolars, and Müller glia. Some amacrine subtypes are also born postnatally, but these are a small portion

of the cells. As discussed previously, a dominant theory is that cells pass through stages of progressive competence and this competence is regulated by the presence of specific transcription factors[84,97,100,127,221–224]. How transcription factors control cell competence and drive specific cell fates is only partially understood.

*OTX2 is a Key Regulator of Cell Fate Competence in the Retina*

A key regulator within late retinal development is the transcription factor *Otx2*. In early development, *Otx2* is necessary for patterning of the eye, and its loss results in major structural defects[225]. If *Otx2* is conditionally knocked out in the retina after initial eye patterning is established, rods and bipolars fail to form and instead supernumerary amacrine cells are produced[138,139,226]. *Otx2* remains on in all mature photoreceptors and bipolar cells and significant evidence shows it is a critical regulator of progenitors' competence to form bipolar cells or photoreceptors[130,138,227,228]. OTX2 is turned on in the majority of late born progenitors as they exit the cell cycle (Fig 1.5)[229]. It will remain on in cells that go onto become bipolars or rods, but may be transiently active and then turned off in nascent amacrine cells (Fig 1.5)[230]. OTX2 is so prevalent in late born post-mitotic cells that it is not clear at this time if it comes on in all of them for at least a short period of time, or only a dominant subset.

PRDM1 and VSX2 work downstream of OTX2 in a gene regulatory network that governs rod photoreceptor and bipolar interneuron fates (Fig 1.5). In the absence of PRDM1, OTX2+ cells precociously upregulate *Vsx2* and excess bipolar cells are generated at the expense of photoreceptors. Indeed, the conditional loss of PRDM1 in postnatal nascent photoreceptors that have already turned on rod-specific markers, results in a transdifferentiation of developing rods into VSX2+ bipolar interneurons[127,128,231]. PRDM1 overexpression results in the opposite

effect, with a decrease in VSX2+ bipolar cells (Fig 1.5)[128,231]. Zinc-finger transcription factors, like PRDM1, primarily work in concert with other transcriptional elements to inhibit the expression of specific genes, though PRDM1 has been shown to have both repressive and activating activity[112]. Taken together with previous studies, it is possible that PRDM1 is not instructing photoreceptor fates, but instead inhibiting bipolar fates, though the mechanisms of PRDM1 action in the retina are not well understood at this time.

VSX2 works in opposition to PRDM1 and drives bipolar fates. Like *Otx2*, *Vsx2* is necessary for early retinal development and its deletion results in microphthalmia and a small neural retina. However, if *Vsx2* is knocked down later in development, the retina fails to generate bipolar cells. Indeed, significant evidence suggests that no bipolar cells will form in the absence of VSX2 (Fig 1.5)[148–150,232]. While VSX2 is on in progenitors, it turns off during late retinal development and appears to be upregulated using a separate mechanism in bipolar cells[233].

OTX2 directly interacts with cell type-specific enhancers that are sufficient to drive expression of the transcription factors *Prdm1* and *Vsx2*. The Brzezinski lab has shown that 6kb upstream of *Prdm1* there is a 139bp cell type-specific enhancer that requires OTX2 binding to drive expression[167]. Approximately 18kb upstream from *Vsx2* there is a 164bp bipolar-specific enhancer sequence that is directly bound by OTX2 and is sufficient to drive expression in nascent bipolar cells[234,235]. OTX2 binds both of these enhancers and is necessary for their expression. However, there are additional binding sites within these enhancers and OTX2 may not be sufficient to drive transcription of either without the aid of additional, as yet unknown, transcriptional regulators.

*PRDM1 and VSX2 Act in a Gene Regulatory Network Downstream of OTX2*

PRDM1 and VSX2 act in a gene regulatory network to produce rod photoreceptor and bipolar interneurons within the OTX2+ lineage, late in retinal development. Upregulation of PRDM1 drives photoreceptor fates and inhibits bipolar fates. Upregulation of VSX2 has the opposite effect. Loss of PRDM1 causes a 1:1 fate shift toward bipolar cells. Loss of VSX2 results in a failure to generate bipolar interneurons. Both genes have sufficient cell type-specific enhancers that are bound by OTX2. Collective evidence suggests that OTX2 establishes competence and then VSX2 and PRDM1 compete in some way to drive specific cell fates or correctly establish the number of rods and bipolar cells.

Despite these similarities, many questions remain about the function of this gene regulatory network. First, rods appear to form even when PRDM1 is absent, though there is a decrease in their numbers[127,231]. Evidence suggests that in the absence of VSX2, bipolar cells are not formed[148–150,232]. PRDM1 is expressed transiently from late embryonic periods through early development, while bipolar-specific VSX2 is activated postnatally and remains on into adulthood[130]. Taken together, it is clear that VSX2 and PRDM1 compete in some way to set OTX2+ cell fate. However, they may have different modes of action.

One hypothesis is that VSX2 and PRDM1 are competing to control the correct number of bipolar cells and rods. Alternatively, they may play a role in stabilizing cell fate after a choice has been made. In the case of *Prdm1*, this would explain its transient activation and likely inhibitory role, as it would be preventing alternative fates and allowing rod transcriptional machinery to take over. Another possibility is that rods or bipolars are a default fate in OTX2+ cells, and that either *Prdm1* or *Vsx2* are working to drive an alternative state.

While this gene regulatory network is primarily implicated in fate choice, it may be playing a role in fate stability. The conditional loss of *Prdm1* in postnatal eyes can cause nascent rods to fate shift to bipolar cells[231]. This shows that for some time after a fate has been selected, cells are capable of a fate shift. I wondered how long this window of malleability exists. For example, will mature bipolar cells change fate to photoreceptors in the presence of PRDM1?

*Determining the Fate of OTX2+ Cells that Lack PRDM1 and VSX2*

I developed a variety of methods to explore this gene regulatory network and ask two broad questions. 1) I asked what happens if OTX2+ cells lacked both PRDM1 and VSX2. Do cells default to bipolars or photoreceptors? Do both cell types fail to form or is there a shift in the number of specific types? 2) What happens to a VSX2+ nascent or mature bipolar cell when PRDM1 is present?

To simultaneously remove PRDM1 and VSX2, I developed a CRISPR/Cas9 system that could be delivered *in vivo* to the developing mouse retina. I created multiple versions of this system. The first directly targeted necessary exons of *Prdm1* and *Vsx2*. The second system targeted the sufficient cell type-specific enhancer of *Prdm1* and *Vsx2*. I reasoned that if these enhancers were not just sufficient but necessary for the expression of these genes in nascent rods or bipolar cells then deletion would result in synchronous loss of PRDM1 or VSX2 at the precise time when it would normally be upregulated within the cell.

In agreement with previous loss-of-function experiments, singly targeting *Prdm1* or *Vsx2* genes affected the formation of photoreceptors or bipolar cells[127,128,148,149]. Targeting either cell type-specific enhancer resulted in an equal reduction of protein expression and an alteration in the fate of the CRISPR targeted cell. Despite the consistency in single loss-of-function

experiments, targeting both enhancers or both genes simultaneously did not have the same result and caused a surprising dysregulation of the gene regulatory network (Chapter III).

Our results shed significant insight on the function of this gene regulatory network, suggesting that rods are the default cell fate for OTX2+ cells, but in the absence of PRDM1, progenitor-derived VSX2 drives excess bipolar cells. I demonstrate that bipolar-specific VSX2 is regulated downstream of the fate-choice-point to become a bipolar cell. Finally, our results show that VSX2 and PRDM1 work in a redundant fashion to restrict competence of OTX2+ cells and loss of both resulted in excess late born amacrines. By targeting multiple genes and cell type-specific enhancers simultaneously, I have gained significant insight into the regulation of this gene regulatory network, as well as the role of redundancy in maintaining progenitor competence restriction in the retina.

*Determining the Stability of Bipolar Cells in the Presence of PRDM1*

I previously mentioned that the loss of PRDM1 in nascent rods is sufficient to cause a fate shift to bipolar cells[130]. I wondered if the presence of PRDM1 in nascent or mature bipolars would be sufficient to cause a fate shift to rods or if VSX2+ bipolar fates were stable once established. To test VSX2+ bipolar cell stability at different developmental time points, I created a CRE-mediated conditional *Prdm1* overexpression mouse. I first utilized a transgenic mouse that expresses Cre in progenitors to see how PRDM1 would affect bipolar fate before VSX2 was present. This early constitutive PRDM1 expression blocked bipolar formation. I next asked if VSX2+ nascent bipolars that had not matured would fate shift in the presence of PRDM1. I electroporated newborn mice conditional PRDM1 overexpression mice with a bipolar-specific Cre plasmid. After seven days, some, but not all, nascent bipolar cells fate shifted to rod

photoreceptors. Finally, I utilized a bipolar-Cre-virus to drive PRDM1 expression in mature bipolar cells. While constitutive expression of PRDM1 did not have any observable effect on mature bipolar cells, it was toxic to rods. This work suggests that there is a window in which nascent bipolar cells will lose VSX2 and become rods in the presence of PRDM1 (Chapter IV). However, mature bipolar cells do not readily change their fate and PRDM1 must be downregulated in mature rods for their survival.

Taken together with the work in Chapter III I have significantly advanced our understanding of this gene regulatory network and shed light on critical developmental paradigms, including the roles of cell type-specific enhancers and timing in regulating cell fate within the retina.

## Chapter I Figures

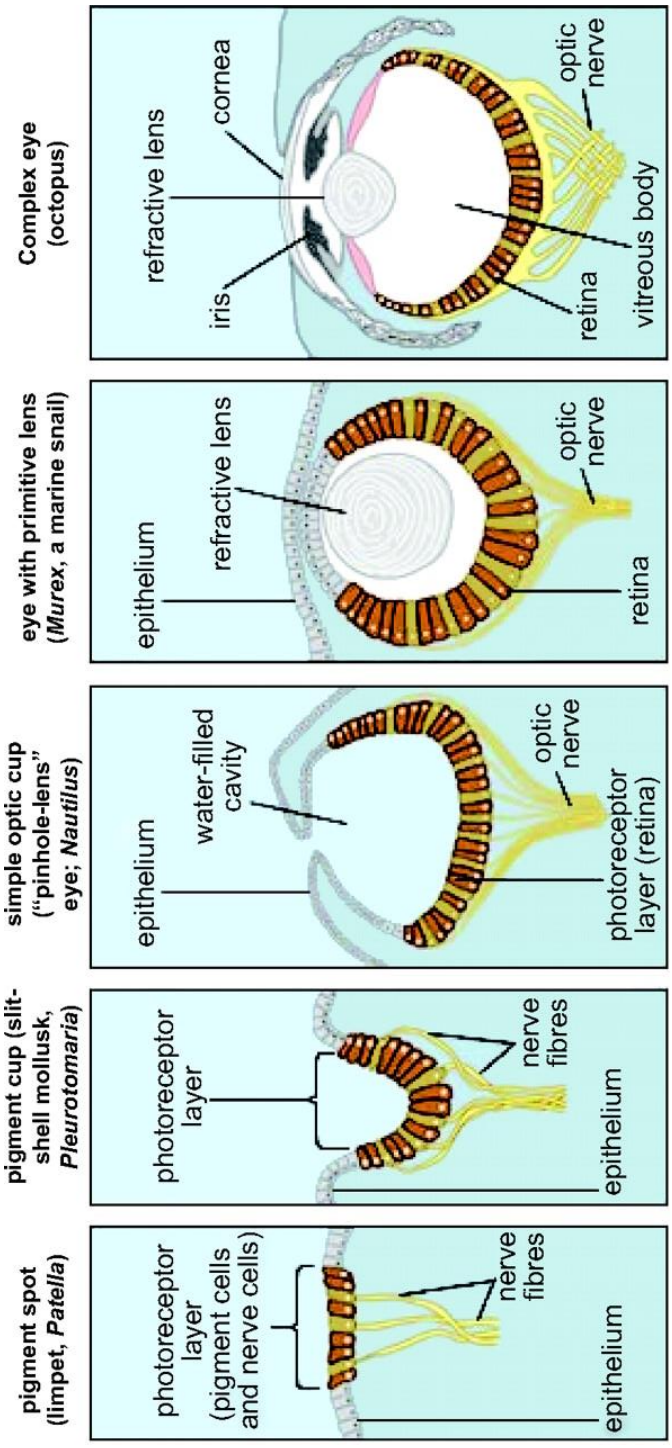



**Figure 1.1 Evolution of the eye**[1]

[1]Portions of figure reproduced from cited literature and used with permission from the copyright holder; copyright 2007 National Academy of Sciences

The eye has evolved multiple times throughout history. Here is an example of the evolution in mollusks, starting with limpets which possess basic light/dark detectors, through to the complex eye of the octopus which is used for camouflage detection and patterning[9].

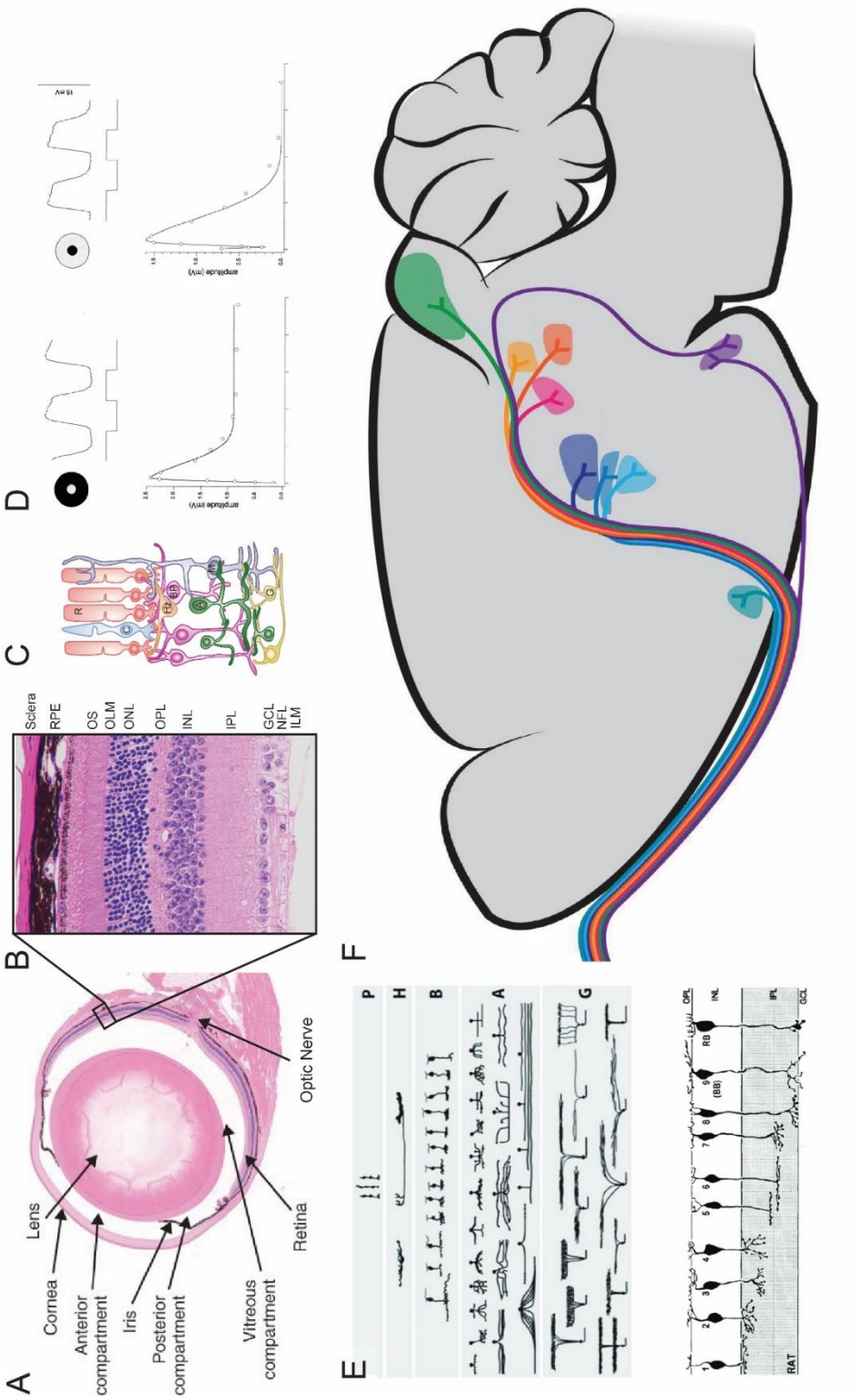



**Figure 1.2 Anatomy, patterning, and cell morphology of mammalian retina neurons**[2]

[2]Portions of figure reproduced from cited literature and used with permission from the copyright holder

**A)** Cross section of mouse eye showing the primary anatomical features[236]. **B)** Zoomed cross section of the retina highlighting the layers including Sclera, Retinal Pigmented Epithelium (RPE), Outer Limiting Membrane (OLM), Outer Segment (OS), Outer Nuclear Layer (ONL), Outer Plexiform Layer (OPL), Inner Nuclear Layer (INL), Inner Plexiform Layer (IPL), Ganglion Cell Layer (GCL), and Inner Limiting Membrane (ILM). **C)** Unique types of cells within the retina including Rods (R), Cones (C), Horizontal Cells (Hz), Bipolar Cells (BP), Amacrine Cells (A), Ganglion Cells (G), and Müller glia. **D)** Center-surround ON and OFF stimulus and signals recorded from bipolar cells in primates[73]. **E)** Morphological diversity of rat retinal neurons including photoreceptors (P), Horizontal Cells (H), Bipolar Cells (B) and bottom area, Amacrine Cells (A), and Ganglion Cells (G)[237]. **F)** Primary projections from the mouse retina and some of the targeted tissue within the brain[238].

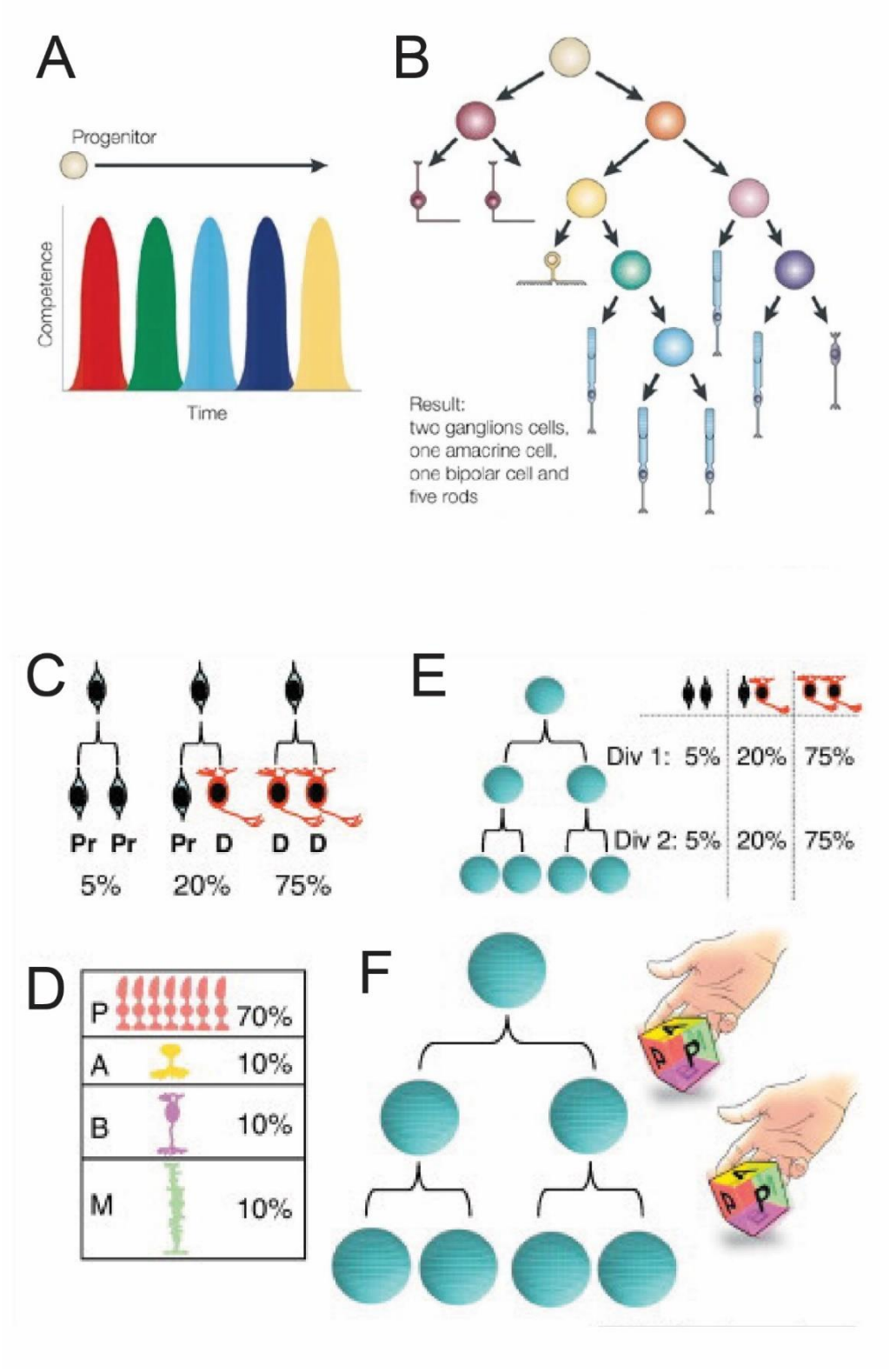



**Figure 1.3 Models of retinal development include intrinsic and stochastic approaches**[3]

[3]Portions of figure reproduced from cited literature and used with permission from the copyright holder

**A)** Under an intrinsic paradigm of development, progenitor cells progress through layers of competence. **B)** The result is that progressive progenitors give rise to the diversity of cells within the retina[84]. **C)** A stochastic model of development in rats predicts fate outcomes based on probability, including **D)** the total numbers of a given cell type and **E)** the percentage probability as development progresses. **F)** At each stage within a stochastic model outcomes are probabilistic, but not fixed, explaining some of the phenomenon observed in higher vertebrate retinas[97]

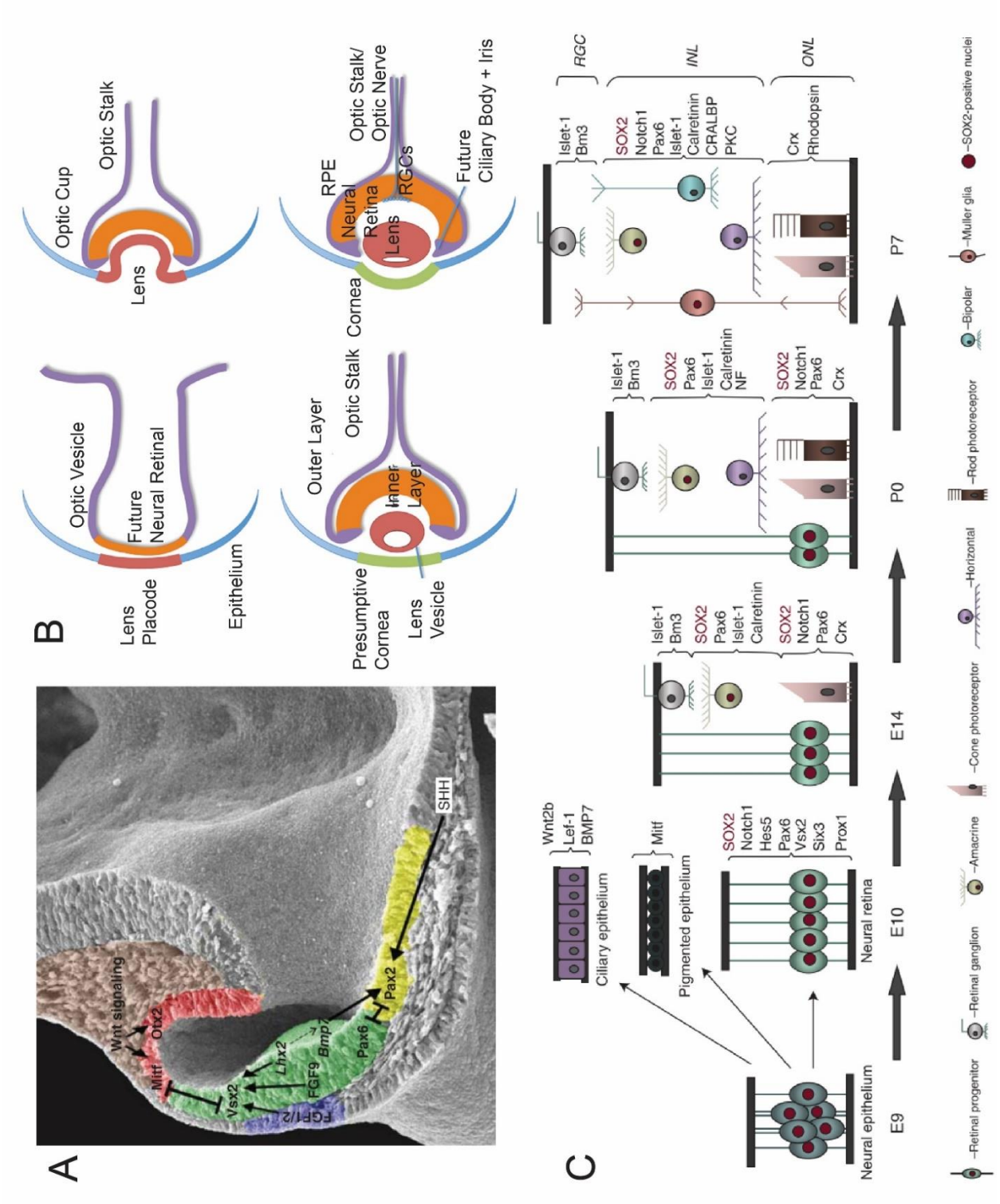



**Figure 1.4 Intrinsic and extrinsic regulation of mammalian retinal development**[4]

[4]Portions of figure reproduced from cited literature and used with permission. Copyright Cold Spring Harbor Laboratory Press.

**A)** The early eye formation is tightly controlled by intrinsic transcription factors like VSX2, PAX6, and OTX2, as well as extrinsic signaling from WNT and SHH[187]. **B)** The optic vesicle extends and contacts the surface epithelium and then invaginates, eventually forming the eye tissue[239]. **C)** Neural epithelium differentiates into neural retina by E10 and then expands into all the major cell types and subtypes by P7, tightly controlled by various transcription factors[187].

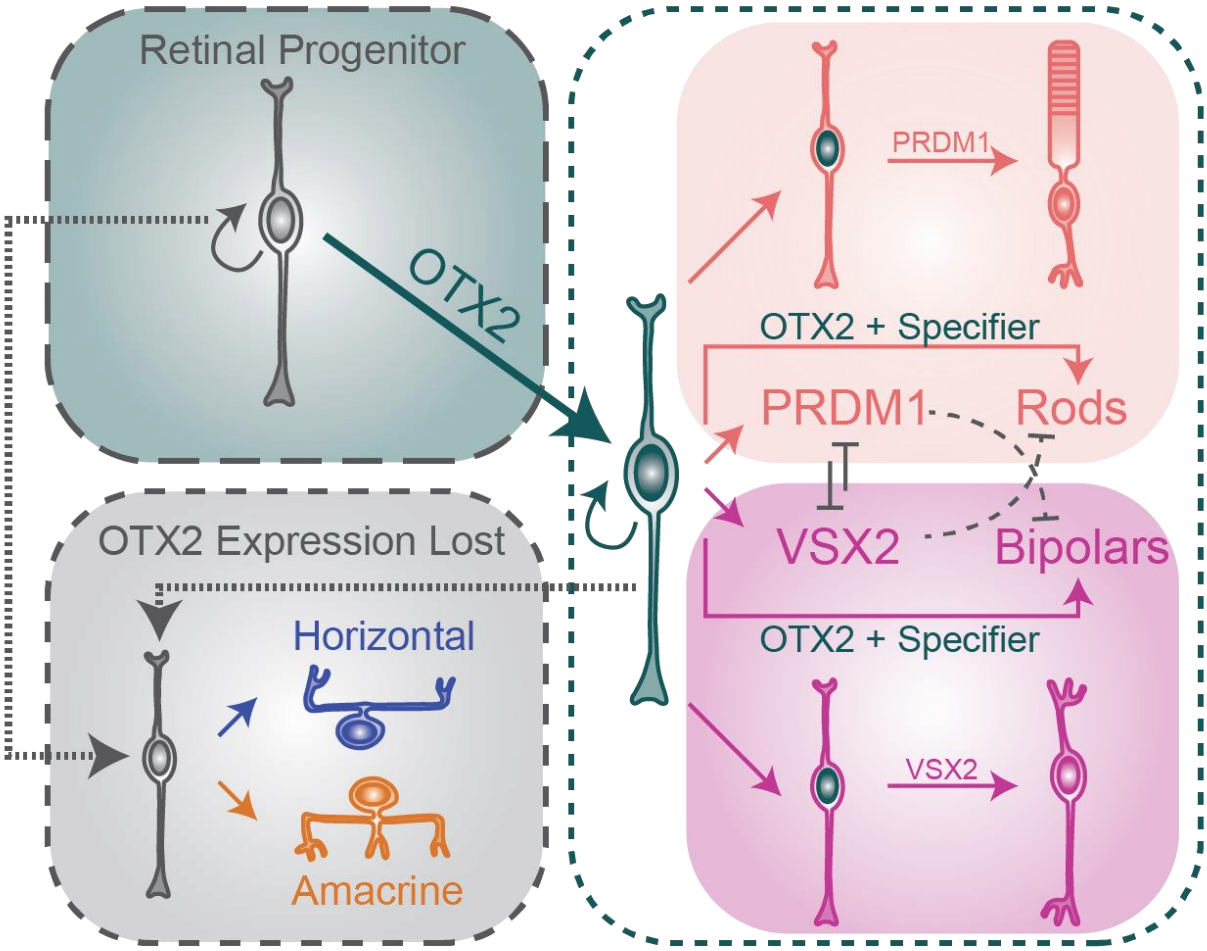



**Figure 1.5 Gene regulatory network governing cell fate in the retina**
Late in development, retinal progenitor cells will turn on OTX2 and permanently exit the cell cycle. They will then activate PRDM1 and become rods or VSX2 and become bipolar cells. OTX2 is necessary to activate either transcription factor but may not be sufficient. PRDM1 and VSX2 may work through direct cross repression or by repressing the alternative cell fate. If OTX2 expression is lost, cells can go on to enter the PTF1a lineage and become amacrine cells late in development or possibly horizontal cells early.

## CHAPTER II

## PRDM13 IS REQUIRED FOR EBF3+ AMACRINE CELL FORMATION IN THE RETINA[5]

### Chapter II Summary

Amacrine interneurons play a critical role in the processing of visual signals within the retina. They are highly diverse, representing 30 or more distinct subtypes. Little is known about how amacrine subtypes acquire their unique gene expression and morphological features. We characterized the gene expression pattern of the zinc-finger transcription factor *Prdm13* in the mouse. Consistent with a developmental role, *Prdm13* was expressed by PTF1a+ amacrine and horizontal precursors. Over time, PRDM13 expression diverged from the transiently expressed PTF1a and marked just a subset of amacrine cells in the adult retina. While heterogeneous, we show that most of these PRDM13+ amacrine cells express the transcription factor EBF3 and the calcium binding protein calretinin. Loss of *Prdm13* did not affect the number of amacrine cells formed during development. However, we observed a modest loss of amacrine cells and increased apoptosis that correlated with the onset timing of EBF3 expression. Adult *Prdm13* loss-of-function mice had 25% fewer amacrine cells, altered calretinin expression, and a lack of EBF3+ amacrines. Forcing *Prdm13* expression in retinal progenitor cells did not significantly increase amacrine cell formation, EBF3 or calretinin expression, and appeared detrimental to the survival of photoreceptors. Our data show that *Prdm13* is not required for amacrine fate as a class, but is essential for the formation of EBF3+ amacrine cell subtypes. Rather than driving

[5]The contents of this chapter have been published and are used with copyright permission: Goodson, N. B. Nahreini, J., Randazzo, G., Uruena, A., Johnson, J. E., and Brzezinski J. A. Prdm13 is required for Ebf3+ amacrine cell formation in the retina. Dev. Biol. 434, 149–163 (2018).

subtype identity, PRDM13 may act by restricting competing fate programs to maintain identity and survival.

**Rationale**

The retina is a thin neural tissue that detects and relays photic information. The mammalian retina has a highly organized structure consisting of alternating nuclear and synaptic (or plexiform) layers. These layers are populated by seven major classes of retinal neurons and glia, each of which are essential for normal vision. These cell types (rod and cone photoreceptors, Müller glia, retinal ganglion cell output neurons, and bipolar, horizontal, and amacrine cell interneurons) are all derived from a common progenitor population during development[82,222,240]. Adding to the complexity of the system, most of the seven cell types can be further divided into more than 60 additional distinct subtypes[66,241,242]. While considerable progress has been made to uncover the transcription factors and signaling molecules that control major cell class development, relatively little is known about how subtypes acquire their identities.

Amacrine cells are primarily inhibitory interneurons. They form synapses with glutamatergic bipolar interneurons and ganglion cell output neurons in the inner plexiform layer (IPL). Their somas are primarily located in the inner nuclear layer (INL), but a small fraction are displaced and localized to the ganglion cell layer (GCL). Amacrines make up only about 8% of retinal cells, but within this population more than 30 distinct subtypes have been described[38,39,206,207]. Amacrines can be grouped into three major categories based on whether they express GAD65/67 (GABAergic, ~43%), GlyT1 (glycinergic, ~43%), or neither of these markers (nGnG, ~15%)[40]. The transcription factor PAX6 is made by all amacrines, but

subpopulations express a wide array of additional markers in a highly heterogeneous fashion. For example, subsets of amacrines are marked by transcription factors (*e.g.* AP2a, EBF3, BHLHB5), calcium binding proteins (*e.g.* calretinin, calbindin), and proteins involved in neurotransmission (*e.g.* ChAT, TH, vGlut3)[30,40–42,44,45,208,209]. Many of these markers overlap in multiple subsets of amacrines, complicating the identification of individual subtypes. This has made interpreting the effects of gain- and loss-of-function experiments on amacrine cell development difficult.

In mice, progenitors that give rise to amacrine interneurons permanently exit the cell cycle (birthdate) from approximately embryonic (E) day 12.5 to postnatal (P) 2[85,211,212]. Progenitors that express the transcription factors FOXN4 and RORB are competent to express PTF1a[107,108,213]. PTF1a is a basic-helix-loop-helix (bHLH) transcription factor that is transiently expressed in postmitotic cells that are restricted to forming amacrines and horizontal cells[108]. Mice that lack *Ptf1a* die at birth and essentially lack horizontal and amacrine cells[108]. Several transcription factors that are expressed by subsets of amacrine cells perturb subtype development when they are mutated. For example, *Isl1* mutants have reduced cholinergic (ChAT+) amacrines, loss of *BHLHB5* decreases GABAergic subtypes, and *Neurod6* loss reduces nGnG (neither GABAergic nor glycinergic) amacrines[40,108,215]. Birthdating experiments show that there is an overlapping genesis order for the major categories of amacrines, such that GABAergic cells are born early followed by glycinergic and nGnG amacrines[40,211,212]. While subtype choice is correlated with cell cycle exit timing, how and when postmitotic PTF1a+ precursors commit to a specific amacrine subtype identity is unclear. Some perturbations, like *Neurod6* loss-of-function, alter subtype distribution without changing the total number of amacrines[40]. This argues that fate choice is progressive, where PTF1a+ cells first adopt amacrine identity before becoming further restricted to a particular subtype identity. To better understand the temporal and spatial

mechanisms that diversify the PTF1a+ precursor population, we looked for factors that act downstream of PTF1a.

We have shown that the zinc finger transcription factor *Prdm13* is genetically downstream of *Ptf1a* in the spinal cord and retina[216]. Within the spinal cord, PTF1a directly activates *Prdm13*, which acts as a transcriptional repressor to promote inhibitory interneuron identity at the expense of excitatory fates[216]. We have demonstrated that PRDM13 is recruited to enhancers by other transcription factors where it acts as a co-repressor[217]. In particular, PRDM13 interacts with bHLH factors like PTF1a in the developing spinal cord to convert transcriptional activators into repressors, helping to silence competing gene expression programs in bistable precursors[217]. These data suggest that PRDM13 acts as a repressor in PTF1a+ retinal cells to control amacrine or horizontal interneuron development.

We investigated the expression of PRDM13 during retinal development using specific antibodies and *Prdm13-GFP* knock-in mice. Consistent with our prediction, PRDM13 was expressed in PTF1a+ amacrine and horizontal precursors throughout development. PRDM13 expression persisted into adulthood, primarily marking a heterogeneous subset of glycinergic and nGnG amacrines. The majority of PRDM13+ cells co-expressed calretinin and EBF3. Of note, the entire population of EBF3+ amacrine cells co-expressed PRDM13. Mice that lacked *Prdm13* died at birth, but showed no deficits in amacrine cell genesis. To bypass lethality, *Prdm13-GFP* mice were bred to mice carrying a hypomorphic *Prdm13* allele (*Prdm13-Δ115*)[217]. These compound heterozygous mice (*Prdm13-GFP/Δ115*) were viable and had fewer amacrine cells in the adult retina compared to control mice. In particular, these mice lacked EBF3+ amacrines and calretinin+ cells that projected their dendrites to the middle of the IPL. Amacrine cell numbers were normal throughout their genesis period and declined only after the normal onset of EBF3

expression in these mutants. Despite early wide-spread expression in PTF1a+ cells, our data show that *Prdm13* is not required for amacrine cell genesis. However, *Prdm13* is required at a later step for amacrine subtype specification. *Prdm13* overexpression did not upregulate EBF3 or calretinin, suggesting that *Prdm13* acts to suppress alternative gene regulatory networks to maintain EBF3+ subtype identity and survival.

## Results

### *PRDM13 Marks Developing Amacrine and Horizontal Cells*

We previously showed that *Prdm13* is expressed by a subset of retinal cells that is similar to those marked by the committed amacrine and horizontal precursor marker *Ptf1a*[108,216]. *Prdm13* in situ hybridization signal was lost in *Ptf1a* mutant embryos, suggesting that *Prdm13* is expressed by developing amacrine and horizontal cells. To better evaluate the spatial and temporal features of PRDM13 expression during retinal development, we immunostained retinal sections with antibodies against PRDM13 and PTF1a. We focused on three time-points (E13.5, E15.5, and birth) when postmitotic nascent horizontals and amacrine cells co-express PTF1a[108]. We observed that both PRDM13 and PTF1a immunostaining formed a mosaic pattern with oval nuclei located between the apical photoreceptor area and the ganglion cell layer (GCL) (Fig 2.1). PRDM13 co-labeled 66.5% (± 21.3% SD) of PTF1a positive cells within the central and peripheral retina at E13.5 and all (100.0% ± 0.0% SD) of the PRDM13+ cells were PTF1a+ (Figs 2.1A-A’’’, D). We observed similar numbers of PRDM13+, PTF1a+, and double labeled cells at E15.5, but at this stage there was a statistically significant decrease in the percentage of PRDM13+ cells that co-expressed PTF1a+ (89.8% ± 6.9% SD, N=7, t-test, $p<0.001$) (Figs 2.1B, D-E). P0 retinas had more PRDM13+ and PRDM13+/PTF1a+ double labeled cells (Figs 2.1C,

E). However, the percentage of PRDM13+ cells that co-expressed PTF1a+ decreased further to 80.0% at P0 (Fig 2.1D). This decreased percentage is consistent with the postnatal loss of PTF1a expression and the persistence of PRDM13 in a subset of postmitotic neurons. To determine whether PRDM13 was expressed in other cell types, we co-stained sections with OTX2, a marker of developing photoreceptors at these time-points[108,130,138,227,228,243]. We observed modest overlap of PTF1a, PRDM13, and OTX2 at all three time-points (Figs 2.1A-C, E). Since the *Ptf1a* lineage lacks photoreceptors, these double and triple labeled OTX2+ cells are already committed to becoming horizontals and/or amacrine cells. This plasticity in the OTX2+ lineage has been observed previously[108,167,231,244,245]. We also co-labeled sections with antibodies against the ganglion cell marker BRN3[246]. No PRDM13+/BRN3+ ganglion cells were observed (data not shown). These data suggest that PRDM13 is expressed downstream of PTF1a, marking only committed amacrine and/or horizontal cells.

*PRDM13 Null Mice Exhibit No Gross Changes in Embryonic Retinal Development*

We created a *Prdm13-GFP* knock-in mouse line to accurately and persistently label *Prdm13*+ cells and conduct loss-of-function analysis[217]. These mice were created by inserting a cytoplasmic GFP cassette followed by a stop codon into exon 1 of the *Prdm13* sequence (Fig 2.2A-A')[217]. We observed that homozygous *GFP/GFP* null mice died at birth while *Prdm13* heterozygous animals showed no overt phenotypes[217]. To track PRDM13 during retinal development, we examined *Prdm13-GFP* heterozygous and homozygous mice at E17.5 (Fig 2.2), around the peak of amacrine cell genesis[212]. The pattern of GFP immunostaining in both *GFP/+* and *GFP/GFP* mice at E17.5 mirrored that seen in wild-type (+/+) retinas labeled with anti-PRDM13 antibodies (Fig 2.1 and data not shown). GFP labeled the cytoplasm, revealing

apical and basal processes in both heterozygous and mutant animals (Fig 2.2). Little if any staining was seen in the ganglion cell layer. There was appreciable basal clustering, with more staining in what will become the inner nuclear layer (Figs 2.2B-E). GFP intensity levels were lower in *GFP/+* mice compared to homozygous mutants, but the total number of GFP+ cells did not vary between the two populations (Fig 2.2F) (N=6, t-test, p=0.9366), suggesting that *Prdm13* is neither required for its own expression nor to maintain cell survival at E17.5.

Next, we stained transgenic mice with antibodies against PRDM13 (Figs 2.2B-C). PRDM13 immunostaining overlapped highly with GFP in *Prdm13* heterozygous mice (Figs 2.2B, G). Some GFP+ cells in the nascent inner nuclear layer lacked PRDM13 staining. This population likely represents neurons that recently inactivated PRDM13 expression, but remained labeled due to the long half-life of GFP. PRDM13 immunostaining was completely absent from *Prdm13-GFP/GFP* null mice (Figs 2.2C, F-G), demonstrating the specificity of the antibody. The number of PRDM13+ cells was equivalent between *Prdm13* heterozygous and wild-type mice (N=6, t-test, p=0.8627) (Fig 2.2G and data not shown).

To determine whether changes in cell fate occurred in *Prdm13* mutants, we examined E17.5 sections with antibodies against OTX2 to mark developing photoreceptors. The immunostaining pattern and intensity of OTX2+ nuclei was unchanged between *Prdm13* heterozygous and homozygous mutant retinas (Figs 2.2B-E). Consistent with our observations above (Fig 2.1), a small number of PRDM13-GFP+ cells co-expressed OTX2 in both heterozygous and mutant animals (Figs 2.2B-E). Nonetheless, the number of these double labeled cells was unchanged between heterozygous and homozygous mice. This suggested that there was no change in photoreceptor genesis in *Prdm13* knockout animals. We then immunostained retinas with antibodies against PTF1a to determine whether *Prdm13* affects the

formation of horizontal and amacrine precursor cells (Figs 2.2D-E). The PTF1a spatial labeling pattern was similar between *Prdm13* heterozygous and null animals (Figs 2.2D-E). The total number of PTF1a+ cells was equivalent between wild-type, heterozygous, and *Prdm13* homozygous mutants (N=8, ANOVA, p=0.8131) (Fig 2.2H). There were no differences in the number of Pft1a+/GFP+ cells between our lines (N=5, t-test, p=0.7193) or the fraction of PTF1a+ cells that co-expressed GFP (N=5, t-test, p=0.3805) (Figs 2.2I-J). Similarly, we observed no differences in the number of cells that co-expressed PTF1a and OTX2 between the three genotypes (N=8, ANOVA, p=0.2510) (Fig 2.2K). These data argue that *Prdm13* is not required for the formation of amacrine and horizontal cell precursors. Though the number of PTF1a cells was unaltered in mutants, the PTF1a staining was typically more intense. This suggests that PRDM13 mediates a negative feedback loop onto *Ptf1a* in the retina, as has been observed in the spinal cord[217]. Lastly, we examined retinas with antibodies against BRN3 to mark ganglion cells, but saw no differences in their numbers between genotypes (data not shown). Taken together, our data suggest that the loss of *Prdm13* does not alter the balance of cell fates formed during embryonic retinal development.

*PRDM13 Labels a Subset of Amacrine Cells in the Adult Retina*

PTF1a is an early postmitotic marker for amacrine and horizontal cell precursors that is necessary for their development[108]. Although PRDM13 initially overlaps with PTF1a, as development progressed PRDM13+/PTF1a negative cells became localized to the nascent inner nuclear layer. This suggested that PRDM13 expression remained in subsets of amacrine or horizontal cells. To test this, we immunostained mature (P30) *Prdm13 GFP/+* retinas for several amacrine and horizontal markers (Fig 2.3). Nearly all of the GFP+ cell bodies were located in the

inner aspect of the INL with cell processes extending into the inner plexiform layer (IPL). There were also a few GFP+ cell bodies within the GCL (Fig 2.3). Immunostaining with PRDM13 antibodies overlapped with the GFP+ somas in heterozygous animals (data not shown). PRDM13 immunostaining was not as robust in adult animals, so we used GFP staining to better characterize the PRDM13+ population. Immunostaining for the pan-amacrine marker PAX6 revealed that 100.0% (± 0.0% SD) of GFP+ cells co-expressed PAX6+ (Fig 2.3E and data not shown). However, only a subset of PAX6+ cells in the INL were GFP positive. These GFP+ cells accounted for 38.6% (± 2.9% SD) of all PAX6+ cells in the INL, suggesting that PRDM13 marks a subset of amacrine cells (Fig 2.3F). No GFP+ cells co-expressed the photoreceptor and bipolar cell marker OTX2 (Fig 2.3E) or the ganglion cell marker BRN3 (data not shown). We then immunostained retinas with markers that define subsets of amacrine and other interneuron populations in the retina. The transcription factor AP2a marks a large subpopulation of amacrine cells (Bassett et al., 2007). We observed that AP2a co-stained 31.3% (± 3.7% SD) of GFP+ cells (Figs. 3A, E). The transcription factor BHLHB5 marks type II cone OFF bipolars and subsets of GABAergic and other amacrine cells[44,108]. BHLHB5 marked 34.9% (± 8.6% SD) of GFP+ cells (Figs 2.3A, D-E). In both cases, only subsets of AP2a+ (23.2% ± 3.5% SD) and BHLHB5+ (27.1% ± 6.5% SD) cells co-expressed GFP (Fig 2.3F and data not shown), reflecting the highly heterogeneous nature of amacrine cells and these markers. Calretinin and calbindin each mark complex subsets of amacrine cells in the mouse[247]. Calretinin stains amacrine and ganglion cell somas in the INL and GCL, as well as three highly stereotypical dendritic sublaminae within the IPL[247,248]. We observed that about half of PRDM13-GFP+ cells co-expressed calretinin (50.3% ± 9.2% SD) (Figs 2.3B, E). In contrast, GFP+ cells rarely co-expressed calbindin (0.6% ± 1.3% SD) and none of the intensely calbindin labeled horizontal cells made GFP (Figs 2.3B, E)[249]. Co-

staining with the glycinergic marker GlyT1 and the GABAergic marker GAD65/67 revealed that most of the PRDM13-GFP+ cells were glycinergic (Figs 2.3C, E)[247,249,250]. GlyT+ glycinergic amacrines accounted for 44.7% (± 9.2% SD) of GFP+ cells, while GABAergic GAD+ cells accounted for only 2.8% (± 2.4% SD) (Fig 2.3E). These percentages may be underrepresented as not all amacrine somas were robustly labeled with these antibodies. Nonetheless, there is a clear preference for glycinergic overlap, consistent with the paucity of GFP+ displaced amacrine cells. We examined cholinergic amacrines by co-staining with Sox2 antibodies[211,251,252] (data not shown). We did not observe any overlap of GFP with Sox2 (Fig 2.3E), showing that PRDM13 does not mark cholinergic amacrine cells. Similarly, we observed no overlap with vGlut3, which marks a small population of mostly glycinergic amacrines or with the dopaminergic amacrine marker TH (data not shown). Lastly, we examined PRDM13-GFP/+ retinas with antibodies to EBF3[43,212]. EBF3 marks glycinergic and nGnG amacrines along with subsets of ganglion cells[40,253]. Many GFP+ cells co-expressed EBF3 (70.4% ± 7.6% SD) (Figs 2.3D-E). To eliminate the possibility of counting EBF3+ ganglion cells, we narrowed our quantification parameters to the EBF3+ cells within the INL and found that 100% (± 0.0% SD) of them co-expressed GFP (Fig 2.3F). This suggests that *Prdm13* marks the entire cohort of EBF3+ amacrines, while also marking a smaller diverse set of non-EBF3+ amacrine cells.

*EBF3+ Amacrine Cells are Absent from Prdm13 Mutants*

Many amacrine subtype differentiation markers appear postnatally. For example, EBF3 expression in amacrine cells is first seen at P4[40]. To overcome the neonatal lethality of *Prdm13-GFP/GFP* null mice, we took advantage of a *Prdm13* allele with a 115bp deletion in the first exon (Fig 2.4A)[217]. This *Prdm13-Δ115* modification was predicted to result in a frame shift with

early truncation of the PRDM13 protein (Fig 2.4A). However, homozygous *Prdm13-Δ115* mice express some PRDM13 protein and were viable, suggesting that the 115bp deletion created a hypomorphic allele[217]. We crossed the *Prdm13-GFP* mouse line with *Δ115* to create mice that had severely reduced *Prdm13* function. These *GFP/Δ115* mice were viable, and adults had a conspicuous loss of GFP+ cells (3.97 cells/100µm ± 1.07 SD) compared to *Prdm13-GFP/+* heterozygotes (16.06 cells/100µm ± 2.66 SD) (Figs 2.4B-C). The GFP+ cells remaining in *GFP/Δ115* mice were localized to the INL and generally had larger cell bodies compared to heterozygous control retinas (Fig 2.4B). We next examined whether this loss of roughly 12 GFP+ cells/100µm was due to a reduction in amacrine cells or GFP expression. PAX6 immunostaining revealed no changes in the number of labeled cells in the GCL (N=6, t-test, p=0.299), but there was a significant decrease in PAX6+ INL cells from 39.68 (SD ± 3.24) cells/100µm in control mice to 31.11 (SD ± 5.71) cells/100µm in *GFP/Δ115* mice (N=6, t-test, p=0.028) (Fig 2.4M and data not shown). This loss of PAX6 staining in the INL was similar in magnitude to the GFP reduction, suggesting that a subset of PRDM13+ amacrine cells were lost in *GFP/Δ115* mice. We then examined whether *Prdm13* perturbation affected specific amacrine subtypes. Immunostaining for GABAergic and glycinergic amacrines revealed no changes in the number of GAD+ cells, but a modest decrease in GlyT+ cells was observed in the *GFP/Δ115* mice (N=9, t-test, p=0.014) (Figs 2.4D-E, M). More conspicuous was the reduced number of GFP+ cells that co-expressed GlyT (N=9, t-test, p=<0.001) (Figs 2.4D-E, N). This suggested that the glycinergic amacrines that normally co-express PRDM13 were selectively reduced in mutants. We observed a slight increase in the number of GFP+ cells that co-expressed GAD65 in *GFP/Δ115* mice (N=9, t-test, p=0.014), but this modest change was not enough to alter the overall number of GABAergic amacrines in the retina (Figs 2.4D-E, M-N). The total number of

AP2a and BHLHB5+ amacrines was significantly reduced in *GFP/Δ115* mice compared to *GFP/+* controls (Figs 2.4F-G, M). The number of GFP+ cells that co-expressed AP2a or BHLHB5 decreased proportionately in *GFP/Δ115* animals (Figs 2.4F-G, M-N). These data suggest that the PRDM13+ subpopulations of AP2a+ and BHLHB5+ amacrines were specifically lost in mutants.

PRDM13 marked large fractions of calretinin and EBF3 expressing amacrine cells in adult retinas (Fig 2.3). As expected, calretinin staining was strikingly different between heterozygous control and *GFP/Δ115* mice (Figs 2.4H-I). There was a conspicuous loss of cell bodies and a change in the distribution of dendritic staining in the IPL (Figs 2.4H-I, L). Calretinin positive somas in the INL decreased from 13.47 (± 3.13 SD) cells/100µm in controls to 9.89 (± 1.52 SD) cells/100µm in the *GFP/Δ115* mice (N=9, t-test, p=0.0019) (Fig 2.4M). There was nearly a total loss of GFP+/calretinin+ cells in the *GFP/Δ115* mice (N=9, t-test, p<<0.001) (Figs 2.4H-I, N). Calretinin strongly marks three (2, 3, and 4) of the five synaptic sublaminae of the IPL[247,253]. Heterozygous control mice displayed this trilaminar calretinin pattern, while the *GFP/Δ115* mice had a thinner IPL that contained only two sublaminae (Figs 2.4H-I, L). The cholinergic amacrine marker ChAT, which labels sublaminae 2 and 4 was normal in *GFP/Δ115* mice (data not shown)[247,254]. There was a minor increase in the number of GFP+ cells that co-expressed calbindin in *GFP/Δ115* mice, but the IPL staining of sublaminae 2 and 4 was normal (Figs 2.4H-I, M-N). These data argue that calretinin positive cells that project to sublamina 3 are lacking in *GFP/Δ115* mice (Fig 2.4L)[247]. Since essentially all EBF3+ amacrine cells co-expressed PRDM13, we expected this population to be the most disrupted in *GFP/Δ115* retinas. Indeed, we observed a nearly complete loss of EBF3+ cells from the INL of these mutants (Figs 2.4J-K, M). This loss of about 10 EBF3+ amacrine cells/100µm was nearly

the same as the loss of PAX6+ INL cells in *GFP/Δ115* mice (Fig 2.4M). Consistent with an amacrine cell-specific deficit, the number of EBF3+ cells in the GCL was unchanged (N=9, t-test, p=0.26). The loss of both EBF3+ and calretinin+ cells suggested that these populations overlap extensively. In adult wild-type mice, we observed that 72.2% (± 12.3% SD, N = 4) of calretinin+ cells in the INL co-expressed EBF3+ and that 64.4% (± 7.0% SD, N = 4) of EBF3+ cells in the INL were calretinin+ (Fig S2.1). Taken together, these data argue that *Prdm13* is required for the formation or survival of EBF3+/calretinin+ cells.

*Loss of the PRDM13+ Amacrine Population Begins at P5*

We did not observe a change in GFP+ cells in E17.5 *Prdm13* mutant mice (Fig 2.2), but P30 *GFP/Δ115* mice had considerably fewer GFP+ cells and about ~25% fewer amacrines (Fig 2.4). There are three general mechanisms that account for this reduction in cell numbers. These include: (1) a reduction in the number formed during development, (2) altered amacrine subtype fate choice, and (3) cell death. To distinguish between these possibilities, we examined mice at intermediate developmental time-points (Fig 2.5). We stained E17.5 *GFP/+* and *GFP/GFP* mice for PAX6 and observed no significant differences in cell number (N=4, t-test, p=0.62) (Figs 2.5A-B). Next, we compared the number of intensely PAX6+ INL cells between *GFP/+* and *GFP/Δ115* retinas at P2, the end of amacrine cell genesis. We observed no statistically significant differences (N=2, t-test, p=0.98) between the genotypes, arguing that amacrine fate specification as a class was unaltered by the loss of *Prdm13* (Figs 2.5C-D).

Many amacrine subtype-specific markers become expressed in the first postnatal week, including EBF3 starting at P4[40]. We next examined P5 GFP/+ control and *GFP/Δ115* mutant retinas for the numbers of GFP, EBF3, and BHLHB5 positive amacrine cells. At P5, nascent IPL

lamination was less delineated and thinner in *GFP/Δ115* retinas (Figs 2.5E-F). Nonetheless, we observed only a modest decrease (N=7, t-test, p=0.027) in the number of GFP+ cells in mutant retinas compared to heterozygous controls (Figs 2.5E-F, I). EBF3 staining was strongly reduced in the INL of *GFP/Δ115* mice, but was abundant in the GCL of both controls and mutants (Figs 2.5E-F). Both control and mutant EBF3+ cells in the INL co-expressed GFP, but there were far fewer EBF3+ nuclei in *GFP/Δ115* retinas (Figs 2.5E-I). The near absence of EBF3+ amacrines in adult mice is also seen at P5, suggesting that EBF3+ cells are not formed in *GFP/Δ115* mice. While the overall number of BHLHB5+ amacrines did not change significantly, more of these cells co-expressed GFP in P5 mutants compared to controls (Figs 2.5E-I). This change suggests that some of the PRDM13-GFP+ cells that would have adopted EBF3+ subtype identity failed to do so and instead express BHLHB5 and perhaps other subtype markers. This is further supported by the modest loss of P5 GFP+ cells compared to adult *GFP/Δ115* retinas. We reasoned that inappropriately specified cells may undergo apoptosis. Staining for activated caspase 3 (AC3) at P5 revealed no appreciable overlap with GFP in control mice, but 28.1% of dying AC3+ cells co-expressed GFP+ in *GFP/Δ115* mutants (N=4, t-test, p=0.029) (Fig 2.5J and data not shown). These data suggest that *Prdm13* is required for EBF3+ amacrine subtype formation and survival.

*Prdm13 Overexpression is not Sufficient to Drive Ectopic Amacrine Formation*

We observed that *Prdm13* is not required for amacrine cell generation as a class. Nonetheless, it could play a redundant role in amacrine genesis and an instructive role in subtype formation. We hypothesized that ectopic expression of *Prdm13* would promote amacrine formation, and in particular, subtypes that express EBF3 and/or calretinin. To test this, we created plasmid expression vectors to drive *Prdm13* (WT), which has been shown to act as a

transcriptional repressor[217,255]. We also created a vector to express a *VP16* fusion with *Prdm13* (VP16) to convert it into a transcriptional activator [216]. Each of these vectors contains the *Ef1α* enhancer to drive ubiquitous expression and an *IRES-Cre* cassette for indirect detection. As a control, we used an *Ef1α* driven nuclear cherry plasmid [256]. Constructs were electroporated into newborn retinas and cultured for 2 or 7 days *in vitro* (DIV) as intact explants. Electroporation preferentially affects retinal progenitor cells, which give rise to photoreceptors and to a lesser extent bipolars, glia, and amacrine cells at this stage[82,85]. Electroporated explants were stained for PAX6 and OTX2 (Fig 2.6) as they mark all cell types (OTX2- photoreceptors and bipolars, PAX6- horizontals, amacrines, glia, and ganglion cells) in the mature retina. At both 2DIV and 7DIV, control cherry cells detected with anti-red fluorescent protein (RFP) antibodies were overwhelmingly OTX2+ photoreceptors and bipolar cells, consistent with the fate distribution of newborn progenitors[82] (Figs 2.6A, D). Only about 20% of electroporated control cells made PAX6, indicative of an amacrine or glial identity (Figs 2.6A, E). In contrast to cherry controls, both PRDM13 WT and VP16 electroporated cells were localized to the nascent INL and were much more likely to co-express PAX6 (N = 26, ANOVA, P=0.0003) than OTX2 at 2 DIV (Figs 2.6A-E). At 2DIV, both WT and VP16 had significantly fewer OTX2+ cells and significantly more PAX6+ cells than cherry control. At 7DIV the overall pattern was similar, such that PRDM13 WT and VP16 transfected cells co-expressed PAX6 more frequently than cherry controls (N=24, ANOVA, 0.0111) (Figs 2.6D-E). However, it was apparent at 7DIV that many PRDM13-VP16 transfected cells co-expressed both OTX2 and PAX6 (Figs 2.6C, D-F). The co-expression of OTX2 and PAX6 was seldom observed in cherry control or PRDM13 WT transfections (Fig 2.6F). Some retinal progenitors appear to transiently co-express PAX6 and OTX2 raising the possibility that VP16 cells remain as undifferentiated progenitors[127,243],.

Alternatively, the VP16 fusion may lead to the inappropriate activation of photoreceptor genes, like *Otx2*. PRDM13 WT transfected cells differed from VP16 and control cells in another way. Many PRDM13 WT transfected cells failed to express either PAX6 or OTX2 (Fig 2.6B-B’’’, inset). These non-PAX6 non-OTX2 cells of unknown identity accounted for nearly 30% of PRDM13 WT transfected cells at 7DIV (Figs 2.6B-B’’’, data not show). Non-PAX6, non-OTX2 electroporated cells were absent from 7DIV Cherry control and PRDM13 VP16 transfections.

The presence of OTX2+/PAX6+ cells in VP16 electroporations and the non-PAX6 non-OTX2 cells in PRDM13 WT conditions raised the possibility that these constructs were deleterious to cell survival. Accordingly, both PRDM13 WT and VP16 fusion transfections resulted in sparse numbers of Cre+ electroporated cells at both 2DIV and 7DIV compared to cherry control transfections (Figs 2.6B-F and data not shown). We observed this reduction at 1, 3, 4, and 10DIV as well (data not shown). Moreover, our initial electroporation experiments with higher concentrations of PRDM13 WT and VP16 plasmids resulted in even fewer Cre+ cells after only 1DIV (data not shown). These findings suggested that overexpression of PRDM13 WT and VP16 conferred an immediate survival disadvantage to transfected cells. The bias towards PAX6 expression and INL localization suggested that both PRDM13 WT and VP16 were especially toxic to OTX2+ photoreceptors and bipolar cells. We also searched for upregulation of EBF3, calretinin, calbindin, and BHLHB5 at 2, 3, 4, and 7DIV. However, we did not observe any ectopic expression of amacrine markers at these time-points (data not shown). Taken together, our data suggest that *Prdm13* is not sufficient to drive ectopic amacrine cell formation in the newborn retina. Instead, *Prdm13* appears to be toxic to nascent photoreceptors.

## Discussion

How the developing retina allocates a set number of amacrine cells and diversifies them into 30+ subtypes is only partially understood. We investigated the transcription factor *Prdm13* and found that it was expressed broadly in developing amacrine cell precursors and became restricted to a heterogeneous subset of amacrines in mature mice. Normal numbers of amacrine cells formed in *Prdm13* mutant mice, but subtype specification was altered in the early postnatal period. EBF3+ amacrine cells were absent and about 25% of amacrine cells were subsequently lost to cell death. Our data show that *Prdm13* does not control amacrine cell genesis as a class, but is instead necessary for subtype fate choice and cell survival. Future work is needed to uncover how *Prdm13* regulates the formation of EBF3+ amacrines and how individual subtypes within this heterogeneous population function in the retina.

### *Prdm13 is Not Necessary for Amacrine Identity*

We observed that *Ptf1a* mutant retinas lack *Prdm13* expression[216]. Therefore, we expected PRDM13 to function downstream of PTF1a in the developing retina. Consistent with this model, 100% of PRDM13+ cells co-expressed PTF1a at E13.5. In the spinal cord, PTF1a drives *Prdm13* expression, which then feeds back to inhibit *Ptf1a* expression[217,257]. This negative feedback loop is also present in the retina since *Prdm13* mutant mice had more intensely stained PTF1a+ cells. Despite this expression increase, *Prdm13* loss did not change the number of PTF1a+ cells in the retina or the number of amacrine cells that were initially formed. This suggests that feedback is important for controlling *Prdm13* levels and in turn, amacrine subtype fate choice and survival (see below). Despite PRDM13 being expressed at early stages in PTF1a+ cells, it was not required for the formation of amacrine or horizontal cell precursors.

Since *Prdm13* acts as a repressor, we reasoned that it acts by blocking competing cell identities in multipotent precursors[216,217]. This was reinforced by our observation that some PTF1a+ and PRDM13+ cells transiently co-expressed OTX2. This overlap is consistent with data suggesting that OTX2+ cells can adopt amacrine and horizontal cell identities[167,231,244,245]. It has been shown that the PTF1a+ lineage contains only horizontal and amacrine cells[108]. We hypothesized that PRDM13 represses *Otx2* expression in PTF1a+ cells to restrict fate choice. However, we did not observe an increase in OTX2+ cells in *Prdm13* mutants or a fate shift to photoreceptors or bipolar cells. This shows that *Prdm13* is not required to suppress photoreceptor identity in PTF1a+ cells. Interestingly, PRDM13-VP16 activator misexpression increased the number of PAX6+ cells that co-expressed OTX2. This raises the possibility that PRDM13 normally inhibits *Otx2* expression in the developing retina. For this to be true, other factors must compensate for or act redundantly with *Prdm13* to suppress *Otx2* expression. Since *Ptf1a* misexpression can promote amacrine identity at the expense of photoreceptors and bipolar cells fate restriction appears to be downstream of PTF1a. The transcription factors AP2a (*Tfap2a*) and AP2b (*Tfap2b*) are both decreased in *Ptf1a* mutants[218,258]. Gain-of-function analysis showed that these factors can promote amacrine formation[258]. However, deletion of both genes simultaneously resulted in only a modest amacrine phenotype and there was no appreciable fate shift to OTX2+ photoreceptors or bipolar cells[97]. It remains unclear how PTF1a+ cells are restricted to horizontal and amacrine cell fates and whether *Prdm13* plays a redundant role in this process.

*PRDM13 Marks Multiple Subtypes of Amacrine Cells*

Amacrine cells are highly diverse, with estimates of 30 or more discrete subtypes in the

mouse retina. About one third of amacrine cells were marked by PRDM13 in the adult retina. PRDM13 labeled a heterogeneous group of glycinergic and nGnG amacrines, but did not mark cholinergic, dopaminergic, or glutamatergic amacrine subtypes. Recent reports have also shown that PRDM13 is made by subsets of amacrine cells. In mice and frogs, most PRDM13+ cells are glycinergic[218,257]. The frog retina has more PRDM13+ cells that are GABAergic compared to mice[218,259]. Our characterization of subtype markers is similar to those described previously in mice, except that we observed far fewer PRDM13+ cells that co-expressed calbindin or GAD65[218]. The reason for these discrepancies is unclear, but may involve differential sensitivities of antibodies used in each study. We examined additional subtype markers, including BHLHB5 and EBF3, to further probe the diversity of PRDM13+ amacrine cells. Strikingly, we found that EBF3+ cells are the only amacrine population that is entirely PRDM13 labeled.

Our experiments revealed that nearly 75% of PRDM13+ amacrine cells co-expressed EBF3. The EBF3+ population is itself heterogeneous, representing a 3-to-1 mix of glycinergic and Neurod6+ nGnG subtypes[40]. The glycinergic subpopulation has narrow, multistratified dendritic fields that project to sublaminae 1-4 in the IPL[40]. The nGnG EBF3+ population has similar morphology, but projects dendrites to sublaminae 1-3[40]. We observed a small number of EBF3+ amacrines that co-expressed BHLHB5, raising the possibility that EBF3+ amacrines can be divided into additional subtype groups. This is supported by single cell profiling experiments that identified three EBF3+ clusters (one glycinergic and two nGnG) in the retina[260]. About one fourth of the PRDM13+ amacrines did not co-express EBF3. There was no conspicuous marker that labeled all of these cells, suggesting they are a heterogeneous population. This likely includes the small number of GABAergic amacrines, the bulk of the BHLHB5+ subtypes, and

additional glycinergic and nGnG amacrines. Going forward, intersecting PRDM13 with other subpopulation markers may uniquely define individual amacrine subtypes and facilitate experiments to uncover their physiology.

*Prdm13 effects amacrine subtype specification*

Despite expression at early time-points in PTF1a+ cells, *Prdm13* mutants had no discernable phenotypes embryonically. In fact, there were no conspicuous changes in *Prdm13* loss-of-function mice until P5. This is after the timing of amacrine cell birth, but overlaps with subtype maturation and culling of excess generated amacrines[40,85,211,212,261–263]. At P5, *Prdm13-GFP/Δ115* mice showed increased apoptosis and largely lacked EBF3+ amacrines. GFP+ cells in these mice were more likely to co-express BHLHB5 than controls, suggesting that subtype specification was altered. This supports a model where *Prdm13* is required for EBF3+ amacrine subtype specification. By the adult stage *Prdm13-GFP/Δ115* mice have fewer amacrines and essentially lack EBF3+ subtypes. This argues that *Prdm13* is also required for the survival of EBF3+ amacrine cells. It is difficult to determine whether *Prdm13* controls amacrine subtype specification, survival, or both. One possibility is that EBF3+ amacrines are still specified in mutants, but die without *Prdm13* due to derepression of genes made by other subtypes. Another possibility is that EBF3+ amacrines fail to become specified in mutants. This is consistent with our observations of a significant reduction of EBF3 expression that precedes cell death. In the absence of subtype specification, cells could die due to a lack of identity or they could adopt a different subtype choice. Though calbindin+ amacrines increased subtly in mutants, most amacrine types profiled (including BHLHB5+ cells) decreased modestly. This argues against a fate shift; however, upwards of 50% of amacrines are normally culled during the early postnatal

period[261–264]. Thus, an excess of improperly specified amacrine subtypes could be masked by apoptosis. In the frog, *Prdm13* morpholinos reduced glycinergic amacrines without increasing GABAergic numbers[259]. Similarly, Watanabe and colleagues observed a reduction of glycinergic amacrines without an increase in other subtypes in *Prdm13* mutant mice carrying a distinct allele to those used here[218]. Taken together, these loss-of-function data are consistent with roles for *Prdm13* in both subtype specification and survival.

Gain-of-function experiments suggest a more active role for *Prdm13* in fate choice. Overexpression of *Prdm13* in the frog retina biased cells towards glycinergic amacrine fate at the expense of bipolar cells and glia[259]. Watanabe and colleagues overexpressed *Prdm13* in newborn mice and observed that most transfected cells adopted amacrine fate[218]. Moreover, *Prdm13* overexpression modestly increased the fraction of transfected cells that expressed calretinin and/or calbindin[218]. These findings argue that *Prdm13* is sufficient to specify amacrine type and subtype identity. Based on these findings, we expected *Prdm13* overexpression to drive ectopic EBF3+/calretinin+ amacrine cell formation. Though we observed that *Prdm13* overexpressing cells were more likely to make PAX6, they did not ectopically express EBF3 or calretinin. Thus, *Prdm13* is not sufficient to instruct EBF3+ amacrine subtype formation. We also observed a strong reduction in the number of Prdm13 transfected cells compared to control. Many transfected cells failed to express OTX2 or PAX6, which mark progenitors and specified retinal cells. These double negative cells are likely poised for apoptosis. Our data suggest that *Prdm13* overexpression is especially toxic to developing photoreceptors and to a lesser extent, progenitors and other cell types. Thus, we may be observing selection instead of fate changes in these gain-of-function experiments. Overexpressing PRDM13-VP16 also appeared toxic in the retina. This toxicity is likely caused by a different mechanism because PRDM13-VP16 increased

the fraction of cells that co-expressed OTX2 and PAX6. This forced activator activity may upregulate competing gene regulatory networks and cause apoptosis. Due to the toxicity we observed, future experiments where *Prdm13* is specifically overexpressed in postmitotic amacrine cell precursors will reveal whether it instructs EBF3+ subtype identity.

North Carolina Macular Dystrophy (OMIM-136550) has been attributed to dominantly inherited mutations in *PRDM13*[129]. This includes duplication of the coding sequence and non-coding point mutations flanking *PRDM13*[129,265,266]. This disorder is developmental in nature and affects the structure of the macula, resulting in a highly variable loss of photoreceptors and central vision. It is unlikely that this represents haploinsufficiency, as *Prdm13* heterozygous mice have no overt deficits in development. Instead, the pathology is consistent with a *PRDM13* gain-of-function. Since PRDM13 is expressed in PTF1a+ committed amacrine and horizontal cell precursors, it seems unlikely that increased *PRDM13* dosage would negatively affect photoreceptor formation. Instead, mutations that drive ectopic *PRDM13* expression in progenitors or nascent photoreceptors could either bias their fate towards amacrine cell identity or cause toxicity. One of these non-coding mutations (V2) creates a potential *OTX2* binding site, which may create a photoreceptor-specific enhancer for *PRDM13*[129,267]. Our initial attempts to examine whether the non-coding mutations create novel retinal enhancers in developing mice were unsuccessful. Though our experiments suggest that *Prdm13* kills nascent photoreceptors, other experiments did not note toxicity[218,259]. Due to the heterogeneity of mutations and the disease severity, it seems likely that more than one mechanism underlies the pathophysiology of North Carolina Macular Dystrophy.

*Function of EBF3+ Amacrine Cells in the Retina*

We and others observed a decrease in the number of calretinin+ amacrines in *Prdm13* mutants[218]. Interestingly, calretinin staining of the IPL was altered such that the prominent sublamina 3 band was absent. This suggests that calretinin+ dendrites that project to sublamina 3 are absent or re-routed. We observed that many EBF3+ amacrine cells co-expressed calretinin (~62%). Since EBF3+ cells are essentially absent in *Prdm13* mutants, we reasoned that EBF3+/calretinin+ cells normally project dendrites to sublamina 3 of the IPL. Kay and colleagues showed that EBF3+ glycinergic amacrines (75%) projected to sublaminae 1-4, whereas EBF3+ nGnG amacrines (25%) have dendrites in sublaminae 1-3[40]. Overexpression of *Neurod6* increased the nGnG fraction of amacrines, creating prominent dendritic bands in sublaminae 1 and 3 of the IPL[40]. Thus, the loss of EBF3+ nGnG amacrines in *Prdm13* mutants may explain the loss of calretinin labeling of sublamina 3. Alternatively, calretinin+ cells that do not co-express EBF3 and project to sublamina 3 may be lost or misrouted. Lastly, it is possible that loss of EBF3+ and other amacrine subtypes in *Prdm13* mutants has a non-autonomous effect on the localization of calretinin+ dendrites.

The profound loss of EBF3+ amacrines and disruption of the IPL should alter the normal physiology of the retina. The complex nature of the cells lost and limited knowledge of amacrine physiology make this difficult to study. Watanabe and colleagues showed that scotopic and photopic electroretinography was normal in *Prdm13* mutants[218]. They also examined optokinetic reflex response behaviors. Interestingly, these behavioral tests showed that *Prdm13* mutant mice had greater spatial and contrast sensitivity than control mice[218]. Since EBF3+ amacrines are most disrupted in *Prdm13* mutants, the loss of these amacrines likely caused the observed sensitivity increases, ostensibly at the expense of other visual functions[268,269]. A more narrow dissection of

the amacrines disrupted in *Prdm13* mutants is needed to determine how defined amacrine subtype(s) contribute to visual behaviors.

**Chapter II Figures**

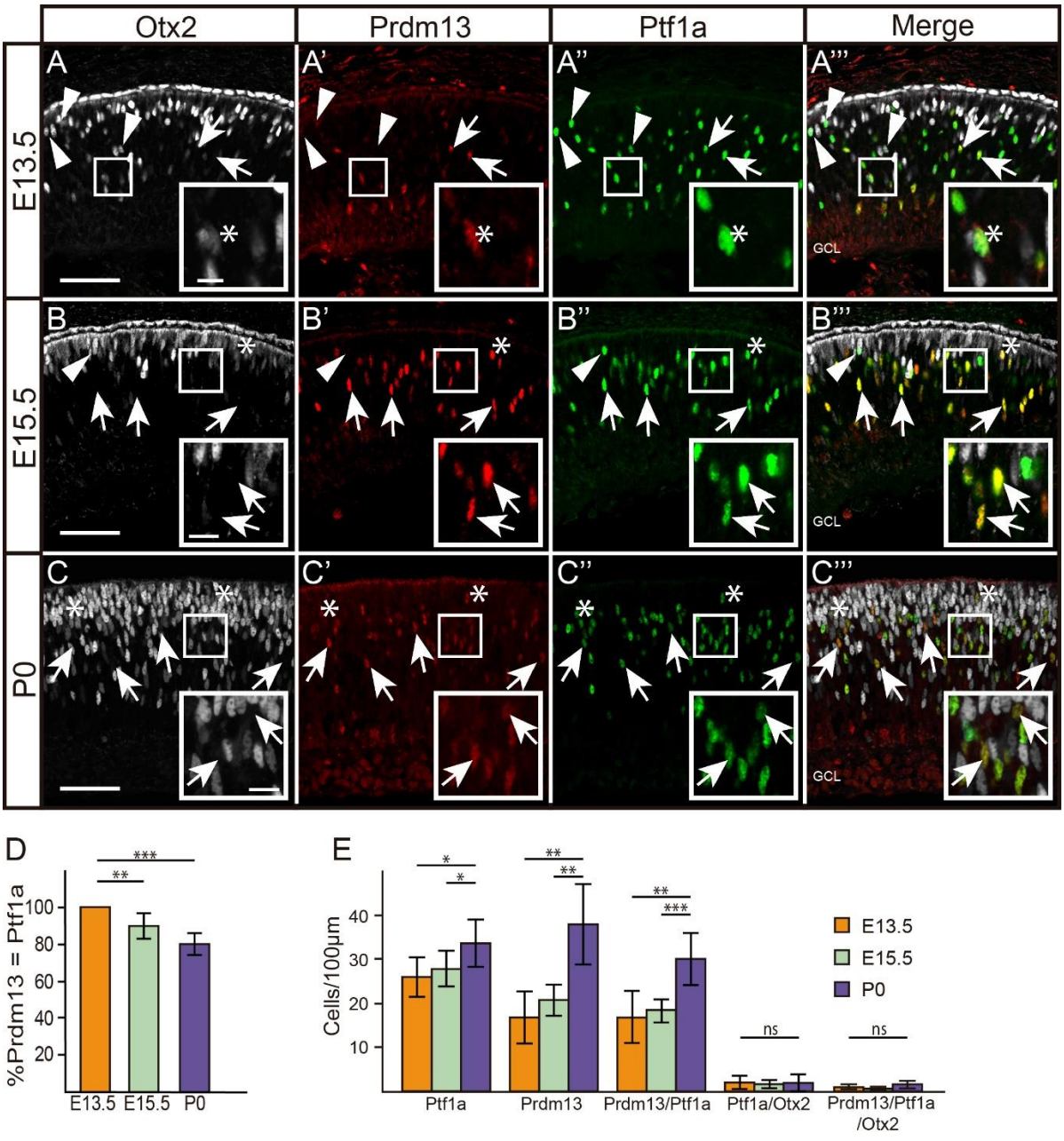



**Figure 2.1 OTX2, PTF1a, and PRDM13 expression within the developing mouse retina**

**(A-A’’’)** PRDM13+ cells (green) in E13.5 retinas overlap with PTF1a (red, arrows). PTF1a/OTX2 (grey, arrowheads) and PRDM13/PTF1a/OTX2 triple positive cells (stars) are always present in low abundance. **(B-B’’’)** A similar pattern of PTF1a, PRDM13 and OTX2 expression are seen at E15.5. **(C-C’’’)** At P0, more PRDM13+ cells are evident and these overlap less frequently with PTF1a (arrows). PTF1a/OTX2 (arrowheads) and triple labeled cells (stars) are present. **(D)** Plot of the percentage of PRDM13+ cells that co-express PTF1a over time. **(E)** Plot of the number of PTF1a, PRDM13, and OTX2 labeled cells at each time-point. Sample sizes at E13.5 and E15.5 are 4 mice each and 3 mice are quantified at P0. Statistical significance determined by unpaired two-sample t-tests: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Error bars represent standard deviation. Scale bar 50μm. Inset scale bar 10μm. GCL, ganglion cell layer; ns, not significant.

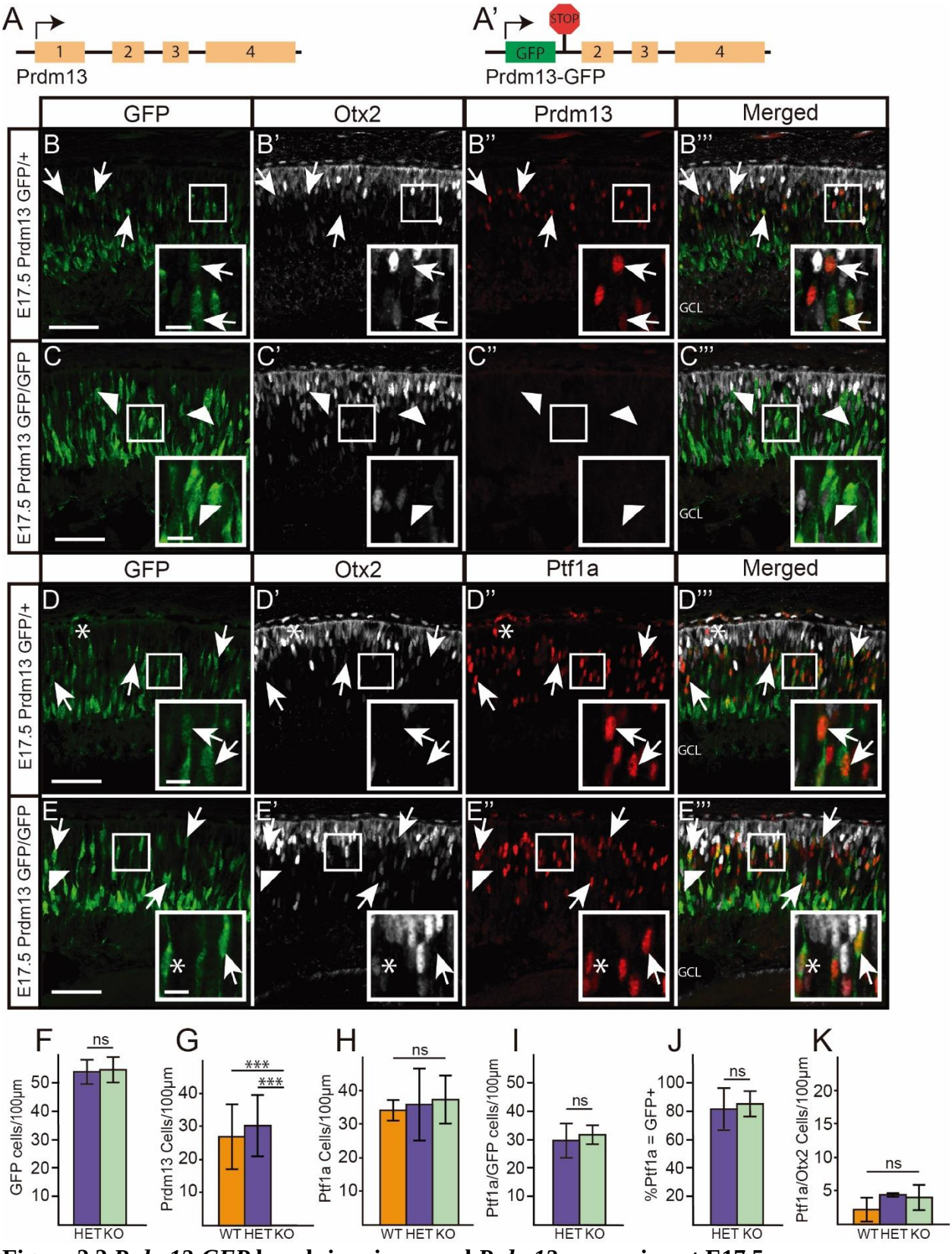



**Figure 2.2 *Prdm13-GFP* knock-in mice reveal *Prdm13* expression at E17.5**

**(A-A')** Schematic of wildtype (A) and *Prdm13-GFP* mice (A'). The first exon of *Prdm13* is replaced with GFP followed by a stop codon. Note that all homozygous *Prdm13-GFP/GFP* null mice die by birth. **(B-B''')** All PRDM13+ (red) cells co-express GFP (green, arrows) in E17.5 *GFP/+* mice. However, some GFP+ cells do not express PRDM13, likely because of the long half-life of GFP. **(C-C''')** Homozygous *GFP/GFP* mutants have a similar number of GFP+ cells compared to heterozygous controls, but lack PRDM13+ cells. A subset of PRDM13-GFP+ cells co-express OTX2 (grey, arrowheads). **(D-E''')** Heterozygous control (D) and mutant (E) retinas stained for GFP (green), OTX2 (grey), and PTF1a (red). The PTF1a and OTX2 staining patterns are equivalent between genotypes. Arrows mark PTF1a+/GFP+ cells, arrowheads mark OTX2+/GFP+ cells, and stars mark triple labeled cells. **(F-G)** Plots showing GFP+ cells in heterozygotes (HET) and *GFP/GFP* knock-outs (KO) (F), and PRDM13+ cells in WT, HET and KO animals (G). **(H-K)** Plots of the number of PTF1a+ cells (H), PTF1a/GFP double labeled cells (I), percentage of PTF1a that express GFP (J), and the number of PTF1a+/OTX2+ cells (K). Sample sizes are 3-4 mice per condition. Statistical significance determined by unpaired two-sample t-tests and 1-way ANOVA: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars represent standard deviation. Scale bar 50μm. Inset scale bar 10μm.

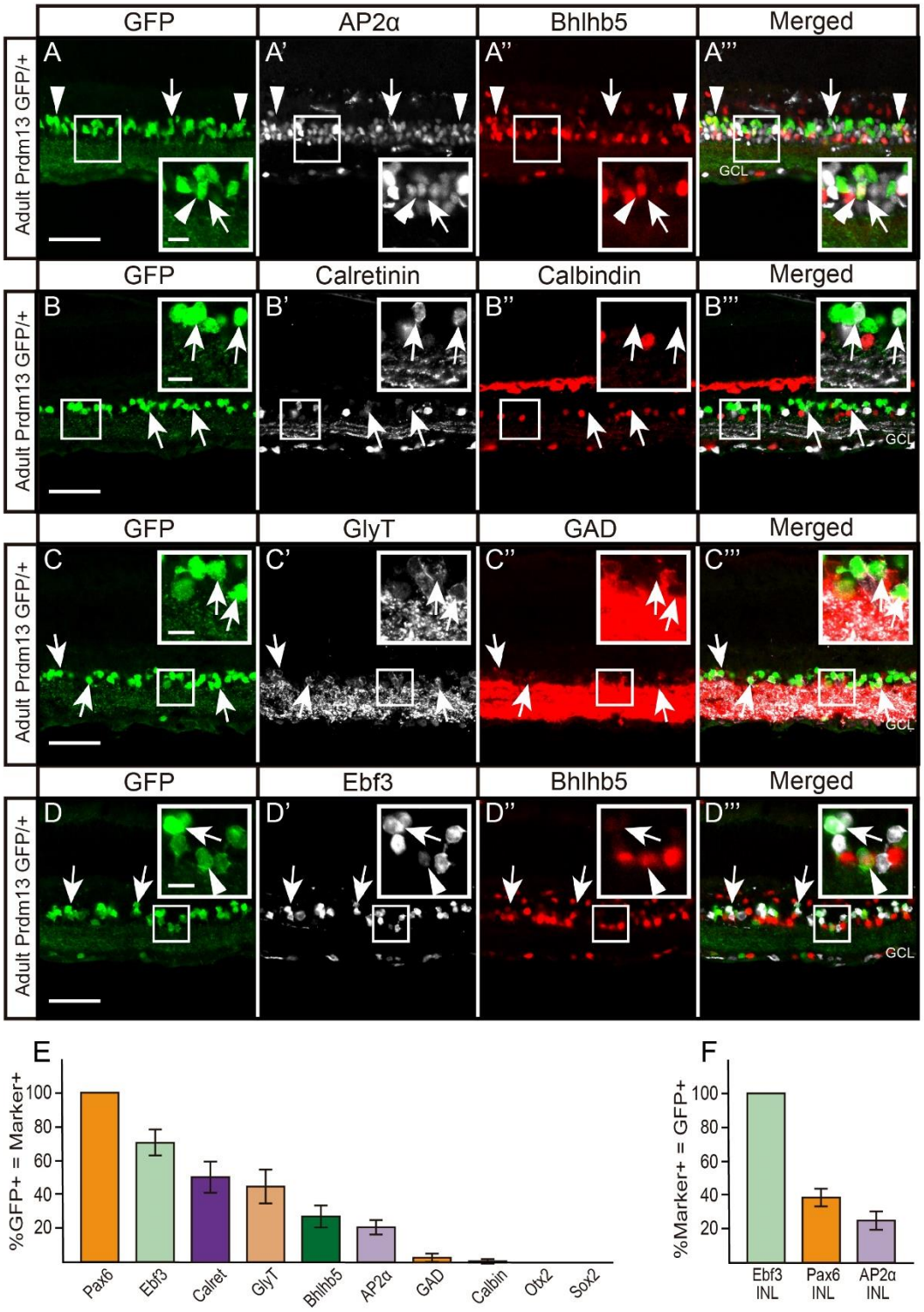



**Figure 2.3 PRDM13 marks a subset of amacrines in the adult retina**

**(A-D’’’)** P30 PRDM13-GFP/+ heterozygous animals co-stained with GFP (green) and amacrine cell markers (grey/red). **(A-A’’’)** A small subset of PRDM13-GFP+ amacrine cells co-express AP2α α(grey, arrows) and BHLHB5 (red, arrowheads). **(B-B’’’)** Nearly half of GFP+ cells co-express calretinin (grey, arrows), while less than 1% of GFP+ cells co-express calbindin (red). No intensely calretinin+ horizontal cells co-express GFP. **(C-C’’’)** A large fraction of GFP+ cells co-express the glycinergic amacrine marker GlyT (grey, arrows), but few GFP+ cells co-express the GABAergic marker GAD65/67 and these were sometimes GlyT+ as well. **(D-D’’’)** The majority of GFP+ cells co-express EBF3 (grey, arrows), but all EBF3+ cells in the INL are GFP+. A minority of GFP+ cells co-express BHLHB5 (red, arrowheads) and cells that co-express BHLHB5, EBF3, and GFP are rarely seen. **(E)** Plot of the percentage of GFP+ cells that co-express a given marker. GFP does not overlap with BRN3, TH, or vGlut3 (not shown). **(F)** Plot showing the percentage of EBF3+ amacrines, PAX6+ INL nuclei, and AP2α+ INL cells that co-express PRDM13-GFP. Sample size was 3 mice per condition. Statistical significance determined by unpaired two-sample t tests: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars represent standard deviation. Scale bar 50µm. Inset scale bar 10µm. INL, inner nuclear layer.

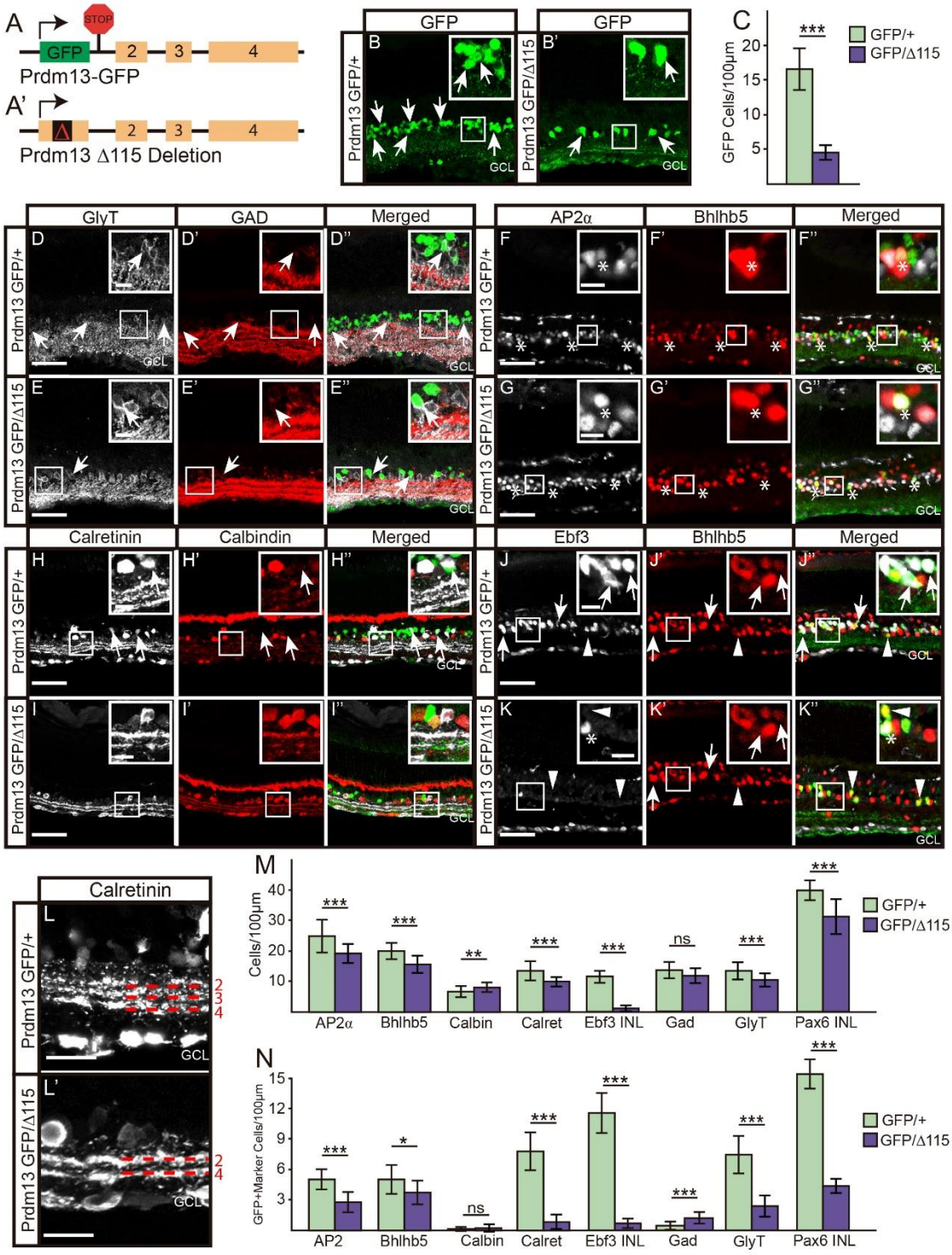



**Figure 2.4 EBF3+ amacrines are lost in adult *Prdm13* mutants**

**(A-A')** Schematics of the two transgenic mice, *Prdm13-GFP* (A) and *Prdm13-Δ115* (A'). The *Δ115* allele has an 115bp deletion in the first exon of *Prdm13*. Unlike homozygous *Prdm13-GFP* mice, homozygous *Δ115/Δ115* mice and compound heterozygous *Prdm13-GFP/Δ115* are viable. **(B-B')** *Prdm13-GFP/Δ115* mice (B') have far fewer GFP+ cells (arrows) than *Prdm13-GFP/+* control mice (B). **(C)** Plot showing the reduction in GFP+ cells between the two genotypes. **(D-K)** *Prdm13-GFP/+* and *Prdm13-GFP/Δ115* retinas stained for GFP (green) and various amacrine cell markers (red, grey). **(D-E'')** The number of GlyT (grey) and GAD+ (red) cells is modestly altered in *Prdm13-GFP/Δ115* mice (E) versus controls (D). The number of GlyT+/GFP+ cells (arrows) is reduced in *Prdm13-GFP/Δ115* mice. **(F-G'')** Staining for AP2α (grey) and BHLHB5 (red) show a small decrease in both populations in *Prdm13-GFP/Δ115* mice (E) compared to controls (F). Cells expressing GFP, AP2α, and BHLHB5 are marked by stars. **(H-I'')** Calbindin (red) staining is similar between genotypes and rarely overlaps with GFP, but calretinin (grey) stains revealed a disrupted IPL in *Prdm13-GFP/Δ115* mice. There are fewer calretinin+ cells in the INL and far fewer GFP+/calretinin+ cells in *Prdm13-GFP/Δ115* mice compared to *Prdm13-GFP* controls (arrows). **(J-K'')** EBF3+ (grey) amacrine cells are almost entirely absent from the INL in *Prdm13-GFP/Δ115* mice. GFP+/EBF3+ cells (arrows) are rare in *Prdm13-GFP/Δ115* mice (K) compared to control (J). Some BHLHB5+/GFP+ cells are seen in both conditions, some of which are EBF3+/BHLHB5+/GFP+ (stars). **(L-L')** Close up views of calretinin staining in control (L) and *Prdm13-GFP/Δ115* (L') retinas. Loss of sublamina 3 and a thinning of the IPL are evident. **(M)** Plot showing the number of marker positive cells in control and *Prdm13-GFP/Δ115* mice. **(N)** Plot showing the number of marker positive cells that co-express GFP. Sample sizes are 3 mice per condition for heterozygotes and 6 mice for mutants. Statistical significance determined by unpaired two-sample t tests: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bar 50μm, inset scale bar 10μm, scale bar for L 20μm. Error bars represent the standard deviation.

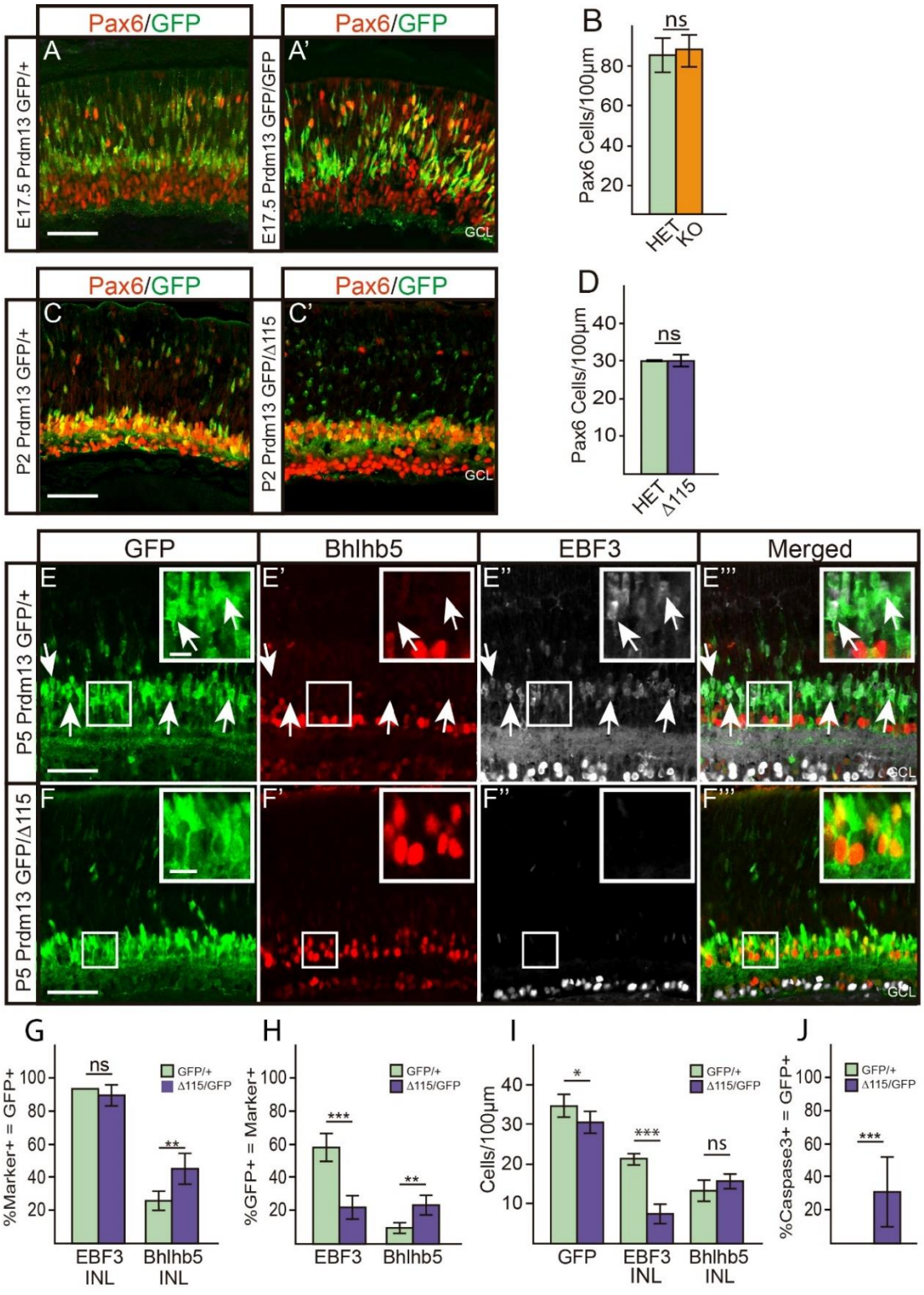



**Figure 2.5 Amacrine cells are initially formed, but subsets are lost in the first postnatal week of *Prdm13* mutants**

**(A-B)** At E17.5, there is no difference in PAX6 (red) numbers between *Prdm13-GFP/+* control (A) and *Prdm13-GFP/GFP* null mice (A'). GFP (green) staining is equivalent between these genotypes. **(C-D)** P2 PRDM13-GFP/+ (C) and *Prdm13-GFP/Δ115* mice (C') have equivalent GFP staining and PAX6 numbers. **(E-F''')** P5 *Prdm13-GFP/+* control (E) and *Prdm13-GFP/Δ115* (F) retinas stained for GFP (green), BHLHB5 (red) and EBF3 (grey). At this age, there are fewer GFP+ cells in *Prdm13-GFP/Δ115* retinas. Controls have many cells that co-express GFP and EBF3 (arrows), but EBF3+ cells are nearly absent from the INL of *Prdm13-GFP/Δ115* mice. A higher percentage of GFP+ cells co-express BHLHB5 in mutants. **(G)** Plot showing the percentage of marker positive cells that co-express GFP at P5. **(H)** Plot showing the percentage of GFP+ cells that co express EBF3 and BHLHB5. **(I)** Plot showing the number of cells that express GFP, EBF3, and BHLHB5. Sample size for E17.5 is one mouse, 3 mice per condition at P2, and 3-4 mice per condition at P5. Statistical significance determined by unpaired two-sample t-tests: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars represent the standard deviation. Scale bar 50µm. Inset scale bar 10µm.

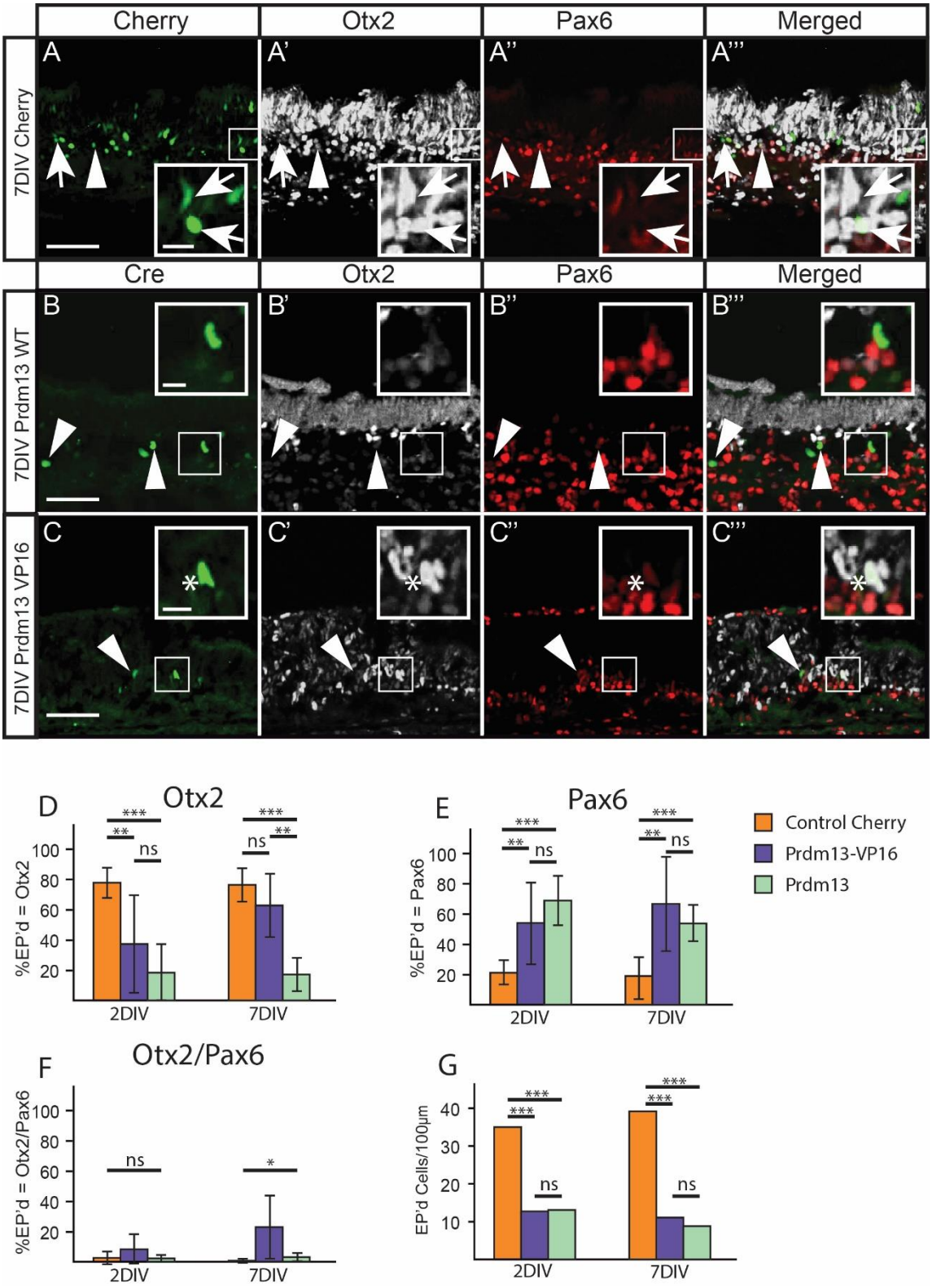
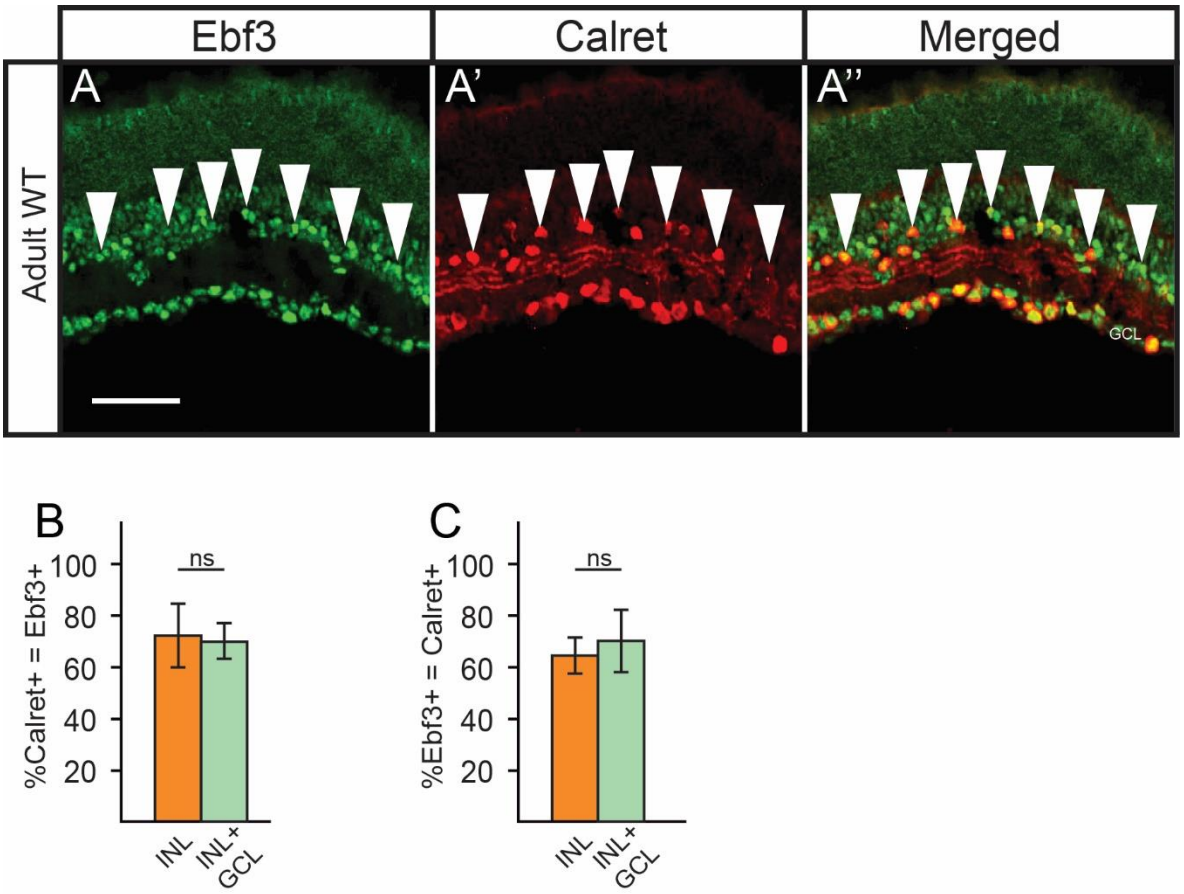



**Figure 2.6 Overexpression of *Prdm13* does not specify amacrine identity and is toxic to photoreceptors**

**(A-C’’’)** Wild-type P0 mouse retinas electroporated (EP’d) with control, wild-type PRDM13, and PRDM13-VP16 expression plasmids and cultured for 7 days *in vitro* (DIV). Electroporated cells are detected with antibodies to Cre (PRDM13 and PRDM13-VP16) or to red fluorescent protein (RFP, control). Sections are stained for the photoreceptor and bipolar cell marker OTX2 (grey) and for PAX6 (red), which marks amacrine cells intensely. **(A-A’’’)** Control Ef1α nuclear Cherry transfections have numerous RFP cells (pseudo-colored green), primarily in the photoreceptor area after 7DIV. Most of these cells co-express OTX2 (arrows), while a minority co-express PAX6 (arrowheads). **(B-B’’’)** Overexpression of wild-type PRDM13 (PRDM13-WT-IRES-Cre) yields few Cre+ (green) cells after 7DIV. These cells are localized to the INL and are typically PAX6+ (arrowheads). In about 30% of cases, Cre+ cells expressed neither PAX6 nor OTX2 (inset). **(C-C’’’)** Electroporation of a PRDM13-VP16 fusion (PRDM13-VP16-IRES-Cre) construct also results in sparse numbers of Cre+ cells. These transfected cells are seen in the INL and co-express PAX6 (arrowheads) at high frequency. Many of these cells also co-express OTX2 (star), which is rarely seen in control or PRDM13 WT transfections. **(D-F)** Plots showing the percentage of electroporated cells at 2DIV and 7DIV that co-express OTX2 (D), PAX6 (E), and OTX2/PAX6 (F). **(G)** Plot showing the number of transfected cells seen after 2DIV or 7DIV. Sample sizes for 2DIV are 8-10 retinas per condition and for 7DIV are 7-9 retinas per condition. Statistical significance is determined by unpaired two-sample t-tests (D, E, G), and ANOVA (F): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars represent the standard deviation. Scale bar 50µm. Inset scale bar 10µm.




**Supplemental Figure 2.1 EBF3 and calretinin co-expression marks a large population of amacrines**

**(A-A'')** P16 wild-type mouse retinas labeled with antibodies to EBF3 (green) and calretinin (red). The majority of both calretinin+ and EBF3+ cells in the INL co-express both markers (arrowheads). **(B-C)** Plots showing the percentage of calretinin+ cells that co-express EBF3 **(B)** and the percentage of EBF3+ cells that co-express calretinin **(C)** in both the INL and within the entire retina. Sample size was 4 retinas. Statistical significance determined by unpaired two-sample t-tests: ns = not significant. Error bars represent the standard deviation. Scale bar 50µm.

# CHAPTER III

# SIMULTANEOUS DELETION OF PRDM1 AND VSX2 ENHANCERS ALTERS RETINAL CELL FATE SPECIFICATION, YET DIFFERS FROM DELTEING BOTH GENES[6]

## Chapter III Summary

The transcription factor *Otx2* is required for photoreceptor and bipolar cell formation in the retina. It directly activates the transcription factors *Prdm1* and *Vsx2* through cell type-specific enhancers. *Prdm1* and *Vsx2* work in opposition, such that *Prdm1* promotes photoreceptor cell fate and *Vsx2* bipolar cell fate. To determine how OTX2+ cell fates are regulated, we deleted *Prdm1* and *Vsx2* or their cell type-specific enhancers simultaneously using a CRISPR/Cas9 *in vivo* retina electroporation strategy. Double gene or enhancer targeting effectively removed PRDM1 and VSX2 protein expression. However, double enhancer targeting favored bipolar fate outcomes whereas double gene targeting favored photoreceptor fates. Both conditions generated excess amacrine cells. Combined, these fate changes suggest that photoreceptors are a default fate outcome in OTX2+ cells and that VSX2 must be present in a narrow temporal window to drive bipolar cell formation. *Prdm1* and *Vsx2* also appear to redundantly restrict the competence of OTX2+ cells, preventing amacrine cell formation. By taking a combinatorial deletion approach of both coding sequences and enhancers, our work provides novel insights into the complex regulatory mechanisms that control cell fate choice.

[6]The contents of this chapter have been submitted for publication and the work was completed in collaboration with co-authors including Michael A. Kaufman, Ko U. Park, Joseph A. Brzezinski IV.

**Rationale**

One of the fundamental questions in development is how progenitor cells give rise to the incredible variety of cell types found in the central nervous system. The retina is an excellent system for studying the mechanisms of fate specification because the major cell types are readily discernable. The seven principal cell types are derived from a population of retinal progenitor cells in a stereotyped overlapping fashion during embryonic and early postnatal development in the mouse[81–83,85,219,220]. Some cell types, like cone photoreceptors, horizontals, and ganglion cells, permanently exit the cell cycle (*i.e.*, they are born) embryonically. Rod photoreceptors, bipolar cells, and Müller glia are primarily born in the postnatal period. Amacrine cells are mostly formed embryonically, but a small population is born postnatally. It is widely thought that retinal progenitors progressively pass through stages where they have the competence (potential) to give rise to certain cell types early and others later[84,97,100,127,221–224]. However, the means by which progenitors change their competence and adopt their final cell fates are only partially understood.

The transcription factor *Otx2* sits at the top of a regulatory network that controls photoreceptor and bipolar cell genesis[130]. It is expressed by all mature photoreceptors and bipolar cells[138,227,228]. Mice that lack this gene do not generate photoreceptors or bipolar cells, but instead produce excess amacrine cells[138,139,226]. OTX2 is first upregulated in a large fraction of retinal progenitors as they permanently exit the cell cycle[229]. OTX2 directly regulates two additional transcription factors, *Prdm1* (*Blimp1*) and *Vsx2* (*Chx10*), through discrete enhancer sequences[167,234,235]. In the absence of *Prdm1*, OTX2+ cells precociously upregulate *Vsx2* and form bipolar cells at the expense of photoreceptors[127,128,231]. Many of these *Prdm1* mutant OTX2+ cells appear by morphology and immunohistochemistry to start off as photoreceptors

and subsequently transition (transdifferentiate) into VSX2+ bipolar cells in the early postnatal period[231]. Conversely, overexpression of PRDM1 suppresses VSX2 and bipolar cell formation[128,231]. These data argue that *Prdm1* does not instruct photoreceptor fate, but instead suppresses bipolar cell formation. *Vsx2* overexpression suppresses photoreceptor genes and *Vsx2* mutant mice fail to generate bipolar cells[148–150,232]. Taken together, this suggests that OTX2 establishes competence for photoreceptor and bipolar cell fates while PRDM1 and VSX2 form a mutually inhibitory network to influence the fate choice made by these competent OTX2+ cells (Fig 3.1A).

Several non-exclusive mechanisms may explain the role of *Prdm1* and *Vsx2* in OTX2+ cell fate determination (Fig 3.1A). *Vsx2* and *Prdm1* could fine-tune the numbers of bipolars and photoreceptors formed by repressing instructive factors (Fig 3.1A). Another possibility is that these transcription factors stabilize specification after a fate choice has occurred. Finally, it is possible that either photoreceptor or bipolar cell identity is a default outcome during retinal development and that *Prdm1* and *Vsx2* are used to break away from such a default state to ensure both fates are formed. We reasoned that removing the function of *Prdm1* and *Vsx2* simultaneously in OTX2+ cells would discriminate between these potential developmental mechanisms.

To simultaneously remove PRDM1 and VSX2, we developed a loss-of-function CRISPR/Cas9 targeted gene disruption system that could be delivered *in vivo* to the developing mouse retina. We created two versions of this system. The first targets the coding region of each gene to remove its function via deletion in a non-cell type-specific manner from transfected retinal cells. The second targets the *Prdm1* and *Vsx2* enhancers that are directly regulated by OTX2. We then delivered these CRISPR/Cas9 plasmids via *in vivo* electroporation into hundreds

of newborn mouse retinas and tracked cell fate under a variety of conditions across multiple time points. Similar to prior loss-of-function experiments[127,128,148,149], we observed that singly targeting *Prdm1* and *Vsx2*, either through their enhancers or coding sequences, affected the number of photoreceptors and bipolar cells that were formed. Removing *Prdm1* and *Vsx2* simultaneously yielded different fate changes depending on how they were removed. From these data we conclude that photoreceptors are a default fate choice during development, but there is a critical temporal window when bipolar fate can be driven by VSX2. We found that VSX2 is dispensable for bipolar fate maintenance and we also observed that *Prdm1* and *Vsx2* act redundantly in OTX2+ cells to restrict their ability to form amacrine cells. By targeting multiple genes and enhancers simultaneously *in vivo*, we have gained novel insights into the complex regulation of cell fate choice within the retina.

**Results**

*Vsx2 and Prdm1 Enhancers are Necessary for Protein Expression at P2 and P7*

*Otx2* is required for the formation of photoreceptors and bipolar cells[138,139]. The transcription factors *Prdm1* and *Vsx2* are direct targets of OTX2 and appear to have a cross-repressive relationship (Fig 3.1A)[127,128,167,231,234]. We reasoned that deleting *Prdm1* and *Vsx2* simultaneously would unmask the processes controlling photoreceptor and bipolar fate choices. To test this, we developed a CRISPR/Cas9 system to specifically delete these transcription factors during the postnatal period, when OTX2+ cells primarily choose between rod photoreceptor and bipolar fates[81,85,220].

We first modified existing plasmid constructs (Ran et al., 2013) to so that: (1) *S. pyogenes Cas9* was driven by a ubiquitous *EF1α* promotor, and (2) in some cases GFP was

replaced with RFP. (Fig 3.1B). Guide RNAs were designed to triply target a key coding exon of *Prdm1* (PC) and *Vsx2* (VC) (Fig 3.1B). By co-electroporating these plasmids into newborn retinas, *Vsx2* and *Prdm1* can be targeted in any transfected cell (Fig 3.1C).

Prior studies identified OTX2 regulated enhancers for *Prdm1* and *Vsx2*. This included a 139bp retina-specific enhancer approximately 6kb upstream of *Prdm1* and a 164bp bipolar-specific enhancer about 19kb upstream of *Vsx2*[167,235,270]. Because *Vsx2* plays a role in promoting retinal progenitor proliferation in addition to its role in bipolar cell genesis[148,149,271], we generated another targeting strategy that would prevent *Vsx2* and *Prdm1* expression specifically within postmitotic OTX2+ cells. To do this we designed a separate trio of guide RNAs to target the *Prdm1* (PE) and *Vsx2* enhancers (VE) (Fig 3.1B). All guides; PC, VC, PE, and VE along with a non-targeting control were cloned into plasmids that expressed either RFP or GFP fluorescent markers.

To test whether this CRISPR/Cas9 strategy effectively lowered PRDM1 and VSX2 protein expression, we electroporated postnatal day 0 (P0) wildtype (WT) CD1 mouse retinas with plasmids (PE, PC, VE, VC or non-targeting control) and raised the pups until the desired age of tissue collection (Fig 3.1C). PC, PE, and control eyes were collected at P2 when PRDM1 is quantifiable via immunohistochemistry (Fig 3.1D). Retinas were stained for PRDM1 and the fluorescent marker (Fig 3.1D, E). We observed a significant decrease in PRDM1+ cells in PC ($p<0.001$) and PE ($p<0.001$) compared to control (Fig 3.1F), but no difference between PC and PE ($p=0.075$). This argues that the *Prdm1* enhancer is necessary for PRDM1 expression in the retina. Next, we conducted electroporations with control, VC, and VE targeting plasmids and collected pups at P7, a time when bipolar-specific VSX2 expression is high (Fig 3.1G-H). The percentage of electroporated cells that co-expressed VSX2 was significantly lower in VC

($p<0.001$) and VE ($p<0.001$) compared to control (Fig 3.1I). There was no difference between VC and VE ($p=0.39$).

Since VSX2 is made by progenitors, it is possible that both VC and VE affect expression in progenitors. To test this, we electroporated embryonic (E) day 13.5 retinal explants with VC, VE, or control guides and cultured them for 72 hours (Fig S3.1A). We then quantified the percentage of electroporated cells (GFP+) that co-expressed VSX2. At this time-point, VSX2 only marks progenitors. The VC targeted cells had significantly reduced co-expression ($p<0.001$), whereas the VE cells co-expressed VSX2 at the same frequency as control electroporated cells (Fig S3.1 B-C). Thus, the *Vsx2* enhancer is only necessary for bipolar-specific VSX2 expression. Taken together, both coding (PC, VC) and enhancer (PE, VE) CRISPR/Cas9 systems were able to equivalently reduce PRDM1 and VSX2 expression.

*Vsx2 and Prdm1 Cell Type-Specific Enhancers Control Fate Choice*

If VE and PE are necessary for VSX2 and PRDM1 expression in OTX2+ cells, then targeting these enhancers should mimic targeting the coding regions and recapitulate the phenotypes reported in *Prdm1* and *Vsx2* loss-of-function studies[127,128,148,149]. To test this, we raised PC, PE, VC, VE, and control electroporated mice to P28 and quantified the fate of electroporated cells (Fig 3.2).

At P28, PE and PC significantly reduced photoreceptors ($p<0.001$ both), but increased bipolar cells (PE $p=0.005$, PC $p<0.001$) (Fig 3.2C,D). There were no changes in the percentage of electroporated amacrine interneurons or Müller glia (Fig 3.2E,F). These data mimic *Prdm1* knockout mice and suggest that the *Prdm1* enhancer is essential for protein expression. PC produced a more robust shift from photoreceptors ($p=0.013$) to bipolars ($p=0.009$) than PE (Fig

3.2C,D). We next examined VC and VE targeted mice at P28 (Fig 3.2). Both conditions had a significant loss of electroporated bipolar cells (VC p=0.011, VE p=0.008) (Fig 3.2D). There was no change in the percentage of electroporated cells that became photoreceptors, amacrines, or Müller glia in VE or VC (Fig 3.2C,E,F). There were more photoreceptors in VE and VC compared to control, however, our methods did not have the power to detect a significant photoreceptor increase (Fig 3.2B-D). Targeting the *Vsx2* coding and enhancer regions affected cell fate equally (p=0.382). These results are similar to *Vsx2* mutants, suggesting that the *Vsx2* enhancer is necessary for bipolar cell genesis[148,149].

*Simultaneous Deletion of Vsx2 and Prdm1 Enhancers Dysregulates Cell Fate*

Targeting *Prdm1* or *Vsx2* was robust, but never reached 100% efficiency in our experiments. To ensure that we knocked-out multiple targets consistently, we electroporated retinas with PCVC or PEVE, such that PC and PE were labeled with GFP and VC and VE were marked by RFP (Fig 3.3). RFP and GFP overlapped 85% of the time regardless of condition (Fig 3.3B,C). Based on this high degree of overlap, we assume throughout the remainder of this paper that a single fluorescent label is sufficient to conclude a cell received multiple constructions.

To determine whether targeting multiple genes was as effective as just one, we stained VC, VE, PCVC, and PEVE for VSX2 expression at P7. We observed an equivalent reduction in the percentage of electroporated cells that co-expressed VSX2 in all four conditions (Fig 3.3D). This suggested that the effect was not diluted by including guides targeting other genes. Next, we asked whether simultaneously targeting *Vsx2* and *Prdm1* (PEVE, PCVC) diluted its effect (Fig 3.3E). We quantified the percentage of electroporated cells that expressed VSX2 and PRDM1 at P4 and observed a significant reduction of VSX2+ and PRDM1+cells in both conditions (Fig

3.3F-H). We concluded that we can simultaneously eliminate PRDM1 and VSX2 using either gene or enhancer targeting. Although effective, we chose to utilize a larger number of biological replicates throughout our studies to overcome the limitations of incomplete targeting that we observed.

With the ability to target *Vsx2* and *Prdm1* simultaneously, we next examined the fate of doubly targeted cells. To do this, we performed *in vivo* electroporations at P0 with control, PCVC, and PEVE plasmids, raised pups to P28, and quantified the fate of electroporated cells based on their morphology and location (Fig 3.4A). Control electroporated cells were mostly photoreceptors and bipolars, but a modest number of OTX2-negative amacrines and Müller glia were also seen (Fig 3.4). Mice targeted with PEVE had a significantly lower percentage of photoreceptors ($p=0.014$) compared to controls (Fig 3.4B). In contrast, PCVC photoreceptors remained indistinguishable from controls ($p=0.366$) (Fig 3.4B). PCVC had a smaller fraction of bipolar cells compared to controls ($p=0.002$), whereas there was no change in the fraction of bipolars in PEVE electroporations ($p=0.261$) (Fig 3.4B,C). These results were in contrast to single VC and VE electroporations that experienced a loss of bipolar cells and single PC and PE electroporations that had a loss of photoreceptors (Fig 3.2). Thus, perturbing both genes or enhancers simultaneously was not simply the sum of the individual electroporations.

We expected that targeting *Prdm1* and *Vsx2* would only alter the fate of OTX2+ cells. However, the sum of bipolar and photoreceptor cells was lower than controls in both PEVE and PCVC conditions (Fig 3.4B,C). This suggested that there should be changes in the number of OTX2-negative Müller glia and amacrine cells. Upon quantification, we saw no changes in the number of Müller Glia (not shown). However, we observed a 2-3 fold increase in electroporated cells that become amacrine cells in booth PCVC and PEVE conditions compared to control

(p<0.001 both) (Fig 3.4D). Both conditions had an equivalent increase in the fraction of amacrines observed (p=0.178) (Fig 3.4E). This increase in amacrines accounted for the reduction in photoreceptors (PCVC) or bipolar cells (PEVE) (p<0.001 both) (Fig 3.4D,E).

One explanation for this unexpected result is that some bipolar cells migrate to the wrong portion of the inner nuclear layer (INL) and display amacrine-like morphology. To determine whether the morphologic characterization was misleading, we examined these mice by immunohistochemistry with cell type-specific markers. Both PCVC and PEVE had significantly fewer OTX2+ cells compared to controls (p<0.001, p<0.001), matching what we observed morphologically (Fig 3.4E-G). Both PCVC and PEVE showed a significant (p<0.001, p<0.001) and equal reduction in VSX2 co-staining compared to control, despite the fact that PEVE did not lose morphologically identified bipolars (Fig 3.4C,H-I). The few bipolars seen in PCVC typically co-expressed VSX2 (rare exception highlighted in center panel, Fig 3.4H). In contrast, PEVE had numerous clear VSX2-negative bipolars (Fig 3.4H). These data argue that bipolar cells could form or survive without VSX2 or that their morphology remained misleading. To test this, we stained with additional bipolar markers (Fig S3.2). We found that co-expression with ISLET1/2 (Cone ON and rod bipolars)[215,272] matched the morphologically categorized bipolars, showing a significant decrease in PCVC (p=0.002) and no change in PEVE compared to controls (Fig S3.2B-C). The co-expression with Secretagogin, which marks a subset of ON and OFF cone bipolars[273], paralleled our observations of VSX2 and showed a significant decrease in PEVE and PCVC compared to control (p<0.001, p=0.003) (Fig S3.2D,E). There was no difference between the percentage of morphological bipolar cells that lacked both ISLET1/2 and Secretagogin between the conditions (Fig S3.2F). Taken together, these data argue that most (perhaps all) morphologically identified bipolar cells co-express other bipolar markers even though they lack

VSX2. Thus, our morphological assessment is robust and suggests that bipolars were formed in the absence of bipolar-specific VSX2. Nonetheless, loss of VSX2 may alter the subtypes of bipolar cells formed.

To corroborate the change in morphologically identified amacrine cells, we stained for the pan-amacrine marker PAX6[209]. The percentage of electroporated cells that co-expressed PAX6 in controls was indistinguishable from the value determined by morphology (Fig 3.4D, J-K). We observed a significant increase in PAX6+ cells to about 20% in both PCVC ($p<0.001$) and PEVE ($p<0.001$) (Fig 3.4J,K). This increase was only observed in the PEVE and PCVC conditions and was remarkably consistent across litters (Figs S3.4F,S3.5E). We next stained for GLYT1 and EBF3, which primarily mark different populations of late-born amacrine cells[211,212,250,274–276]. Like PAX6, the percentage of electroporated cells that co-expressed EBF3 doubled in both PCVC ($p=0.054$) and PEVE ($p=0.051$) compared to controls (Fig S3.3B,C). Similarly, the percentage of electroporated cells that co-expressed GLYT1 was strongly increased in both PCVC ($p<0.001$) and PEVE ($p<0.001$) conditions (Fig S3.3D, E). Thus, morphologically identified amacrines express pan-specific and late-born markers. We also observed that the number of cells co-expressing EBF3 increased in proportion to the total number of amacrines (Fig S3.3F). The extra amacrines in PEVE and PCVC conditions appear to proportionally adopt late-born subtype fates, similar to control amacrines formed postnatally.

*Fate Changes Do Not Result from Selective Survival*

We reasoned that our *Prdm1* and *Vsx2* perturbations could alter cell survival, biasing the observed fate changes. Because of the temporally precise nature of cell death markers and our sparse-label technique, we were not able to directly test differences in cell death. Instead, we

took three complementary indirect approaches to show that cell survival was equivalent between conditions. First, we quantified the number of cells per image across six litters of control and six litters of PEVE (63 total mice and 116 images) and found no differences between conditions (Fig 3.5A). This suggests that cell survival is comparable at P28 between the two conditions. There was one exception where a control litter had significantly more cells per image compared to the rest of the controls and PEVE mice (Fig 3.5A, S3.4). However, the variance within a litter was equal to or greater than between litters (Fig 3.5B). Second, we asked whether variance in the total number of electroporated cells measured in any given litter skews the assessment of cell fate. Across six control and six PEVE litters we observed no differences in the percentages within a condition and significant differences between conditions in the number of photoreceptors and amacrines (Fig S3.4). Indeed, we could compare any control litter to any test litter and arrive at the same conclusion. Third, we asked whether there was a progressive loss in electroporated cells by quantifying the number of cells per image at intermediate developmental time-points. In no condition were there significantly more cells at P7 or P14 compared to P28 (Fig 3.5C), arguing against a progressive loss of electroporated cells. Taken together, our data strongly suggests that cell survival is unaffected by our perturbations and changes are a result of shifting cell fates.

*Cell Fate Changes Are Seen at Different Times in Development*

Perturbing *Prdm1* and *Vsx2* could alter cell fate early. Alternatively, doubly targeted cells may stall in an intermediate or confused state. To explore this question, we repeated PC, PE, VC, VE, PCVC, PEVE, and control electroporations at P0 and collected animals at P7 and P14. Electroporated cells were screened by morphology to determine if fate shifts occurred early (by

P7) or were delayed, suggesting that fate choice is stalled (Fig 3.5). We considered all radially oriented cells that lacked clear neuronal morphology to be progenitors, undifferentiated neurons, or Müller glia. For simplicity, we pooled all of these cells in our quantification of electroporated retinas (Fig 3.5G).

Targeting PC and PE revealed an early fate shift, such that there were more bipolars and less photoreceptors at all time-points (Fig 3.5D, E). In contrast, perturbing VC and VE only showed a loss of bipolars at the P28 time-point (Fig 3.5E). PCVC and PEVE animals showed an increase in amacrines at all time points (Fig 3.5F), which was not observed in the single loss-of-function conditions (Fig 3.5D-G). The number of progenitor-like cells progressively decreased in all conditions suggesting that some electroporated cells had not fully differentiated by P7 or P14 (Fig 3.5G). This likely reflects a delay in these cells adopting clear photoreceptor morphology (Fig 3.5D). In particular, the PEVE and PCVC conditions had excess undifferentiated cells at the P7 time-point compared to controls and most of the single mutant conditions. This correlates with the early deficit and protracted accumulation of photoreceptors in PCVC and PEVE conditions. Taken together, our data suggest that cell fate changes seen in *Prdm1* mutants occur early, *Vsx2* mutants were delayed, and double mutants were a mix of both.

*Birthdating Shows an Increase in Late-Born Amacrines*

The double mutant conditions may perturb cell-cycle exit timing and thus fate choice. To explore this possibility, we conducted an EdU birthdating experiment. Eight litters of P0 pups were electroporated with control or PEVE and subsequently received a single EdU pulse at either P0, P2, P4, or P6 (Fig 3.6A). All mice were raised to P28 and examined by immunohistochemistry to determine the fate of birthdated (EdU+) cells. The total number and

percentage of electroporated cells marked by EdU did not vary between control and PEVE conditions (Fig 3.6B,C). We concluded there were no major differences in cell cycle exit timing between PEVE and control. Nonetheless, there were differences in the fate of cells born at P0, P2, and P4 (Fig 3.6, S5). Significantly fewer photoreceptors were born in PEVE compared to controls at P0 ($p=0.008$), P2 ($p=0.025$), and P4 ($p=0.038$) (Fig 3.6D). The fraction of birthdated photoreceptors was reduced to a similar degree over time, suggesting that the fate shift was independent of cell cycle exit timing (Fig 3.6D). In contrast, there was no difference at any time-point in the number of bipolar cells born (Fig 3.6E). The birthdating pattern of PEVE closely matched controls and the unchanged number of morphologically identified bipolars that we observed (Fig 3.4,5,6E). However, when we stained for VSX2 we saw a significant decrease in birthdated VSX2+ cells in PEVE compared to controls at P0 ($p<0.001$), P2 ($p<0.001$), and P4 ($p=0.005$) (Fig 3.6G, S5C). This paralleled the decrease in VSX2+ bipolars that we observed in PEVE conditions (Fig 3.4H). There were slightly more Müller glia born in the PEVE condition at P0 ($p=0.035$), although this did not result in significantly more total glia and appeared to be caused by three outliers (Fig 3.6I, S4G-PEVE litter 1). Taken together, these data indicate that cell-cycle exit timing is not contributing to the differences seen in photoreceptor and bipolar cell numbers in PEVE.

Next, we examined amacrine cell birthdates. We observed an increase in birthdated amacrines in PEVE compared to controls at P0 ($p=0.048$), P2 ($p=0.004$), and P4 ($p=0.004$) (Fig 3.6F). This increase matched the loss of photoreceptors at each time-point (Fig 3.6D). Unlike photoreceptors (Fig 3.6D), the birthdating profiles of control and PEVE amacrine cells were not parallel, with PEVE amacrines extending beyond their normal window. We next asked if the percentage of electroporated and birthdated PAX6+ amacrines exceeded the total percentage of

all birthdated amacrines in PEVE. Indeed, there were far more electroporated and birthdated amacrines (Marker+, PAX6+, EDU+) at P0 ($p<0.001$, pairwise t-test), P2 ($p<0.001$), and P4 ($p=0.005$) compared to the percentage of all birthdated (EDU+ PAX6+) cells in those same images (Fig 3.6H, S5D). Together, these data reveal that many of the increased amacrines in PEVE conditions are born later than normal, likely at the direct expense of rods.

*Bipolar Fate is Selected Prior to VE Activation and Does Not Require VSX2 for Maintenance*

Previous loss-of-function work[148,149] and our own study showed that VSX2 is necessary for bipolar formation, leading to the conclusion that bipolar-specific VSX2 is a fate-determining regulatory element. Yet, our PEVE data show that in the absence of PRDM1, bipolar-specific VSX2 is not necessary to form or maintain bipolar cell identity. We wondered if VE is in fact downstream of the bipolar cell fate choice and that this outcome has been obscured by the effects of PRDM1.

To test this, we designed a system to compare the timing of bipolar fate choice with the expression of the *Vsx2* enhancer. We built a plasmid containing the *Vsx2* bipolar-specific enhancer driving Cre recombinase (VE-CRE) to fate map newly formed bipolar cells (Fig 3.7A). We co-electroporated this construct along with control or VC-Cas9-GFP targeting plasmids into newborn *ROSA-RFP* reporter mice[277]. In this system, control and VC targeted cells will be GFP+, but only cells that activated the *Vsx2* enhancer will become permanently RFP+ (Fig 3.7). Mice were examined at P7 for RFP, GFP, and VSX2 (Fig 3.7B,I). Since the control and VC plasmids are expressed under the ubiquitous *EF1α* enhancer, there were more GFP+ cells in both conditions than RFP+ cells (Fig 3.7B). We saw no differences in the percentage of total GFP+ cells that co-expressed RFP+ between control and VC conditions ($p=0.169$) or in the percentage

of RFP+/GFP+ cells in the INL (p=0.199) (Fig 3.7C-D). RFP+ cells in control animals overwhelmingly adopted bipolar cell morphology, although about 1 in 7 cells showed rod photoreceptor morphology (Fig 3.7B). This is consistent with VE becoming activated in cells that are poised between photoreceptor and bipolar fates with most adopting a bipolar fate. We then examined the VC condition to determine whether there was a fate change in the VE lineage. If VSX2 acts only after the onset of VE activity, we expected to see an increase in the fraction of lineage traced cells that become photoreceptors at the expense of bipolars. However, we observed the opposite in VC conditions. An even higher fraction of the VC RFP+ cells had bipolar morphology (p=0.017) (Fig 3.7E), which came at the direct expense of cells with photoreceptor morphology in the outer nuclear layer (ONL) (p=0.017) (Fig 3.7F).

This result suggested that VSX2 can act before the onset of VE activity. We next expanded our analysis by asking if the RFP+ cells within the INL made VSX2. In the control condition 98.9% of the RFP+ cells in the INL co-expressed VSX2+, as expected for this bipolar cell maker (Fig 3.7G,I). However, only 15.1% of RFP+ cells in the INL co-expressed VSX2+ in the VC electroporated retinas (p<0.001) (Fig 3.7G). Thus, RFP+ cells with bipolar morphology persisted in the INL of VC electroporations despite the absence of VSX2. This, along with our PEVE findings (Fig 3.4), argues that VSX2 is not required for bipolar cell maintenance. We also observed that a small percentage of the RFP+ cells were GFP-negative in the VC condition and hence untargeted (Fig 3.7D). Not only were there far fewer RFP+ photoreceptors in the ONL of the VC condition (Fig 3.7F), of those RFP+ cells, only three across all images co-expressed GFP+ (Fig 3.7H, p=0.003). Thus, when VC is targeted, VE gives rise to a purely bipolar cell lineage instead of one poised between photoreceptor and bipolar fates. Taken together, these data

argue that targeting VSX2 can alter the fate of OTX2+ cells *before* the decision to activate VE (Fig 3.7J).

**Discussion**

OTX2 activates two downstream transcription factors (*Prdm1* and *Vsx2*) to influence whether cells adopt bipolar or photoreceptor fates (Fig 3.8A). Lineage tracing experiments of the *Prdm1* (PE) and *Vsx2* bipolar (VE) enhancers show that each can give rise to bipolars and photoreceptors[167,233,234]. This argues that OTX2+ cells are poised between fates and that *Prdm1* and *Vsx2* compete to regulate the choice. To investigate how these genes interact, we used a CRISPR/Cas9 approach to simultaneously remove *Prdm1* and *Vsx2* activity from the retina. Our results suggest that: (1) the PE and VE elements are necessary in the retina, (2) photoreceptors are a default outcome in OTX2+ cells, (3) there is a critical period where VSX2 is needed to drive bipolar fate choice, (4) bipolar fate maintenance does not require VSX2, and (5) PRDM1 and VSX2 act redundantly to suppress amacrine formation. Together, our findings provide a framework for how deletion of necessary enhancers and their target genes can cause divergent results.

*Prdm1 and Vsx2 Enhancers Are Necessary for Gene Expression*

*Otx2* regulates both the *Prdm1* and *Vsx2* enhancers[167,231,233,234]. Targeting the *Prdm1* enhancer was equally effective at reducing protein expression compared to targeting its coding sequence (Fig 3.1). Targeting PE and PC each caused the same type of cell fate changes, arguing that the *Prdm1* enhancer is necessary for PRDM1 expression (Fig 3.2, 8C-D). Our results parallel other work that explored the necessity of the PE sequence indirectly[233]. Despite effecting

protein expression equally during development, targeting PE and PC did not result in the same degree of fate changes between photoreceptors and bipolars at P28 (Fig 3.2, 8C-D). It is unclear why this was the case. One possibility that accounts for the milder phenotype in the PE condition is that targeting PE may result in slightly later timing of PRDM1 loss, allowing more cells to develop normally.

Targeting the VE sequence significantly reduced VSX2 and matched targeting the coding region. Correspondingly, there was no difference in the effect on cell fate between VE and VC conditions (Figs 3.1-2). In contrast to VC, targeting VE did not affect VSX2 in progenitors (Fig S3.1). Taken together, this suggests that the VE region is necessary for VSX2 expression in bipolars (Fig 3.8F,G). This is in line with a recent report where a large (~32kb) upstream region of *Vsx2* (including VE) was deleted, resulting in mice that lacked bipolar cells[278]. Here we show that targeting the 164bp VE enhancer was sufficient to prevent bipolar formation (Fig 3.2D).

*Photoreceptors Are the Default Outcome in OTX2+ Cells*

Targeting *Prdm1* or *Vsx2* resulted in changes in the numbers of photoreceptors and bipolar cells (Fig 3.8). Targeting the coding regions of *Prdm1* and *Vsx2* simultaneously resulted in retinas that had reduced numbers of bipolar cells (Fig 3.4,8I). One interpretation is that fate choice in double mutant cells is moderately biased against forming bipolar cells. However, the percentage of bipolars formed in PCVC electroporations matched the percentage of cells that failed to lose VSX2 expression (Fig 3.4C,I). We almost never observed VSX2-negative bipolars in PCVC, unlike what we saw in the PEVE condition. This strongly suggests that morphological bipolars seen in the PCVC condition were predominantly cells that escaped *Vsx2* targeting. This indicates that OTX2+ cells that lacked both PRDM1 and VSX2 overwhelmingly adopted

photoreceptor fates, leading to the conclusion that photoreceptors are the default outcome of postnatal OTX2+ cells (Fig 3.9A). This aligns with our findings and other work showing that bipolar cells only form if VSX2 is present[148,149,278]. Further, our data argue there are additional regulatory components that contribute to the specification of OTX2+ cells (Fig 3.1A). The identity of these factors and how they work remains to be uncovered.

*VSX2 is Not Required for Bipolar Cell Maintenance*

Loss-of-function experiments argue that *Vsx2* is necessary to form bipolar cells. Throughout our study, the bipolars that formed in PCVC, VC, and VE conditions closely aligned with the number of VSX2+ cells. We concluded that the bipolars observed in these conditions were a function of the escape rate within our CRISPR/Cas9 technique and not representative of what happens in the absence of VSX2. In contrast, many bipolars in the PEVE conditions lacked VSX2, yet robustly displayed bipolar morphology (Fig 3.4), bipolar subtype markers (Fig S3.2), and were born in a similar pattern (Fig 3.6). These data argue that at some point soon after an OTX2+ cell has decided to become a bipolar, it does not need VSX2 to maintain its identity.

This raises the question; why do adult bipolar cells express VSX2? Under normal conditions, mature bipolars express VSX2[25]. When we quantified the subtype fates of the PEVE bipolars, we observed that ON type bipolar cell markers were comparable to controls, while Secretagogin positive cone bipolars (types 2-6, 8)[273] were strongly reduced. This suggests that the formation or maintenance of some cone bipolar subtypes requires VE mediated VSX2 expression. This may be due to complex negative interactions between VSX2 and its paralog VSX1, which is needed for the proper formation of cone bipolars[279–283]. Another possibility is

that VSX2 is used to maintain the physiologic functions of mature bipolar cells. Further studies are needed to understand the role of VSX2 in mature bipolar cells.

*VSX2 Affects Bipolar Fate Choice over a Critical Window of Time*

We were surprised to discover that simultaneously targeting *Prdm1* and *Vsx2* coding regions did not yield the same result as targeting their enhancers. In contrast to PCVC electroporations, PEVE cells were more likely to be bipolars and less likely to be rod photoreceptors (Fig 3.8J). We reasoned that the most probable explanation for this difference is that there was some VSX2 present in the electroporated cells. Since targeting VE has no effect on VSX2 made in progenitors (Fig S3.1), it is likely that some VSX2 is carried over as cells become postmitotic (Fig 3.8J). This residual VSX2 expression could drive bipolar fate, but only in the absence of PRDM1 due to its ability to suppress bipolar formation in OTX2+ cells (Fig 3.8, 9B). This would not be the case in PCVC conditions, where VSX2 was eliminated in progenitors before it has a chance to carry-over into OTX2+ cells and affect fate (Fig 3.8I). The decrease in photoreceptors in the PEVE condition is likely explained by the ability of progenitor-derived VSX2 to promote bipolar fate at the expense of rods. Further support of this carry-over model (Fig 3.8, 9B) was found when we traced the *Vsx2* enhancer lineage in control and *Vsx2* coding mutant cells. The VE lineage did not gain photoreceptors when VSX2 was targeted, suggesting that the choice between photoreceptor and bipolar fates can occur before VE is activated (Fig 3.7J). This further suggests that there is a temporal window where VSX2 carry-over is sufficient to rescue bipolar cell formation, but only in the absence of PRDM1 (Fig 3.8, 9B). The presence of photoreceptors in the PEVE condition may reflect that only a subset of

OTX2+ cells carry-over sufficient VSX2 to rescue bipolar fate. Alternatively, targeting PE may not remove PRDM1 quickly or completely enough to prevent photoreceptor formation.

In the absence of PRDM1, progenitor VSX2 carry-over appears sufficient to rescue bipolar cell formation (Fig 3.9). However, VC and VE single targeting equivalently reduced bipolar cell formation (Fig 3.2) and mice lacking a 32kb region of DNA upstream of *Vsx2* completely lack bipolars[278]. This shows that VSX2 expression driven by VE is essential for bipolar fate choice when PRDM1 is present. We also observed that bipolar cells as a class do not require VSX2 to maintain their fate. Taken together, these data argue that there is a transient period where VSX2 is required for bipolar cell formation. This critical period spans a relatively narrow window before and after the onset of VE activity. The mechanisms that VSX2 uses during this window and its interactions with PRDM1 and other factors remain to be determined.

*Prdm1 and Vsx2 Combine to Suppress Amacrine Formation*

Since *Prdm1* and *Vsx2* are downstream of *Otx2*, we expected that mutating these genes would simply alter the proportion of photoreceptors and bipolar cells that were formed. Indeed, mutating *Vsx2* or *Prdm1* on their own changed the numbers of OTX2+ cell types that formed. We were surprised to see a proportional increase in amacrine cells when we perturbed *Prdm1* and *Vsx2* (PEVE and PCVC) simultaneously. This mimics what is seen in *Otx2* null retinas, which form excess amacrine cells at the expense of photoreceptors and bipolar cells[138,139].

OTX2+ cells have the competence to become photoreceptors and bipolar cells, but some of these cells likely give rise to OTX2-negative amacrines and horizontals[130,167,230,231] (Fig 3.1A). We observed that single mutants of *Prdm1* or *Vsx2* did not alter the number of amacrine cells formed. Thus, it seems unlikely that *Prdm1* or *Vsx2* are necessary for restricting amacrine

competence in OTX2+ cells. Since double mutants have excess amacrine cells that are born outside of their normal temporal window, it is possible that *Prdm1* and *Vsx2* act redundantly to restrict amacrine competence in OTX2+ cells (Fig 3.8K). It is unclear why only a subset of these double mutant cells shifted to amacrines. One possibility is that there are other factors beyond *Prdm1* and *Vsx2* that contribute to inhibiting amacrine formation. Another possibility is that signals promoting amacrine identity are limited in the postnatal retinal environment. Future experiments with *Prdm1* and *Vsx2* double enhancer mutant mice will help decipher how competence is regulated in the OTX2+ cell population.

## Chapter III Figures

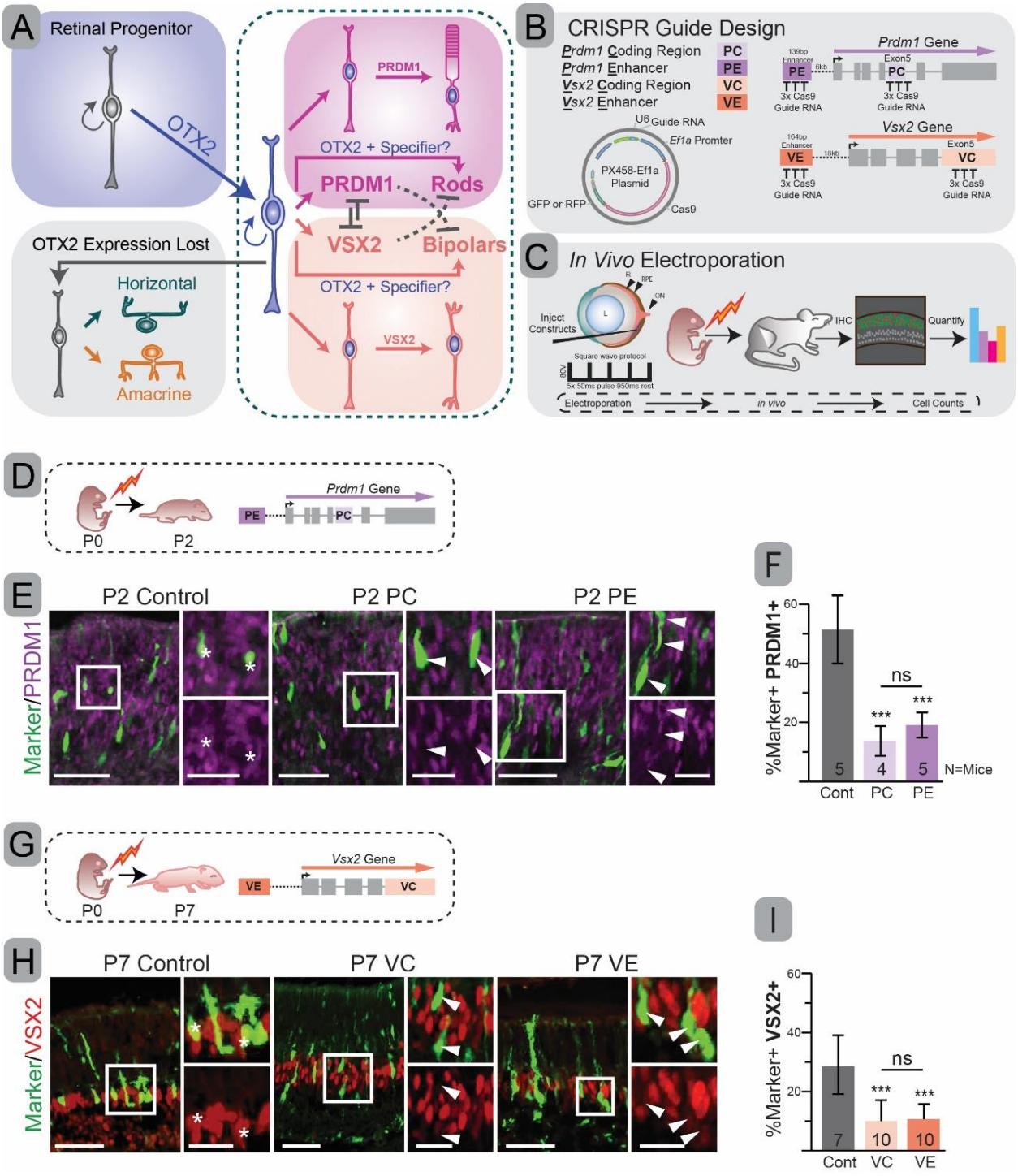



**Figure 3.1 Deletion of *Prdm1* and *Vsx2* enhancers blocks protein expression**

**(A)** Schematic for the Otx2 gene regulatory network. **(B**) CRISPR targeting guide design and nomenclature. **(C)** Schematic of experimental approach. **(D)** Experiment schematic. **(E, H)** Immunohistochemistry from electroporated cells. **(F)** Percentage of electroporated cells that also express PRDM1. **(G)** Experiment schematic. **(I)** Quantification of electroporated cells that also express VSX2. Error bars=standard deviation. ns=not significant, ***p<0.001. bars=50µm, inset bars=25µm. Stars=double-labeled cells, Arrows=single-labeled cells. N=number of mice utilized for statistics.

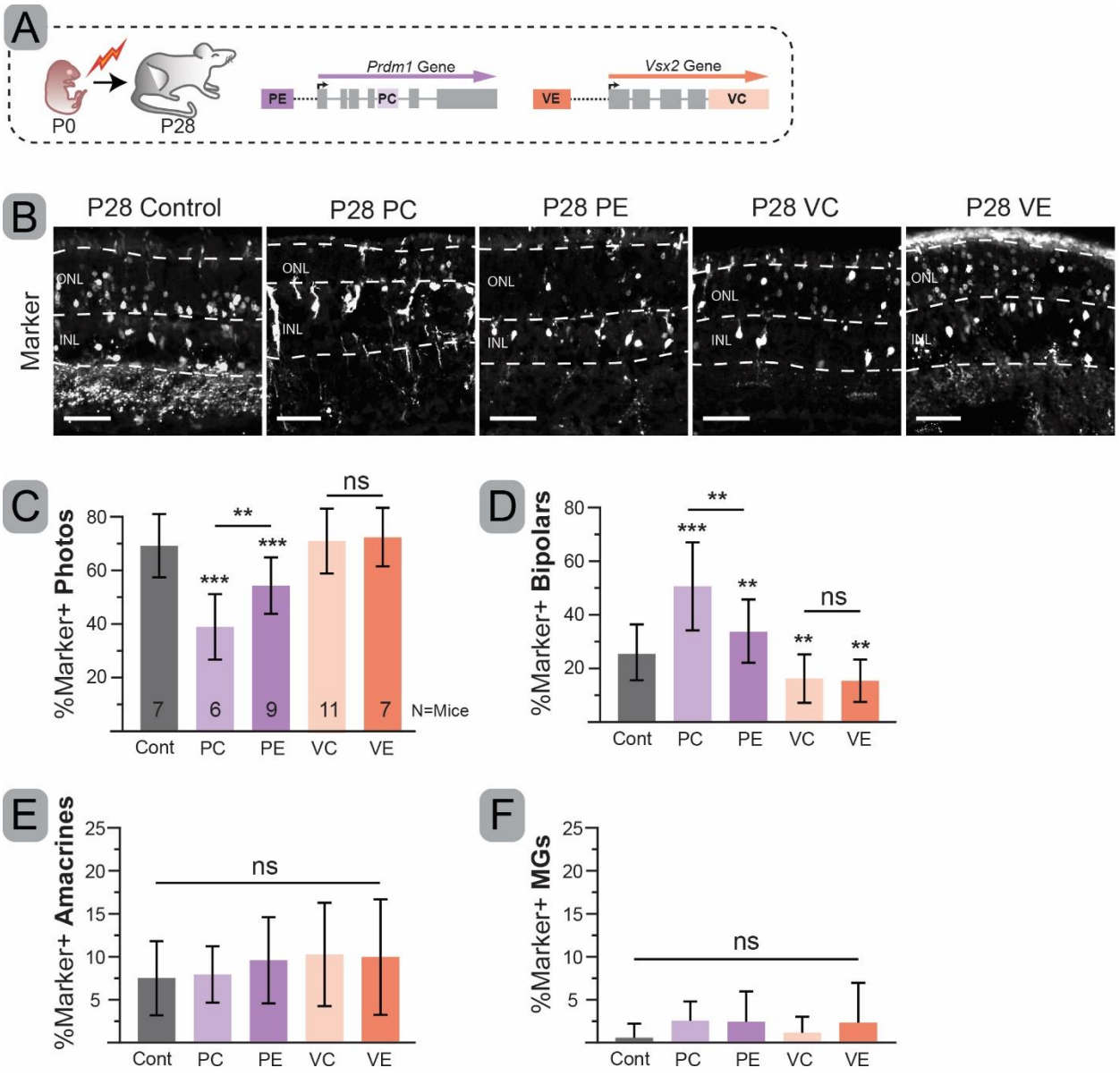



**Figure 3.2 Targeting PC, PE, VC, or VE shifts cell fates in mature retinas**
**(A)** Experimental schematic. **(B)** Representative immunohistochemistry of electroporated cells (grey) for each condition. **(C-F)** Percentage of electroporated cells that are photoreceptors **(C)**, bipolars **(D)**, amacrines **(E)**, and Müller glia **(F)**. **p<0.01, ***p<0.001. Bars=50µm, inset bars=25µm.

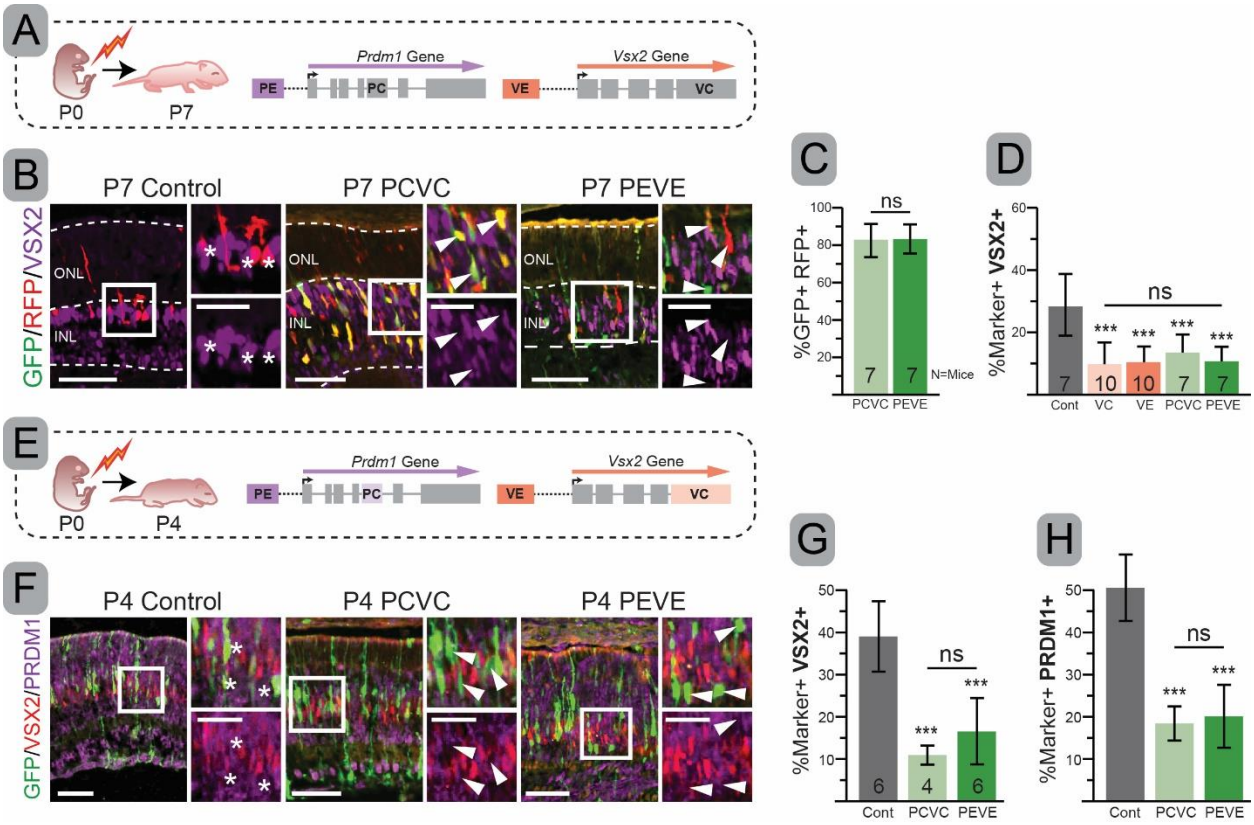



**Figure 3.3 Double targeting is as efficient as single at blocking expression**
**(A)** Experimental schematic. **(B)** Representative immunohistochemistry of electroporated (RFP or GFP) cells co-stained with VSX2. **(C)** Percentage overlap between GFP and RFP. **(D)** Percentage of electroporated cells (marker+) that co-express VSX2. **(E)** Experimental schematic. **(F)** Electroporated cells co-stained with VSX2 and PRMD1. **(G-H)** Percentage of electroporated cells that co-express VSX2 **(G)** or PRDM1 **(H)**. ***p<0.001. Bars=50µm, inset bars=25µm. Stars=double-labeled cells, Arrows=electroporated without additional co-labeling.

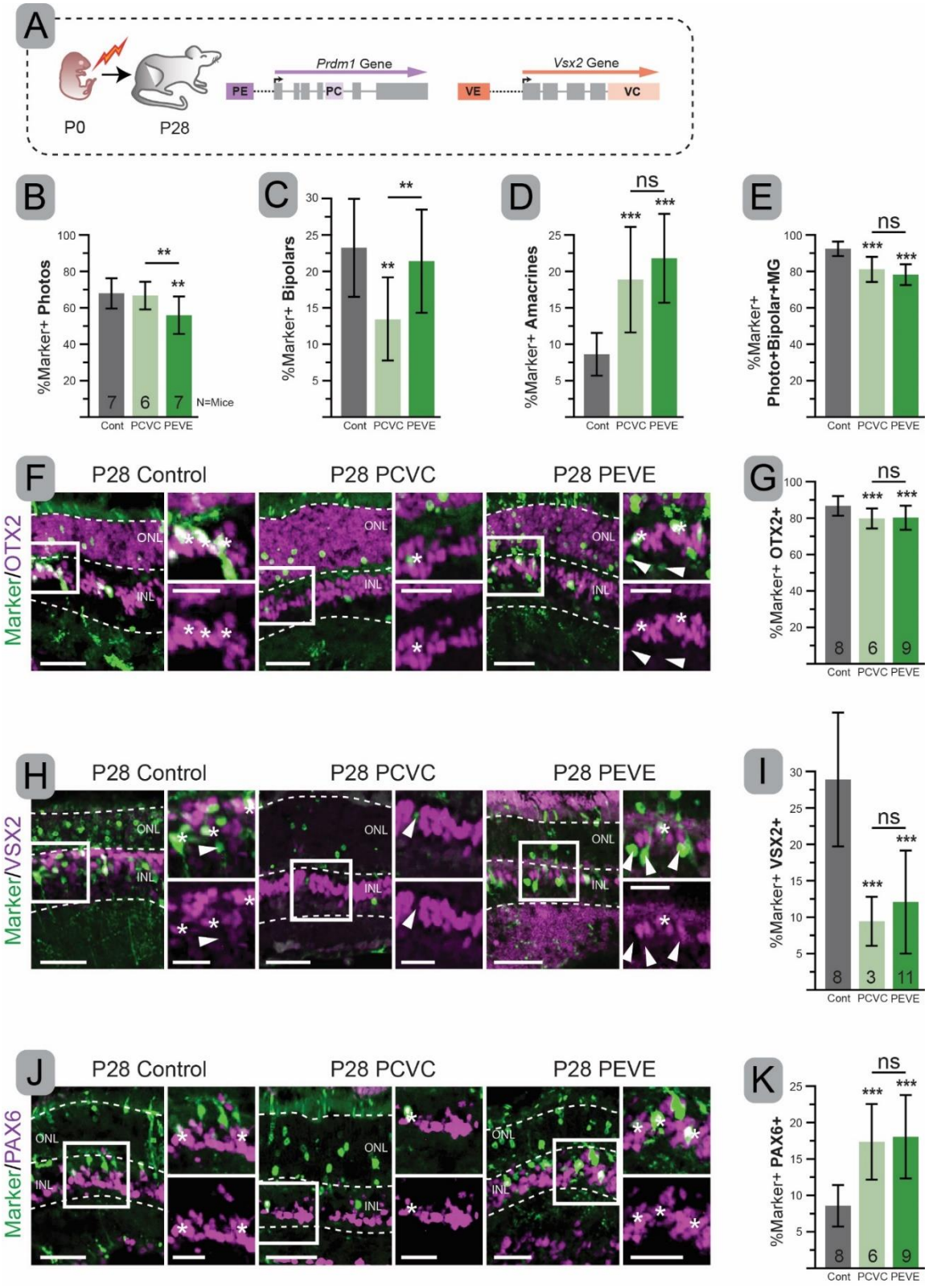



**Figure 3.4 PCVC and PEVE targeting increases amacrines, but have divergent bipolar and photoreceptor changes**

**(A)** Experimental schematic. **(B-D)** Percent of electroporated cells with photoreceptor **(B)**, bipolar **(C)**, or amacrine **(D)** cell morphology. **(E)** Combined percentage of electroporated cells that are photoreceptors, bipolars, and Müller glia. Representative immunohistochemistry of electroporated retinas co-stained with OTX2 **(F)**, VSX2 **(H)**, and PAX6 **(J)**. Percentage of electroporated cells that are also OTX2+ (G), VSX2+ (I), and PAX6+ (K) **p<0.01, ***p<0.001. Bars=50µm, inset bars=25µm.

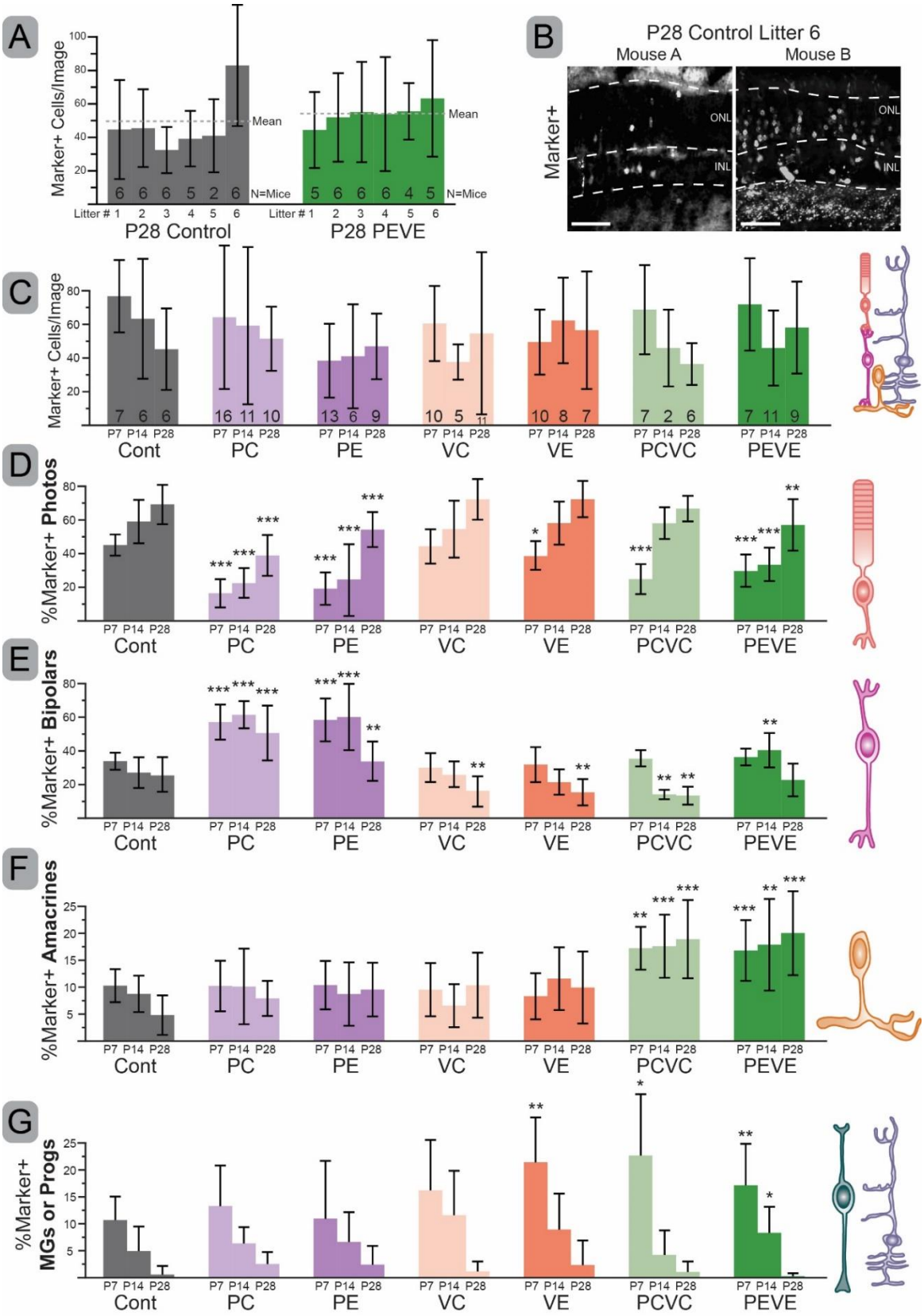



**Figure 3.5 Cell fates quantified across all conditions from P7, P14, and P28**

**(A)** Number of electroporated cells per image showing the variability both within and between conditions. These are the average cells per image across 12 unique litters electroporated at P0 and collected at P28 along with the number of mice with quantifiable electroporations. **(B)** Representative immunohistochemistry of two mice from the same litter showing electroporation efficiency differences within a condition. **(C)** Average electroporated cells per image by condition over time. **(D-G)** Percentage of electroporated cells with photoreceptor **(D)**, bipolar **(E)**, amacrine **(F)**, or glial/progenitor **(G)** morphologies by condition over time. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Bars=50µm.

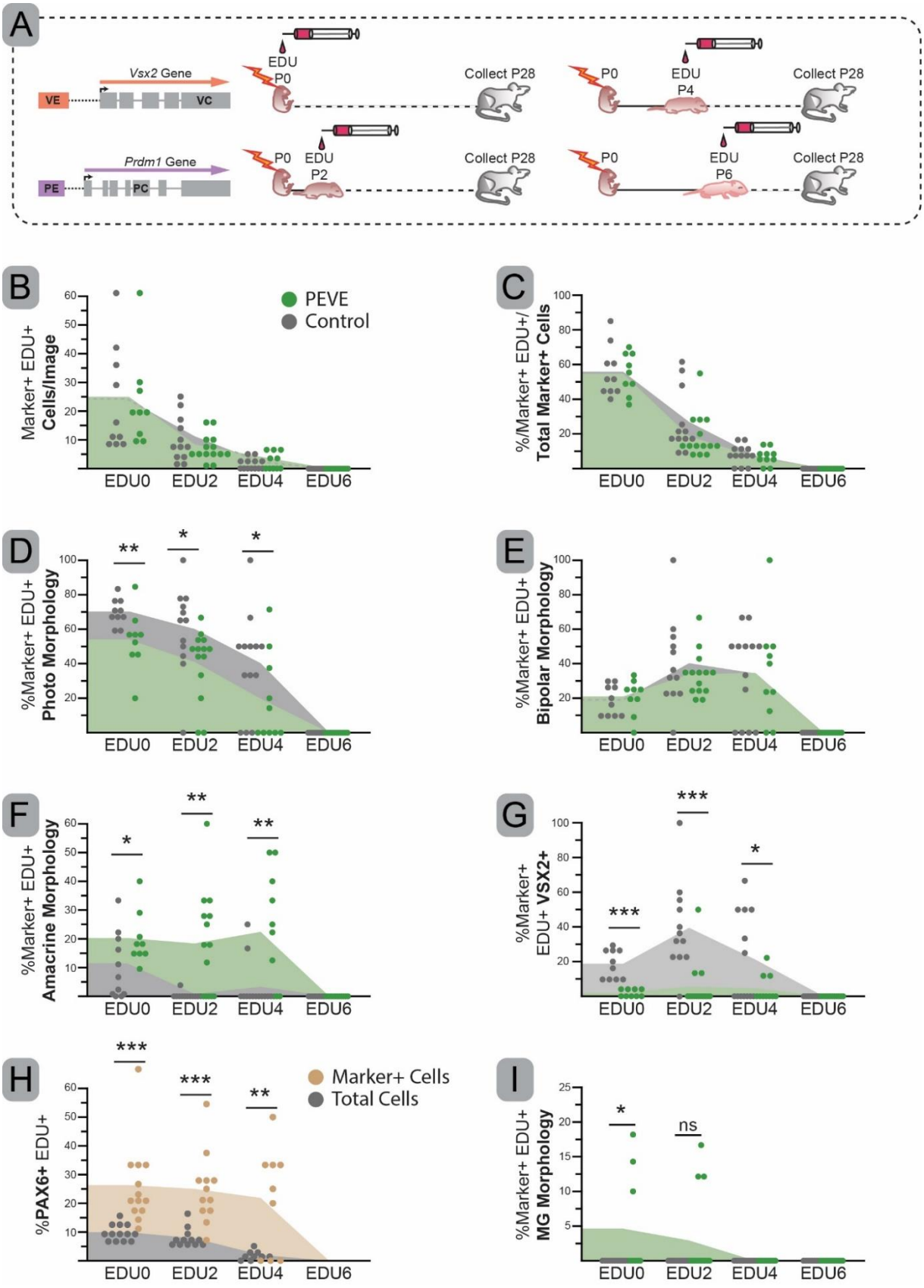



**Figure 3.6 Birthdating of control and PEVE electroporated cells**

**(A)** Schematic of experimental design. **(B-I)** Dots represent specific quantified images while the top of the shaded area represents the mean. **(B-C)** The total number **(C)** and the percentage **(D)** of electroporated cells that are also EdU+ over time. (D-F, I) Percentage of electroporated and EdU+ cells with photoreceptor **(D)**, bipolar **(E)**, and amacrine cell **(F)**, or Müller glial (MG) **(I)** morphologies. **(G-H)** Percentage of electroporated and EdU+ cells that co-express VSX2. **(H)** Percentage of electroporated and EdU+ cells that express PAX6 compared to the percentage of all EdU+ cells that expressed PAX6 in the same image. N for EDU0: Cont=6, PEVE=6; for EDU2: Cont=7, PEVE=6; for EDU4: Cont=7, PEVE=6; and for EDU6: Cont=5, PEVE=7. *p<0.05, **p<0.01, ***p<0.001. Paired t-test for panel H.

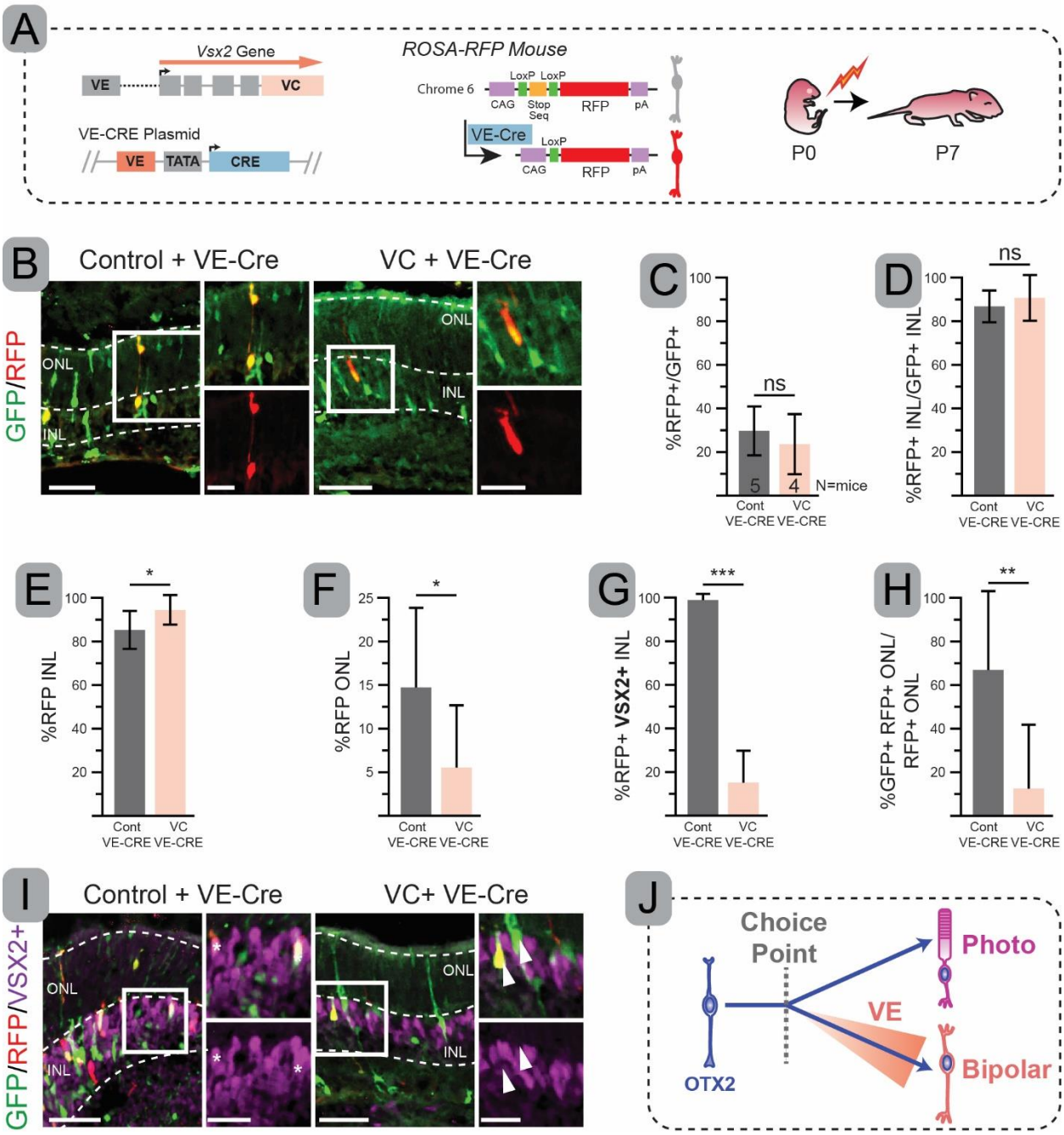



**Figure 3.7 VSX2 enhancer lineage tracing in control and *Vsx2* targeted cells**

**(A)** Schematic of the experimental design. **(B)** Immunohistochemistry showing RFP+ (lineage trace) and GFP+ (control or VC CRISPR/Cas9) electroporated cells at P7. VE-CRE driven RFP had a low efficiency that resulted in small numbers of cells and higher than normal standard deviations. **(C)** Percentage of GFP+ cells that are RFP+. **(D)** RFP+ cells within the INL as a percentage of GFP+ cells within the INL. **(E-F)** Percentage of RFP+ cells in the INL **(E)** and the ONL **(F)**. **(G)** Percentage of RFP+ cells in the INL that are co-stained for VSX2. **(H)** Cells (photoreceptors) that co-stain for RFP and GFP in the ONL as a percentage of the total RFP+ cells located in the ONL. There were only 3 GFP+/RFP+ cells in ONL of the VC VE-CRE condition across all images. **(I)** Immunohistochemistry showing GFP, RFP, and VSX2 overlap at P7. **(J)** Schematic showing that VE enhancer activation can occur after the decision to become a bipolar cell. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Bars=50µm, inset bars=25µm. Stars=VSX2+ cells, Arrows=VSX2- cells.

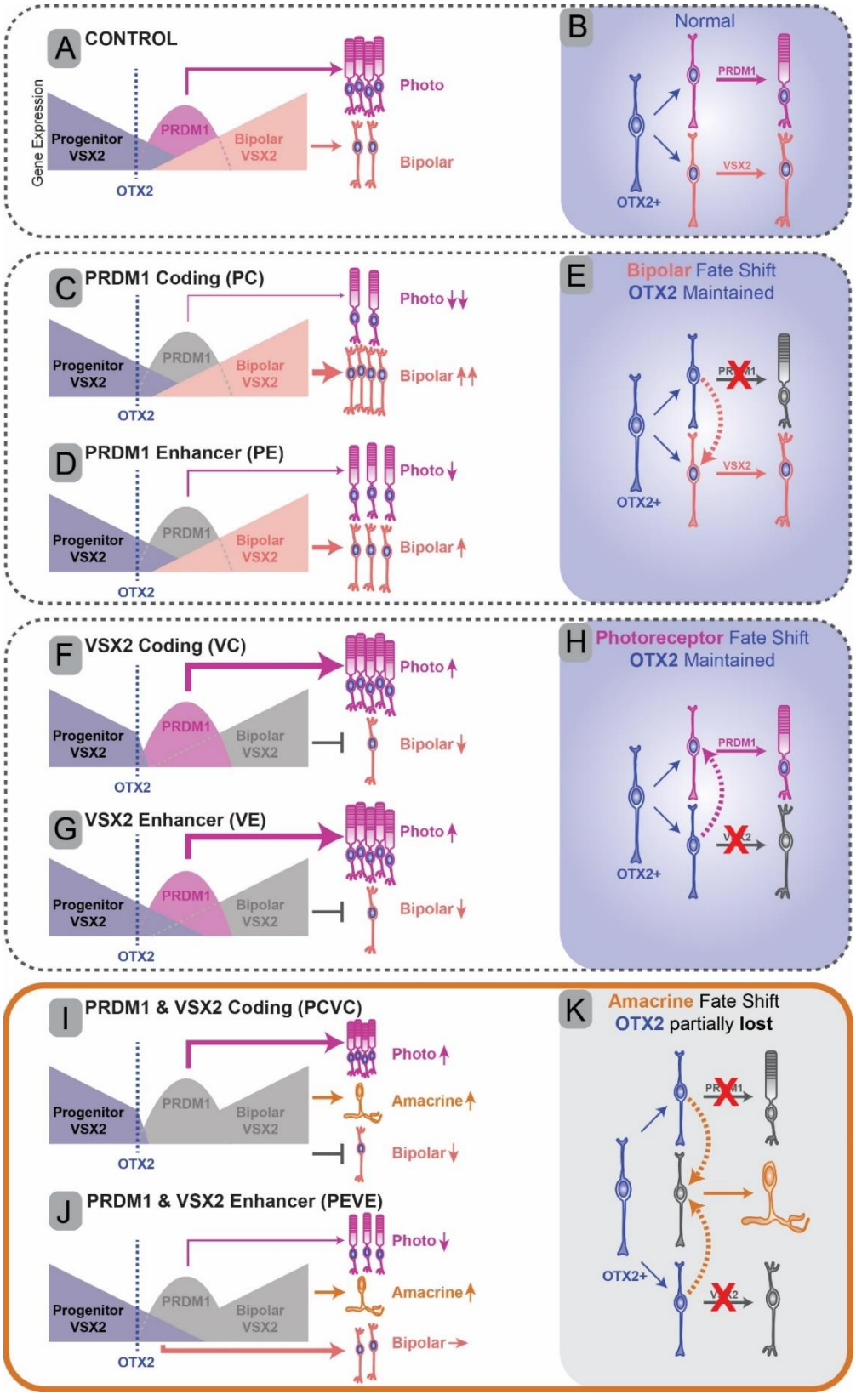



**Figure 3.8 Summary of results from single and double CRISPR knockouts**

**(A)** Under control conditions, progenitors express VSX2 and activate OTX2 as they exit the cell cycle. These OTX2+ cells generate both photoreceptors and bipolars. The presence of PRDM1 and bipolar VSX2 control the numbers of each cell that forms. **(B)** OTX2 drives PRDM1 and VSX2 expression, leading to photoreceptor and bipolar fates respectively. **(C)** PC leads to a major loss of photoreceptors and VSX2 is able to drive excess bipolars. **(D)** PE leads to a more modest loss of photoreceptors and increase in bipolars. **(E)** OTX2+ cells without Prdm1 shift and adopt bipolar fates. **(F-G)** VC and VE cause a loss in bipolars which may become rods. **(H)** OTX2+ cells are unable to adopt bipolar fate and are likely to adopt rod fate instead as *Prdm1* is intact. **(I)** PCVC blocks VSX2 and PRDM1 expression. Without VSX2 carry-over from progenitors, OTX2+ cells adopt photoreceptor identity. Some cells become amacrines and lose OTX2 expression. **(J)** PEVE targeted cells retain progenitor-derived VSX2, resulting in a preference for bipolars in the absence of *Prdm1*. **(K)** In the absence of both VSX2 and PRDM1, some cells fail to maintain OTX2 expression and become amacrines. Thus, *Vsx2* and *Prdm1* may redundantly suppress amacrine competence in OTX2+ cells.

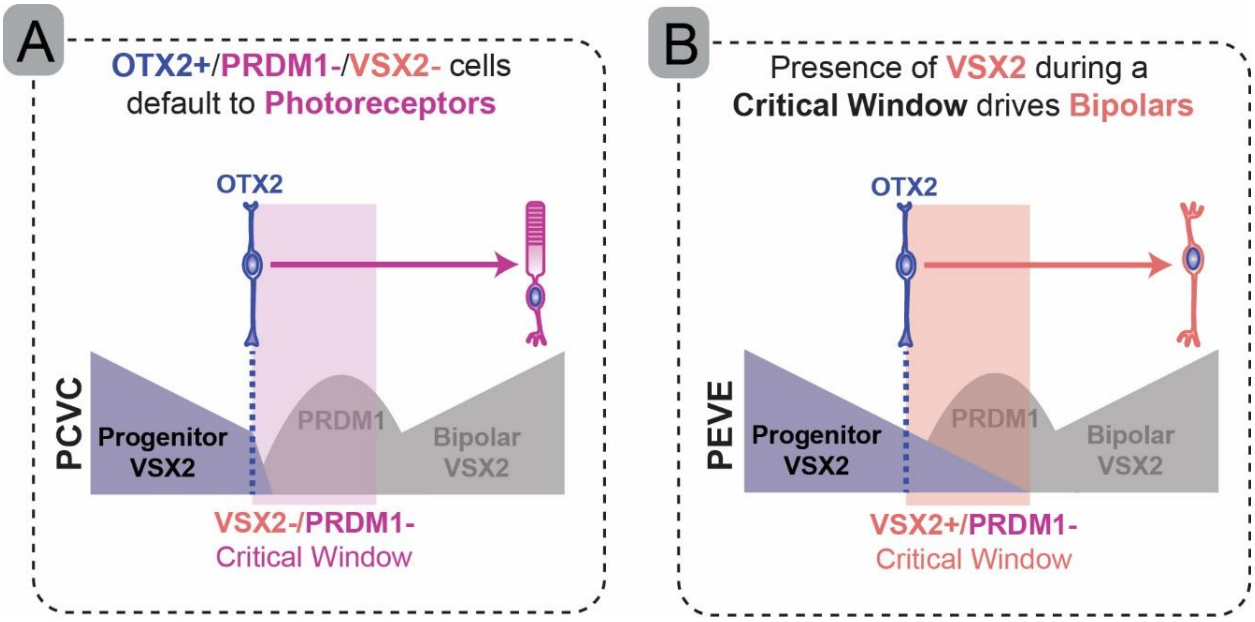
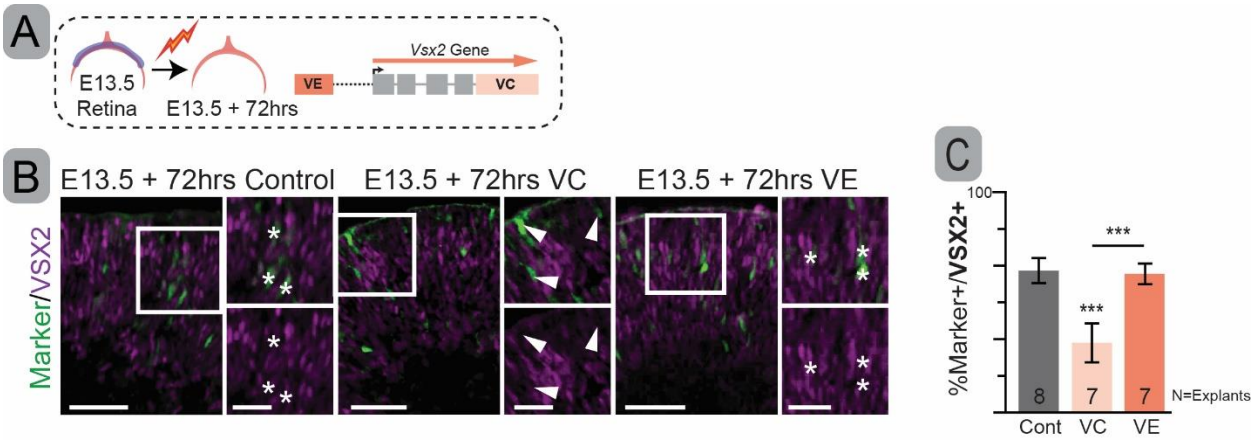
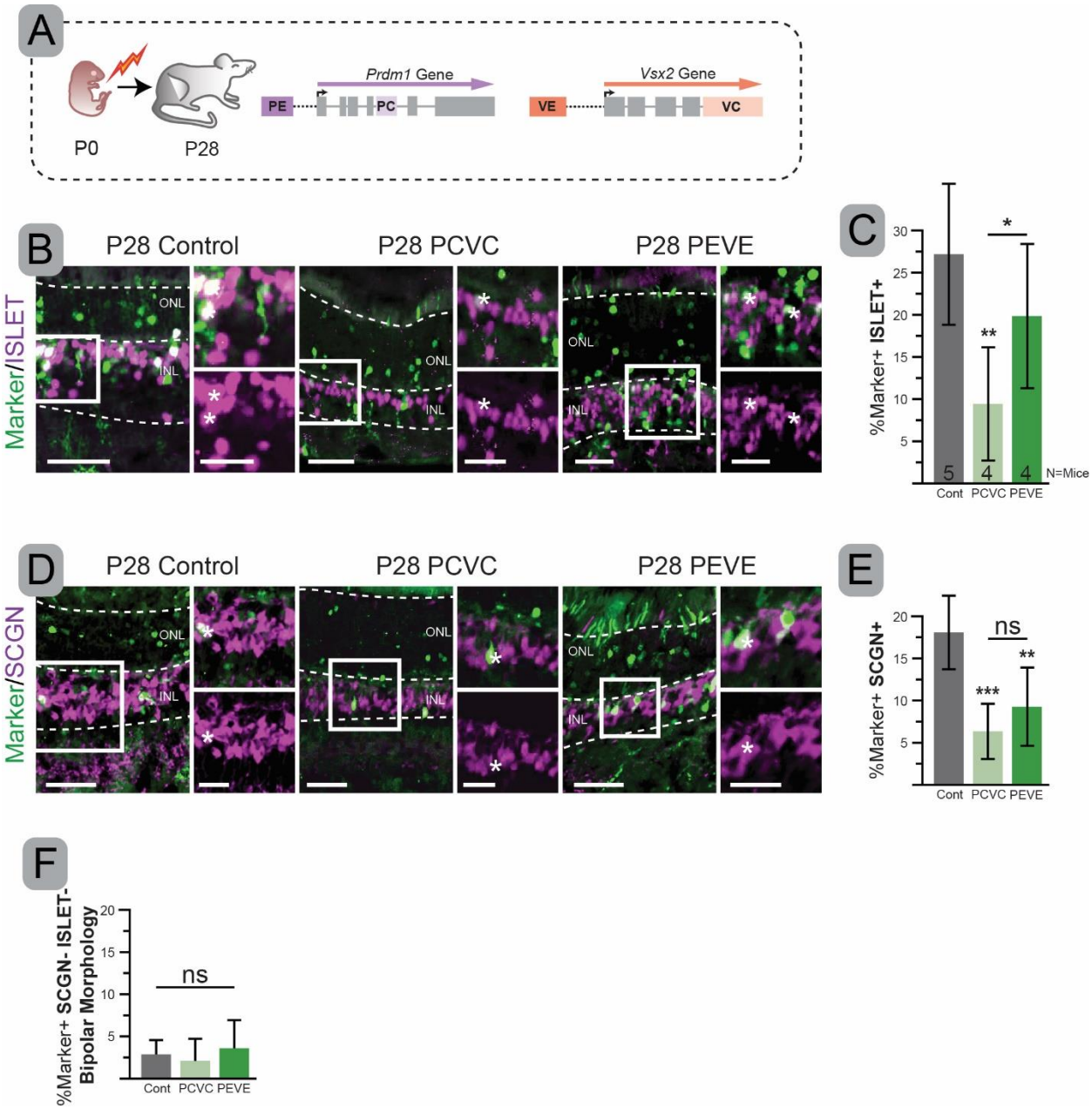


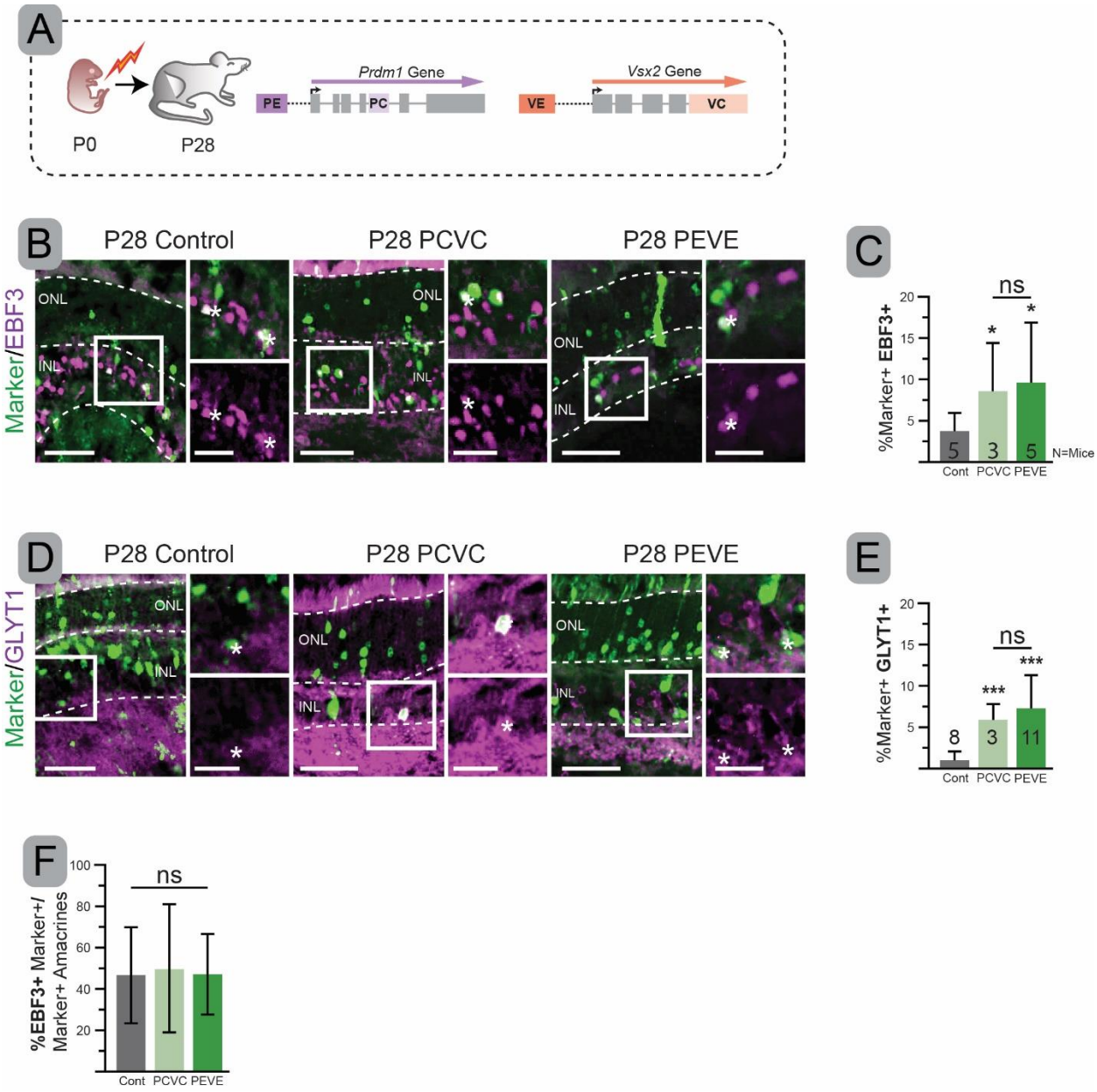

**Figure 3.9 Summary of changes in OTX2+ cell fates**
**(A)** Loss of PRDM1 and VSX2 leads to a photoreceptor default state in OTX2+ cells. **(B)** The presence of VSX2 in a critical period when PRDM1 is absent can instruct bipolar fate.



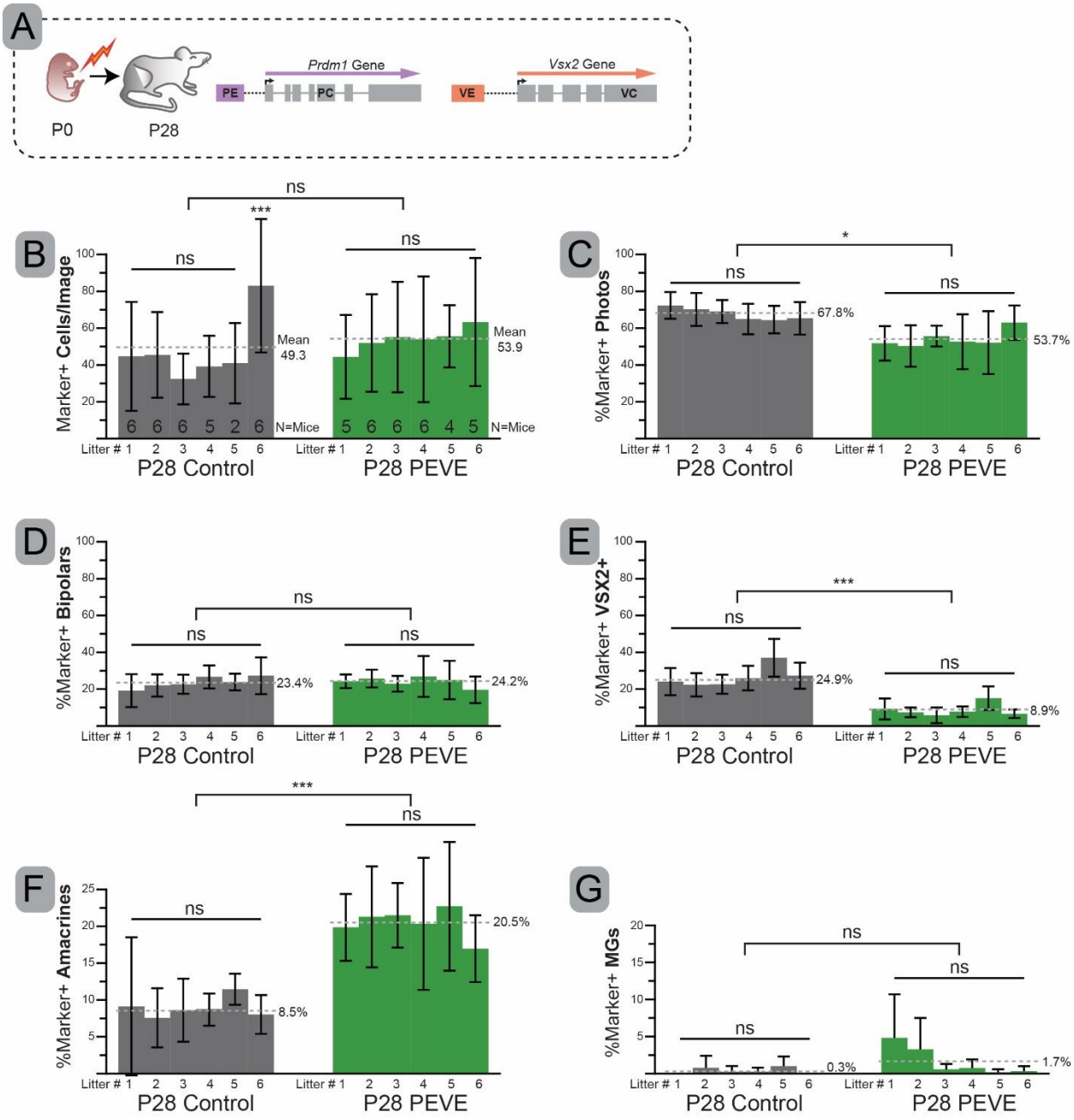

**Supplemental Figure 3.1 VE does not affect progenitor-derived VSX2 expression**
**(A)** Schematic of experimental design. **(B)** Immunohistochemistry of electroporated cells (marker) and VSX2 in cultured retinal explants. **(C)** Percentage of electroporated cells that are VSX2+. Error bars=standard deviation. ns=not significant, ***p<0.001. Bars=50µm, inset bars=25µm. Stars=VSX2+ cells, Arrows=VSX2- cells.




**Supplemental Figure 3.2 Bipolar subtype marker expression in doubly targeted retinas**
**(A)** Schematic of experimental design. **(B, D)** Immunohistochemistry of electroporated (marker) cells co-stained with the pan ON-bipolar marker ISLET1/2 **(B)** or the cone bipolar marker Secretagogin (SCGN) **(D)**. **(C, E)** Percentage of electroporated cells that are co-labeled with ISLET1/2 **(C)** or SCGN **(E)**. **(F)** Percentage of electroporated cells that appear to be bipolar cells by morphology but lacked ISLET1/2 and Secretagogin co-staining. *p<0.05, **p<0.01, ***p<0.001. Bars=50µm, inset bars=25µm. Stars=double labeled cells.

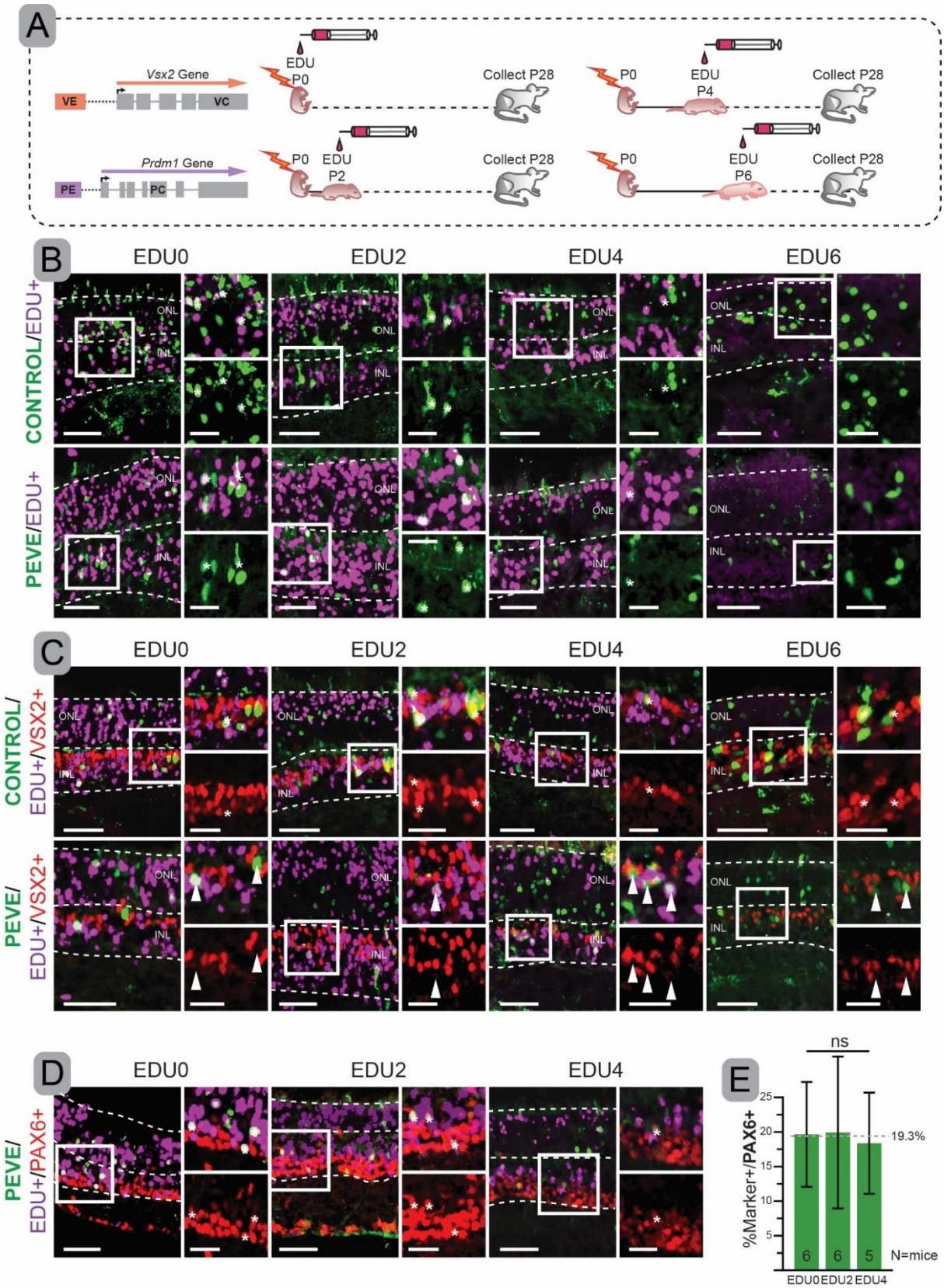



**Supplemental Figure 3.3 Amacrine subtype marker expression in doubly targeted retinas** **(A)** Schematic of experimental design. **(B, D)** Immunohistochemistry of electroporated (marker) cells co-stained with two markers of different late-born amacrine populations. EBF3 **(B)** primarily marks non-glycinergic, non-GABAergic amacrines while GLYT1 (D) marks the glycinergic population. **(C, E)** Percentage of electroporated cells that are co-labeled with EBF3 **(C)** or GLYT1 **(E)**. **(F)** Percentage of electroporated cells that appear amacrine by morphology but lacked EBF3 or GLYT1 co-staining. *p<0.05, **p<0.01, ***p<0.001 Bars=50µm, inset bars=25µm. Stars=double labeled cells.




**Supplemental Figure 3.4 Comparison of cell quantification from control and PEVE electroporated litter at P28**

**(A)** Schematic of experimental design. **(B)** Number of average cells per image across 12 litters of mice. **(C-D, F-G)** Percentage of electroporated cells with photoreceptor **(C)**, bipolar **(D)**, amacrine **(F)**, and Müller glial **(G)** morphologies. **(E)** Percentage of electroporated cells that co-statin for VSX2. *p<0.05, ***p<0.001.




**Supplemental Figure 3.5 Representative images from birthdated control and PEVE electroporations**

**(A)** Schematic of experimental design. **(B-D)** Control and PEVE electroporated retinas birthdated with EdU at four time-points (P0, P2, P4, and P6). **(B)** P28 retinas showing electroporated (marker) cells in green and EdU+ cells in purple. **(C-D)** Control and PEVE electroporated retinas at P28 additionally stained with VSX2 **(C)** and PAX6 **(D)** to mark bipolars and amacrine cells, respectively. **(E)** Percentage of electroporated PEVE cells that co-express PAX6 across three litters. Bars=50µm, inset bars=25µm. Stars=cells with all markers, arrows=cells without all markers.

## Chapter III Supplemental Tables

| Title | Sequence |
|---|---|
| Sequencing Primer | GAGGGCCTATTTCCCATGATTCC |
| Vsx2_Exon5_1.1 | caccgTGAGTCGGGAAGGAAGCCCG |
| Vsx2_Exon5_1.2 | aaacCGGGCTTCCTTCCCGACTCAC |
| Vsx2_Exon5_2.1 | caccgCTGGAGAAGAGCAGTTCCG |
| Vsx2_Exon5_2.2 | aaacCGGAACTGCTCTTCTCCAGC |
| Vsx2_Exon5_3.1 | caccgGTCTGTTTCTAGAGTCGTG |
| Vsx2_Exon5_3.2 | aaacCACGACTCTAGAAACAGACC |
| Vsx2_Enhancer_1.1 | caccgCTATGTGGTAAGACCATGG |
| Vsx2_Enhancer_1.2 | aaacCCATGGTCTTACCACATAGC |
| Vsx2_Enhancer_2.1 | caccgAAACAGAAAGTGGAGTACGG |
| Vsx2_Enhancer_2.2 | aaacCCGTACTCCACTTTCTGTTTC |
| Vsx2_Enhancer_3.1 | caccgAAAACGTCTAACCCCTTAGG |
| Vsx2_Enhancer_3.2 | aaacCCTAAGGGGTTAGACGTTTTC |
| Prdm1_Exon5_1.1 | caccgTTGGAACTAATGCCGTACGG |
| Prdm1_Exon5_1.2 | aaacCCGTACGGCATTAGTTCCAAC |
| Prdm1_Exon5_2.1 | caccgGATAGGATAAACCACCCGA |
| Prdm1_Exon5_2.2 | aaacTCGGGTGGTTTATCCTATCC |
| Prdm1_Exon5_3.1 | caccgAATGTTTCCTATGGTTCCG |
| Prdm1_Exon5_3.2 | aaacCGGAACCATAGGAAACATTC |
| Prdm1_Enhancer_1.1 | caccgATTCAAATCAGTGTCTCGGA |
| Prdm1_Enhancer_1.2 | aaacTCCGAGACACTGATTTGAATC |
| Prdm1_Enhancer_2.1 | caccgAGAGCTAAGCCCACAACGG |
| Prdm1_Enhancer_2.2 | aaacCCGTTGTGGGCTTAGCTCTC |
| Prdm1_Enhancer_3.1 | caccgCATCCCTGAATACAATTAGG |
| Prdm1_Enhancer_3.2 | aaacCCTAATTGTATTCAGGGATGC |

**Supplemental Table 3.1 List of guide primers utilized in CRISPR/Cas9 experiments**

List of all primers utilized for CRISPR constructs.

# CHAPTER IV

# *PRDM1* OVEREXPRESSION CAUSES A PHOTORECEPTOR FATE-SHIFT IN NASCENT, BUT NOT MATURE BIPOLAR CELLS[7]

## Chapter IV Summary

The transcription factors *Prdm1* (*Blimp1)* and *Vsx2 (Chx10)* work downstream of *Otx2* to regulate photoreceptor and bipolar cell fates in the developing retina. Mice that lack *Vsx2* fail to form bipolar cells while *Prdm1* mutants form excess bipolars at the direct expense of photoreceptors. Excess bipolars in *Prdm1* mutants appear to derive from rods, suggesting that photoreceptor fate remains mutable for some time after cells become specified. Here we tested whether bipolar cell fate is also plastic during development. To do this, we created a system to conditionally misexpress *Prdm1* at different times during bipolar cell development. We found that *Prdm1* blocks bipolar cell formation if expressed before the fate choice decision occurred. When we misexpressed *Prdm1* just after the decision to become a bipolar cell was made, some cells were reprogrammed into photoreceptors. In contrast, *Prdm1* misexpression in mature bipolar cells did not affect cell fate. Surprisingly, we also found that sustained misexpression of *Prdm1* was selectively toxic to photoreceptors. Our data show that bipolar fate is malleable, but only for a short temporal window following fate specification. *Prdm1* and *Vsx2* act by stabilizing photoreceptor and bipolar fates in developing OTX2+ cells of the retina.

[7]The contents of this chapter have been submitted for publication and the work was completed in collaboration with co-authors including Ko U. Park, Jason S. Silver, Vince A. Chiodo, William W. Hauswirth, and Joseph A. Brzezinski IV

**Rationale**

During development, a population of retinal progenitor cells gives rise to all six major classes of neurons within the eye: rod and cone photoreceptors, ganglion, amacrine, bipolar, and horizontal cells, as well as Müller glia[82,83]. Retinal progenitors permanently exit the cell cycle (their birthdate) and generate these cell fates in a stereotyped overlapping fashion from approximately embryonic (E) day 11.5 to postnatal (P) day 7 in mice[85,219,220,284]. These progenitors have more than one fate option they can select from at nearly any given time in retinal development. Nonetheless, it is unclear how these cells choose their fate and subsequently make that decision permanent.

Mouse retinal progenitors in the postnatal period choose between four fates: amacrine cells, Müller glia, bipolar cells, and rod photoreceptors[82,85]. Of these, rods and bipolars are the most abundant and both types express the key transcription factor *Otx2*[25,138,227,228]. *Otx2* is required for the formation of photoreceptors and bipolar cells[138,139]. Its expression is activated in the final cell cycle and precedes the decision to adopt photoreceptor versus bipolar cell fates[243]. Otx2 directly regulates two downstream transcription factors, *Vsx2* (*Chx10*) and *Prdm1* (*Blimp1*), through defined enhancer sequences[167,231,233,234]. When *Prdm1* is knocked out in the developing retina, there is a severe reduction in the number of photoreceptors that form[127,128,231]. In these mutants, there is a 1:1 fate shift within OTX2+ cells such that bipolars are increased at the expense of photoreceptors. Overexpression of *Prdm1* in early development suppresses bipolar cell formation[127,128]. No bipolar cells are formed when *Vsx2* is mutated[148,149,285]. In contrast, when *Vsx2* is overexpressed it inhibits photoreceptor gene expression and increases bipolar cell formation at the expense of rods[150,232]. These data suggest that there is a mutually inhibitory gene regulatory network within OTX2+ cells where *Prdm1* represses bipolar fate and

*Vsx2* blocks photoreceptor formation. Correspondingly, loss of *Prdm1* results in precocious and excess expression of *Vsx2* in the retina[127,128,231]. We observed that bipolar cells in *Prdm1* mutants could be derived from cells that make rod-specific markers[231]. This suggests that photoreceptor fate is transiently plastic after it has been selected and is subsequently stabilized as a rod or superseded by the bipolar cell program. In *Vsx2* mutant retinas, non-functional *Vsx2* mRNA remains and was found in developing photoreceptors[150]. This raises the possibility that OTX2+ cells destined for bipolar fate can have their identity superseded by the photoreceptor program. Here, we asked whether and when bipolar fate can be superseded in developing OTX2+ cells.

Since *Prdm1* can block bipolar cell formation, we misexpressed PRDM1 at different stages of retinal development to determine whether bipolar fate is plastic. To do this, we first created a mouse line that allowed us to conditionally drive *Prdm1* expression via CRE-mediated recombination. We then drove constitutive PRDM1 expression using three distinct sources of CRE during unique timeframes in development: (1) Before bipolar fate choice is made, (2) just after bipolar fate selection, and (3) in mature bipolar cells. *Prdm1* had different effects at each of these developmental stages. It blocked bipolar formation if present before the choice, partially converted bipolars into photoreceptors just after bipolar specification, and had no effect on the fate of mature bipolar cells. Surprisingly, we also observed that long-term misexpression of *Prdm1* was toxic, but only to photoreceptors. Taken together, our data show that bipolar cells are transiently plastic after their fate is selected. Nonetheless, shortly after fate selection bipolar cells become stabilized and refractory to the effects of PRDM1. One role of the interplay between *Prdm1* and *Vsx2* in OTX2+ cells is to stabilize fate choices made during retinal development.

**Results**

Photoreceptor and bipolar cell formation requires the transcription factor *Otx2*[138,139]. OTX2 directly activates two downstream transcription factors, *Prdm1* and *Vsx2*, which have been hypothesized to play a cross-repressive role to set the balance of photoreceptors and bipolar cells formed[127,128,130,167,231,233,270]. If so, constitutive expression of PRDM1 in OTX2+ cells should block bipolar genesis and force these cells to adopt photoreceptor fate. Since *Prdm1* is not permanently expressed in photoreceptors, there may only be a transient period when *Prdm1* can affect fate choice. To test how *Prdm1* affects cell fate, we built systems to misexpress *Prdm1* in a spatially and temporally controlled fashion. To drive *Prdm1* expression, we first constructed a CRE-inducible *Prdm1* mouse line. We replaced the red fluorescent protein sequence (tdTomato) in the *Ai9* targeting vector with the cDNA for mouse *Prdm1*[277]. This targeting vector was used to make knock-in mice, resulting in the insertion of a *Lox-stop-Lox Prdm1* allele into the *ROSA26* locus (Fig 4.1A). Throughout, we refer to these *ROSA-Prdm1* knock-in animals as *PRDM1* mice. As detailed below, we permanently misexpressed *Prdm1* in different spatial and temporal patterns by combining these *PRDM1* mice with multiple CRE expression systems.

*Constitutive Prdm1 expression alters cell fates within the retina*

To test that our misexpression system worked and that *Prdm1* can induce cell fate changes, we crossed *PRDM1* mice with α*PAX6-Cre-IRES-GFP* mice (*PAX6-CRE*) (Fig 4.1A)[141]. The *PAX6-CRE* line drives CRE and GFP expression in nearly all retinal progenitors of the peripheral retina starting before the onset of neurogenesis and persisting in a subset of amacrine cells across the whole mature retina[141,286]. This transgene also drives expression in the developing ciliary structure of the eye that is immediately adjacent to the retina and within a

small number of peripheral retinal pigmented epithelial (RPE) cells. We collected *PAX6-CRE/PRDM1* experimental and *PRDM1/+* control mice at P0. Immunostaining for PRDM1 showed a dramatic increase in the number and distribution of labeled cells in the peripheral retinas of *PAX6-CRE/PRDM1* eyes compared to controls (Fig 4.1B). This included PRDM1+ cells in the RPE and ciliary body, which were never observed in controls (Fig 4.1B). Thus, the *PRDM1* mice operate as expected, leading to robust PRDM1 misexpression following CRE-mediated recombination in the eye.

Misexpressing PRDM1 throughout the periphery at P0 had no observable effect on retinal architecture (Fig 4.1B-D). Despite the increase in PRDM1 expression in experimental mice, immunostaining retinal progenitors in the P0 peripheral retina with VSX2 showed no changes in gross morphological pattern or overlap with PRDM1+ cells compared to control (Fig 4.1C-D)[148–150,287]. This is consistent with a role for PRDM1 in controlling cell fate choice in OTX2+ cells, rather than proliferative progenitors.

To examine cell fate choice, we next collected mice at P7, when bipolar cells are readily detectable in the retina (Fig 4.2A). PAX6-*CRE/PRDM1* mice had morphologically similar peripheral retinas compared to CRE-negative controls (Fig 4.2B-C). We stained P7 sections with multiple markers for bipolar cells. These include: (1) OTX2, which marks newly postmitotic rods and bipolars at higher intensity than mature photoreceptors, (2) VSX2, which marks bipolars intensely and progenitors weakly, (3) ISLET1/2, which marks cone ON and rod bipolar cells, and (4) Secretagogin (SCGN), which marks a subset of ON and OFF cone bipolars[25,138,149,215,227,272,273]. *PAX6-CRE/PRDM1* mice had fewer intensely labeled VSX2+ and OTX2+ cells (Fig 4.2B-E). The overall number of OTX2+ cells was slightly, but not significantly, reduced (Fig 4,2F). We observed mosaic patterns of VSX2+ bipolar cell reduction

(Fig 4.2D) and continuous stretches of near complete bipolar cell loss (Fig 4.2E). When we quantified bright VSX2 cells, which generally represent bipolar-specific expression, we observed a significant decrease in our test condition compared to control (Fig 4.2D-E, G, $p<0.001$). This was consistent with a reduction in bipolar cells. We found that SCGN+ cone bipolar cells were reduced in experimental retinas compared to control (Fig 4.2D-E, H, $p<0.001$). All areas that lacked intense OTX2 or VSX2 staining also lacked SCGN or ISLET1/2 staining (Fig 2B-E, H-I). ISLET1/2+ bipolar cells showed a severe loss of labeling in *PAX6-CRE/PRDM1* retinas compared to control (Fig 4.2B-C, I, $p<0.001$). In contrast, we saw no differences in the number of amacrine and ganglion cells that were also labeled by ISLET1/2 between conditions (Fig 4.2B-C, J, $p=0.355$)[215,272]. Our results show that misexpressing *Prdm1* before the onset of bipolar cell formation can prevent their genesis. This aligns with previous findings where overexpression of *Prdm1* in postnatal retinal progenitors suppressed bipolar cell formation[127,128]. Moreover, our results show that *Prdm1* misexpression does not significantly interfere with other cell fate choices that occur in retinal development.

We next tested whether *Prdm1* could affect fate choice in OTX2+ cells that have already decided to become bipolar cells. To do this, we first obtained *VSX2-CRE* mice from Jackson Labs[288]. This line uses sequences upstream of *Vsx2* to drive CRE in a bipolar cell-specific fashion starting shortly after they become specified in the early postnatal period[288]. We then crossed the *VSX2-CRE* mice with our *PRDM1* animals to test whether *Prdm1* misexpression at the onset of bipolar fate specification could alter fate choice (Fig S4.1A). However, we consistently had trouble breeding these mice. When litters were obtained, they were small (3-5 pups) and no *VSX2-CRE/PRDM1* double-transgenic mice were seen across dozens of crosses. Since *Prdm1* promotes the germ cell lineage at the expense of somatic development[289–291], we

suspected that CRE expression might be occurring early in development and preventing embryogenesis. To test this, we crossed the *VSX2-CRE* mice with *ROSA-RFP* (RFP) reporter mice (Fig S4.1)[277]. When mature *VSX2-CRE/RFP* retinas were stained for CRE, the pattern of expression was limited to bipolar cells as expected (Fig S4.1D). However, these mice had visibly red skin and their entire eye was RFP+, including the lens and surrounding ocular tissues (Fig S4.1D). When the *VSX2-CRE* was maternally derived, all pups (even those that were *VSX2-CRE*-negative) ubiquitously expressed RFP (Fig S4.1E). When *VSX2-CRE* was paternally inherited, *VSX2-Cre/RFP* pups had mosaic, yet global, RFP expression (Fig S4.1F). We concluded that *VSX2-CRE* mice have CRE activity in retinal bipolar cells, but also at very early stages of development. When crossed with *PRDM1* mice, the *VSX2-CRE* allele will drive widespread PRDM1 expression. Based on *Prdm1's* early role in development, this is incompatible with embryogenesis. Early PRDM1 misexpression explains why we never recovered any *VSX2-CRE/PRDM1* mice, which necessitated a different approach to misexpress *Prdm1* in bipolar cells. To do this, we designed viral and plasmid-electroporation strategies to deliver *CRE* recombinase to mature and developing bipolar cells, respectively.

*AAV driven PRDM1 does not alter bipolar fate choice and is toxic to mature photoreceptors*

To ascertain if PRDM1 expression in mature bipolar cells is sufficient to cause a fate shift to photoreceptors we created adeno-associated viruses (AAV) that drive CRE-recombinase under the control of an ON bipolar cell-specific *PCP2* enhancer (Bipolar-CRE-AAV)[292,293]. *PRDM1* mice were crossed with *RFP* mice to generate trans-heterozygous experimental (*RFP/PRDM1*) and control (*RFP/+*) mice. Bipolar-CRE-AAV was injected into the vitreous of P28 eyes and the mice were raised till P60 (Fig 4.3A). This allowed us to track cells that

expressed the bipolar-specific CRE because they will permanently express RFP. We then compared *RFP/+* controls to *RFP/PRDM1* mice by immunostaining for RFP. Cell fates were assessed by morphology and location within the retina. In addition to RFP+ bipolar cells, we also observed RFP+ ganglion cells, amacrines, Müller glia, and photoreceptors (Fig 4.3B). We quantified cells and found that both control mice and their *RFP/PRDM1* littermates had an equal number of amacrines and ganglion cells, which were proximal to the site of the intravitreal injections (Fig 4.3D). This suggested that amacrine and ganglion cell labeling was the result of transient off-target CRE expression, as previously noted with similar viruses[293]. Thus, we excluded amacrine and ganglion cells from further calculations. While off-target expression accounts for RFP labeling of photoreceptors and Müller glia in controls, we quantified them to determine whether any cell fate changes occurred upon PRDM1 misexpression. When summed, the number of RFP+ bipolars and photoreceptors were similar between conditions (Fig 4.3C). If PRDM1 reprogrammed bipolar cell fate, we expected to see an increase in RFP+ photoreceptors at the expense of bipolars. However, when we specifically examined each cell fate, we observed fewer photoreceptors and more bipolar cells in our *Prdm1* misexpression condition compared to our controls, exactly the opposite of our hypothesis (Fig 4.3E-G). Indeed, there were almost no RFP+ photoreceptors found in the *RFP/PRDM1* condition (Fig 4.3E).

We next tested whether PRDM1 misexpression repressed VSX2 expression in RFP+ bipolar cells. VSX2 stains revealed that significantly more cells in *RFP/PRDM1* mice were VSX2+ compared to controls (Fig 4.3H-I). However, we did not observe diminished VSX2 staining intensity compared to neurons in the control condition or in RFP-negative bipolar cells within the same image. We concluded that constitutive PRDM1 expression cannot alter the fate

of mature bipolar cells, does not affect VSX2 expression, and is toxic only to mature photoreceptors.

*Overexpression of PRDM1 in nascent bipolar cells causes a fate shift to rods*

In the absence of *Prdm1*, VSX2 is precociously upregulated in the retina[127,128,231]. Early overexpression of *Prdm1* reduces VSX2 and bipolar cell formation (Fig 4.2)[127,128]. Nonetheless, long-term misexpression of *Prdm1* in adult bipolar cells does not repress VSX2 expression or change cell fate (Fig 4.3). We hypothesized that there is a critical period in OTX2+ cells where PRDM1 can suppress VSX2 and promote photoreceptor fate at the expense of bipolar cell development.

To test this, we designed a plasmid containing a 164bp bipolar-specific *Vsx2* enhancer driving CRE-recombinase expression that we called VE-CRE (Fig 4.4A)[270]. Previous work showed that this enhancer is sufficient to drive bipolar-specific expression and our recent findings (See Chapter III) suggest it is necessary for VSX2 expression and bipolar cell formation[270]. We electroporated VE-CRE into the retina of P0 *RFP/+* controls and *RFP/PRDM1* littermates and raised the pups to P7 (Fig 4.4A). RFP+ cells were quantified based on morphology and location within the retina. As expected, the majority of RFP+ cells were bipolars (Fig 4.4B, D). However, about 12% of RFP+ cells were photoreceptors, arguing that some VE-expressing cells are poised between bipolar and photoreceptor fates (Fig 4C). We also observed a small number of amacrines and Müller glia in control electroporations, which likely reflects off-target CRE activity (Fig 4.4B, E, H). VE-CRE electroporations in *RFP/PRDM1* mice had three times more RFP+ photoreceptors compared to control, which came at the direct expense of RFP+ bipolar cells (Fig 4.4B-D). We stained for VSX2 and found that the loss of

RFP+ bipolar cells perfectly matched the loss of RFP+/VSX2+ cells (Fig 4.4E-F). The presence of VSX2 in morphologically identified bipolar cells suggested that they were accurately quantified and had repressed photoreceptor fate despite the misexpression of PRDM1. Photoreceptors did not co-express VSX2 in either condition. Thus, overexpression of PRDM1 in VSX2+ nascent bipolars was sufficient to force a fate change in some (~25%), but not all of the cells. Upon quantification, we observed no differences in the number of amacrines or Müller glia between conditions (Fig 4.4G, data not shown). We also stained for OTX2 and the pan-amacrine marker PAX6 and found no differences in either between conditions (Fig 4.4H-J)[209]. This suggests that *Prdm1* misexpression only alters fate choice within OTX2+ cells.

*Constitutive PRDM1 expression is toxic to mature photoreceptors*

PRDM1 is expressed by OTX2+ cells throughout retinal development but is downregulated in the early postnatal period and becomes undetectable by immunohistochemistry between P6 to P10[127]. Although PRDM1 is important for photoreceptor formation, it is not appreciably expressed by mature rods or cones. When *Prdm1* is turned on earlier in developing bipolars, it can cause a fate shift to photoreceptor identity (Fig 4.4). However, our Bipolar-CRE-AAV experiment suggests that expressing *Prdm1* in mature photoreceptors is toxic (Fig 4.3). This suggested that constitutive PRDM1 expression would eventually kill photoreceptors that were formed from nascent bipolar cells.

To test this, we electroporated P0 *RFP/+* control pups and *RFP/PRDM1* littermates with the VE-CRE plasmid and raised them to P28 when the retina is mature. We assessed the fate of RFP+ cells by morphology and immunohistochemistry (Fig 4.5A). Unlike at P7, we did not observe differences in the number of RFP+ photoreceptors and bipolar cells between control and

*RFP/PRDM1* conditions (Fig 4.5B-D). The percentage of electroporated cells that were photoreceptors at P28 in the *RFP/PRDM1* condition matched controls at both P7 and P28 (~10%). This argues that the increase in photoreceptors caused by *Prdm1* misexpression was lost by P28. While it is likely that photoreceptor cell death is the cause, the sparse label nature of our approach and the narrow time frame of cell death markers prevented us from directly measuring cell death. Additionally, there was large variation in the total number of electroporated cells per eye that prevented us from determining if there were significantly fewer cells in the *RFP/PRDM1* condition at P7 and P28. We saw no differences in the number of RFP+ amacrines or Müller glia at P28 (Fig 4.5G, data not shown). There were also no differences in the number of RFP+ cells that co-expressed VSX2+, OTX2+, or PAX6+ between conditions (Fig 4.5E-J). Taken together with the Bipolar-CRE-AAV findings, we conclude that sustained *Prdm1* expression in photoreceptors is toxic. Nonetheless, it appears that *Prdm1* misexpression in other cell types does not affect their survival.

**Discussion**

During late retinogenesis, OTX2+ cells decide between rod photoreceptor and bipolar fates. This is driven in part by the actions of OTX2's downstream targets, *Vsx2* and *Prdm1* (Fig 4.6D). The loss of *Prdm1* from the retina results in a fate shift to VSX2+ bipolar cells, even after cells have started to show photoreceptor-like morphology and markers (Brzezinski et al., 2013). This suggests that there is some window wherein developing OTX2+ cells have selected a specific fate but remain susceptible to signals driving an alternative identity. Here, we tested whether OTX2+ cells can change their identity at different stages of maturity (Fig 4.6A-C). Our findings show that some specified bipolar interneurons can be shifted to photoreceptor fates by

PRDM1 (Figs 4.4, 4.6B). However, there is a narrow temporal limit to this fate conversion, and mature bipolars do not change their fate in response to PRDM1 (Fig 4.3, 4.6C). While *Prdm1* is critical for photoreceptor development, we found that if PRDM1 is constitutively expressed in mature photoreceptors it will cause their death (Figs 4.3, 4.5, 4.6C).

*Bipolar cell fate choice is plastic over a narrow window of development*

We used *Prdm1* misexpression to ask whether bipolar cell fate can be superseded at three different stages of retinal development. Using *PAX6-CRE* mice, we were able to drive *Prdm1* misexpression before the decision to become a bipolar cell is normally made. As predicted from prior work, this early *Prdm1* misexpression was highly effective at preventing bipolar cell formation (Figs 4.1-4.2, 4.6A)[127,128]. Next, we used an electroporation approach to permanently activate *Prdm1* in newly specified bipolar cells. Misexpression at this stage blocked a portion of bipolar cell formation while increasing the number of photoreceptors (Fig 4.4). Thus, bipolar fate can be superseded by *Prdm1* around the time of specification, although at a modest efficacy compared to early misexpression (Fig 4.6A-B). This fate plasticity is similar to what is seen in *Prdm1* conditional knock-out retinas, where a substantial number of rod photoreceptors appear to directly transition into bipolar cells in the early postnatal period[231]. Lastly, we activated *Prdm1* expression in mature bipolar cells using an AAV strategy. This had no discernable effect on bipolar cell fate stability (Figs 4.3, 4.6C). Taken together, our results suggest that bipolar cell fate is malleable. Nonetheless, plasticity is limited to a narrow time period around the bipolar fate specification event (Fig 4.6).

Bipolar cell plasticity is similar to what has been observed in rods. Knocking out the rod-instructive factor *Nrl* prevents rod formation and massively increases cone genesis if done early

in development[294,295]. Removing *Nrl* shortly after birth incompletely converts rods into cones, while targeting *Nrl* in the adult retina has a more modest effect that is limited to changes in rod- and cone-specific gene expression[295–297]. These data argue that OTX2+ neurons are transiently plastic and progressively alter their epigenetic states to "lock-in" their identities (Fig 4.6E)[295]. The ability to reprogram bipolar cell identity around the time of fate specification appears more modest than what occurs to photoreceptors in *Nrl* or *Prdm1* knockouts. This raises the possibility that some cell types, such as photoreceptors, take longer to "lock-in" their identities. This may correlate with the highly specialized chromatin architecture that slowly forms in rods over several weeks in postnatal mice[298]. In this model, *Prdm1* may inhibit bipolar fate while rods develop their "locked-in" stabilized state. This could explain why PRDM1 is only active for a relatively short period during photoreceptor development.

Our previous loss-of-function data and current PRDM1 gain-of-function experiments show that cell fate choice is plastic in developing OTX2+ cells (Fig 4.6E)[231]. While this plasticity appears to be short-lived in newly specified bipolar cells, it is unclear how long photoreceptors remain plastic. As PRDM1 expression is low in photoreceptors by P7, it is unlikely that plasticity extends beyond the first postnatal week (Fig 4.6E). Since fate plasticity is only widespread in genetically perturbed conditions, it is likely that PRDM1 and VSX2 robustly stabilize fate choices in OTX2+ cells. How PRDM1 and VSX2 interact with OTX2 and other factors to select and maintain cell fate decisions remains to be determined.

*PRDM1 acts in a context-specific fashion in OTX2+ cells*

OTX2 directly activates the expression of *Prdm1* and *Vsx2* through essential retina-specific enhancer elements (see Chapter III)[167,231,233,270]. Gain- and loss-of-function experiments

argue that the balance of photoreceptors and bipolar cells is controlled by direct cross-repression between *Prdm1* and *Vsx2*[127,128,150,231]. Overexpression of *Prdm1* early in development blocked the intense VSX2 labeling that is characteristic of bipolar cells (Fig 4.2). However, permanently misexpressing *Prdm1* at later times did not block VSX2 expression in morphologically identified bipolar cells (Figs 4.3-4.5). There are several possible explanations for this observation. As mentioned above, one possibility is that mature bipolar cells become "locked-in" and can no longer respond to PRDM1. This could be through changes in the epigenetic state that prevents PRDM1 from silencing *Vsx2* in mature bipolar cells. This could also occur if there are unique enhancers that maintain VSX2 expression in mature bipolar cells that are not regulated by PRDM1. We observed that misexpression of *Prdm1* in early development did not block VSX2 expression in progenitor cells (Fig 4.1C-D), which would be expected to severely limit retinal progenitor proliferation[148–150,287]. This argues that PRDM1 regulates *Vsx2* expression in a context-specific fashion. This could occur if PRDM1 has no regulatory effect on *Vsx2* enhancers that control progenitor-specific expression, while binding the bipolar-specific enhancer and suppressing its activity. However, the 164bp bipolar-specific *Vsx2* enhancer sequence lacks a high-confidence PRDM1 binding site, raising the possibility that PRDM1 silences *Vsx2* by binding at a different site or by acting indirectly on *Vsx2* expression[128,270]. We observed that most retinal cell types were unaffected by *Prdm1* misexpression during development or in adults (Figs 4.1-4.5). This further argues that *Prdm1* acts in a context-specific fashion, only affecting fate in cells that are competent to become bipolar cells. Other cells are either unaffected by PRDM1 activity or "locked-in" and refractory to its influence. Additional studies examining PRDM1 and VSX2 binding sites in the developing retina are needed to understand the mechanisms these two factors use to control photoreceptor and bipolar fate choice.

*PRDM1 is toxic to mature photoreceptors*

In the retina, *Prdm1* is transiently expressed in OTX2+ cells. Expression does not persist into mature photoreceptors[127]. There are several other systems where transient PRDM1 expression affects fate determination, such as primordial germ cell genesis in mice as well as slow twitch muscle and neural crest development in zebrafish[289,290,299–302]. We presumed that *Prdm1* expression was transient because it was no longer needed in mature photoreceptors. However, we were surprised to see that sustained *Prdm1* expression was toxic to photoreceptors. This was in contrast to other retinal cell types, including mature bipolar cells, which appeared unaffected by *Prdm1* misexpression. Thus, the apparent toxicity of PRDM1 was not simply due to its overexpression, but rather because of a specific effect on photoreceptors (Fig 4.6C). We did not observe acute toxicity with *Prdm1* overexpression at P0 or P7. This suggests that toxicity occurs relatively late, during or after the process of maturation. It is unclear why sustained PRDM1 expression is toxic only in photoreceptors. One possibility is that by blocking bipolar formation, PRDM1 also inhibits photoreceptor maturation. The inability to mature may then cause photoreceptor cell death. Another possibility is that PRDM1 expression is incompatible with the formation or maintenance of the specialized rod nuclear architecture that forms in the postnatal period[298]. Due to chromatin accessibility differences between photoreceptors versus other retinal cell types, PRDM1 may have the ability to regulate deleterious gene expression networks only in photoreceptors. Lastly, alterations in the dosage of key transcription factors may destabilize gene regulatory networks required for photoreceptor function and survival. In support of this possibility, a recent report showed that overexpressing OTX2 in photoreceptors caused mild toxicity within rods[226]. Taken together, these data suggest that careful control of

transcription factor expression timing and levels are essential for rod survival. The mechanisms that underlie PRDM1-induced photoreceptor toxicity remain to be uncovered.

**Chapter IV Figures**

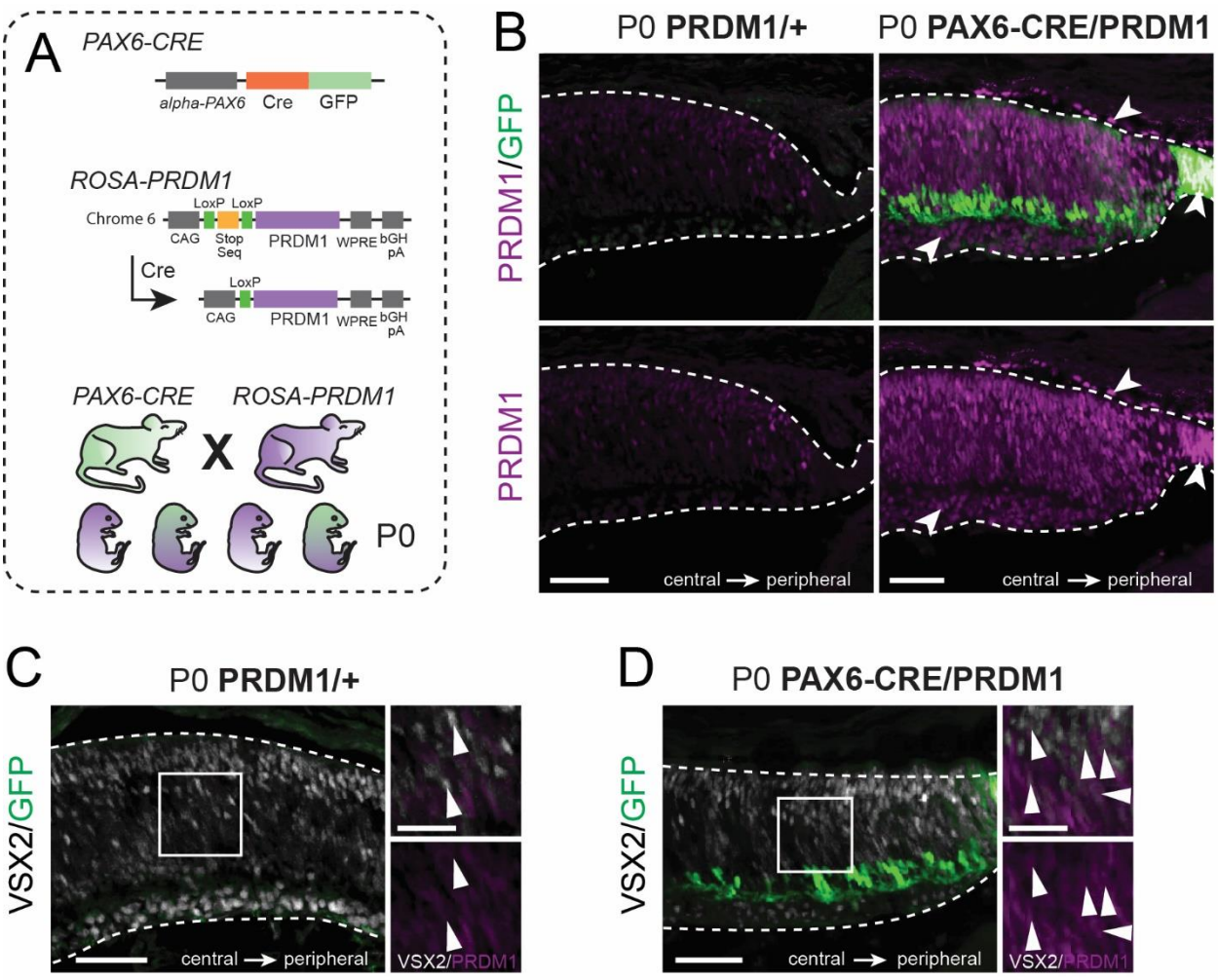



**Figure 4.1. Constitutive PRDM1 expression driven by *PAX6-CRE* does not prevent progenitor VSX2 expression**

**A)** Schematic of transgenic mice utilized. To make the *PRDM1* strain, the *ROSA26* locus on chromosome 6 was targeted to insert the *Prdm1* cDNA downstream of a CAG enhancer and a *LoxP-stop-LoxP* sequence. Further downstream is a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and a bovine growth hormone polyadenylation (bGH pA) site. Introduction of CRE removes the stop sequence and allows for permanent PRDM1 expression. **B)** Representative immunohistochemistry of *PRDM1/+* control compared to *PAX6-CRE/PRDM1* retinas stained for PRDM1 (purple) and GFP (green) and imaged at the same exposure level. Note the ectopic expression of PRDM1 in the RPE and ciliary body of PAX6-CRE/PRDM1 eyes (arrowheads). **C-D)** P0 *PRDM1/+* control compared to *PAX6-CRE/PRDM1* retinas stained for VSX2 (white), GFP (green), and PRDM1 (purple, inset-only). Arrowheads mark PRDM1+/VSX2+ cells. There is VSX2 background staining in the ganglion cell layer in both conditions. bars=50µm, inset bars=25µm.

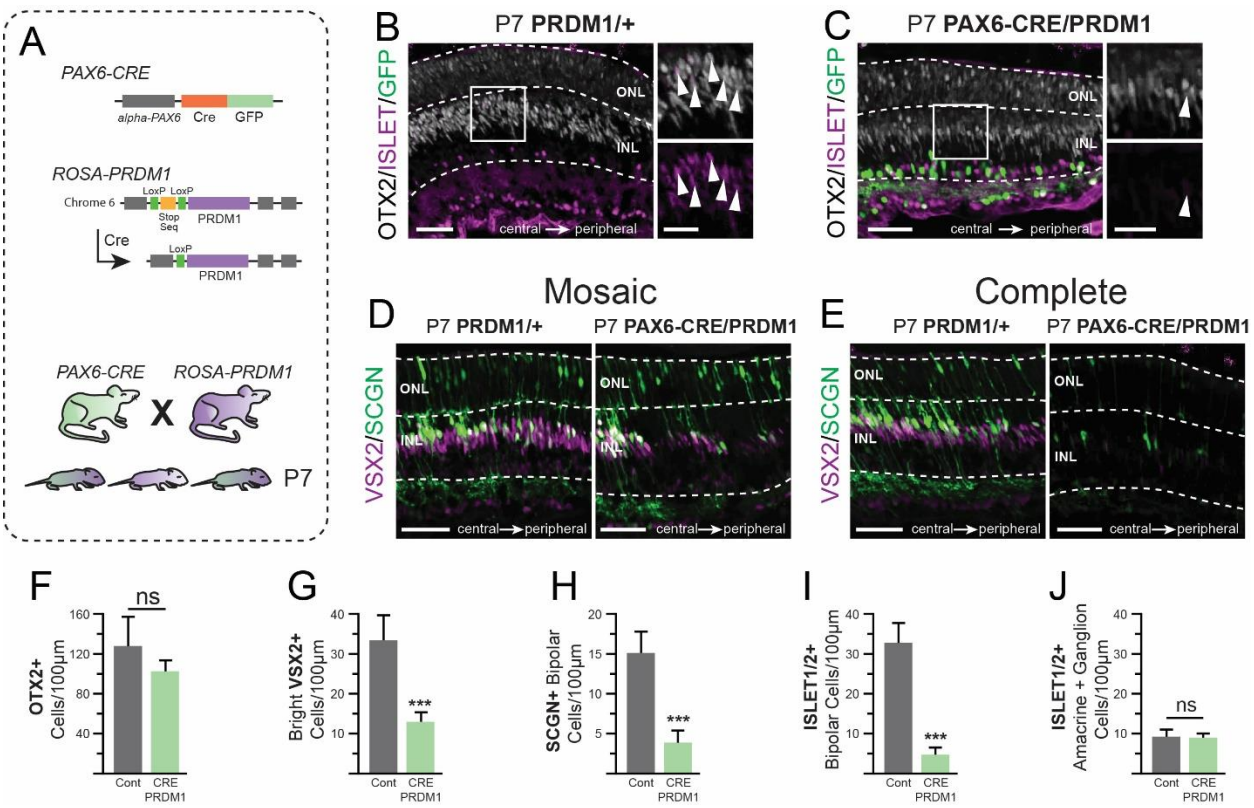



**Figure 4.2. Constitutive PRDM1 expression driven by *PAX6-CRE* prevents bipolar cell formation at P7**

**A)** Schematic of transgenic mice utilized. **B-C)** P7 *PRDM1/+* control compared to *PAX6-CRE/PRDM1* retinas stained for ISLET1/2+ (purple), OTX2 (white), and GFP (green). Stain of VSX2 (purple) and SCGN (green) with **D)** mosaic loss and E**)** complete loss of bipolars in the peripheral retina. Note that SCGN marks some photoreceptors at this stage. **F)** The total number of OTX2+ cells (bright and faint) is not significantly decreased. **G-I)** Quantification of bipolar cell markers. There are fewer **G)** bright VSX2+ cells, **H)** SCGN+ bipolars, and **I)** ISLET1/2+ bipolars in in PAX6-CRE/PRDM1 retinas compared to controls. **J)** There is no difference in the number of ISLET1/2+ amacrines or ganglion cells between conditions. Statistics calculated based on number of mice (N), Cont N=4, *PAX6-CRE/PRDM1* N=5. Error bars=standard deviation. ns=not significant, ***p<0.001. bars=50µm, inset bars=25µm, INL=Inner Nuclear Layer, ONL=Outer Nuclear Layer.

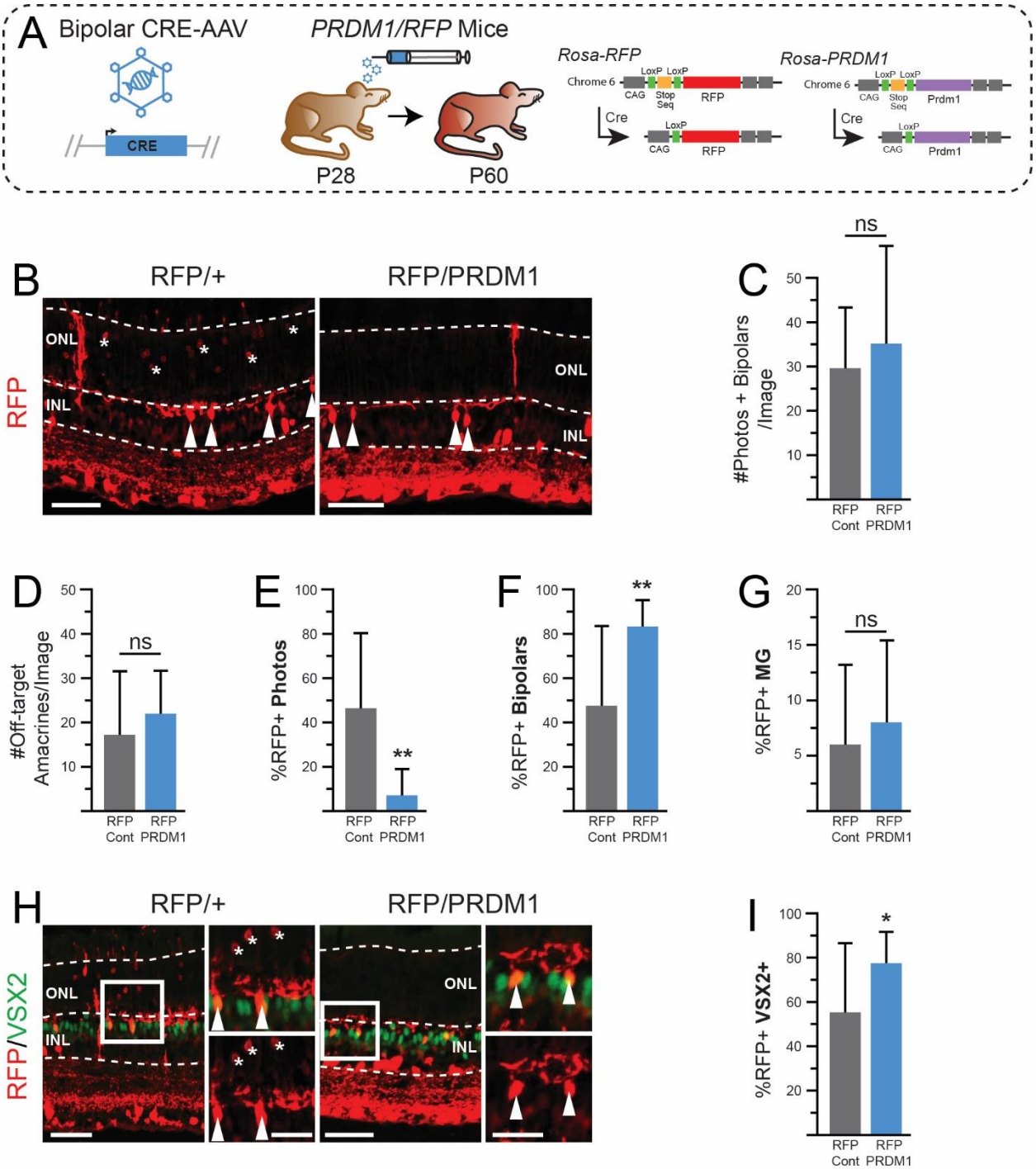



**Figure 4.3. Overexpression of PRDM1 in mature retinas driven by bipolar-CRE-AAV**

**A)** Schematic of experimental design. *ROSA-RFP* and *PRDM1* mice are as described in figure 1. **B)** Representative immunohistochemistry of *RFP/+* controls compared to *RFP/PRDM1* littermates. Arrowheads mark bipolar cells and asterisks mark photoreceptors. **C-D)** There is no difference in the total number of RFP+ bipolars and photoreceptors when summed, or in the total number of amacrines and ganglion cells. Amacrines and ganglion cells were excluded from the rest of the calculations. *RFP/PRDM1* mice have **E)** significantly fewer RFP+ photoreceptors, **F)** increased bipolar cells, **G)** and no change in Müller glia compared to controls. **H)** There is no observable difference in VSX2 levels (red) in RFP+ bipolars and **I)** there are significantly more VSX2+ bipolars in *RFP/PRDM1* mice. Statistics calculated based on number of eyes (N), Cont (*RFP/+*) N=6, *RFP/PRDM1* N=11. Error bars=standard deviation. ns=not significant, *p<0.05, **p<0.01. bars=50µm, inset bars=25µm, INL=Inner Nuclear Layer, ONL=Outer Nuclear Layer.

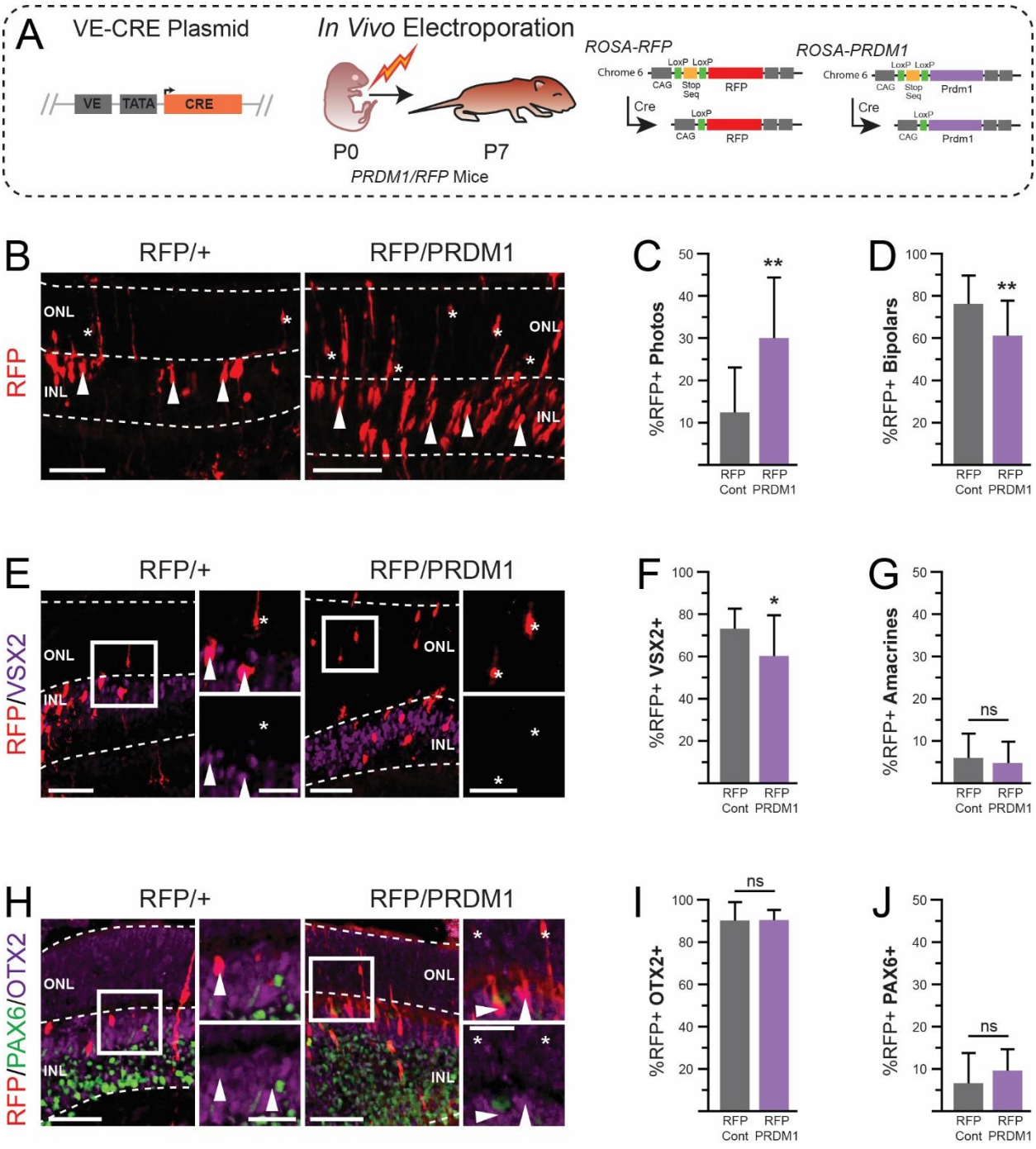



**Figure 4.4. Overexpression of PRDM1 in developing retinas driven by VE-CRE at P7**

**A)** Schematic of experimental design. Mice are as described in figure 1. **B)** Representative immunohistochemistry of *RFP/+* controls compared to *RFP/PRDM1* littermates. Arrowheads mark bipolar cells and asterisks mark photoreceptors. *RFP/PRDM1* pups have **C)** significantly more RFP+ photoreceptors, **D)** significantly fewer bipolar cells, and **G)** no change in amacrines compared to controls. **E)** Stains of electroporated cells (red) and VSX2 (purple). **F)** Quantification showing a significant loss of VSX2+ bipolar cells, but no change in the levels of VSX2 in remaining bipolars of the *RFP/PRDM1* mice. **H)** Stains for electroporated cells (red) that express PAX6 (green) or OTX2 (purple). **I-J)** There is no difference in the expression patterns of PAX6 and OTX2. Statistics calculated based on number of mice (N), RFP/+ N=6, *RFP/PRDM1* N=3. Error bars=standard deviation. ns=not significant, ***p<0.001. bars=50µm, inset bars=25µm, INL=Inner Nuclear Layer, ONL=Outer Nuclear Layer.

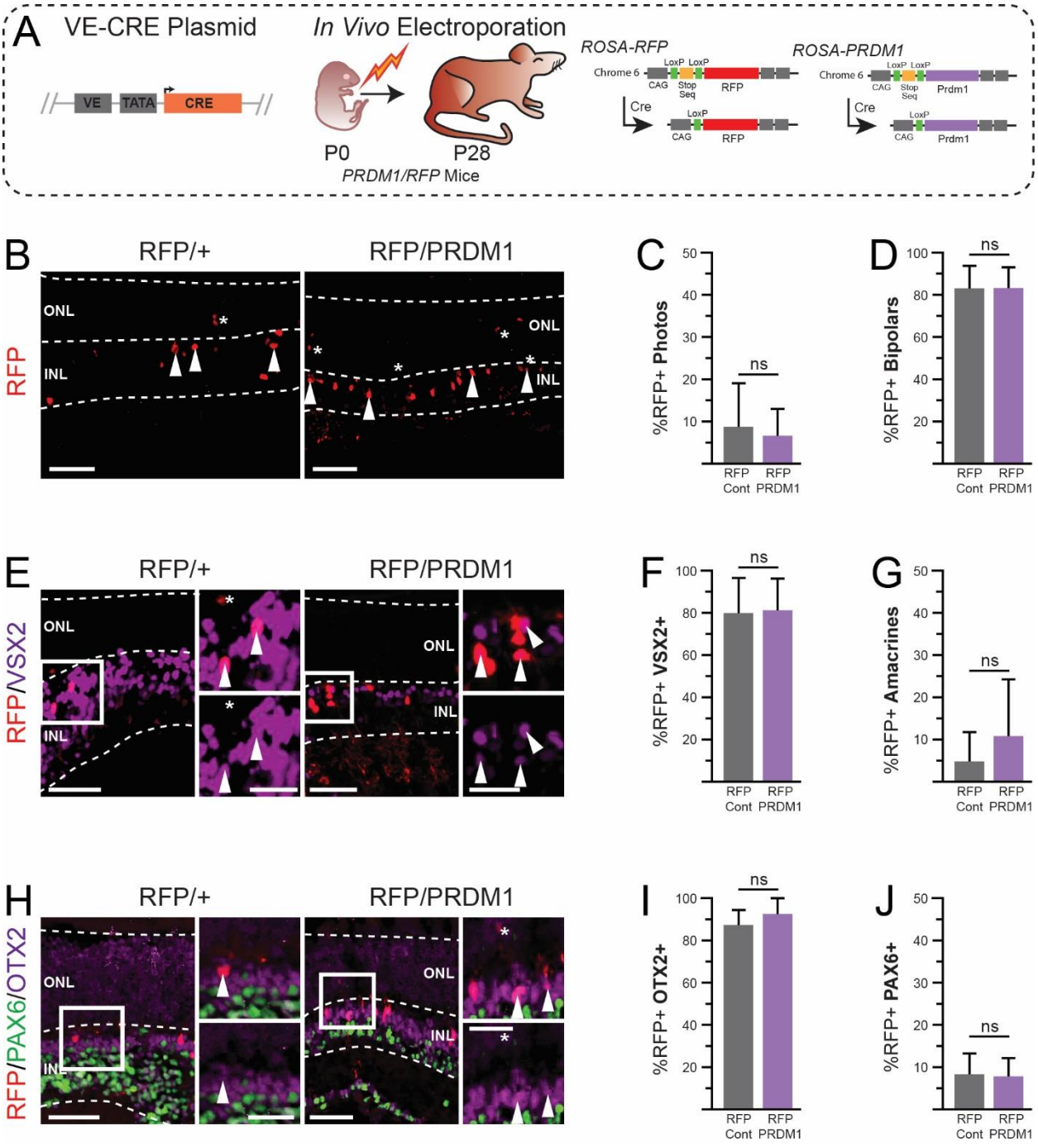



**Figure 4.5. Overexpression of PRDM1 in developing retinas driven by VE-CRE at P28**

**A)** Schematic of experimental design. Mice are as described in figure 1. **B)** Representative immunohistochemistry of *RFP/+* controls compared to *RFP/PRDM1* littermates. Arrowheads mark bipolar cells and asterisks mark photoreceptors. *RFP/PRDM1* pups have no differences in **C)** RFP+ photoreceptors, **D)** bipolar cells, or **G)** amacrines compared to controls. **E)** Stains of electroporated cells (red) and VSX2 (purple). **F)** Quantification showing no change in the number of VSX2+ cells. **H)** Stains for electroporated cells (red) that express PAX6 (green) or OTX2 (purple). **I-J)** there is no difference in the expression patterns of PAX6 and OTX2. Statistics calculated based on number of mice (N), *RFP/+* N=6, *RFP/PRDM1* N=4. Error bars=standard deviation. ns=not significant, bars=50µm, inset bars=25µm, INL=Inner Nuclear Layer, ONL=Outer Nuclear Layer.

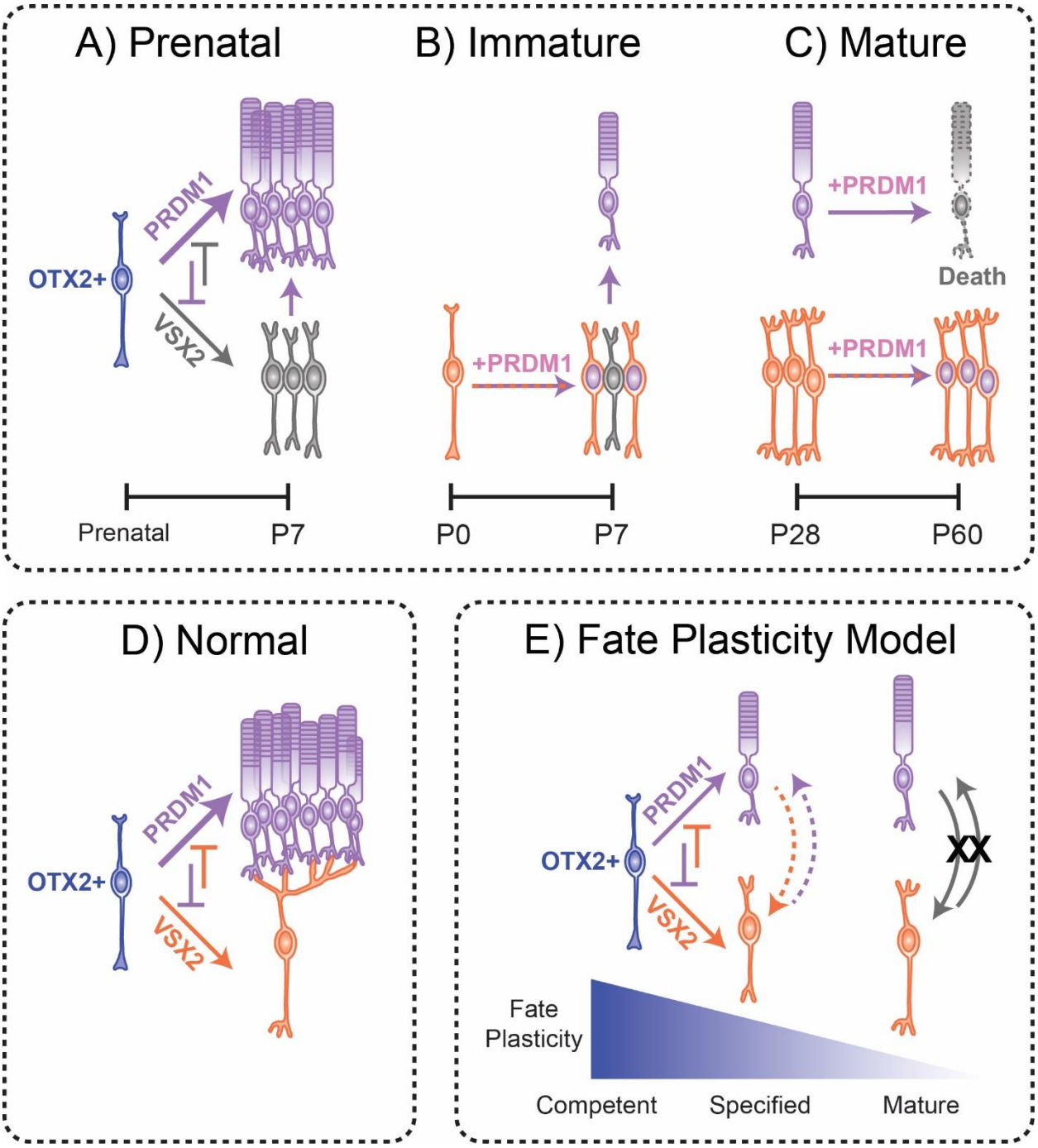
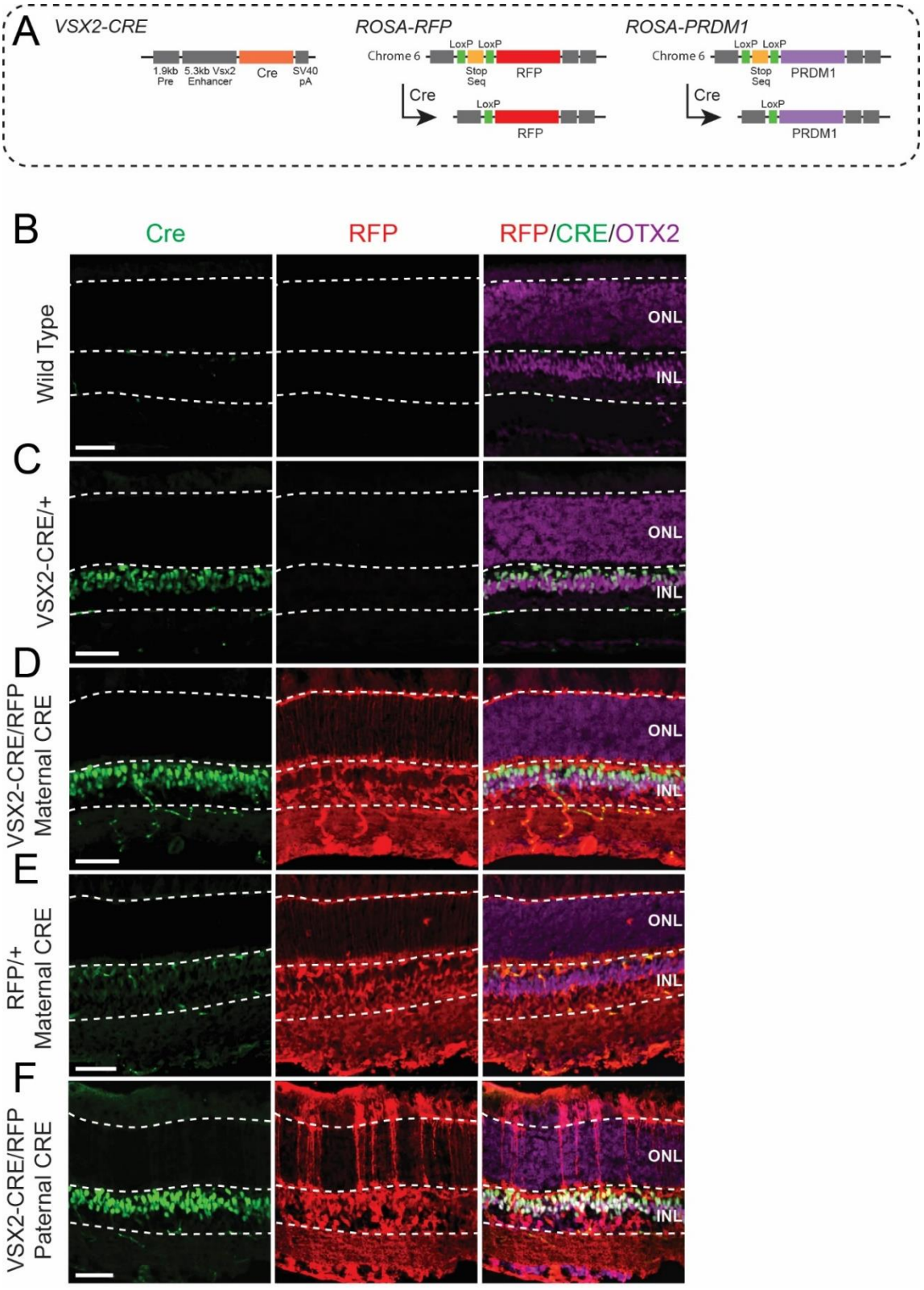



**Figure 4.6. Summary of chapter IV findings**

**A)** Turning on PRDM1 in prenatal retinas prevents bipolar cell formation, increasing rods. **B)** Misexpressing PRDM1 in nascent bipolar cells causes some to convert into rods by P7. **C)** Activating PRDM1 in a mature retina has no apparent effect on bipolar cell fate but is toxic to rods. These PRDM1-expressing bipolar cells still make VSX2, however, it is possible these bipolar cells have abnormal gene expression patterns despite their normal morphology. **D)** During normal development, OTX2+ cells express PRDM1 and VSX2. These transcription factors interact to control the decision between rod photoreceptor and bipolar cell interneuron fates. This is done, in part, by stabilizing fate decisions. **E)** OTX2+ cells are initially competent to form rods and bipolars. Rod and bipolar fates are plastic even after fate is specified, but this plasticity is rapidly lost as the cells mature.

**Chapter IV Supplemental Figures**




**Supplemental Figure 4.1. Early CRE expression in *VSX2-CRE* mice leads to a broad recombination pattern**

**A)** Schematic of experimental design. The *VSX2-CRE* mice are designed to drive bipolar-specific CRE expression. *ROSA-RFP* and *PRDM1* mice are as described in figure 1. **B-F)** Adult mice stained for CRE (green), RFP (red) and OTX2 (purple). **B)**. No CRE or RFP staining is present in wildtype mice. **C)** *VSX2-CRE* retinas have CRE expression in OTX2+ bipolar cells but lack RFP staining. The green vascular staining is non-specific background. **D)** The *VSX2-CRE/RFP* mice have bipolar CRE expression, but pan-retinal RFP expression. **E)** An RFP/+ mouse, without *VSX2-CRE* or CRE staining, has global RFP expression when the mother carried a *VSX2-CRE* allele. **F)** Paternally inherited *VSX2-CRE* mice also show global, yet mosaic RFP expression. Bars=50μm, INL=Inner Nuclear Layer, ONL=Outer Nuclear Layer.

## CHAPTER V

## CCTSI TL1 CLINICAL EXPERIENCE IN OPHTHALMOLGY

Throughout my doctoral work, I maintained an interest in clinical applications of basic research and the translation of our fundamental understanding into the practice of medicine. During my second full year in the Brzezinski lab, I was afforded the opportunity to shadow several clinicians in the Sue Anschutz-Rodgers Eye Center. My work started with Dr. Scott Oliver, and I saw a range of patients from pediatric through geriatric with a variety of ocular cancers, including melanomas and retinoblastoma. I next spent time with Dr. Naresh Mandava, exploring clinical approaches to age related macular degeneration. I then worked with Dr. Alan Palestine in a clinic focusing on immune-mediated disease of the retina.

This diverse clinical experience led to my application to the Colorado Clinical and Translational Sciences Institute (CCTSI) TL1 training program. I recognized a gap in our understanding of the fundamental mechanisms of uveitis, an autoimmune mediate inflammation of the eye and was awarded a grant to study "Cell type specific degeneration in retinal inflammatory disease." The objective of the TL1 training program is to provide pre-doctoral trainees the opportunity to develop and refine their understanding of clinical and translational efforts in team science by exposing them to a broad curriculum of research across the health spectrum. Thus, my work incorporated two parts, first I conducted a series of experiments in lab on the effects of repetitive uveitis inflammatory events on the mouse retina. Second, I spent more than 100 hours in clinic with Dr. Alan Palestine, observing the clinical treatment of uveitis and other inflammatory diseases of the eye.

To satisfy the course requirements for the TL1 certification in clinical and translational research, I completed the following courses: BIOS 6606 Statistics for the Basic Sciences, CLSC

6260 Conducting Clinical Trials for Investigators, IDPT 8890 Clinical Experience for CTSI PhD Students, NRSC 7661 Grant Proposal Writing, NRSC 7610 Fundamentals of Neurobiology, NRSC 7600 Cell/Molecular Neurobiology, PHCL 7605 Ethics in Research. In addition to this training, I participated in the Responsible Conduct of Research and HIPAA training through the CCTSI and department of Ophthalmology. I presented work funded through the TL1 training program at international conference for the Association for Research in Vision and Ophthalmology (ARVO), held in May 2019, in Vancouver, Canada, where I presented a poster entitled "Utilizing CRISPR to perturb photoreceptor/bipolar cell fate decisions in the mouse retina." This work, along with the significant training in team science and exposure to diverse fields of research significantly enriched my graduate training experience and prepared me for a career in clinical and translational research.

**CCTSI Clinical Experience**

The work with Dr. Alan Palestine in the ophthalmology clinic exposed me to over 100 patients aged 7-89, male and female, with a variety of symptoms and diseases. Here we list a few of the patients and their disease symptoms and treatment paradigms.

78 y/o, African American, female. This patient had post-operative uveitis. This occurs in some surgeries where the body responds with an autoimmune mediated inflammation of the anterior or posterior chamber. The patient was given the corticosteroid Kenalog as a sub-Tenon's injection.

12 y/o, white, male. The patient was diagnosed previously with Pars Planitis. This disease is characterized by inflammation of the pars plana, which is the narrow field between the iris and choroid[303]. The patient was on 0.6mL of Methotrexate once a week. He had received

injections for approximately 6 months and the inflammation was beginning to decrease. There was decreased center and peripheral leakage and patient reported decreased vitreous floaters.

86 y/o, white, female. Patient had intraocular pressure (IOP) of 38, questionable glaucoma, and severe anterior uveitis. It was unclear if high IOP was related to uveitis, glaucoma, or both. Suggested diagnosis was Uveitis-Glaucoma-Hyphema Syndrome. The syndrome is a complication of intraocular chafing from an implanted intraocular lens[304]. The proposed treatment was a combination of topical corticosteroids and IOP lowering systemic medication.

30 y/o, white, male. Patient experience an Acute Retinal Necrosis event ~10 weeks prior. These events are broadly thought to be caused by Zoster Simplex Virus infecting the retina and causing direct neuronal death as well as secondary death from frequent retinal detachment[305]. No cutaneous zoster simplex was detected. Patient was on 30mg of prednisone. No further retinal detachments or holes were detected during visit. Vision had stabilized with some blind spots.

**CCTSI Translational Research**

Uveitis, a condition marked by inflammation of the eye, is responsible for up to 10% of visual impairments globally, with 35% of those afflicted experiencing significant visual loss or blindness[306]. A variety of etiologies result in uveitis, but a common mechanism is inflammation and organ-specific immune responses that cause permanent vision loss due to retinal cell death[306,307]. Experimental animal work has addressed the immune pathways involved in various forms of uveitis, but little work has been done to understand how these conditions affect the retinal tissue itself[308–312]. We set out to investigate the effects of intraocular inflammation on the retina, probing the histological and physiological changes in parallel.

Uveitis involves inflammation of the eye's most vascular layer, the uvea. It can affect both the anterior and posterior segments of the eye, but all inflammations have the potential to affect the entire organ[306,307,313,314]. For individuals with uveitis, it is critical to provide retinal neural protection to prevent permanent visual loss. Clinically, many patients with uveitis experience bouts of acute inflammation with major intrusions of immune cells into the eye with longer intervals of chronic low to moderate inflammation[313]. Clinical evidence suggests that repetitive acute events are more likely to lead to permanent deficits, but little laboratory work has been done to validate or explain the cell type specificity of this retinal degeneration[307,313,315]. Loss of retinal tissue is the cause of permanent visual deficits in uveitis, but which cell types of the retina are most effected by inflammation remains unknown. Understanding, in detail, the effects acute ocular inflammation on specific cell types within the retina is a key step in fighting uveitis.

To test how uveitis affects unique populations of retinal neurons, we employed endotoxin induced uveitis (EIU), a common reproducible rodent model where intraocular injection of bacterial lipopolysaccharide endotoxins results in uveitis[314]. After injection, acute uveitis peaks at 18 hours, but chronic inflammation will persist for up to a week afterward[314] (Fig 4.1A). We induced EIU in adult mice and took their retinal tissue at acute (18 hour, Fig 5.1B-i), chronic (1 week, Fig 5.1B-ii), or resolved (1 month, Fig 5.1B-iii) time points and utilized immunohistochemistry for various cell type-specific markers to assess the effects of EIU (Fig 4.1G)[230]. We observed obvious inflammation of the retina and accumulation of inflammatory immune cells in the vitreous of EIU mice at 18 hours and 7 days post EIU event (Fig 5.2C-B). Our analysis for this work is ongoing.

Our goal was to directly connect the work in mice to clinical applications. In order to do this, we utilized three techniques available in the clinic and the laboratory on all mice weekly during the experiments. 1) The scalar visual assessment (SVA) of inflammation, which involves examining the anterior chamber based on specific visual criteria, provides a gross assessment of uveitis severity on a scale of zero to five[316] (Fig 5.1D). 2) Electroretinography (ERG) was used to test the electrical function of retinal neurons *in vivo* and will show how functional the retina remains after EIU (Fig 5.1E). 3) Optical coherence tomography (OCT) utilizes light to non-invasively image the cross-sectional structure of the retina *in vivo* and was used to reveal any major structural disruptions without sacrificing the animal (Fig 4.1F)[317]. We established all of these techniques in lab and employed them in our EIU mouse model.

Our next goal was to quantify the differential loss of retinal cells in individual versus repetitive uveitis events**.** In general, multiple acute uveitis events lead to greater retinal degradation in humans[306,307]. It is possible that increased degradation progresses through the same cellular mechanisms, compounded by frequency. Alternatively, repetitive events may cause certain cell types (*e.g.* rod photoreceptors) to die more readily than other types, indicating unique sensitivity of some neuronal subtypes to inflammation. To how repetitive acute uveitis events differentially affect cell types within the retina we directly compared the effects of single verses multiple uveitis events. We created three cohorts of mice: 1) A single EIU group (Fig 5.1C-i), 2) a group with two EIU events two weeks apart (Fig 5.1C-ii), and 3) a group with EIU events weekly for four weeks (Fig 5.1C-iii). All groups were tested pre-EIU and then weekly for scalar visual assessment, OCT, and ERG (Fig 5.1C-F). Five weeks after initial injection, tissue was collected, and the retinas assessed utilizing IHC for cell type-specific changes compared to each other as well as sham injected littermates (Fig 5.1C, G). While these experiments were

performed and showed robust deficits via ERG, OCT, and IHC we have made no conclusions that this time (Fig 5.2). Our analysis for this work is ongoing.

*Broad Translational Applications of Work*

Outside of this direct translational work, our other work on the fundamental mechanisms of retinal development and fate-stability may have critical applications for approaches to curing human disease. We highlight these applications in Chapter VII.

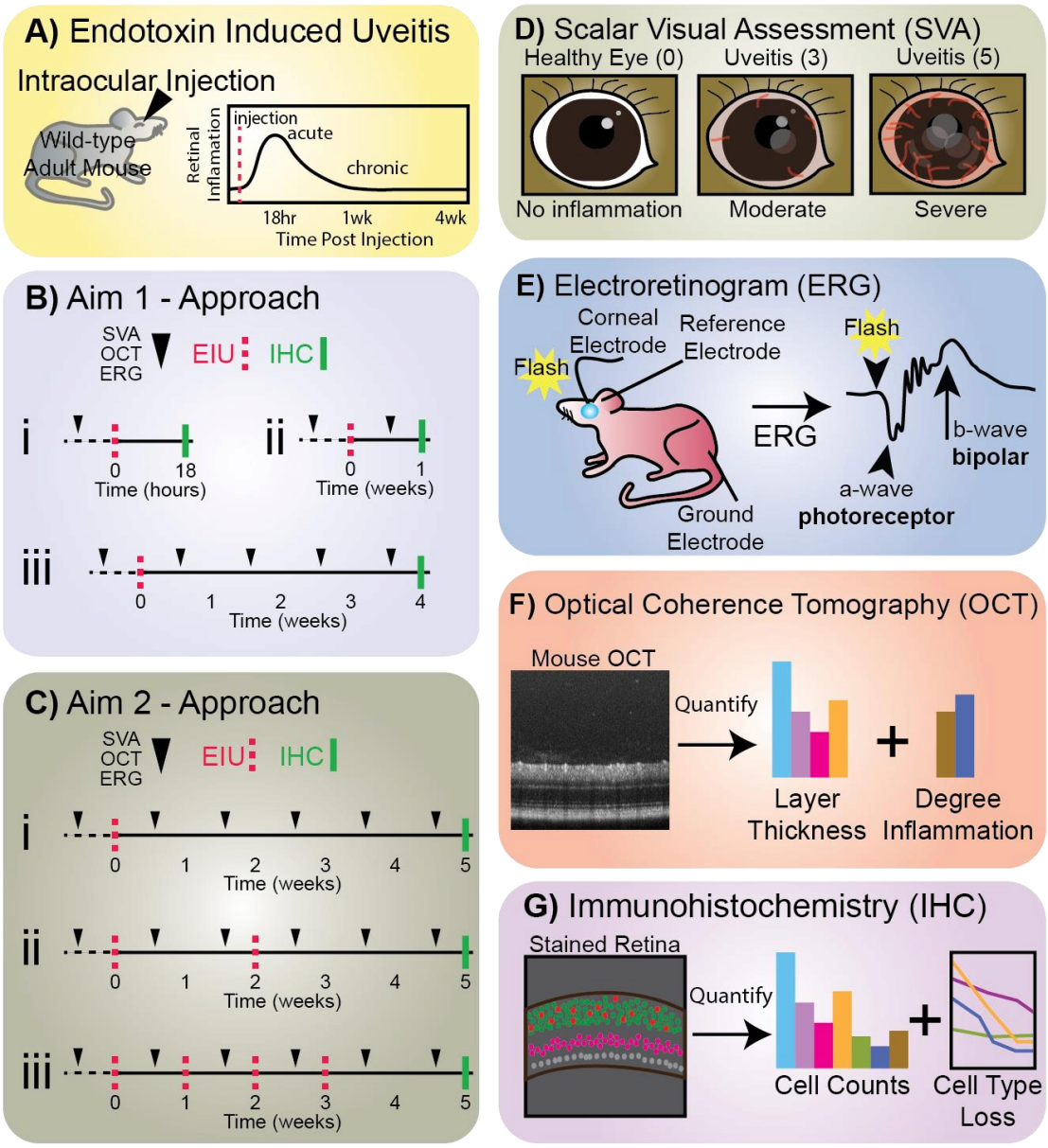



**Figure 5.1 Endotoxin induced uveitis (EIU) approach and assessments in mouse model.** A) The EIU model involves intraocular injection of endotoxin producing retinal inflammation. B) Aim 1 includes examining retinal tissues at acute (B-i), chronic (B-ii) and resolved (B-iii) times. C) Aim 2 compares single (C-i), double (C-ii), or four EIU events (C-iii). D) example of scalar visual assessment (SVA) under different levels of inflammation. E) Electroretinogram (ERG) tests neuronal functionality within the retina and allows separation of cell type-specific responses. F) Optical Coherence Tomography visualizes the retinal structure in vivo and can be quantified by layer thickness as well as degree of inflammation over time. G) Immunohistochemistry allows us to selectively label and count specific cell types within the retina and calculate cell type loss over time.

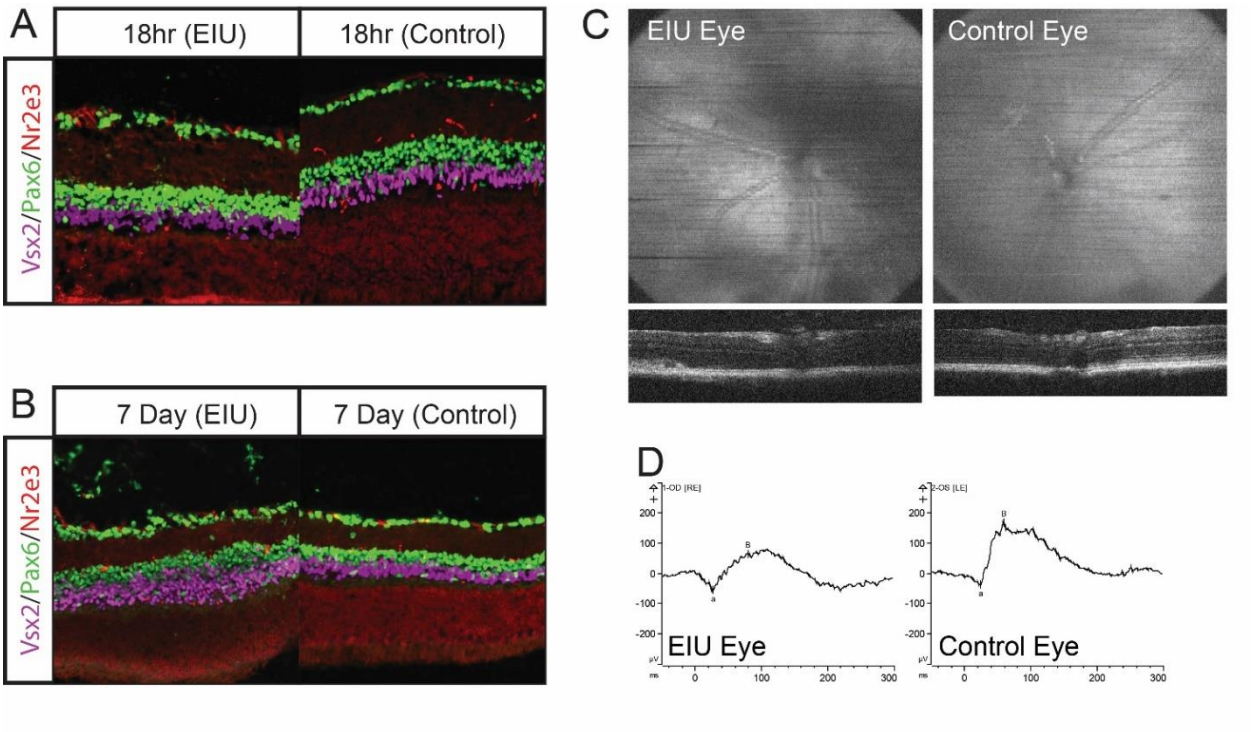



**Figure 5.2 Endotoxin induced uveitis (EIU) causes inflammation in mice**
A) EIU eyes show distinct inflammation 18 hours post injection compared to contralateral control. Early intrusion of cells into the vitreous and a distinct inflammation of the retina were observed. B) EIU eye has major intrusion of inflammatory immune cells into the vitreous after 7 days compared to contra-lateral control. C) OCT of EIU and control eye after 7 days showing major inflammation of the vitreous and enlarged or inflamed retina cross section. D) ERG showing distinct loss of function in EIU vs control eye. There is obvious loss of visual function in this inflamed eye.

# CHAPTER VI

# MATERIALS AND METHODS

## Mice

Mice were used in accordance with procedures approved by the local IACUCs at the University of Colorado Denver and the University of Texas Southwestern Medical Center (UTSW). Wild-type mice, *C57BL/6J*, were obtained from The Jackson Labs (Bar Harbor, ME). *ROSA-RFP* mice (*B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J*) (Strain #007914)[277] and *VSX2-CRE* mice (*129S1.Cg-Tg(Vsx2-cre)2690Chow/J*) (strain #026200)[288] were obtained from Jackson Labs (#026200) (Bar Harbor, ME, USA). The *αPax6-Cre-IRES-GFP* mice were a gift from Dr. Ruth Ashery-Padan (Tel Aviv University, Israel)[141]. All pups were housed with their parent and sibling following electroporation until collected. Animals older than P28 were separated by sex and housed in groups of 3-5 mice until tissue was collected

### *Prdm13-GFP and Prdm13-Δ115 Mouse Construction*

*Prdm13* gene targeted mouse lines used in chapter II were created at UTSW[217]. Briefly, *Prdm13-GFP* (*GFP/GFP)* mice were developed using zinc-finger nuclease targeting technology such that a GFP cassette followed by a stop codon was inserted into the first exon of *Prdm13* (Fig. 2.2A). Homozygous mutant mice died at or before birth. Mice were genotyped by PCR at 60°C annealing with three primers: 5'-GCTGCTCCTGGTTCTGTCA-3', 5'-CCTTTTCTCTGCTGCTCGTC-3' and 5'-GCTGGAGTACAACTACAACAGCCA-3' to generate 313bp wild-type and 549bp mutant bands (Mona et al., 2017). A presumed hypomorphic allele of *Prdm13* (Δ115) was the result of creating a 115bp deletion within the first exon of *Prdm13* (Fig. 2.4A'). While this deletion was predicted to create a null allele,

homozygous Δ*115* /Δ*115* mice live to adulthood and express PRDM13 protein in the developing neural tube[217]. The features of the two *Prdm13* alleles used here and those of two additional mutant lines are described in detail by Mona and colleagues[217]. PCR genotyping was done at 60°C annealing with two primers: 5'-GCTGCTCCTGGTTCTGTCA-3' and 5'-CCTTTTCTCTGCTGCTCGTC-3' to yield 313bp wild-type and 198bp mutant bands.

ROSA-PRDM1 *Mouse Construction*

To build the *ROSA-PRDM1* (*PRDM1*) mouse line in chapter IV, we first obtained the *Ai9* targeting vector from Dr. Hongkui Zeng (Allen Institute, Seattle, WA, USA)[277]. This construct is designed to insert a CAG enhancer/promoter, LoxP-stop-LoxP sequence, and a tdTomato cassette into the *ROSA26* locus (Madisen et al., 2010). We cut the *Ai9* vector with *Fse*I to remove the *tdTomato* sequence and re-ligated the plasmid. We PCR-amplified the mouse *Prdm1* cDNA sequence and used IN FUSION cloning (Takara Bio, Mountain View, CA, USA) to insert it into the remaining *Fse*I site in the modified *Ai9* vector. This drives the expression of an 823 amino acid PRDM1 protein (NCBI: NP_031574.2). The targeting vector was validated by Sanger sequencing and used to target *C57BL/6* embryonic stem cells with the assistance of the University of Colorado Bioengineering Core facility. After selection for neomycin resistance, several properly targeted clones were obtained and two were karyotyped. One normal karyotyped embryonic stem cell line was then used to generate chimeras. From this, one chimera was used to establish the *ROSA-PRDM1* line. *RFP* and *PRDM1* mice were genotyping using primers for both wildtype and mutant genes, including the forward primer 5'-CTCTGCTGCCTCCTGGCTTCT and the reverse primer 5'-CGAGGCGGATCACAAGCAATA for wildtype or 5'-TCAATGGGCGGGGGTCGTT for

mutants. PCR was performed using 35 cycles of 94°C for 25", 61°C for 30", and 72°C for 30". Cre PCR genotyping was performed at 59°C annealing as previously described[127].

### *Prdm13* Plasmid Creation

A *Prdm13* expression plasmid for work in chapter II was made by inserting the wild type mouse *Prdm13* cDNA sequence under the control of a ubiquitous human Ef1α enhancer, followed by IRES2 sequence and cDNA for Cre recombinase and a β-globin polyadenylation sequence. The *Prdm13-VP16* construct was cloned similarly, but used a previously constructed C-terminal VP16 fusion protein sequence[216]. Both constructs were validated by Sanger sequencing and Cre immunostaining of transfected explants (data not shown). We used a plasmid containing an Ef1α enhancer driving nuclear localized cherry fluorescent protein as an electroporation control[256].

### CRISPR Guide Design and Production

CRISPR constructs utilized in chapter III were designed based on a plasmid from Dr. Feng Zhang's Lab; pSpCas9(BB)-2A-GFP (PX458) (Addgene plasmid # 48138)[318]. The original PX458 plasmid was modified by exchanging the *CMV* promoter for an *Ef1α* promoter driving *Cas9*. In some conditions, the eGFP was replaced with Cherry. Guide RNAs were designed using the CRISPR Guide tool in Benchling (Benchling.com). Potential guides were sorted for their on-target and off-target scores[319,320]. Three non-overlapping guides with the highest aggregate score were selected per condition (Supplemental Table 3.1). Guides were inserted into the modified PX458 plasmid (above) utilizing Golden Gate Assembly[318].

**CRE-AAV Production**

Adeno-Associated Virus (AAV) vector preparations for work in chapter IV were produced by the 2-plasmid, co-transfection method[321,322]. Briefly, one Cell Stack (Corning Inc., Corning, NY, USA) with approximately 1 X $10^9$ HEK 293 cells was cultured in Dulbecco's Modified Eagle's Medium (Hyclone Laboratories, Logan UT, USA), supplemented with 5% fetal bovine serum and antibiotics. A $CaPO_4$ transfection precipitation was done by mixing a 1:1 molar ratio of recombinant (r) AAV vector plasmid DNA and serotype-specific rep–cap helper plasmid DNA. For the virus payload, we used a 1.65kb DNA sequence upstream of the human *PCP2* gene (also known as Ple155) to drive bipolar cell-specific expression of *Cre* recombinase[292,293]. The capsid was engineered to generate AAV2 vectors with the following substitutions: Y272F, Y444F, Y500F, Y730F, and T491V (AAV2, quad Y-F + T471V)[323]. After transfection, the cells grew at 37°C, 7% $CO_2$, for 60 hours and were then harvested and lysed by three freeze/thaw cycles. The crude lysate was clarified by centrifugation and the resulting vector-containing supernatant was divided among four discontinuous iodixanol step gradients. The gradients were centrifuged at 350,000g for 1 hour, and 5 mL of the 60–40% step interface was removed from each gradient and combined. This iodixanol fraction is further purified and concentrated by column chromatography on a 5-mL HiTrap Q Sepharose (anion exchange) column using a Pharmacia AKTA FPLC system (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The vector was eluted from the column using 215 mM NaCl, pH 8.0, and the rAAV peak collected. The vector-containing fraction was then concentrated and buffer exchanged in Alcon BSS with 0.014% Tween 20, using a Biomax 100K concentrator (Millipore, Billerica, MA, USA). The vector was then titered for DNase-resistant vector genomes by Real-Time PCR relative to a standard. The purity of the vector was validated using three standard assays. First by

silver-stained SDS– PAGE (the three AAV capsid proteins are the only visible protein bands in an acceptable prep). The second assay screens for bioburden by adding 10 µL of the final product to 15 mL of non-selective LB and monitored for 5 days. Lastly, the sample was assayed for Endotoxin using an Endosafe-PTS test system (Charles River, Durham, NC, USA). Passing criteria was ≤ 5 EU/mL.

**AAV Injections**

*In Vivo* CRE-AAV injections in chapter IV were performed on P28 mice utilizing common intraocular injection methods. In short, the concentrated CRE-AAV stock (7.28 X $10^{13}$ vector genomes per mL) was diluted 1:10 in sterile nuclease-free water. Mice were anesthetized utilizing 2% isoflurane mixed with oxygen and were kept under utilizing a nose cone giving a continuous 2% isoflurane and oxygen mixture (NDC 66794001725). Under a dissecting microscope and light the mice were held steady and a 31G needle was used to create a perforation of the sclera nasally near the junction with the cornea. A Hamilton syringe (Hamilton Company, Reno, NV) was inserted into the vitreous and 0.5µL of diluted AAV solution was injected. The mouse was allowed to recover on a heat block before being returned to housing.

***In Vivo* Electroporation**

*In Vivo* electroporation's in chapters III and IV were performed on P0 pups following previously described methods[324]. All electroporated DNA was delivered at a concentration of 2µg/µL. Mice were cryoanesthesized for approximately five minutes until all response to external stimulation ceased. Under a dissecting microscope and light, the mice were placed on a frozen block and held steady while a 31G needle was used to create an opening along the eye-

crease. The lid was then opened and a second 31G needle was utilized to create a perforation of the sclera nasally near the junction with the cornea. A Hamilton syringe (7653-01, Hamilton Company, Reno, NV) was inserted into the opening and pressed medially back against the central anterior portion of the retina. 0.5µL of 2µg/µL DNA in sterile $H_2O$ was then injected between the retina and underlying membrane. The syringe was removed. The mouse's head was placed between a tweezertrode electrode (BTX, Holliston, MA) and electroporated with 5 80V square wave pulses for 50ms with a 950ms delay between each pulse using a Bio-Rad Gene Pulser Xcell (BioRad, California, USA). Neosporin was placed on the surface of the eyelid to prevent infection. The mouse was brought back up to normal body temperature on a heat pad and returned to the mother.

### *In Vitro* Electroporation and Culturing

For work in chapters II, III, and IV, Newborn eyes were collected immediately after animals were sacrificed. The eyes were extracted with forceps, dissected in cold HBSS+ (HBSS, Ca2+, Mg2+, 6 mg/mL glucose, and 0.05M HEPES), and transferred to calcium and magnesium free phosphate buffer solution (PBS) for electroporation[167,230]. Retinas were oriented with the photoreceptor side up and 1µL of 2µg/µL DNA in 30% glycerol with methyl green was pipetted onto their surface. They were then electroporated with five 50ms square-wave pulses of 50mV at 125ms intervals from a BioRad Gene Pulser Xcell (BioRad, Hercules, CA, USA). Retinas were cultured in Neurobasal media, with 1X N2 supplement, 1X L-glutamine, 1X penicillin/streptomycin, and 1% FBS (Gibco/ThermoFisher Scientific, Waltham, MA, USA)[167,230]. For 1 DIV cultures, retinas were placed in 12 well plates floating in 1mL of media. For all other cultures, retinas were flat mounted with their photoreceptor surface facing up on

0.4µm Milicell CM cell culture inserts (Milipore, Billerica, MA, USA) in 6 well plates with 1mL of media in the well, such that retinas were maintained at the air-media interface. Half the media was changed every other day. All cultures were kept at 37°C and 5% $CO_2$.

**PRDM13 Antibody Creation**

Three different rabbit antibodies used in chapter II against PRDM13 were generated in the Johnson lab at UTSW: PRDM13-Ab1 (ZF) PA6658, PRDM13-Ab1 (ZF) PA6659, and PRDM13-Ab2 (FL) TX970[217]. The antigens were bacterially expressed C-terminal domain of PRDM13 including amino acids 622 to 755 (ZF) or full length protein (FL), respectively. PA6658, PA6659, and TX970 were validated by western blot and tested on embryonic retinas. All three antibodies showed an equivalent expression pattern, but preparation PA6658 was the most robust reagent. To validate the specificity of the antibody, we immunostained (see below) E17.5 *Prdm13-GFP/GFP* null animals with the PA6658 antiserum and observed a total loss of signal compared to equivalently stained heterozygous E17.5 retinas (Fig 2.2).

**Retina collection and Immunohistochemistry**

For work throughout, under a dissecting scope, a 31G needle was used to create 3-4 perforations in the sclera near the junction of the cornea ensuring fluid access to the vitreous, the eyes were then placed in 2% paraformaldehyde for 2-4 hours, followed by cryoprotection at 4°C with an increasing concentration series (10-30%) of sucrose solutions in PBS[230]. Eyes were stored in 30% sucrose overnight and flash frozen in OCT (Sakura Finetech, Torrance, CA, USA). Eyes were cryosectioned at 12µm and transferred to Shandon Colorfrost Plus microscope slides (ThermoFisher Scientific, Waltham, MA, USA). Slides were stored in a -20°C freezer until

immunohistochemistry was performed.

Immunohistochemistry procedures were conducted as previously[103,127,167,230]. Slides were washed in PBS, blocked for two hours in 5% milk block (the supernatant of a solution of 5% powdered milk, 0.5% TX100, 0.2% $NaN_3$, in PBS), and placed in primary antibody in 5% milk block overnight at room temperature. The next day slides were washed with PBS then 5% milk block along with secondary antibodies was applied and slides left two hours in the dark. Slides were washed in PBS and covered with Flouromount-G (eBioscience, San Diego, CA, USA) and a glass coverslip.

The following primary antibodies: AP2α (1:400; 5E4-c, DSHB Iowa City, Iowa USA); goat anti-BHLHB5 (1:500; sc-6045, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA); mouse anti-BRN3a (1:500; sc-6026, Santa Cruz Biotechnology, Inc); rabbit anti-Calbindin (1:400; AB1778, Millipore, Temecula, CA, USA); mouse anti-Calretinin (1:500; MAB1568. Millipore); rabbit anti-ChAT (1:400; AB143, Millipore); mouse anti-CRE (1:250; MAB3120, Millipore); mouse anti-EBF3 (1:400; H00253738-M05, Abnova, Taipei, Taiwan); rabbit anti-GAD65/67 (1:400; AB1511, Millipore, Burlington, MA, USA); rabbit anti-GAD65/67 (1:400; AB1511, Millipore); mouse anti-GFP (1:1000; ab13970, Abcam, Cambridge, MA, USA); goat anti-GLYT1 (1:500; AB1770, Millipore); mouse anti-ISLET1/2 (1:200, 39.4D5, Developmental Studies Hybridoma Bank, Iowa City, IA USA); goat anti-OTX2 (1:250; AF1979, Bio-Techne Corporation, Minneapolis, MN, USA); mouse anti-LHX1 (1:400; 4F2-c, DSHB); rabbit anti-PAX6 (1:500; PRB-278P, Covance, Princeton, NJ, USA); guinea pig anti-PTFA1a (Hori et al., 2008); rabbit anti-PRDM13 (see above) (1:250); rabbit anti-recoverin (1:500; AB5585, Abcam Inc); chicken anti-RFP (1:100; 600-901-379, Rockland, Gilbertsville, PA, USA); chicken anti-RFP (1:100; 600-901-379, Rockland Antibodies & Assays); mouse anti-RFP (1:1000; ab65856,

Abcam); Rabbit anti-Secretagogin (1:2500, RD181120100; Biovendor LLC, Ashville, NC, USA); and goat anti-SOX2 (1:500; sc-17320, Santa Cruz Biotechnology, Dallas, TX, USA); rabbit anti-tyrosine hydroxylase (TH) (1:100, ab152, Milipore); rabbit anti-vGlut3 (1:100; Cat# 135 203, Synaptic Systems, Göttingen, Germany); sheep anti-VSX2 (1:400; X1179P, Exalpha Biologicals, Shirley, MA, USA). EdU staining was done according the manufacturer's instructions (ThermoFisher, Waltham, MA, USA) after immunostaining.

**Imaging, Cell Counts, and Statistics**

Across all chapters, slides were imaged following immunohistochemical staining with a 20x objective on a Nikon C2 laser scanning confocal scope (Melville, NY, USA). Retinas were imaged at multiple layers 1.5 µM thick and 4-6 images were collected per a section as a Z-stack and then compressed to a single layer utilizing a maximum intensity stack compression in ImageJ[325]. All images were minimally processed in Adobe Photoshop (San Diego, CA, USA), and cell counts were conducting utilizing manual counting.

Images were taken from the eyes of both male and female mice. Bipolar-CRE-AAV images were taken in both central and peripheral retina but no differences were observed between these regions. Images from *PAX6-CRE* mice were taken in the peripheral retina. Due to the nature of *in vivo* retina electroporations, most sections covered central or mid-peripheral retina, however, some mice had electroporated cells that extended to the periphery. In the case of electroporations, which require a subretinal injection, counts were only conducted on sections of relatively intact retina, and disrupted sections were excluded from quantification. We also excluded images with less than 10 cells. Retinas were imaged in 2-6 locations and the counts pooled to calculate the mean and standard deviation.

For plots throughout this work, error bars represent standard deviation. Generally, one-tailed unpaired t-test with the assumption of heteroscedasticity were used for statistics. The degrees of freedom were based on the number (N) of eyes in AAV experiments and the number of animals examined when electroporations or transgenic mice were involved. In Chapter II, two-tailed unpaired t-tests with the assumption of heteroscedasticity were utilized for statistical comparisons, and the degrees of freedom are based on the number (N) of mice examined or the number (N) of retinas electroporated. Additionally, in Chapter II for multiple comparisons, one-way ANOVA was used. In chapters III and IV, one-tailed unpaired t-test with the assumption of heteroscedasticity were used for statistics except for a specifically noted paired t-test. Throughout, $p<0.05$ was considered significant, and was calculated utilizing GraphPad Prism 8 (GraphPad Software, La Jolla California, USA). Dotplots were generated using ggplot in R[326].

# CHAPTER VII

# CONCLUSION

The development of retinal neural tissue is a complex and multifaceted processes. Here I have explored mechanisms that impact cell fate choice, competence of progenitor cells, stability of mature neurons, and the functions of a gene regulatory network. I will now look at how our findings impact developmental biology as well as the implications for therapeutic treatment of human diseases of the eye.

## The Roles of PRDM13 in Developing and Maturing Amacrine Cells

The developing retina is able to specify 30+ morphological subtypes of amacrine interneurons which in turn may represent dozens to hundreds of specific roles in the neural circuits of the retina. I found that the zinc-finger transcription factor *Prdm13* turns on in a subset of developing interneurons of the mouse retina (Fig 2.1-2.2). It then remains on in some cells, which go on to become a heterogenous group of amacrine interneurons (Fig 2.3). The largest group are co-labeled with the transcription factor EBF3 and the calcium binding protein calretinin. When PRDM13 is reduced using hypomorphic mice, there are no obvious immediate developmental effects embryonically (Fig 2.5). However, when amacrines would normally turn on EBF3 and mature in early postnatal mouse eyes, approximately 25% of the cells go through apoptosis, resulting in a deficit in amacrine interneurons and the failure to form the third lamina of the IPL (Fig 2.4-2.5).

### *Prdm13 in Early Amacrine Development*

When PRDM13 was knocked down with hypomorphs, there were no immediate effects

on cell fate. Despite the fact that it comes on as early as E13.5, no phenotypes were observed till P5 (Figs 2.1, 2.5). Our study was not able to identify the purpose of PRDM13 embryonically in developing amacrine cells. While it is clear that PRDM13 is necessary to for a subpopulation of amacrines to mature and correctly laminate the IPL, it was not obvious why it was on for more than a week and a half within the retina before any observable phenotype manifested.

It was previously observed that *Prdm13* is directly downstream of *Ptf1a* in the spinal cord[216]. As early as E13.5 I observed robust PRDM13 expression in a subset of PTF1a cells, and all PRDM13+ cells descended from this lineage. PTF1a expression decreased over time and was likely negatively regulated by *Prdm13*, as it is in the spinal cord[217,257]. However, when *Prdm13* was absent, there were no observable changes in the numbers or fate of PTF1a+ cells during embryonic development.

In opposition to this lack of change in *Prdm13* loss-of-function experiments, gain-of-function experiments showed increased PAX6+/OTX2+ cells, suggesting a potential role of *Prdm13* in suppressing *Otx2* fates. I hypothesized that perhaps there are redundant mechanisms for restricting amacrine/horizontal cell fates within the PTF1a+ lineage. Our double knockout experiments with PRDM1 and VSX2 led credence to this possibility. While the specific timing and gene regulatory networks are not the same, the fact that double loss-of-function experiments late caused an increase in amacrine fates, suggests that a similar mechanism *could* be in place in early retina development to restrict *Ptf1a* fates.

Many of these experiments were performed before the prevalence of single-cell RNAseq technology. An obvious experiment is to utilize our GFP+ labeled *Prdm13* mice as well as the hypomorph knock downs in a scRNAseq experiment. This could be performed by taking the retinas at various developmental time-points and flow-sorting the cells to only analyze GFP+

cells, then comparing the transcriptional profile of hypomorphs to their control littermates. This could elucidate more about the role of PRDM13 in nascent amacrine cells as well as highlight transcriptional regulators that may be able to account for relatively normal early amacrine development in *Prdm13* mutants.

Throughout development, transcription factors are used repetitively in different contexts to perform unique, and sometimes contradictory operations in developing cells. I suspect this may be the case with *Prdm13*. Early, it may play a role in restricting amacrine fates by repressing OTX2 expression. In the postnatal development it may be required in a subset of amacrine cells to activate the EBF3 subtype machinery. An alternative explanation is that developing neurons pass through stages of progressive activation of transcription factors that lead to subtype fates. The deficits in PRDM13 embryonically in our hypomorph mice could have led to a failure to engage a set of transcriptional machinery that only had consequences when the cells differentiated into mature neurons. Our experiments were not able to discriminate between these mechanisms.

*The Future of Retinal Amacrine Subtype Research*

Our experiments showed that PRDM13 was present in about 30% of the amacrines and clearly marked multiple subtypes. While only the EBF3+ population died in the *Prdm13* hypomorph, it is likely that PRDM13 is playing other roles in the cells that survived. All the PRDM13+ cells in the adult retina were from late born amacrine fates, including glycinergic and nGnG amacrines. It is possible that PRDM13 is restricting amacrine subtype fates. Conditional knock out mice need to be created to test if there are distinct early and late roles for *Prdm13.* However, if the characteristics of other transcription factors are indicators of general themes in

retinal development, I suspect that *Prdm13* will be shown to possess a variety of roles across developmental time frames.

Three out of four mature PRDM13+ amacrines co-expressed EBF3. While EBF3+ amacrines represent at least two and possibly three unique subtypes, this limited population remains an excellent target for future studies[40]. A group of EBF3+ amacrines are glycinergic and project dendrites to both ON and OFF lamina 1-4 of the IPL, while the nGnG population only projects to the OFF lamina 1-2 and partially to the mixed lamina 3, which contains both ON and OFF projections[40]. These subtypes of PRDM13+/EBF3+ amacrines may play a distinct role in retinal circuits. A fluorescently labeled *Ebf3* mouse line could be an excellent system for attempting to understand an additional amacrine circuit as it could empower studying the physiological function of these cells.

There are a variety of reasons to study EBF3+ amacrines. First, in their absence, the IPL fails to laminate properly. I was not able to discern if this was because the cells died, the axons were rerouted, or their entire lamina failed to properly pattern. By studying the role of these EBF3+ amacrines in IPL patterning, a deeper understanding of cell-cell signaling as well as lamination of the retina could be uncovered. Second, EBF3+ amacrines are multistratified with narrow dendritic fields[40]. This suggests that they may be playing a role in more localized circuits compared to the far-field SACs. I am curious why the glycinergic population project arbors to both ON and OFF lamina. Are they mitigating signals to control specific retinal output? What ganglion cell population do these amacrines interact with during signal processing? Finally, are all dendritic laminations of a given EBF3+ cell expressed uniformly, or do they vary depending on if they project to an ON or OFF layer of the INL? Unpacking these differences and understanding the role of EBF3+ amacrine cells could help further elucidate the diverse circuit

machinery at work within the retina.

Retinal ganglion cells have been implicated in 49 unique signaling patterns[55]. This suggests a huge range of retinal circuits remain unstudied. These circuits may provide insights into the fundamental mechanisms of sensory signal processing. When *Prdm13* mutants were examined by another group, they showed that scotopic and photopic electroretinography was normal in *Prdm13* mutants[218]. They also examined optokinetic reflex response behaviors. For some unexplained reason, *Prdm13* mutant mice had greater spatial and contrast sensitivity than control mice[218]. Since EBF3+ amacrines are most disrupted in *Prdm13* mutants, the loss of these amacrines likely caused the observed sensitivity increases, ostensibly at the expense of other visual functions[268,269]. This suggests that EBF3+ amacrines may be playing a role in setting the sensitivity of contrast discrimination. If true, it could explain why EBF3+ amacrines project to both ON and OFF lamina of the IPL. One possibility is that they perform an inhibitory role in identifying edges or calculating the severity of contrast. SACs shunt signals across lateral space to precisely discriminate motion in a directionally selective way. EBF3+ amacrines may be performing similarly complex calculations over a narrow, or even columnar space. Distinguishing EBF3+ amacrine populations and studying their physiological role in retinal circuits may provide novel insights into the processing of visual information by the mammalian central nervous system.

**Gene Regulatory Networks in the Developing Retina**

In early eye formation, OTX2 plays a role in patterning of the eye cup, and distinguishing RPE from the neural retina. Later it comes on in progenitors around the time they exit the cell cycle. If OTX2 remains on in late born progenitors, they will be competent to form bipolar cells

or rods. Previous work shows that this fate decision is influenced by a gene regulatory network which includes the zinc-finger transcription factor *Prdm1* and the homeodomain transcription factor *Vsx2*. In general, cells that turn on *Vsx2* become bipolars, and cells that turn on *Prdm1* become rods, however lineage tracing shows that under normal developmental circumstances, some cells will turn on one, but then deactivate it and become the alternative fate[167,233,234]. This suggests that there is some form of competition within the cells to determine the specific fate. In this section I will explore the implications of several of our findings. Specifically, I look at the role of cell type-specific enhancers in development, how redundant mechanisms may impact cell fate and competence, the critical role of timing in developmental patterns, and finally the inhibitory and instruction roles of transcription factors in gene regulatory networks.

*Necessary Cell Type-Specific Enhancers Govern Cell Fate*

Evidence accumulated from various systems over years of research has shown that long distance enhancers play a critical role in gene expression[153–155]. This regulation may be particularly important when it comes to transcription factors. I have highlighted how transcription factors may be turned on early with one effect, later with a second role, and even a third or fourth time. Each activation causes that transcriptional regulator to play a unique role as the chromatin ultrastructure as well as associated transcriptional regulators may be different. As the epigenetic state of a cell shifts, the role of a specific transcription factor may also change. This is certainly the case with *Prdm13*, which plays an inhibitory role via the recruitment of associated factors to a specific region of DNA and performs various roles across developing tissue[217,257].

I show that two cell type-specific enhancers are in fact necessary for the expression of VSX2 and PRDM1 downstream of OTX2 in late retinal development. When a short long-distance necessary enhancer regions were removed from the genome of developing neurons, the effect in protein loss was equal to that of removing the genes themselves (Fig 3.1). Without these enhancers, the gene regulatory network breaks down and cells have dysregulated fate-choice. To our knowledge, this is the first work to precisely target multiple cell type-specific enhancers within a developing system. It is possible that this type of temporally precise, systemically limited, cell type-specific enhancer is unique to the developing retina, but evidence from other systems suggests this is not the case[153,327]. This may highlight a general mechanism of cellular diversity and specification. It is possible that nearly every transcription factor in every system has multiple long-distance regulatory elements or it could be that fewer regulatory elements are precisely layered with a suite of transcriptional regulators at specific times in development. If most transcription factors are regulated by multiple cell type-specific enhancers it could explain, to some extent, their diverse and precise activation and repression across diverse developmental contexts.

Our work highlights a critical technical advancement in our ability to target systems in a developmentally precise way. For example, transgenic mice that lack *Prdm1* are embryonically lethal, as previously discussed (Chapter IV). But, by targeting cell type-specific enhancers that only affect later development an elegant means of testing the effect of one or more enhancers on a specific system becomes available for future studies. Our work here provides a general strategic approach; by deleting putative enhancers and their genes *in vivo* or *in vitro* with CRISPR/Cas9 and comparing protein expression, others may be able to identify additional necessary enhancers and the upstream regulatory regions that govern developing cell fates.

*Prdm1 and Vsx2 Redundantly Maintain Restricted Cell Competence*

There were two findings in our work that surprised us so much that I went back and recounted our images twice, assuming I had made an error. 1) Targeting *Prdm1* and *Vsx2* at the same time resulted in a doubling of amacrine cells at the expense of OTX2+ cells. 2) Even more surprising was the finding that deletion of *Prdm1* and *Vsx2* genes simultaneously differed from deleting their necessary cell type-specific enhancers.

I did not expect double deletion to increase the number of amacrine cells (Fig 3.4). Indeed, no previous evidence suggested there would be any outcome other than excess bipolars, excess photoreceptors, or cells that were "confused" between these states. Previous work, including that in Chapter II, has shown that progenitors can express OTX2, then lose expression and go on to become amacrines and horizontals[130,167,230,231]. Additionally, mice with inactivated *Otx2* have no bipolar cells or rods and supernumerary amacrines[138,139]. Taken together, this suggests that OTX2 restricts cell fates to rods and bipolars, but loss of OTX2 may de-restrict fate.

There are several possible mechanistic explanations for this finding. First, VSX2 and PRDM1 may act directly on OTX2 to maintain its expression. In this case, if at least one of the two were present, *Otx2* would continue to be transcribed and restrict the competence window. Alternatively, they may be acting through a similar mechanism, but upstream of OTX2 and not on the gene directly. In this scenario, VSX2 and PRDM1 *indirectly* maintain OTX2 expression by somehow preserving cell competence, which includes *Otx2* expression. Both mechanisms would have an identical outcome and allow amacrine cells to form when VSX2 and PRDM1 were simultaneously absent. Studies examining the binding of VSX2 and PRDM1 to OTX2

enhancers could show if the first explanation were possible. A third possibility is that they work through directly restricting amacrine fates. PRDM1 and VSX2 may be acting on some amacrine activators by restricting their ability to upregulate pro-amacrine transcriptional regulators. Alternatively, it may be that OTX2+ cells which fail to activate *Prdm1* or *Vsx2* turn on amacrine machinery, which subsequently downregulates *Otx2*. Finally, there may be a mixed effect between several of these mechanisms, and while VSX2 and PRDM1 both restrict cells to rod and bipolar fates, they do it through unique mechanisms. While functionally similar in outcome, each of these possibilities present a unique mechanistic explanation for the role of PRDM1 and VSX2 in redundantly maintaining OTX2+ cell identity. In my opinion, the most probable explanation is that the two genes indirectly maintain OTX2 identity, which in turn represses amacrine identity.

How and why PRDM1 and VSX2 work redundantly to restrict OTX2+ cell identity presents a curious conundrum, highlighting the fact that most transcription factors play more than one role at a given time. Since only a portion of the population go on to become amacrines, perhaps only a subset of OTX2+ cells are susceptible to adopting amacrine fates, though the mechanisms whereby this occurs are unclear. An obvious experiment to test all of these hypotheses is to perform serial scRNAseq analysis on developing tissue and compare *Prdm1/Vsx2* loss-of-function cells to their normal counterparts. What transcription regulators change? What is the difference between the cells that become amacrines and those that adopt OTX2+ fates? What other transcriptional regulators are playing a role upstream of *Vsx2* and *Prdm1* as well as downstream to direct cell fates? The fact that loss of two genes within a gene regulatory network causes a shift in competence restriction suggests there may be critical mechanisms for biological control of developing cells still to be discovered within the *Otx2/Prdm1/Vsx2* network.

*Development Occurs within Critical Temporal Windows*

Overexpressing PRDM1 in nascent VSX2+ bipolar cells results in a cell fate-shift early, but not late (Chapter IV). If VSX2 is lost early, bipolar cells will not form, but if VSX2 and PRDM1 are lost via enhancer targeting, transient progenitor-derived VSX2 is sufficient to drive bipolar fates (Figs 3.4, 3.8). Additionally, some bipolar cells form normally without VSX2 once they have made it through a critical developmental window (Chapter III). The same PRDM1 that causes bipolar cells to fate shift early will have no observable effect on mature bipolar cells. Yet the PRDM1 that drives rod fates early is toxic to them if it remains on or is constitutively activated in mature rods.

These findings are in contrast to those of Prdm13, which is normally activated as early as E13.5 and comes on in a large population of PTF1a+ nascent interneurons. Yet, loss of PRDM13 did not appear to have any effect on these cells in terms of number, patterning, or markers, until the cells began to mature. As discussed above, PRDM13 may be playing distinct roles embryonically and in mature amacrines.

Thus, in some circumstances, a transcriptional regulator can be lost with no observable impact on the developing tissue. At other times, loss or overexpression may result in an immediate and dramatic change. Our results confirm broad findings across neural systems that regulation of development is often tied to specific temporal windows[328,329]. Once neurons of the retina are mature, they do not readily shift fates. A transcription factor like PRDM1 is critical to generating the correct number of rods in the retina yet must be downregulated before the cells mature (Figs 4.2-4.4). Conversely, the very cells it inhibits, bipolar cells, appear to be unaffected by its presence late after they have matured (Fig 4.2).

Here I have shown that VSX2+ nascent bipolar cells have a specific temporal window in which the loss of VSX2 or the presence of PRDM1 are both sufficient to cause a fate shift. However, once that window is closed, the environment of the cell is changed in such a way that PRDM1 is no longer able to inhibit the bipolar machinery and loss of VSX2 does not stop the cells from maturing into bipolars. It is possible that there is some inhibition of correct circuit functions in these cells that I was not able to observe, but the general fate as assessed via immunohistochemistry remained stable.

Future studies employing advanced computational methods may be required to better understand what constitutes a competence or fate restriction window and how these can be manipulated. The systems examined here could provide an excellent starting place for studies attempting to understanding the timing and complex regulation of competence restriction and permanent fate choice within the retina.

*Inhibitory and Instructive Transcription Factors in Retinal Fates*

Targeting the coding regions for *Prdm1* and *Vsx2* resulted in the dominance of rod photoreceptor fates. It was only when the enhancers were targeted and there was transient VSX2 from progenitors that bipolar fates were formed. Indeed, this finding agrees with all previous work suggesting that VSX2 must be present for bipolar cells to form[148,149,278]. It is not clear if VSX2 has a primarily inhibitory, fate-driving, or mixed role in bipolar fates. However, it does appear to have a functionally unique role from *Prdm1* within this gene regulatory network.

PRDM1 is expressed over a short temporal window and may play a predominately inhibitory role. Indeed, leaving *Prdm1* active is toxic to photoreceptors. PRDM1 appears to work in this short temporal window to inhibit bipolar fates, not necessarily instruct photoreceptor

fates. The absence of PRDM1 seemed to have no effect on rod formation, unless there is VSX2 present. This is in contrast to VSX2 which is required for bipolar cells to form. Bipolar cells did form without bipolar-specific VSX2, but there was still VSX2 within the system and they only did this in the absence of PRDM1.

In addition, it is possible there is cross repressive activity. Previous studies showed that conditional loss of PRDM1 in nascent rods was sufficient to turn off rod machinery and cause a fate shift to bipolar cells. Indeed, lineage tracing of PRDM1+ cells shows that in some cases, PRDM1 is deactivated and VSX2 machinery takes over. The same is true in VSX2+ cells. We specifically demonstrated that when constitutive PRDM1 is driven by the bipolar-specific VSX2 enhancer, cells will fate shift to rods. This suggests that PRDM1 is able to inhibit, directly or indirectly, VSX2 expression, and VSX2 is certainly able to downregulate photoreceptor machinery, though it is not clear if it does this by acting on PRDM1 directly.

Rods developed later in evolution than cones and bipolar cells[17,84,150]. One can imagine a situation in which VSX2 was on in progenitors and later drove the expression of bipolar cells. With the advent of rods, a means of inhibiting this VSX2 from progenitors so that more rods could form could become necessary. Transient PRDM1 expression could then act as an inhibitory transcriptional regulator to block the formation of excess bipolar cells. This retina would now have too many rods. In this scenario, the retina would need a novel solution for maintaining the correct number of bipolar cells, while not stopping the formation of rods. We mentioned previously that cis-regulatory enhancers are thought to be a major means of driving evolution[155]. A “simple” mechanistic solution to balancing rod and bipolar fate numbers would be to create a bipolar-specific VSX2 enhancer. Whether this hypothetical order of events matches evolution is beyond the scope of this thesis. However, it is clear that these two

transcription factors are critical to setting the correct numbers of rods and bipolar cells in OTX2+ cells of the mouse retina.

Taken together, we suspect that PRDM1 and VSX2 are acting through unique mechanisms to set the correct balance of rods and bipolar cells within this circuit. PRDM1 appears to be primarily inhibitory, blocking the bipolar-specific machinery and allowing rods to develop, then turning off as the cell matures. VSX2 is necessary for bipolar cell formation and in its absence bipolars will not form. However, once bipolars pass through a specific temporal window, VSX2 can be deactivated, however certain subtypes may not develop as a result. Rods will form in the absence of PRDM1 and we broadly classify this as an inhibitory transcription factor that blocks specific fate outcomes. VSX2 is an instructive transcription factor that actively drives bipolar fates, though it may mechanistically work through transcriptional inhibition. Future studies may reveal if PRDM1 and VSX2 interact directly or are working through other, as yet unknown mechanisms, to regulate the fate of cells in the retina.

*Types, Subtypes, and Transcriptional Regulation of Fate*

We previously noted that transcription factors may often play unique roles at different times in a developing tissue. I was not able to define the role of PRDM13 in embryonic retinas, but we know that in its absence, 25% of amacrines die when they should normally mature (Figs 2.5). One mechanistic explanation for this is that PRDM13 is necessary to correctly drive amacrine subtype formation and in the absence of this, they do not integrate correctly into retinal circuits and are culled.

VSX2 normally remains on in nearly all bipolar interneurons. We also know that it is necessary to form bipolar cells[130,148–150,232]. However, once bipolar cells passed through a

temporal window, they were able to robustly express a suite of bipolar-specific markers and maintain apparently normal morphology (Fig 3.4). However, one noticeable change was that there was a shift in the subtypes of bipolar cells and a loss of Secretagogin+ cone bipolars (Fig S3.2D-E). This suggests that VSX2 may remain on for a reason, and that some bipolar diversity is lost when VSX2 is absent. Unlike in our PRDM13 model, these cells were not lost. It was not clear if this difference is because the bipolar cells still form a meaningful cell type and incorporate into a circuit or if using a CRISPR/Cas9 sparse label approach generated different outcomes from transgenic mice. It could simply be that PRDM13 and VSX2 have vastly different roles. Finally, we were not able to test if cells that shifted fates were fully functional mature neurons, or just retained correct morphology and cellular markers. Future studies will be needed to better understand how fate-shifted neurons integrate into retinal tissue.

**Retinal Development and Neural Regenerative Therapies**

On December 17th, 2017 the FDA approved Luxterna. This was the first *in vivo* gene therapy approved to treat a genetic heritable disease in humans. The treatment utilizes a viral system to deliver human *RPE65* cDNA to the RPE supporting tissue of the retina. This therapy not only stops a disease state, it results in restoration of visual function and elimination of night-blindness in patients with *RPE65* Leber's congenital amaurosis.

This remarkable treatment was the result of years of research into the fundamental mechanisms of retinal degenerative diseases[330–334]. It was also a hallmark advancement in our ability to deliver genetic material to mature cells within human tissue. Despite expenditures in the billions relatively few new gene therapies have subsequently been developed and delivered. However, significant ongoing efforts are currently in place attempting to devise approaches to

treating neural degradation in the retina, autosomal dominant diseases, and development of neural protective strategies. In this section we explore the therapeutic applications currently in development around the globe to cure retinal disease and restore vision and highlight the implications of our findings for these fields of inquiry. While not all of our work has direct applications in moving clinical and translational research forward, many of our findings have implications that we highlight throughout this section.

*Challenges in Neural Regeneration*

A central challenge in all neural protective treatments, gene therapy, and regenerative medicine, is that mature neurons in the mammalian central nervous system are resistant to change. Not only do neurons in mammalian retinas fail to reproduce in response to injury they often fail to regrow their axons whether in the spinal cord or retina[335,336]. Significant work has been done exploring the role of the extracellular matrix in both driving and inhibiting axonal regrowth after injury, however no solutions have been generated to date[336,337].

The challenges of neural regeneration do not exist in all systems. Neurons of the olfactory system regenerate throughout the lifetime of most mammals, including humans. Toxic application of zinc sulfate to the olfactory epithelium causes cell death[338–341]. Yet, these neurons readily divide, generate new cells, and project their axons to the correct glomeruli resulting in a cohesive sense of smell, despite loss of all original sensory neurons[342,343]. Years of work into the regenerative capacity of these neurons has highlighted the fact that mature neurons of the central nervous system can regenerated at least in certain specific tissue contexts[344].

This regenerative capacity is not limited to the surface facing olfactory sensory neurons. Extensive studies have explored the effects of transection of the olfactory nerve. When cut, the

projection neurons from the olfactory bulb crossing the cribriform plate experience retrograde degeneration and apoptosis[345,346]. Despite the enormous loss of cells resulting from nerve bundle severing, the cells can be regenerated and re-project their axons, resulting in a nearly complete recovery of functional olfactory sensory tissue[347,348]. However, it is important to note that both of these sensory neuron and projection neuron regenerative processes involve a variety of extrinsic cell signaling molecules, including various Bone Morphogenetic Proteins (BMPs), Transforming Growth Factors (TGFs), and Insulin-like Growth Factors (IGFs)[344].

If new neurons in the central nervous system can be born, migrate to the correct tissue, specify into a subtype of sensory neuron, and project axons to the correct target in the olfactory system, why do they fail to perform similar functions in other CNS tissues like the retina? A simple argument is that the retina cannot function properly when the neurons are regenerating. This could be the case if, for example, mapping of ganglion cells axonal projections into the lateral geniculate nucleus of the thalamus failed to function properly in a regenerative context. Olfactory sensory neurons target specific glomeruli based on the olfactory signals to which they respond[296]. This is not the case with visual processes which encode a wide array of visual information before ganglion cells project through to the thalamus and send signals on to the visual cortex or other neural targets[349]. Making computational sense of visual signals requires precise spatio-temporal mapping of the signals and that suggests re-wiring could be more complex in the retina than the olfactory bulb. However, non-mammalian retinas continue to grow throughout the lifetime of the animal and can be regenerated in response to injury. Thus, there is not a clear explanation for why neural regeneration varies by tissue within mammals. However, there are a number of approaches currently being pursued to surmount these difficulties, including inducing retinal regeneration, causing stable neurons to fate change, implanting neural

tissue into a degenerating retina, and growing entirely new eyes through the use of retinal organoids.

*Neural Regeneration in the Retina*

Most neural degenerative diseases of the retina will only cause a dysfunction in one cell type, for example rods, or a portion of the phototransduction cascade, as is the case in *RPE65* Leber's congenital amaurosis. However, this single degrading event can lead to a cascade of cell death that ultimately effects the entire tissue[334,350,351]. Permanent loss of visual function due to cell death occurs in all cases of physical ablation of the retina in mammals. But this is not the case non-mammalian vertebrate, despite the fact that most vertebrate retinas have roughly similar lamination and all share the common major cell types[352].

In urodeles (salamanders), the entire retina can be physically removed or chemically ablated and it will regrow. In these circumstances, the RPE will re-enter the miotic cycle and transition from epithelial tissue into laminated neural tissue[353–356]. Fish grow throughout their lifetime and their eyes grow along with the rest of their body. This leads to a steady formation of new cells at the periphery of their retina[357]. This process is separate and distinct from the injury response in fish retinas. When damage occurs in a fish retina, the Müller glia will undergo what is referred to as a gliosis response and give rise to a variety of neurons, including rods, as well as amacrine, bipolar, and ganglion cells[352]. The new neurons are able to migrate properly, network with existing retinal tissue, and form fully functional circuits[357,358].

In contrast to studies within olfactory regeneration, which have largely looked at the impact of extrinsic signals driving cells through a series of steps to transform them into sensory or project neurons, studies within the retina have largely focused on the roles of intrinsic

signaling in driving the transformation of Müller glia into progenitors. It is known that retinal progenitors respond to some extrinsic signals like EGFs, FGFs, and SHH[359]. But most work has focused in on the transcription factors ASCL1 and PAX6, which have been shown to be critical to these processes, though the full mechanism is not yet fully understood[109,173,359,360]. However, additional factors have been shown to be upregulated in transdifferentiated Müller glia including Notch, HES5, SOX2, FOXN4, and PEA3[173]. It is not clear why non-mammalian vertebrates are so much more capable of regeneration.

Müller glia of higher vertebrates may have lost their regenerative capacity. But the key genes upregulated in fish, salamanders, and avians are also critical to mammalian progenitors, especially *Notch1, Pax6*, and *Ascl1*[352]. This has led to the belief by some, that given the right cocktail, mammalian Müller glia could be stimulated to regenerative potential. However, there are currently no efficient strategies for causing regeneration within mammalian systems.

One explanation for the regenerative resistant in mammalian Müller glia could be redundancy. We showed that loss of PRDM1 or VSX2 alone was not sufficient to dysregulate the function of OTX2+ cells. While they did fate shift, they remained within a specific competence window, choosing between rods and bipolar cells. Only when both VSX2 and PRDM1 were simultaneously absent did OTX2+ cells shift their competence, lose OTX2, and adopt amacrine fates. It is possible that the regenerative capacity of Müller glia is restricted in a similar redundant fashion. If this were the case, then studies attempting to downregulate or upregulate a single transcriptional regulator may fail to generate results. Under this paradigm only when two or more critical factors were shifted simultaneously would Müller glia transdifferentiate through gliosis into regenerative progenitors.

Identifying such a mechanism through trial and error would be a monumental endeavor. However, one approach could be to explore cross-species single-cell RNAseq profiles between zebrafish and mice, specifically asking how zebrafish Müller glia differ from those in mice and by targeting the transcriptional changes that occur in response to a gliosis transdifferentiation initiating event. Since there are many conserved developmental paradigms across mammals there may be meaningful insight provided by this computational line of inquiry. Indeed, initial studies in this field have already been published on BioRxiv and suggest a possible role for NFI family transcription factors in zebrafish proliferative capabilities[361]. Ultimately, this could provide a means of identifying specific regulatory changes that have resulted in the loss of regenerative capacity in mammalian retinal neural tissue.

*Neurons of the Retina Are Resistant to Fate Change*

In the absence of regenerative therapies, one potential line of inquiry is causing cells to fate convert. This is particularly relevant to certain degenerative diseases like retinitis pigmentosa. In this disease state, loss of rods leads to cone death. Loss of cones is particularly challenging to humans because we use high acuity cone vision for most of our behavioral functions[362]. One hypothesis is that if you cause rods in retinitis pigmentosa patients to covert to cones, then you could prevent the catastrophic breakdown of the outer nuclear layer that ultimately leads to blindness in these patients[295]. The cost of this would be immediate night-blindness or at least a significant loss of low light visual function but this eventually occurs in these patients regardless. This is a tradeoff most patients would be willing to take as it could theoretically preserve some day-light vision long term, rather than suffering complete blindness.

The transcription factor *Nrl* is required for the formation of rods but not cones. One group took advantage of this and created a conditional *Nrl* knockout mouse and crossed it with a known retinitis pigmentosa mouse model. They then knocked out *Nrl* in mature retinas[295]. Surprisingly, the cells converted from rods into cone-like photoreceptors. The mice had a significant reduction in night-vision but were able to maintain robust daytime visual function as assessed by electroretinogram (ERG). The new cells were not fully cones and maintained some of their rod like functions, however they did not die off as they normally would in this disease state suggesting there was a neural protective effect[295].

This study indicates the significant therapeutic potential of converting one cell type into another in order to prevent degeneration in a disease state. However, it also highlights a key challenge in fate-conversation studies: Mature neurons are resistant to change. This is likely because mature neurons, unlike developing ones, have a strongly established chromatin ultrastructure and conversion to an alternative fate will likely require more than a single change. Indeed, the simplest protocols for generating induced pluripotent stems cells (iPSCs), involves at least four Yamanaka factors[363]. That does not include any of the steps needed to re-differentiate cells into alternative fates.

Our work builds on these findings in a novel system. We showed that driving PRDM1 expression during retinal development can shift bipolar cells to rod fates, even after they make their initial fate choice (Fig 4.3). However, we also saw that mature bipolar cells do not respond in any observable way to the presence of PRDM1 suggesting that their fate was stabilized and resistant to change (Figs 4.2, 4.4). We also showed that cells lacking VSX2 and PRDM1 can mature into bipolar cells once their fate is set or mature into photoreceptors when neither is present (Chapter III). Within this complex network it is clear that once retinal cells pass through

a specific transient fate choice window they will no longer readily shift their identity. Taken together, it is likely that significant more work into the fundamental mechanisms of cell fate and maturation will be required before any functional therapies are realized.

*Transplantation of Neural Tissue into a Mature Retina*

The prospect of successful and complete cell fate conversion within the retina remains somewhat elusive, particularly in a therapeutic context. While it is possible that one day we will develop the capability to genetically manipulate Müller glia to regrow a retina or fully fate shift mature rods to cones, substantial barriers still exist. This has led to significant work into transplantation strategies. The prevailing thought is that most retinal degenerative diseases have already caused some permanent loss of function before the disease is detected or treated. A wide range of diseases could be theoretically treated by the implantation of novel neural tissue into the retina provided it appropriately differentiated and integrated into the existing circuits. There are number of strategies for transplantation that include 1) transplantation of stem cells from embryonic, adult, or induced sources, 2) transplantation of mature cells and 3) transplantation of whole tissue.

Initial transplant studies were done using entire pieces of mouse tissue and grafting them into an extant retina. These had limited success in generating functional neural tissue, but there was some formation of outer-segment like structures that were promising[364–367]. This was followed by work attempting to place photoreceptors alone into a healthy or degraded retina. While these approaches generally had tissue survival, there was little restored visual function[368–371]. These studies were followed by work transplanting retinal progenitor cells[369]. Unfortunately, there is little evidence that transplanted retinal progenitor cells into a mature retina causes any

meaningful recovery of vision or indeed the generation of novel healthy retinal neurons[372–375]. Despite this evidence, clinical trials are in place inserting retinal progenitor cells into human retinas (jCYTE; www.clinicaltrials.gov; ID: NCT02320812)[376]. Others have grafted whole sections of human fetal retina into retinitis pigmentosa patients. While the tissue survived and there were no autoimmune effects, there was no change in visual function[377].

The transplantation of retinal progenitor cells or other stem cell derivatives had little to no functional integration into healthy or degraded retinas across numerous studies[364]. However, early post-mitotic nascent photoreceptors seem to have far more success in surviving when transplanted than mature photoreceptors or progenitors[378]. Little functional change has been shown in healthy retinas in response to transplantation. However, a few studies have shown that rod transplantation into a night-blind mouse model resulted in both restoration of electroretinograms (ERGs) and ability to perform low-light visual tasks[379,380]. Follow up studies using female/male transplants revealed that the transplanted cells were not in fact integrating, but their genetic or protein material was taken up into existing photoreceptors, leading to false conclusions based on fluorescent labels[381–383]. Taken together this suggests that restoration of vision in transplantation mouse models may be a result of material transfer from functional cells to host mutant cells rather than integration of transplanted cells. On the surface, this presents a major challenge to transplantation studies. However, if true, this could lead to a potential mechanism for delivering novel genetic material to rods, beyond those currently employed in gene therapy. It is possible that studies focused on transplantation will ultimately lead to the discovery of a mechanistic tool for gene therapy or cell trans-differentiation. However, to date, there are no studies showing conclusive evidence for the successful restoration of vision in a

disease model via the integration of transplanted tissue, whether it be whole tissue sections, progenitors, stem cells, immature postmitotic cells, or mature photoreceptors.

I suspect that further advancements in this field necessitate a better understanding of how neural circuits are created within the retina. I identified the fact that EBF3+ amacrines are necessary for properly laminated the IPL of the retina. There are no known mechanistic reasons for this. Future studies that attempt to define the neural circuits that EBF3+ amacrines form as well as their cell-cell communications and how that might impact IPL patterns are excellent lines of inquiry. Insights into the underlying biological drivers of retinal patterning and circuit formation could provide understanding into the means by which new neurons from implanted tissue might be integrated into an existing retina.

*Retinal Organoids and Regenerative Therapy*

There are variety of excellent vertebrate models for studying retinal development. However, many of them differ significantly from humans. Zebrafish have regenerative capabilities and their retinas grow throughout their lifetime. Mice and rats are primarily nocturnal and do not have a fovea or macula, the central part of the retina critical to human high acuity vision. This has led to the search for an alternative means of studying human retinal development. Human eyes develop significantly slower than mice or rats and studies on human fetal tissue are problematic for a variety of reasons. Human embryonic stem cells (ESCs) and iPSC have provided some insights into retinal development, but their uses are limited as neurons in a dish often vary significantly from those integrated into a laminated tissue[384,385]. Years of work lead to the ability to develop human iPSC derived organoids in culture[386,387]. Given the

right series of treatments and growth factors, small organized tissue sections can be grown that replicate aspects a developing organ[388].

There are a variety of critical developmental insights that organoids empower and a host of human-specific genetic modification approaches that are now available for testing but much of this work is still under development. Obstructions include the challenges spoken of previously regarding limitations in transplantation technology. Additionally, while organoids can mimic the development of human tissue, the process is not yet fully refined and critical differences have been shown by lamination, cell types, cell markers, and single-cell RNA sequencing compared to a normal developing tissue[388–390].

Fundamental to all the regenerative therapies and associated technologies discussed here is the need for increased understanding of intrinsic and extrinsic regulation of retinal development. How do cells choose a cell fate and how are those fates maintained? Under what circumstances is a cells fate maintained or lost? How is the retina patterned and how are plexiform layers formed? The work elucidated within this document sheds light on all of these questions and provides insights into the mechanisms of retinal cell fate regulation in developing and mature neurons, maintenance of cell identity, transcriptional regulation, and laminar patterning of tissue.

In mice, *Prdm13* derived EBF3+ amacrine cells are necessary to correctly laminate the IPL. Current organoid technologies have focused on driving the production of photoreceptors yet they frequently suffer from issues in the correct cell types and proper lamination [388]. It is possible that by changing the media, growth context, or birthdate order of progenitors the proper neuronal subtypes could form, resulting in a more robust and complete neural tissue. Conversely, if a group wanted to grow a rod-rich organoid tissue, our findings in chapter III may provide a

mechanism. I showed evidence that OTX2+ cells default to photoreceptors in the absence of VSX2 and show that deletion of a 164bp enhancer element is sufficient to stop bipolar formation. If a similar enhancer were identified in humans, a cell line could be created without the necessary bipolar-specific enhancer and could result in tissue that defaulted to rod-fates.

**Closing Remarks**

I have explored the developmental roles of a number of key transcriptional regulators that contribute to human disease including *Prdm13, Vsx2*, and *Otx2*. I have also unpacked more of the fate stabilizing functions played by *Prdm1*, a gene implicated in numerous developmental diseases and cancers. This work highlights the enormous challenges associated with causing a fate change within neurons of a mature retina and the temporally precise regulation of cell fate when the tissue is developing. The finding that simultaneous loss of PRDM1 and VSX2 produces amacrine cells and has alternative outcomes based on the way the gene is deleted provides insight into potential unforeseen complexities gene therapeutic approaches may face in the future. The fact that *Prdm13* mutants share common fate outcomes with humans afflicted by *Prdm13* North Carolina Macular Dystrophy suggests a future potential solution, provided an *in-utero* gene therapy were available. Cures such as this were once thought impossible. Actualizing them will be challenging. However, I can envision a future, built on the foundations of fundamental mechanisms of retinal development, in which no person suffers from permanent blindness.

I see this work as a modest contribution to the lifechanging global effort to let blind eyes see. The findings here represent an incremental advance in our understanding of retinal neural

development. Yet, I hope it will empower the work of dedicated scientists around the world who want to know, how do you make an eye?

## REFERENCES


1. Eye Disease Statistics - American Academy of Ophthalmology. Available at: https://www.aao.org/eye-disease-statistics. (Accessed: 4th March 2020)
2. Eye Health Data and Statistics | National Eye Institute. Available at: https://www.nei.nih.gov/learn-about-eye-health/resources-for-health-educators/eye-health-data-and-statistics. (Accessed: 4th March 2020)
3. Bowmaker, J. K. Evolution of vertebrate visual pigments. *Vision Res.* **48**, 2022–2041 (2008).
4. Breitmeyer, B. G. *Blindspots : the many ways we cannot see*. (Oxford University Press, 2010).
5. Thoen, H. H., How, M. J., Chiou, T. H. & Marshall, J. A different form of color vision in mantis shrimp. *Science (80-. ).* **343**, 411–413 (2014).
6. Fernald, R. D. The Evolution of Eyes. *Brain. Behav. Evol.* **50**, 253–259 (1997).
7. Greiner, B. Adaptations for Nocturnal Vision in Insect Apposition Eyes. *International Review of Cytology* **250**, 1–46 (2006).
8. Nilsson, D.-E. Vision Optics and Evolution. *Bioscience* **39**, 298–307 (1989).
9. Ayala, F. J. Darwin's greatest discovery: Design without designer. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 8567–8573 (2007).
10. Lamb, T. D., Collin, S. P. & Pugh, E. N. Evolution of the vertebrate eye: Opsins, photoreceptors, retina and eye cup. *Nature Reviews Neuroscience* **8**, 960–976 (2007).
11. Wong-Riley, M. Energy metabolism of the visual system. *Eye Brain* **2**, 99 (2010).
12. Strauss, O. The retinal pigment epithelium in visual function. *Physiological Reviews* **85**, 845–881 (2005).
13. Agathocleous, M. & Harris, W. A. From Progenitors to Differentiated Cells in the Vertebrate Retina. *Annu. Rev. Cell Dev. Biol.* **25**, 45–69 (2009).
14. Grimes, W. N., Zhang, J., Graydon, C. W., Kachar, B. & Diamond, J. S. Retinal Parallel Processors: More than 100 Independent Microcircuits Operate within a Single Interneuron. *Neuron* **65**, 873–885 (2010).
15. Masland, R. H. R. *No Title*. *Neuron* **76**, 266–280 (2012).
16. Amini, R., Rocha-Martins, M. & Norden, C. *Neuronal Migration and Lamination in the Vertebrate Retina*. *Frontiers in Neuroscience* **11**, (Frontiers Media S.A., 2018).
17. Lamb, T. D. Evolution of phototransduction, vertebrate photoreceptors and retina. *Prog. Retin. Eye Res.* **36**, 52–119 (2013).
18. Reingruber, J., Holcman, D. & Fain, G. L. How rods respond to single photons: Key adaptations of a G-protein cascade that enable vision at the physical limit of perception. *BioEssays* **37**, 1243–1252 (2015).

19. Srinivasan, S., Guixà-González, R., Cordomí, A. & Garriga, P. Ligand Binding Mechanisms in Human Cone Visual Pigments. *Trends Biochem. Sci.* **44**, 629–639 (2019).

20. Ingram, N. T., Sampath, A. P. & Fain, G. L. Why are rods more sensitive than cones? *Journal of Physiology* **594**, 5415–5426 (2016).

21. Dowling, J. E. Retina: An Overview. in *Reference Module in Biomedical Sciences* (Elsevier, 2015). doi:10.1016/b978-0-12-801238-3.04749-8

22. Pang, J. J., Gao, F. & Wu, S. M. Light-evoked current responses in rod bipolar cells, cone depolarizing bipolar cells and AII amacrine cells in dark-adapted mouse retina. *J. Physiol.* **558**, 897–912 (2004).

23. Pang, J. J. *et al.* Direct rod input to cone BCs and direct cone input to rod BCs challenge the traditional view of mammalian BC circuitry. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 395–400 (2010).

24. Tsukamoto, Y. & Omi, N. Classification of mouse retinal bipolar cells: Type-specific connectivity with special reference to rod-driven AII amacrine pathways. *Front. Neuroanat.* **11**, (2017).

25. Shekhar, K. *et al.* Comprehensive Classification of Retinal Bipolar Neurons by Single-Cell Transcriptomics. *Cell* **166**, 1308-1323.e30 (2016).

26. Balasubramanian, R. & Gan, L. Development of Retinal Amacrine Cells and Their Dendritic Stratification. *Curr. Ophthalmol. Rep.* **2**, 100–106 (2014).

27. Shelley, J. *et al.* Horizontal cell receptive fields are reduced in connexin57-deficient mice. *Eur. J. Neurosci.* **23**, 3176–3186 (2006).

28. Hombach, S. *et al.* Functional expression of connexin57 in horizontal cells of the mouse retina. *Eur. J. Neurosci.* **19**, 2633–2640 (2004).

29. Janssen-Bienhold, U. *et al.* Connexin57 is expressed in dendro-dendritic and axo-axonal gap junctions of mouse horizontal cells and its distribution is modulated by light. *J. Comp. Neurol.* **513**, 363–374 (2009).

30. Haverkamp, S. & Wässle, H. Immunocytochemical analysis of the mouse retina. *J. Comp. Neurol.* **424**, 1–23 (2000).

31. Jackman, S. L., Babai, N., Chambers, J. J., Thoreson, W. B. & Kramer, R. H. A positive feedback synapse from retinal horizontal cells to cone photoreceptors. *PLoS Biol.* **9**, e1001057–e1001057 (2011).

32. Kamermans, M. *et al.* Hemichannel-mediated inhibition in the outer retina. *Science (80-. ).* **292**, 1178–1180 (2001).

33. Vessey, J. P. *et al.* Proton-mediated feedback inhibition of presynaptic calcium channels at the cone photoreceptor synapse. *J. Neurosci.* **25**, 4108–4117 (2005).

34. Thoreson, W. B., Babai, N. & Bartoletti, T. M. Feedback from horizontal cells to rod photoreceptors in vertebrate retina. *J. Neurosci.* **28**, 5691–5695 (2008).

35. Messersmith, E. K. & Redburn, D. A. Kainic acid lesioning alters development of the outer plexiform layer in neonatal rabbit retina. *Int. J. Dev. Neurosci.* **8**, 447–461 (1990).

36. Tykocinski, L. O. *et al.* Epigenetic regulation of promiscuous gene expression in thymic medullary epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 19426–19431 (2010).

37. Janssen-Bienhold, U. *et al.* Ablation of retinal horizontal cells from adult mice leads to rod degeneration and remodeling in the outer retina. *J. Neurosci.* **32**, 10713–10724 (2012).

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

39. MacNeil, M. A. & Masland, R. H. Extreme diversity among amacrine cells: Implications for function. *Neuron* **20**, 971–982 (1998).

40. Kay, J. N., Voinescu, P. E., Chu, M. W. & Sanes, J. R. Neurod6 expression defines new retinal amacrine cell subtypes and regulates their fate. *Nature Neuroscience* **14**, 965–972 (2011).

41. Bassett, E. A. *et al.* Conditional Deletion of Activating Protein 2 (AP-2 ) in the Developing Retina Demonstrates Non-Cell-Autonomous Roles for AP-2 in Optic Cup Development. *Mol. Cell. Biol.* **27**, 7497–7510 (2007).

42. Feng, L. *et al.* Requirements for Bhlhb5 in the specification of amacrine and cone bipolar subtypes in mouse retina. *Development* **133**, 4815–4825 (2006).

43. Ghosh, K. K., Bujan, S., Haverkamp, S., Feigenspan, A. & Wässle, H. Types of Bipolar Cells in the Mouse Retina. *J. Comp. Neurol.* **469**, 70–82 (2004).

44. Huang, L. *et al.* Bhlhb5 is required for the subtype development of retinal amacrine and bipolar cells in mice. *Dev. Dyn.* **243**, 279–289 (2014).

45. Kondo, H., Kuramoto, H., Wainer, B. H. & Yanaihara, N. Discrete distribution of cholinergic and vasoactive intestinal polypeptidergic amacrine cells in the rat retina. *Neurosci. Lett.* **54**, 213–218 (1985).

46. J Lettvin, H. M. W. M. W. P. What the frog's eye tells the frog's brain. *Proc. IRE* **47**, 1940–1951 (1959).

47. BG Cleland, W. L. Brisk and sluggish concentrically organized ganglion cells in the cat's retina. *J. Physiol.* **240**, 421–456 (1974).

48. CW Oyster, H. B. Direction-selective units in rabbit retina: distribution of preferred directions. *Science (80-. ).* **155**, 841–842 (1967).

49. SH Devries, D. B. Mosaic arrangement of ganglion cell receptive fields in rabbit retina. *J. Neurophysiol.* **78**, 2048–2060 (1997).

50. K Farrow, R. M. Physiological clustering of visual channels in the mouse retina. *J. Neurophysiol.* **105**, 1516–1530 (2011).

51. J Coombs, D. van der L. G.-Y. W. L. C. Morphological properties of mouse retinal ganglion cells. *Neuroscience* **140**, 123–136 (2006).

52. Sümbül, U. A genetic and computational approach to structurally classify neuronal types. *Nat. Commun.* **5**, 3512 (2014).

53. B Völgyi, S. C. S. B. Tracer coupling patterns of the ganglion cell subtypes in the mouse retina. *J. Comp. Neurol.* **512**, 664–687 (2009).

54. J-H Kong, D. F. R. R. R. M. Diversity of ganglion cells in the mouse retina: unsupervised morphological classification and its limits. *J. Comp. Neurol.* **489**, 293–310 (2005).

55. Baden, T. *et al.* The functional diversity of retinal ganglion cells in the mouse. *Nature* **529**, 345–350 (2016).

56. E Robles, E. L. H. B. The retinal projectome reveals brain-area-specific visual representations generated by ganglion cell diversity. *Curr. Biol.* **24**, 2085–2096 (2014).

57. Lawrence, P. M. & Studholme, K. M. Retinofugal projections in the mouse. *J. Comp. Neurol.* **522**, 3733–3753 (2014).

58. A Reichenbach, A. B. New functions of Müller cells. *Glia* **61**, 651–678 (2013).

59. Bringmann, A. *et al.* Müller cells in the healthy and diseased retina. *Progress in Retinal and Eye Research* **25**, 397–424 (2006).

60. Newman, E. & Reichenbach, A. The Muller cell: A functional element of the retina. *Trends in Neurosciences* **19**, 307–312 (1996).

61. K Ghai, C. Z. A. F., Ghai, K., Zelinka, C. & Fischer, A. J. Notch signaling influences neuroprotective and proliferative properties of mature Müller glia. *J. Neurosci.* **30**, 3101–3112 (2010).

62. Fischer, A. J. & Reh, T. A. Potential of Müller glia to become neurogenic retinal progenitor cells. *Glia* **43**, 70–76 (2003).

63. Gallina, D., Todd, L. & Fischer, A. J. A comparative analysis of Müller glia-mediated regeneration in the vertebrate retina. *Exp. Eye Res.* **123**, 121–130 (2014).

64. Baden, T., Schubert, T., Berens, P. & Euler, T. The Functional Organization of Vertebrate Retinal Circuits for Vision. (2018). doi:10.1093/ACREFORE/9780190264086.013.68

65. T Breuninger, C. P. S. H. T. E. Chromatic bipolar cell pathways in the mouse retina. *J. Neurosci.* **31**, 6504–6517 (2011).

66. Euler, T., Haverkamp, S., Schubert, T. & Baden, T. *Retinal bipolar cells: Elementary building blocks of vision*. *Nature Reviews Neuroscience* **15**, 507–519 (Nature Publishing Group, 2014).

67. C Puller, E. I. T. E. S. H. T. S. OFF bipolar cells express distinct types of dendritic glutamate receptors in the mouse retina. *Neuroscience* **243**, 136–148 (2013).

68. Wässle, H., Puller, C., Müller, F., Haverkamp, S. & H Wässle, C Puller, F Müller, S. H. Cone contacts, mosaics, and territories of bipolar cells in the mouse retina. *J. Neurosci.* **29**, 106–117 (2009).

69. Lee, S., Zhang, Y., Chen, M. & Zhou, Z. J. Segregated Glycine-Glutamate Co-

transmission from vGluT3 Amacrine Cells to Contrast-Suppressed and Contrast-Enhanced Retinal Circuits. *Neuron* **90**, 27–34 (2016).

70. Grimes, W. N., Hoon, M., Briggman, K. L., Wong, R. O. & Rieke, F. Cross-synaptic synchrony and transmission of signal and noise across the mouse retina. *Elife* **3**, e03892 (2014).

71. Pan, F. *et al.* Inhibitory masking controls the threshold sensitivity of retinal ganglion cells. *J. Physiol.* **594**, 6679–6699 (2016).

72. Ivanova, E., Müller, U. & Wässle, H. Characterization of the glycinergic input to bipolar cells of the mouse retina. *Eur. J. Neurosci.* **23**, 350–364 (2006).

73. Dacey, D. *et al. Center surround receptive field structure of cone bipolar cells in primate retina*. *Vision Research* **40**, 1801–1811 (Pergamon, 2000).

74. Diamond, J. S. Inhibitory Interneurons in the Retina: Types, Circuitry, and Function. *Annu. Rev. Vis. Sci.* **3**, 1–24 (2017).

75. Zhang, A.-J. & Wu, S. M. Receptive Fields of Retinal Bipolar Cells Are Mediated by Heterogeneous Synaptic Circuitry. *J. Neurosci.* (2009). doi:10.1523/JNEUROSCI.4984-08.2009

76. Hubel, D. H. Single unit activity in striate cortex of unrestrained cats. *J. Physiol.* **147**, 226–238 (1959).

77. HB Barlow, R. H. W. L. Rabbit retinal ganglion cells responding selectively to direction and speed of image motion in the rabbit. *J. Physiol.* **173**, 377–407 (1964).

78. Cruz-Martín, A. *et al.* A dedicated circuit links direction-selective retinal ganglion cells to the primary visual cortex. *Nature* **507**, 358–361 (2014).

79. Mauss, A. S., Vlasits, A., Borst, A. & Feller, M. Visual Circuits for Direction Selectivity. *Annu. Rev. Neurosci.* **40**, 211–230 (2017).

80. Masland, R. H. The fundamental plan of the retina. *Nature Neuroscience* **4**, 877–886 (2001).

81. Carter-Dawson, L. D. & LaVail, M. M. Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. *J. Comp. Neurol.* **188**, 263–72 (1979).

82. DL Turner, C. C. *et al.* A common progenitor for neurons and glia persists in rat retina late in development. *Nature* **328**, 131–136 (1987).

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

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

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

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

87. CP Austin, D. F. J. I. C. C. Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* **121**, 3637–3650 (1995).

88. MJ Belliveau, C. C. Extrinsic and intrinsic factors control the genesis of amacrine and cone cells in the rat retina. *Development* **126**, 555–556 (1999).

89. MJ Belliveau, T. Y. C. C. Late retinal progenitor cells show intrinsic limitations in the production of cell types and the kinetics of opsin synthesis. *J. Neurosci.* **20**, 2247–2254 (2000).

90. McConnell, S. Fates of visual cortical neurons in the ferret after isochronic and heterochronic transplantation. *J. Neurosci.* **8**, 945–974 (1988).

91. Briscoe, J. Homeobox gene Nkx2.2 and specification of neuronal identity by graded sonic hedgehog signalling. *Nature* **398**, 622–627 (1999).

92. J Ericson, S. M. A. K. H. R. T. J. Two critical periods of sonic hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661–673 (1996).

93. AR Desai, S. M. Progressive restriction in fate potential by neural progenitors during cerebral cortical development. *Development* **127**, 2863–2872 (2000).

94. MA Selleck, M. B.-F. The genesis of avian neural crest cells: a classic embryonic induction. *Proc. Natl Acad. Sci. USA* **93**, 9352–9357 (1996).

95. Wong, L. L. & Rapaport, D. H. Defining retinal progenitor cell competence in Xenopus laevis by clonal analysis. *Development* **136**, 1707–1715 (2009).

96. Gomes, F. L. A. F. *et al.* Reconstruction of rat retinal progenitor cell lineages in vitro reveals a surprising degree of stochasticity in cell fate decisions. *Development* **138**, 227–235 (2011).

97. Bassett, E. A. & Wallace, V. A. Cell fate determination in the vertebrate retina. *Trends Neurosci.* **35**, 565–573 (2012).

98. Ohsawa, R. & Kageyama, R. *Regulation of retinal cell fate specification by multiple transcription factors*. *Brain Research* **1192**, 90–98 (Elsevier, 2008).

99. Harris, W. A. Cellular diversification in the vertebrate retina. *Curr. Opin. Genet. Dev.* **7**, 651–658 (1997).

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

101. Narlikar, G. J., Fan, H. Y. & Kingston, R. E. Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108**, 475–487 (2002).

102. Lan, X., Glass, C. K. & Rosenfeld, M. G. Coactivator and corepressor complexes in nuclear receptor function. *Curr. Opin. Genet. Dev.* **9**, 140–147 (1999).

103. Brzezinski, J. A., Prasov, L. & Glaser, T. Math5 defines the ganglion cell competence

state in a subpopulation of retinal progenitor cells exiting the cell cycle. *Dev. Biol.* **365**, 395–413 (2012).

104. Amoutzias, G. D., Robertson, D. L., Oliver, S. G. & Bornberg-Bauer, E. Convergent evolution of gene networks by single-gene duplications in higher eukaryotes. *EMBO Rep.* **5**, 274–279 (2004).

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

106. Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F. & Kageyama, R. Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. *EMBO J.* **19**, 5460–72 (2000).

107. Li, S. *et al.* Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. *Neuron* **43**, 795–807 (2004).

108. Fujitani, Y. *et al.* Ptf1a determines horizontal and amacrine cell fates during mouse retinal development. *Development* **133**, 4439–4450 (2006).

109. Akagi, T. *et al.* Requirement of multiple basic helix-loop-helix genes for retinal neuronal subtype specification. *J. Biol. Chem.* **279**, 28492–28498 (2004).

110. Keller, A. D. & Maniatis, T. Identification and characterization of a novel repressor of β-interferon gene expression. *Genes Dev.* **5**, 868–879 (1991).

111. Huang, S. Blimp-1 is the murine homolog of the human transcriptional repressor PRDI-BF1. *Cell* **78**, 9 (1994).

112. Hohenauer, T. & Moore, A. W. The Prdm family: Expanding roles in stem cells and development. *Development (Cambridge)* **139**, 2267–2282 (2012).

113. Fumasoni, I. *et al.* Family expansion and gene rearrangements contributed to the functional specialization of PRDM genes in vertebrates. *BMC Evol. Biol.* **7**, 187–187 (2007).

114. Kinameri, E. *et al.* Prdm proto-oncogene transcription factor family expression and interaction with the Notch-Hes pathway in mouse neurogenesis. *PLoS One* **3**, e3859–e3859 (2008).

115. Miyauchi, Y. *et al.* The Blimp1-Bcl6 axis is critical to regulate osteoclast differentiation and bone homeostasis. *J. Exp. Med.* **207**, 751–762 (2010).

116. Hayashi, K., Yoshida, K. & Matsui, Y. A histone H3 methyltransferase controls epigenetic events required for meiotic prophase. *Nature* **438**, 374–378 (2005).

117. Fears, S. *et al.* Intergenic splicing of MDS1 and EVI1 occurs in normal tissues as well as in myeloid leukemia and produces a new member of the PR domain family. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 1642–1647 (1996).

118. Mandelbaum, J. *et al.* BLIMP1 Is a Tumor Suppressor Gene Frequently Disrupted in Activated B Cell-like Diffuse Large B Cell Lymphoma. *Cancer Cell* **18**, 568–579 (2010).

119. Küçük, C. *et al.* PRDM1 is a tumor suppressor gene in natural killer cell malignancies. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 20119–20124 (2011).

120. Behrends, U. *et al.* Novel tumor antigens identified by autologous antibody screening of childhood medulloblastoma cDNA libraries. *Int. J. Cancer* **106**, 244–251 (2003).

121. Kouzarides, T. Chromatin Modifications and Their Function. *Cell* **128**, 693–705 (2007).

122. Huang, S., Shao, G. & Liu, L. The PR domain of the Rb-binding zinc finger protein RIZ1 is a protein binding interface and is related to the SET domain functioning in chromatin-mediated gene expression. *J. Biol. Chem.* **273**, 15933–15939 (1998).

123. Bartholomew, C., Kilbey, A., Clark, A. M. & Walker, M. The Evi-1 proto-oncogene encodes a transcriptional repressor activity associated with transformation. *Oncogene* **14**, 569–577 (1997).

124. Harper, J., Mould, A., Andrews, R. M., Bikoff, E. K. & Robertson, E. J. The transcriptional repressor Blimp1/Prdm1 regulates postnatal reprogramming of intestinal enterocytes. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 10585–10590 (2011).

125. Kurimoto, K. *et al.* Complex genome-wide transcription dynamics orchestrated by Blimp1 for the specification of the germ cell lineage in mice. *Genes Dev.* **22**, 1617–1635 (2008).

126. Bikoff, E. K. & Robertson, E. J. One PRDM is not enough for germ cell development. *Nature Genetics* **40**, 934–935 (2008).

127. Brzezinski IV, J. A., Lamba, D. A. & Reh, T. A. Blimp1 controls photoreceptor versus bipolar cell fate choice during retinal development. *Development* **137**, 619–629 (2010).

128. Katoh, K. *et al.* Blimp1 suppresses Chx10 expression in differentiating retinal photoreceptor precursors to ensure proper photoreceptor development. *J. Neurosci.* **30**, 6515–6526 (2010).

129. Small, K. W. *et al.* North Carolina Macular Dystrophy Is Caused by Dysregulation of the Retinal Transcription Factor PRDM13. *Ophthalmology* **123**, 9–18 (2016).

130. Brzezinski, J. A. & Reh, T. A. *Photoreceptor cell fate specification in vertebrates*. *Development* **142**, 3263–3273 (Company of Biologists Ltd, 2015).

131. Gommans, W. M., Haisma, H. J. & Rots, M. G. Engineering zinc finger protein transcription factors: The therapeutic relevance of switching endogenous gene expression on or off at command. *Journal of Molecular Biology* **354**, 507–519 (2005).

132. Affolter, M., Slattery, M. & Mann, R. A lexicon for homeodomain-DNA recognition. *Cell* **133**, (2008).

133. Wigle, J. T. & Eisenstat, D. D. Homeobox genes in vertebrate forebrain development and disease. *Clinical Genetics* **73**, 212–226 (2008).

134. Zagozewski, J. L., Zhang, Q., Pinto, V. I., Wigle, J. T. & Eisenstat, D. D. *The role of homeobox genes in retinal development and disease*. *Developmental Biology* **393**, 195–208 (Academic Press Inc., 2014).

135. Gonzalez-Rodriguez, J. *et al.* Mutational screening of CHX10, GDF6, OTX2, RAX and SOX2 genes in 50 unrelated microphthalmia-anophthalmia-coloboma (MAC) spectrum cases. *Br. J. Ophthalmol.* **94**, 1100–1104 (2010).

136. Martinez-Morales, J., Dolez, V., Rodrigo, I., Zaccarini, R. & Leconte, L. OTX2 activates the molecular network underlying retina pigment epithelium differentiation. *J. Biol. Chem.* **278**, (2003).

137. Martinez-Morales, J., Signore, M., Acampora, D., Simeone, A. & Bovolenta, P. Otx genes are required for tissue specification in the developing eye. *Development* **128**, (2001).

138. Nishida, A. *et al.* Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. *Nat. Neurosci.* **6**, 1255–1263 (2003).

139. Koike, C. *et al.* Functional Roles of Otx2 Transcription Factor in Postnatal Mouse Retinal Development. *Mol. Cell. Biol.* **27**, 8318–8329 (2007).

140. Tzoulaki, I., White, I. M. S. & Hanson, I. M. PAX6 mutations: Genotype-phenotype correlations. *BMC Genet.* **6**, (2005).

141. Marquardt, T. *et al.* Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* **105**, 43–55 (2001).

142. Oron-Karni, V. *et al.* Dual requirement for Pax6 in retinal progenitor cells. *Development* **135**, 4037–4047 (2008).

143. Zou, C. & Levine, E. M. *Vsx2 Controls Eye Organogenesis and Retinal Progenitor Identity Via Homeodomain and Non-Homeodomain Residues Required for High Affinity DNA Binding*. *PLoS Genetics* **8**, e1002924 (Public Library of Science, 2012).

144. Dorval, K., Bobechko, B., Ahmad, K. & Bremner, R. Transcriptional activity of the paired-like homeodomain proteins CHX10 and VSX1. *J Biol Chem* **280**, (2005).

145. Chow, R. L. *et al.* Vsx1, a rapidly evolving paired-like homeobox gene expressed in cone bipolar cells. *Mech. Dev.* **109**, 315–322 (2001).

146. Liu, I. S. C. *et al.* Developmental expression of a novel murine homeobox gene (Chx10): Evidence for roles in determination of the neuroretina and inner nuclear layer. *Neuron* **13**, 377–393 (1994).

147. Nguyen, M. & Arnheiter, H. Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. *Development* **127**, (2000).

148. Green, E., Stubbs, J. & Levine, E. Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. *Development* **130**, (2003).

149. Burmeister, M. *et al.* Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. *Nat. Genet.* **12**, 376–384 (1996).

150. Livne-Bar, I. *et al.* 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**, 4988–93 (2006).

151. Carroll, S. Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. *Cell* **134**, 25–36 (2008).

152. C-T Ong, V. C. Enhancer function: new insights into the regulation of tissue-specific gene expression. *Nat. Rev. Genet.* **12**, 283–293 (2011).

153. Levine, M. Transcriptional enhancers in animal development and evolution. *Curr. Biol.* **20**, R754–R763 (2010).

154. M Bulger, M. G. Functional and mechanistic diversity of distal transcription enhancers. *Cell* **144**, 327–339 (2011).

155. Wittkopp, P. J. & Kalay, G. Cis-regulatory elements: Molecular mechanisms and evolutionary processes underlying divergence. *Nature Reviews Genetics* **13**, 59–69 (2012).

156. Zuckerkandl, E. & Pauling, L. Evolving Genes and Proteins. (1965).

157. Britten, K. H. Mechanisms of Self-Motion Perception. *Annu. Rev. Neurosci.* **31**, 389–410 (2008).

158. M-C King, A. W. Evolution at two levels in humans and chimpanzees. *Science (80-. ).* **188**, 107–116 (1975).

159. Savinkova, L. TATA box polymorphisms in human gene promoters and associated hereditary pathologies. *Biochemistry* **74**, 117–129 (2009).

160. Ghiasvand, N. M. *et al.* Deletion of a remote enhancer near ATOH7 disrupts retinal neurogenesis, causing NCRNA disease. *Nat. Neurosci.* **14**, 578–588 (2011).

161. JW Hong, D. H. M. L. Shadow enhancers as a source of evolutionary novelty. *Science (80-. ).* **321**, 1314 (2008).

162. Wray, G. The evolutionary significance of cis-regulatory mutations. *Nat. Rev. Genet.* **8**, 206–216 (2007).

163. Abdulrehman, D. Yeastract: providing a programmatic access to curated transcriptional regulatory associations in Saccharomyces cerevisiae through a web services interface. *Nucleic Acids Res.* **39**, D136–D140 (2011).

164. Roy, S. Identification of functional elements and regulatory circuits by Drosophila modENCODE. *Science (80-. ).* **330**, 1787–1797 (2010).

165. Visel, A. ChIP–seq accurately predicts tissue-specific activity of enhancers. *Nature* **457**, 854–858 (2009).

166. CM Ghim, S. L. S. T. R. M. The art of reporter proteins in science: past, present and future applications. *BMB Rep.* **43**, 451–460 (2010).

167. Mills, T. S. *et al.* Combinatorial regulation of a Blimp1 (Prdm1) enhancer in the mouse retina. *PLoS One* **12**, 1–27 (2017).

168. GD Stormo, Y. Z. Determining the specificity of protein–DNA interactions. *Nat. Rev.*

*Genet.* **11**, 751–760 (2010).

169. Drewett, V. DNA-bound transcription factor complexes analysed by mass-spectrometry: binding of novel proteins to the human c-fos SRE and related sequences. *Nucleic Acids Res.* **29**, 479–487 (2001).

170. SJ Maerkl, S. Q. A systems approach to measuring the binding energy landscapes of transcription factors. *Science (80-. ).* **315**, 233–237 (2007).

171. Furukawa, T. *et al.* No Title. *Neuron* **26**, 383–394 (2000).

172. Reh, T. A. Neural Stem Cells: Ocular. in *Encyclopedia of Neuroscience* 247–251 (Elsevier Ltd, 2009). doi:10.1016/B978-008045046-9.01024-X

173. S Hayes, B. N. B. B. T. R., Hayes, S., Nelson, B. R., Buckingham, B. & Reh, T. A. Notch signaling regulates regeneration in the avian retina. **312**, 300–311 (2007).

174. Fuhrmann, S. *Wnt signaling in eye organogenesis*. *Organogenesis* **4**, 60–67 (Landes Bioscience, 2008).

175. Malicki, J. Cell fate decisions and patterning in the vertebrate retina: The importance of timing, asymmetry, polarity and waves. *Current Opinion in Neurobiology* **14**, 15–21 (2004).

176. Stump, R. J. W. *et al.* A role for Wnt/β-catenin signaling in lens epithelial differentiation. *Dev. Biol.* **259**, 48–61 (2003).

177. Fujimura, N., Taketo, M. M., Mori, M., Korinek, V. & Kozmik, Z. Spatial and temporal regulation of Wnt/β-catenin signaling is essential for development of the retinal pigment epithelium. *Dev. Biol.* **334**, 31–45 (2009).

178. Fujimura, N. WNT/β-Catenin Signaling in Vertebrate Eye Development. *Front. Cell Dev. Biol.* **4**, 138 (2016).

179. Esteve, P. & Bovolenta, P. Secreted inducers in vertebrate eye development: More functions for old morphogens. *Current Opinion in Neurobiology* **16**, 13–19 (2006).

180. Wang, Y., Dakubo, G. D., Thurig, S., Mazerolle, C. J. & Wallace, V. A. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. *Development* **132**, 5103–5113 (2005).

181. Erskine, L. & Herrera, E. The retinal ganglion cell axon's journey: Insights into molecular mechanisms of axon guidance. *Developmental Biology* **308**, 1–14 (2007).

182. Mason, C. A. & Sretavan, D. W. Glia, neurons, and axon pathfinding during optic chiasm development. *Curr. Opin. Neurobiol.* **7**, 647–653 (1997).

183. Sánchez-Camacho, C., Rodríguez, J., Ruiz, J. M., Trousse, F. & Bovolenta, P. Morphogens as growth cone signalling molecules. *Brain Research Reviews* **49**, 242–252 (2005).

184. Morcillo, J. *et al.* Proper patterning of the optic fissure requires the sequential activity of BMP7 and SHH. *Development* **133**, 3179–3190 (2006).

185. Sánchez-Camacho, C. & Bovolenta, P. *Autonomous and non-autonomous Shh signalling mediate the in vivo growth and guidance of mouse retinal ganglion cell axons*. *Development* **135**, 3531–3541 (The Company of Biologists Ltd, 2008).

186. Traiffort, E., Moya, K. L., Faure, H., Hässig, R. & Ruat, M. High expression and anterograde axonal transport of aminoterminal sonic hedgehog in the adult hamster brain. *Eur. J. Neurosci.* **14**, 839–850 (2001).

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

188. Byerly, M. S. & Blackshaw, S. Vertebrate retina and hypothalamus development. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **1**, 380–389 (2009).

189. Hägglund, A. C., Dahl, L. & Carlsson, L. Lhx2 is required for patterning and expansion of a distinct progenitor cell population committed to eye development. *PLoS One* **6**, (2011).

190. Ashery-Padan, R., Marquardt, T., Zhou, X. & Gruss, P. Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev.* **14**, 2701–2711 (2000).

191. Pontoriero, G. F. *et al.* Co-operative roles for E-cadherin and N-cadherin during lens vesicle separation and lens epithelial cell survival. *Dev. Biol.* **326**, 403–417 (2009).

192. Akagi, T. *et al.* Otx2 homeobox gene induces photoreceptor-specific phenotypes in cells derived from adult iris and ciliary tissue. *Investig. Ophthalmol. Vis. Sci.* **45**, 4570–4575 (2004).

193. Chuang, J. C. & Raymond, P. A. Embryonic origin of the eyes in teleost fish. *BioEssays* **24**, 519–529 (2002).

194. Tajima, T. *et al.* OTX2 loss of function mutation causes anophthalmia and combined pituitary hormone deficiency with a small anterior and ectopic posterior pituitary. *J. Clin. Endocrinol. Metab.* **94**, 314–319 (2009).

195. Fossat, N., Chatelain, G., Brun, G. & Lamonerie, T. Temporal and spatial delineation of mouse Otx2 functions by conditional self-knockout. *EMBO Rep.* **7**, 824–830 (2006).

196. Dateki, S. *et al.* OTX2 mutation in a patient with anophthalmia, short stature, and partial growth hormone deficiency: Functional studies using the IRBP, HESX1, and POU1F1 promoters. *J. Clin. Endocrinol. Metab.* **93**, 3697–3702 (2008).

197. Ragge, N. K. *et al.* Heterozygous mutations of OTX2 cause severe ocular malformations. *Am. J. Hum. Genet.* **76**, 1008–1022 (2005).

198. Burkitt Wright, E. M. M. *et al.* VSX2 in microphthalmia: A novel splice site mutation producing a severe microphthalmia phenotype. *British Journal of Ophthalmology* **94**, 386–388 (2010).

199. Percin, E. F. *et al.* Human microphthalmia associated with mutations in the retinal homeobox gene CHX10. **25**, 397–401 (2000).

200. Liang, L. & Sandell, J. H. Focus on molecules: Homeobox protein Chx10. *Experimental*

*Eye Research* **86**, 541–542 (2008).

201. Bar-Yosef, U., Abuelaish, I., Harel, T., Hendler, N. & Ofir, R. CHX10 mutations cause non-syndromic microphthalmia/anophthalmia in Arab and Jewish kindreds. *Hum Genet* **115**, (2004).

202. Morrison, D. *et al.* National study of microphthalmia, anophthalmia, and coloboma (MAC) in Scotland: Investigation of genetic aetiology. *J. Med. Genet.* **39**, 16–22 (2002).

203. Macular Dystrophy - AllAboutVision.com. Available at: https://www.allaboutvision.com/conditions/macular-dystrophy.htm. (Accessed: 6th March 2020)

204. Michaelides, M., Hunt, D. M. & Moore, A. T. The genetics of inherited macular dystrophies. *Journal of Medical Genetics* **40**, 641–650 (2003).

205. OMIM Entry - # 136550 - MACULAR DYSTROPHY, RETINAL, 1, NORTH CAROLINA TYPE; MCDR1. Available at: https://www.omim.org/entry/136550. (Accessed: 6th March 2020)

206. Badea, T. C. & Nathans, J. Quantitative analysis of neuronal morphologies in the mouse retina visualized by using a genetically directed reporter. *J. Comp. Neurol.* **480**, 331–351 (2004).

207. Vaney, D. I. Chapter 2 The mosaic of amacrine cells in the mammalian retina. *Progress in Retinal Research* **9**, 49–100 (1990).

208. Sharma, R. K., O'Leary, T. E., Fields, C. M. & Johnson, D. A. Development of the outer retina in the mouse. *Dev. Brain Res.* **145**, 93–105 (2003).

209. de Melo, J., Qiu, X., Du, G., Cristante, L. & Eisenstat, D. D. Dlx1, Dlx2, Pax6, Brn3b, and Chx10 homeobox gene expression defines the retinal ganglion and inner nuclear layers of the developing and adult mouse retina. *J. Comp. Neurol.* **461**, 187–204 (2003).

210. Bassett, E. A. *et al.* Overlapping expression patterns and redundant roles for AP-2 transcription factors in the developing mammalian retina. *Dev. Dyn.* **241**, 814–829 (2012).

211. Cherry, T. J., Trimarchi, J. M., Stadler, M. B. & Cepko, C. L. Development and diversification of retinal amacrine interneurons at single cell resolution. *Proc Natl Acad Sci U S A* **106**, 9495–9500 (2009).

212. Voinescu, P. E., Kay, J. N. & Sanes, J. R. Birthdays of retinal amacrine cell subtypes are systematically related to their molecular identity and soma position. *J. Comp. Neurol.* **517**, 737–750 (2009).

213. Wang, L., Lang, L. L., Wang, Y., Shi, S. & Liu, L. Prostaglandin E2 enhances proliferation, dedifferentiation and stem-like properties of rat retinal müller glial cells in vitro. *Ophthalmic Res.* **49**, 100–107 (2013).

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

215. Elshatory, Y. *et al.* Islet-1 Controls the Differentiation of Retinal Bipolar and Cholinergic Amacrine Cells. *J. Neurosci.* **27**, 12707–12720 (2007).

216. Chang, J. C. *et al.* Prdm13 Mediates the Balance of Inhibitory and Excitatory Neurons in Somatosensory Circuits. *Dev. Cell* **25**, 182–195 (2013).

217. Mona, B. *et al.* Repression by PRDM13 is critical for generating precision in neuronal identity. *Elife* **6**, (2017).

218. Watanabe, S. *et al.* Prdm13 regulates subtype specification of retinal amacrine interneurons and modulates visual sensitivity. *J. Neurosci.* **35**, 8004–8020 (2015).

219. Sidman, R. L. Histogenesis of mouse retina studied with thymidine H3. in *Structure of the Eye* (ed. Smelser, G. K.) 487–506 (Academic Press, 1961).

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

221. Reh, T. A. & Cagan, R. L. Intrinsic and extrinsic signals in the developing vertebrate and fly eyes: viewing vertebrate and invertebrate eyes in the same light. *Perspect Dev Neurobiol* **2**, 183–190 (1994).

222. Cepko, C. Intrinsically different retinal progenitor cells produce specific types of progeny. *Nature Reviews Neuroscience* **15**, 615–627 (2014).

223. Boije, H., MacDonald, R. B. & Harris, W. A. Reconciling competence and transcriptional hierarchies with stochasticity in retinal lineages. *Curr. Opin. Neurobiol.* **27**, 68–74 (2014).

224. Wu, F. *et al.* Onecut1 is essential for horizontal cell genesis and retinal integrity. *J. Neurosci.* **33**, 13053–13065 (2013).

225. Heavner, W. & Pevny, L. No Title. *Cold Spring Harb. Perspect. Biol.* **4**, a008391 (2012).

226. Yamamoto, H., Kon, T., Omori, Y. & Furukawa, T. Functional and Evolutionary Diversification of Otx2 and Crx in Vertebrate Retinal Photoreceptor and Bipolar Cell Development. *Cell Rep* **30**, 658-671 e5 (2020).

227. Fossat, N. *et al.* A new GFP-tagged line reveals unexpected Otx2 protein localization in retinal photoreceptors. *BMC Dev. Biol.* **7**, (2007).

228. Beby, F. & Lamonerie, T. *The homeobox gene Otx2 in development and disease*. *Experimental Eye Research* **111**, 9–16 (Academic Press, 2013).

229. Muranishi, Y. *et al.* No Title. **31**, (2011).

230. Goodson, N. B. *et al.* Prdm13 is required for Ebf3+ amacrine cell formation in the retina. *Dev. Biol.* **434**, 149–163 (2018).

231. Brzezinski, J. A., Uoon Park, K. & Reh, T. A. Blimp1 (Prdm1) prevents re-specification of photoreceptors into retinal bipolar cells by restricting competence. *Dev. Biol.* **384**, 194–204 (2013).

232. Dorval, K., Bobechko, B., Fujieda, H., Chen, S. & Zack, D. CHX10 targets a subset of photoreceptor genes. *J Biol Chem* **281**, (2006).

233. Wang, S., Sengel, C., Emerson, M. M. & Cepko, C. L. A gene regulatory network controls the binary fate decision of rod and bipolar cells in the vertebrate retina. *Dev. Cell* **30**, 513–527 (2014).

234. Kim, I. J. *et al.* Molecular identification of a retinal cell type that responds to upward motion. *Nature* **452**, 478–482 (2008).

235. Wang, S., Sengel, C., Emerson, M. M. M. & Cepko, C. L. L. No Title. *Dev. Cell* **30**, 513–527 (2014).

236. c12f012.jpg (412×289). Available at: https://i2.wp.com/veteriankey.com/wp-content/uploads/2017/10/c12f012.jpg?w=960. (Accessed: 11th March 2020)

237. Dowling, J. E. A Life in Vision. *Annu. Rev. Vis. Sci.* **4**, 1–23 (2018).

238. Retinorecipient.jpg (2178×1149). Available at: http://schwartzlab.feinberg.northwestern.edu/wp-content/uploads/sites/3/2019/06/Retinorecipient.jpg. (Accessed: 11th March 2020)

239. image-01.png (1200×1150). Available at: https://basicscience.ucdmc.ucdavis.edu/Brown_Lab/images/research/large/image-01.png. (Accessed: 11th March 2020)

240. Wu, S., Chang, K. C. & Goldberg, J. L. Retinal Cell Fate Specification. *Trends in Neurosciences* **41**, 165–167 (2018).

241. Masland, R. H. Neuronal diversity in the retina. *Current Opinion in Neurobiology* **11**, 431–436 (2001).

242. Sanes, J. R. & Masland, R. H. The Types of Retinal Ganglion Cells: Current Status and Implications for Neuronal Classification. *Annu. Rev. Neurosci.* **38**, 221–246 (2015).

243. Muranishi, Y. *et al.* An essential role for RAX homeoprotein and NOTCH-HES signaling in Otx2 expression in embryonic retinal photoreceptor cell fate determination. *J. Neurosci.* **31**, 16792–16807 (2011).

244. Baas, D. *et al.* The subcellular localization of OTX2 is cell-type specific and developmentally regulated in the mouse retina. *Mol. Brain Res.* **78**, 26–37 (2000).

245. Das, G., Choi, Y., Sicinski, P. & Levine, E. E. M. Cyclin D1 fine-tunes the neurogenic output of embryonic retinal progenitor cells. **4**, (2009).

246. Cheng, H. L., Sullivan, K. A. & Feldman, E. L. Immunohistochemical localization of insulin-like growth factor binding protein-5 in the developing rat nervous system. *Dev. Brain Res.* **92**, 211–218 (1996).

247. Haverkamp, S., Grünert, U., Wässle, H. & S Haverkamp, U Grünert, H. W. The cone pedicle, a complex synapse in the retina. *Neuron* **27**, 85–95 (2000).

248. Béby, F. *et al.* Otx2 gene deletion in adult mouse retina induces rapid RPE dystrophy and slow photoreceptor degeneration. *PLoS One* **5**, 1–8 (2010).

249. Gonzälez-soriano, J. Morphological types of horizontal cell in rodent retinae: A

comparison of rat, mouse, gerbil, and guinea pig. *Vis. Neurosci.* **11**, 501–517 (1994).

250. Pow, D. V. & Hendrickson, A. E. Distribution of the glycine transporter glyt-1 in mammalian and nonmammalian retinae. *Vis. Neurosci.* **16**, 231–239 (1999).

251. Surzenko, N., Crowl, T., Bachleda, A., Langer, L. & Pevny, L. SOX2 maintains the quiescent progenitor cell state of postnatal retinal Müller glia. *Dev.* **140**, 1445–1456 (2013).

252. Taranova, O. V. *et al.* SOX2 is a dose-dependent regulator of retinal neural progenitor competence. *Genes Dev.* **20**, 1187–1202 (2006).

253. Lee, E. S., Lee, J. Y. & Jeon, C. J. Types and density of calretinin-containing retinal ganglion cells in mouse. *Neurosci. Res.* **66**, 141–150 (2010).

254. Voigt, T. Cholinergic amacrine cells in the rat retina. *J. Comp. Neurol.* **248**, 19–35 (1986).

255. Homma, K. *et al.* Developing rods transplanted into the degenerating retina of Crx-knockout mice exhibit neural activity similar to native photoreceptors. *Stem Cells* **31**, 1149–1159 (2013).

256. Wilken, M. S. *et al.* DNase i hypersensitivity analysis of the mouse brain and retina identifies region-specific regulatory elements. *Epigenetics and Chromatin* **8**, (2015).

257. Hanotel, J. *et al.* The Prdm13 histone methyltransferase encoding gene is a Ptf1a-Rbpj downstream target that suppresses glutamatergic and promotes GABAergic neuronal fate in the dorsal neural tube. *Dev. Biol.* **386**, 340–357 (2014).

258. Jin, K. *et al.* Tfap2a and 2b act downstream of Ptf1a to promote amacrine cell differentiation during retinogenesis. *Mol. Brain* **8**, 28 (2015).

259. Bessodes, N., Parain, K., Bronchain, O., Bellefroid, E. J. & Perron, M. Prdm13 forms a feedback loop with Ptf1a and is required for glycinergic amacrine cell genesis in the Xenopus Retina. *Neural Dev.* **12**, 1–15 (2017).

260. Macosko, E. Z. *et al.* Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell* **161**, 1202–1214 (2015).

261. Voyvodic, J. T., Burne, J. F. & Raff, M. C. Quantification of Normal Cell Death in the Rat Retina: Implications for Clone Composition in Cell Lineage Analysis. *Eur. J. Neurosci.* **7**, 2469–2478 (1995).

262. Young, R. W. Cell death during differentiation of the retina in the mouse. *J. Comp. Neurol.* **229**, 362–373 (1984).

263. Strettoi, E. & Volpini, M. Retinal organization in the bcl-2-overexpressing transgenic mouse. *J. Comp. Neurol.* **446**, 1–10 (2002).

264. Péquignot, M. O. *et al.* Major role of BAX in apoptosis during retinal development and in establishment of a functional postnatal retina. *Dev. Dyn.* **228**, 231–238 (2003).

265. Bowne, S. J. *et al.* North Carolina macular dystrophy (MCDR1) caused by a novel tandem duplication of the PRDM13 gene. *Mol. Vis.* **22**, 1239–1247 (2016).

266. Manes, G. *et al.* A novel duplication of PRMD13 causes North Carolina macular dystrophy: overexpression of PRDM13 orthologue in drosophila eye reproduces the human phenotype. *Hum. Mol. Genet.* **26**, 4367–4374 (2017).

267. Bunt, J. *et al.* Joint binding of OTX2 and MYC in promotor regions is associated with high gene expression in medulloblastoma. *PLoS One* **6**, (2011).

268. Bowrey, H. E. & James, M. H. Commentary:"Prdm13 regulates subtype specification of retinal amacrine interneurons and modulates visual sensitivity". *Front. Cell. Neurosci.* **9**, (2015).

269. Sugita, S. *et al.* Successful Transplantation of Retinal Pigment Epithelial Cells from MHC Homozygote iPSCs in MHC-Matched Models. *Stem Cell Reports* **7**, 635–648 (2016).

270. Kim, D. S., Matsuda, T. & Cepko, C. L. A core paired-type and POU homeodomain-containing transcription factor program drives retinal bipolar cell gene expression. *J Neurosci* **28**, 7748–7764 (2008).

271. Sigulinsky, C. L. *et al.* Genetic chimeras reveal the autonomy requirements for Vsx2 in embryonic retinal progenitor cells. *Neural Dev* **10**, 12 (2015).

272. Elshatory, Y., Deng, M., Xie, X. & Gan, L. Expression of the LIM-homeodomain protein Isl1 in the developing and mature mouse retina. *J Comp Neurol* **503**, 182–197 (2007).

273. Puthussery, T., Gayet-Primo, J. & Taylor, W. R. Localization of the calcium-binding protein secretagogin in cone bipolar cells of the mammalian retina. *J. Comp. Neurol.* **518**, 513–525 (2010).

274. Menger, N., Pow, D. V & Wassle, H. Glycinergic amacrine cells of the rat retina. *J Comp Neurol* **401**, 34–46 (1998).

275. Kay, J. N. *et al.* Retinal ganglion cells with distinct directional preferences differ in molecular identity, structure, and central projections. *J. Neurosci.* **31**, 7753–7762 (2011).

276. Jin, K., Jiang, H., Mo, Z. & Xiang, M. Early B-cell factors are required for specifying multiple retinal cell types and subtypes from postmitotic precursors. *J Neurosci* **30**, 11902–11916 (2010).

277. Madisen, L. *et al.* A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci* **13**, 133–140 (2010).

278. Norrie, J. L. *et al.* Nucleome Dynamics during Retinal Development. *Neuron* **104**, 512-528 e11 (2019).

279. Shi, Z. *et al.* Vsx1 regulates terminal differentiation of type 7 ON bipolar cells. *J. Neurosci.* **31**, (2011).

280. Chow, R. L. *et al.* Control of late off-center cone bipolar cell differentiation and visual signaling by the homeobox gene Vsx1. *Proc Natl Acad Sci U S A* **101**, 1754–1759 (2004).

281. Ohtoshi, A. *et al.* Regulation of retinal cone bipolar cell differentiation and photopic vision by the CVC homeobox gene Vsx1. *Curr. Biol.* **14**, 530–536 (2004).

282. Sigulinsky, C., Green, E., Clark, A. & Levine, E. Vsx2/Chx10 ensures the correct timing and magnitude of Hedgehog signaling in the mouse retina. *Dev Biol* **317**, (2008).

283. Shi, Z., Jervis, D., Nickerson, P. E. & Chow, R. L. Requirement for the paired-like homeodomain transcription factor VSX1 in type 3a mouse retinal bipolar cell terminal differentiation. *J Comp Neurol* **520**, 117–129 (2012).

284. Carter-Dawson, L. D., Lavail, M. M., Carter-Dawson, L. D. & Lavail, M. M. Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. **188**, 245–262 (1979).

285. Phillips, M. J. *et al.* Modeling human retinal development with patient-specific induced pluripotent stem cells reveals multiple roles for visual system homeobox 2. *Stem Cells* **32**, 1480–1492 (2014).

286. Lefebvre, J. L., Zhang, Y., Meister, M., Wang, X. & Sanes, J. R. gamma-Protocadherins regulate neuronal survival but are dispensable for circuit formation in retina. *Development* **135**, 4141–51 (2008).

287. Sigulinsky, C. L. *et al.* Genetic chimeras reveal the autonomy requirements for Vsx2 in embryonic retinal progenitor cells. *Neural Dev.* **10**, 12 (2015).

288. Nickerson, P. E. B. *et al.* A transgenic mouse line expressing cre recombinase in undifferentiated postmitotic mouse retinal bipolar cell precursors. *PLoS One* **6**, e27145 (2011).

289. Magnúsdóttir, E. *et al.* A tripartite transcription factor network regulates primordial germ cell specification in mice. *Nat. Cell Biol.* **15**, 905–15 (2013).

290. Ohinata, Y. *et al.* Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* **436**, 207–213 (2005).

291. Sybirna, A., Wong, F. C. K. & Surani, M. A. Genetic basis for primordial germ cells specification in mouse and human: Conserved and divergent roles of PRDM and SOX transcription factors. *Curr. Top. Dev. Biol.* **135**, 35–89 (2019).

292. de Leeuw, C. N. *et al.* Targeted CNS Delivery Using Human MiniPromoters and Demonstrated Compatibility with Adeno-Associated Viral Vectors. *Mol. Ther. Methods Clin. Dev.* **1**, 5 (2014).

293. Scalabrino, M. L. *et al.* Intravitreal delivery of a novel AAV vector targets ON bipolar cells and restores visual function in a mouse model of complete congenital stationary night blindness. *Hum. Mol. Genet.* **24**, 6229–39 (2015).

294. Mears, A. J. *et al.* Nrl is required for rod photoreceptor development. *Nat. Genet.* **29**, 447–452 (2001).

295. Montana, C. L. *et al.* Reprogramming of adult rod photoreceptors prevents retinal degeneration. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 1732–1737 (2013).

296. Yu, C. R. & Wu, Y. Regeneration and rewiring of rodent olfactory sensory neurons. *Experimental Neurology* **287**, 395–408 (2017).

297. Zhu, J. *et al.* Gene and mutation independent therapy via CRISPR-Cas9 mediated cellular reprogramming in rod photoreceptors. *Cell Research* **27**, 830–833 (2017).

298. Solovei, I. *et al.* Nuclear architecture of rod photoreceptor cells adapts to vision in mammalian evolution. *Cell* **137**, 356–68 (2009).

299. Baxendale, S. *et al.* The B-cell maturation factor Blimp-1 specifies vertebrate slow-twitch muscle fiber identity in response to Hedgehog signaling. *Nat. Genet.* **36**, 88–93 (2004).

300. Bikoff, E. K., Morgan, M. A. & Robertson, E. J. *An expanding job description for Blimp-1/PRDM1*. **19**, 379–385 (Elsevier Current Trends, 2009).

301. Hernandez-Lagunas, L. *et al.* Zebrafish narrowminded disrupts the transcription factor prdm1 and is required for neural crest and sensory neuron specification. *Dev. Biol.* **278**, 347–357 (2005).

302. C Powell, A. G. E. C. D. G. Analysis of DNA methylation reveals a partial reprogramming of the Müller glia genome during retina regeneration. *Proc. Natl Acad. Sci. USA* **110**, 19814–19819 (2013).

303. Pars planitis | Genetic and Rare Diseases Information Center (GARD) – an NCATS Program. Available at: https://rarediseases.info.nih.gov/diseases/7339/pars-planitis. (Accessed: 10th March 2020)

304. Uveitis-Glaucoma-Hyphema Syndrome - EyeWiki. Available at: https://eyewiki.aao.org/Uveitis-Glaucoma-Hyphema_Syndrome. (Accessed: 10th March 2020)

305. Lau, C. H., Missotten, T., Salzmann, J. & Lightman, S. L. Acute Retinal Necrosis. Features, Management, and Outcomes. *Ophthalmology* **114**, 756-762.e1 (2007).

306. Tsirouki, T. *et al.* A Focus on the Epidemiology of Uveitis. *Ocul. Immunol. Inflamm.* **26**, 2–16 (2018).

307. Jabs, D. A., Nussenblatt, R. B., Rosenbaum, J. T. & Standardization of Uveitis Nomenclature (SUN) Working Group. Standardization of uveitis nomenclature for reporting clinical data. Results of the First International Workshop. *Am. J. Ophthalmol.* **140**, 509–16 (2005).

308. Klaska, I. P. & Forrester, J. V. Mouse models of autoimmune uveitis. *Curr. Pharm. Des.* **21**, 2453–67 (2015).

309. Koizumi, K. *et al.* Contribution of TNF-α to Leukocyte Adhesion, Vascular Leakage, and Apoptotic Cell Death in Endotoxin-Induced Uveitis In Vivo. *Investig. Opthalmology Vis. Sci.* **44**, 2184 (2003).

310. Rosenbaum, J. T. & Angell, E. Paradoxical effects of IL-10 in endotoxin-induced uveitis. *J. Immunol.* **155**, 4090–4 (1995).

311. Kogiso, M., Tanouchi, Y., Mimura, Y., Nagasawa, H. & Himeno, K. Endotoxin-induced uveitis in mice Induction of uveitis and role of T lymphocytes. *Jpn. J. Ophthalmol.* **36**, 281–90 (1992).

312. Li, Q., Peng, B., Whitcup, S. M., Jang, S. U. & Chan, C.-C. Endotoxin induced uveitis in the mouse: Susceptibility and genetic control. *Exp. Eye Res.* **61**, 629–632 (1995).

313. McCluskey, P. J., Towler, H. M. & Lightman, S. Management of chronic uveitis. *BMJ* **320**, 555–8 (2000).

314. Yadav, U. C. S. & Ramana, K. V. Endotoxin-induced uveitis in rodents. *Methods Mol. Biol.* **1031**, 155–162 (2013).

315. Palestine, A. G. *et al.* Ophthalmic involvement in acquired immunodeficiency syndrome. *Ophthalmology* **91**, 1092–9 (1984).

316. Agarwal, R. K., Silver, P. B. & Caspi, R. R. Rodent models of experimental autoimmune uveitis. *Methods Mol. Biol.* **900**, 443–69 (2012).

317. Chu, C. J. *et al.* Multimodal analysis of ocular inflammation using the endotoxin-induced uveitis mouse model. *Dis. Model. Mech.* **9**, 473–81 (2016).

318. Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* **8**, 2281–2308 (2013).

319. Hsu, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* **31**, 827–832 (2013).

320. Doench, J. G. *et al.* Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol* **34**, 184–191 (2016).

321. Zolotukhin, S. *et al.* Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods* **28**, 158–167 (2002).

322. Zolotukhin, S. *et al.* Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther.* **6**, 973–85 (1999).

323. Kay, C. N. *et al.* Targeting photoreceptors via intravitreal delivery using novel, capsid-mutated AAV vectors. *PLoS One* **8**, e62097 (2013).

324. de Melo, J. & Blackshaw, S. In vivo electroporation of developing mouse retina. *J Vis Exp* (2011). doi:10.3791/2847

325. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671–675 (2012).

326. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag New York, 2016).

327. Hnisz, D., Day, D. S. & Young, R. A. Insulated Neighborhoods: Structural and Functional Units of Mammalian Gene Control. *Cell* **167**, 1188–1200 (2016).

328. Javed, A. & Cayouette, M. Temporal Progression of Retinal Progenitor Cell Identity: Implications in Cell Replacement Therapies. *Front. Neural Circuits* **11**, 105 (2017).

329. Rossi, A. M., Fernandes, V. M. & Desplan, C. Timing temporal transitions during brain development. *Current Opinion in Neurobiology* **42**, 84–92 (2017).

330. den Hollander, A. I., Roepman, R., Koenekoop, R. K. & Cremers, F. P. M. Leber congenital amaurosis: Genes, proteins and disease mechanisms. *Progress in Retinal and Eye Research* **27**, 391–419 (2008).

331. Acland, G. M. *et al.* Gene therapy restores vision in a canine model of childhood blindness. *Nat. Genet.* **28**, 92–95 (2001).

332. Bennicelli, J. *et al.* Reversal of blindness in animal models of leber congenital amaurosis using optimized AAV2-mediated gene transfer. *Mol. Ther.* **16**, 458–465 (2008).

333. Acland, G. M. *et al.* Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Mol. Ther.* **12**, 1072–1082 (2005).

334. Stein, L., Roy, K., Lei, L. & Kaushal, S. Clinical gene therapy for the treatment of RPE65-associated Leber congenital amaurosis. *Expert Opin. Biol. Ther.* **11**, 429–439 (2011).

335. Richardson, P. M., Issa, V. M. K. & Aguayo, A. J. Regeneration of long spinal axons in the rat. *J. Neurocytol.* **13**, 165–182 (1984).

336. Liu, K., Tedeschi, A., Park, K. K. & He, Z. Neuronal Intrinsic Mechanisms of Axon Regeneration. *Annu. Rev. Neurosci.* **34**, 131–152 (2011).

337. Chen, Z.-L., Yu, W.-M. & Strickland, S. Peripheral Regeneration. *Annu. Rev. Neurosci.* **30**, 209–233 (2007).

338. Mulvaney, B. D. & Heist, H. E. Regeneration of rabbit olfactory epithelium. *Am. J. Anat.* **131**, 241–251 (1971).

339. Matulionis, D. H. Ultrastructural study of mouse olfactory epithelium following destruction by ZnSO4 and its subsequent regeneration. *Am. J. Anat.* **142**, 67–89 (1975).

340. Matulionis, D. H. Light and electron microscopic study of the degeneration and early regeneration of olfactory epithelium in the mouse. *Am. J. Anat.* **145**, 79–99 (1976).

341. Burd, G. D. Morphological study of the effects of intranasal zinc sulfate irrigation on the mouse olfactory epithelium and olfactory bulb. *Microsc. Res. Tech.* **24**, 195–213 (1993).

342. Moulton, D. G. DYNAMICS OF CELL POPULATIONS IN THE OLFACTORY EPITHELIUM. *Ann. N. Y. Acad. Sci.* **237**, 52–61 (1974).

343. Moulton, D. G., Çelebi, G. & Fink, R. P. Olfaction in Mammals-Two Aspects: Proliferation of Cells in the Olfactory Epithelium and Sensitivity to Odours. in 227–250 (2008). doi:10.1002/9780470715369.ch12

344. Schwob, J. E. Neural regeneration and the peripheral olfactory system. *Anat. Rec.* **269**, 33–49 (2002).

345. Monti Graziadei, G. A. & Graziadei, P. P. C. Neurogenesis and neuron regeneration in the olfactory system of mammals. II. Degeneration and reconstitution of the olfactory sensory neurons after axotomy. *J. Neurocytol.* **8**, 197–213 (1979).

346. Holcomb, J. D., Mumm, J. S. & Calof, A. L. Apoptosis in the Neuronal Lineage of the

Mouse Olfactory Epithelium: Regulationin Vivoandin Vitro. *Dev. Biol.* **172**, 307–323 (1995).

347. Costanzo, R. M. & Graziadei, P. P. C. A quantitative analysis of changes in the olfactory epithelium following bulbectomy in hamster. *J. Comp. Neurol.* **215**, 370–381 (1983).

348. Coelho, D. H. & Costanzo, R. M. Posttraumatic olfactory dysfunction. *Auris Nasus Larynx* **43**, 137–143 (2016).

349. Piscopo, D. M., El-Danaf, R. N., Huberman, A. D. & Niell, C. M. Diverse visual features encoded in mouse lateral geniculate nucleus. *J. Neurosci.* **33**, 4642–4656 (2013).

350. Jones, B. W. *et al.* Retinal remodeling triggered by photoreceptor degenerations. *J. Comp. Neurol.* **464**, 1–16 (2003).

351. Fariss, R. N., Li, Z. Y. & Milam, A. H. Abnormalities in rod photoreceptors, amacrine cells, and horizontal cells in human retinas with retinitis pigmentosa. *Am. J. Ophthalmol.* **129**, 215–223 (2000).

352. Lamba, D. A., Karl, M. O. & Reh, T. A. Strategies for retinal repair: cell replacement and regeneration. *Progress in Brain Research* **175**, 23–31 (2009).

353. Reh, T. A., Jones, M. & Pittack, C. Common mechanisms of retinal regeneration in the larval frog and embryonic chick. *Ciba Foundation symposium* **160**, 192–208 (1991).

354. Araki, M. Regeneration of the amphibian retina: Role of tissue interaction and related signaling molecules on RPE transdifferentiation. *Development Growth and Differentiation* **49**, 109–120 (2007).

355. Reh, T. A., Nagy, T. & Gretton, H. Retinal pigmented epithelial cells induced to transdifferentiate to neurons by laminin. *Nature* **330**, 68–71 (1987).

356. Sakami, S., Etter, P. & Reh, T. A. Activin signaling limits the competence for retinal regeneration from the pigmented epithelium. *Mech. Dev.* **125**, 106–116 (2008).

357. Bernardos, R. L., Barthel, L. K., Meyers, J. R., Raymond, P. A. & RL Bernardos, L. B. J. M. P. R. Late-stage neuronal progenitors in the retina are radial Müller glia that function as retinal stem cells. **27**, 7028–7040 (2007).

358. Jadhav, A. P. *et al. Development and neurogenic potential of Müller glial cells in the vertebrate retina*. *Progress in Retinal and Eye Research* **28**, 249–262 (2009).

359. Nelson, B. R., Hartman, B. H., Georgi, S. A., Lan, M. S. & Reh, T. A. Transient inactivation of Notch signaling synchronizes differentiation of neural progenitor cells. *Dev. Biol.* **304**, 479–498 (2007).

360. Fischer, A. J., Reh, T. A., AJ Fischer, T. R., Fischer, A. J. & Reh, T. A. Exogenous growth factors stimulate the regeneration of ganglion cells in the chicken retina. *Dev. Biol.* **251**, 367–379 (2002).

361. Hoang, T. *et al.* Comparative transcriptomic and epigenomic analysis identifies key regulators of injury response and neurogenic competence in retinal glia. *bioRxiv* 717876 (2019). doi:10.1101/717876

362. Hartong, D. T., Berson, E. L. & Dryja, T. P. Retinitis pigmentosa. *Lancet* **368**, 1795–1809 (2006).

363. Seki, T. Methods of induced pluripotent stem cells for clinical application. *World J. Stem Cells* **7**, 116 (2015).

364. Santos-Ferreira, T. F., Borsch, O. & Ade, M. Rebuilding the missing Part—A review on photoreceptor transplantation. *Frontiers in Systems Neuroscience* **10**, 105 (2017).

365. Notter, M. F. D. *et al.* Intraretinal Transplantation for Rod-Cell Replacement in Light-Damaged Retinas. *J. Neural Transplant.* **1**, 1–10 (1989).

366. Gouras, P. *et al.* Survival and Synapse Formation of Transplanted Rat Rods. *J. Neural Transplant. Plast.* **2**, 91–100 (1991).

367. Du, J., Gouras, P., Kjeldbye, H., Kwun, R. & Lopez, R. Monitoring photoreceptor transplants with nuclear and cytoplasmic markers. *Exp. Neurol.* **115**, 79–86 (1992).

368. Coles, B. L. K. *et al.* Facile isolation and the characterization of human retinal stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 15772–15777 (2004).

369. Klassen, H. J. *et al.* Multipotent retinal progenitors express developmental markers, differentiate into retinal neurons, and preserve light-mediated behavior. *Investig. Ophthalmol. Vis. Sci.* **45**, 4167–4173 (2004).

370. Merhi-Soussi, F. *et al.* High Yield of Cells Committed to the Photoreceptor Fate from Expanded Mouse Retinal Stem Cells. *Stem Cells* **24**, 2060–2070 (2006).

371. Ballios, B. G. *et al.* A Hyaluronan-Based Injectable Hydrogel Improves the Survival and Integration of Stem Cell Progeny following Transplantation. *Stem Cell Reports* **4**, 1031–1045 (2015).

372. Cicero, S. A. *et al.* Cells previously identified as retinal stem cells are pigmented ciliary epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 6685–6690 (2009).

373. Gualdoni, S. *et al.* Adult ciliary epithelial cells, previously identified as retinal stem cells with potential for retinal repair, fail to differentiate into new rod photoreceptors. *Stem Cells* **28**, 1048–1059 (2010).

374. Czekaj, M. *et al.* In vitro expanded stem cells from the developing retina fail to generate photoreceptors but differentiate into myelinating oligodendrocytes. *PLoS One* **7**, (2012).

375. Ringuette, R. *et al.* Combinatorial hedgehog and mitogen signaling promotes the in vitro expansion but not retinal differentiation potential of retinal progenitor cells. *Investig. Ophthalmol. Vis. Sci.* **55**, 43–54 (2014).

376. Klassen, H. Stem cells in clinical trials for treatment of retinal degeneration. *Expert Opinion on Biological Therapy* **16**, 7–14 (2016).

377. Radtke, N. D., Seiler, M. J., Aramant, R. B., Petry, H. M. & Pidwell, D. J. Transplantation of intact sheets of fetal neural retina with its retinal pigment epithelium in retinitis pigmentosa patients. *Am. J. Ophthalmol.* **133**, 544–550 (2002).

378. Gust, J. & Reh, T. A. Adult donor rod photoreceptors integrate into the mature mouse retina. *Investig. Ophthalmol. Vis. Sci.* **52**, 5266–5272 (2011).

379. Pearson, R. A. *et al.* Restoration of vision after transplantation of photoreceptors. *Nature* **485**, 99–103 (2012).

380. Barber, A. C. *et al.* Repair of the degenerate retina by photoreceptor transplantation. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 354–359 (2013).

381. Pearson, R. A. *et al.* Donor and host photoreceptors engage in material transfer following transplantation of post-mitotic photoreceptor precursors. *Nat. Commun.* **7**, (2016).

382. Santos-Ferreira, T. *et al.* Retinal transplantation of photoreceptors results in donor-host cytoplasmic exchange. *Nat. Commun.* **7**, (2016).

383. Singh, M. S. *et al.* Transplanted photoreceptor precursors transfer proteins to host photoreceptors by a mechanism of cytoplasmic fusion. *Nat. Commun.* **7**, (2016).

384. Thomson, J. A. Embryonic stem cell lines derived from human blastocysts. *Science (80-. ).* **282**, 1145–1147 (1998).

385. Takahashi, K. *et al.* Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **131**, 861–872 (2007).

386. Ader, M. & Tanaka, E. M. Modeling human development in 3D culture. *Current Opinion in Cell Biology* **31**, 23–28 (2014).

387. Zhu, Y. *et al.* Three-Dimensional Neuroepithelial Culture from Human Embryonic Stem Cells and Its Use for Quantitative Conversion to Retinal Pigment Epithelium. *PLoS One* **8**, (2013).

388. Llonch, S., Carido, M. & Ader, M. Organoid technology for retinal repair. *Developmental Biology* **433**, 132–143 (2018).

389. Gasparini, S. J., Llonch, S., Borsch, O. & Ader, M. Transplantation of photoreceptors into the degenerative retina: Current state and future perspectives. *Progress in Retinal and Eye Research* **69**, 1–37 (2019).

390. Sridhar, A. *et al.* Single-Cell Transcriptomic Comparison of Human Fetal Retina, hPSC-Derived Retinal Organoids, and Long-Term Retinal Cultures. *Cell Rep.* **30**, 1644-1659.e4 (2020).