Epigenomic Regulation of Development and Regeneration in the Mammalian Retina

Leah S. VandenBosch

A Dissertation
Submitted in Partial Fulfilment
of the Requirements for the Degree of

Doctor of Philosophy

University of Washington
2019

Reading Committee:
Thomas A. Reh, Chair
Olivia Bermingham-McDonogh
Andrea Wills

Program Authorized to Offer Degree:
Molecular and Cellular Biology

Abstract

Epigenomic Regulation of Development and Regeneration in the Mammalian Retina
Leah S. VandenBosch
Chair of the Supervisory Committee:
Thomas A. Reh
Department of Biological Structure

Diseases and damage to the retina lead to broad losses in retinal neurons and eventual visual impairment. Although the mammalian retina has no inherent regenerative capabilities, fish have robust regeneration from Müller glia (MG). Recently, the Reh lab has shown that driving expression of the proneural transcription factor, Ascl1 in adult mouse MG stimulates neurogenesis from these cells—*in vitro* and *in vivo*—similar to that which occurs in fish. The regeneration observed in the mouse is limited however, by the types of neurons that can be derived from the MG; Ascl1-expressing MG primarily generate bipolar cells. Additionally, Ascl1-based regeneration in the mouse retina is restricted temporally as the MG develop. To better understand the limits to mammalian MG reprogramming, I used RNA-seq and ATAC-seq to compare FACS purified cells. I firstly compared reprogramming treatment combinations in adult regeneration, as well as comparing highly neurogenic newborn mouse progenitors to developing MG. Additionally, I explored the epigenomic roles on fate decisions by comparing glial type cells to retinal neurons, and by modulating the epigenome with small molecule inhibitors ex vivo. I found that while there were many similarities between MG and progenitors, E-box regulatory regions lose accessibility as NFI binding domains increase in accessibility. Analysis of young glia reveals an intermediate epigenomic and transcriptomic profile that directs intermediate reprogrammability of glia as the young retina develops. Similarly, increasing reprogramming factors increased neurogenesis-related accessibility that relates directly to adult MG regeneration outcomes. Broader modifications to the epigenome can thus redirect fate decisions as MG redifferentiate *ex vivo.* Fate decisions do not appear to be directed, however, by inherent epigenomic similarity to retinal neurons, but instead by pioneering factor preference for binding sites. Overall, my analysis demonstrates the roles in which epigenomic accessibility reveals glial potential for neurogenesis during Ascl1-induced regenerative responses in mammalian retinas.

Acknowledgements

Throughout the course of my dissertation work I have received a great amount of support. I would like to thank my advisor, Tom Reh for his mentorship throughout my degree. His support and scientific guidance has been crucial in the process of developing my project, and his encouragement for me to attempt new techniques and analyses has lead me down unexpected and valuable paths. Tom's persistent enthusiasm for science has bolstered my hope for future endurance and prosperity in the field.

I would also like to thank the members of the Reh lab for all of their support and guidance over the years. Specifically, Stefanie Wohl was an invaluable wet lab partner, training me on many techniques including her Fluorescence-Activated Cell Sorting protocol, and for encouraging me to believe in myself. Throughout my tenure in the lab, Nik Jorstad's presence as a colleague and collaborator has been invaluable for personal and scientific support. Our projects have worked in a complementary fashion, and without his guidance, my work would not have produced the same results. Matthew Wilken, who was the senior graduate student in the lab when I began, and existing epigenomics expert. His aid in starting me on computational analysis of epigenomic data was instrumental in giving me a solid foundation for my analysis. I would also like to thank Brent Wilkerson and Alex Chitsazan for their guidance in computational analysis and patience with all of my questions. Without the support of my lab, and surrounding colleagues, I would not have been as successful or thorough.

For their dedicated support and advice over the years, I thank my supervisory committee. Olivia Bermingham-McDonogh has been present for all of my lab meetings, and has always offered her honest feedback and constant support. Andrea Wills' boundless scientific enthusiasm bolsters my spirits, and her guidance with ATAC-seq data collection and analysis has been invaluable. B. Cole Trapnell has been a valuable voice of technical support, and motivated me to hold my data analysis to a higher standard. Rachel Wong has provided important guidance and context for the retinal system. Celeste Berg, my Graduate School Representative, has not only been an excellent developmental advisor, but has also provided crucial personal support to me throughout my time in graduate school.

Of course, no graduate student in the MCB program would easily get through their time in graduate school without the people running the program. Both current and former program specialists, Maia Low and MaryEllin Robinson have been vital go-to people, and they have always fiercely advocated for all the MCB students. Our community engagement and communications specialist Teresa Swanson has given me invaluable professional advice, and I wish her the best as she moves on to her new position as a leader for #MeTooSTEM. Additionally, for his guidance as a director of MCB, Rich Gardner has helped me to find my footing as a young scientist and is always a cheerful supporter of students throughout their graduate careers. The MCB program also was able to provide me with two years of funding through the Cell and Molecular Biology training grant, and I was additionally funded by the Developmental Biology Training Grant for one year.

Finally, I have an incredible support system outside of the lab that have kept my spirits up continuously and have been there for me throughout my education. My dearest friends whom I have known from K-12 public schooling, especially Keelia Silvis, Maria Starr, and Hannah Haverkamp have been there for me every step of the way while they all have also become incredible and talented individuals. Their support keeps me grounded, they remind me to speak up and take care of myself, and each of them inspires me to do great things every day. Many friends from undergraduate and graduate school have been there for me for coffee breaks, general angst and complaining, and emotional support. In particular, Becca Martin has been at the ready to get me out of the lab when times are stressful, and has encouraged unhealthy coping mechanisms as we worked to get our names on the Whiskey Wall of Fame at the Barrel Thief. Of course, I have also been lucky enough to have a very scientific family that have supported me in many ways. My siblings, Geoffrey VandenBosch, Dawn Gundermann, and Kolin Frantz, have provided unwavering support and patience and I am grateful to have them in my life. My parents, Tom Frantz and Kathryn VandenBosch have acted as additional mentors to me and are always available for both scientific and personal advice while persistently believing in my ability to succeed. Lastly, for her continuous and unconditional love and support, I would like to thank my partner, Allison Davis. I deeply appreciate the many ways in which she has sustained me and made sure I believed in myself throughout all aspects of my life. Her enthusiasm and unending belief in me has given me more belief in myself to do more. I am excited to move on with the next phases of our lives together. Without all of these people I would not have done as much or been as successful throughout my PhD.

Table of Contents

List of Figures and Tables

Glossary

Accessible Domain: Regions of accessibility in the genome or within a dataset refers specifically to peaks called by HOMER on datasets from ATAC-seq. These regions are accessible for binding and cleavage activity by Tn5 transposase, and are presumed associated with euchromatic regions where transcriptional machinery may bind.

ANT: Ascl1 in combination with NMDA damage and the small molecule Trichostatin-A are used as a triple factor cocktail to induce regeneration in the adult murine retina. This cocktail is abbreviated to ANT for simplicity in discussion.

Ascl1: The neurogenic pioneering transcription factor Achaete-scute homolog 1 is necessary for retinal development and regeneration. The transcription factor contains a bHLH domain and binds to E-box domains in cis to regulate neurogenic transcription.

ATAC: An Assay for Transposase-Accessible Chromatin which utilizes a Tn5 transposase to preferentially cleave open domains of chromatin which are subsequently sequenced by next generation sequencing methods.

bHLH: A basic-helix-loop-helix domain is a structural component of transcription factors which binds to cis-regulatory regions in the genome and regulates transcriptional activity.

Bipolar Cell: The bipolar cell is a retinal neuron found in the inner nuclear layer of the retina. It acts by accepting signals primarily from photoreceptors and sending signals to the retinal ganglion cells.

ChIP: Chromatin Immunoprecipitation is a technique for assaying the epigenome. The use of antibodies against proteins, including histone modifications, in sheared chromatin allows for isolation of genomic regions where specific proteins are bound. These genomic regions are then purified and either sequenced or assayed by quantitative polymerase chain reaction.

Cis-Regulatory: Cis-regulatory elements are genomic regions in close proximity to a gene that are necessary for regulation of gene expression. These regions contain binding motifs for transcription factors that participate in the activity of transcriptional machinery.

Chromatin: Genomic regions are compacted using structural proteins to generate chromatin. The level of compaction is affected by modifications to the genome and to chromatin proteins to generate dense heterochromatin and loose euchromatin.

DNase: DNase-sequencing is an alternative method for assaying open chromatin. This assay uses DNA-cleaving enzymes that preferentially act on regions of open chromatin and yield short reads of DNA that can be assayed similarly to ATAC-sequencing. This method requires higher numbers of cells but can result in much higher depth of sequencing.

E-Box: Enhancer box domains are genomic response elements found nearby genes that bind to transcription factors with bHLH domains with relative specificity. E-boxes are a broader class with differential specificity, though all contain the CANNTG DNA sequence.

Gene Ontology: Gene ontology refers to categorization of genes with similar functions into distinct categories. For the purposes of this dissertation, I will specifically be discussion biological process gene categories. To acquire gene ontology categories, a list of genes is input into an ontology ranking program and the most statistically relevant ontology categories are selected and ranked according to a chosen metric such as p value.

GREAT: The Genomic Regions of Annotation Tool (GREAT) is a gene ontology tool designed for epigenomic datasets. A set of genomic regions (such as ATAC peaks) is input and annotated to neighboring genes. These annotated genes are then assigned to significant ontology categories, or the gene annotations can be output for other analyses.

HDAC: The epigenetic regulatory enzyme class of histone deacetylases (HDACs) remove acetyl marks from histone tails and contributes to the closing of chromatin.

HOMER: The Hypergeometric Optimization of Motif EnRichment (HOMER) software is a suite of tools used for analysis of ChIP, DNase, and ATAC datasets. It is Perl and C++ based and is run in the command line. The software suite contains tools relevant for peak calling, analysis of read and motif enrichment, and many other related functions.

Motif: A motif refers to a short conserved genomic sequence found in cis-regulatory elements. This conserved sequence is associated with DNA binding by specific transcription factor protein elements. Each motif has specificity towards specific classes of transcription factors.

Müller Glia: Müller glia (MG) are the primary structural and chemical support glia of the retina. These glia are radial-like in morphology and are born from the same common progenitor as all retinal neurons at the end of the neurogenic period.

NFI: Nuclear Factor I (NFI) is a family of transcription factors that bind DNA to regulate expression and directs gliogenesis in the brain (Heng et al. 2015; Deneen et al. 2006; Glasgow et al. 2017).

NMDA: N-methyl-D-aspartate is an amino acid derivative with similarities to the excitatory neurotransmitter glutamate and binds to the same receptors. For the purposes of this dissertation, NMDA is used to induce excitotoxic damage which kills retinal ganglion cells when injected into the vitreous of the eye in high concentrations.

Peaks: In this dissertation, "peaks" refers to genomic regions of high read density in epigenomic datasets such as ChIP and ATAC. These peaks are called through the use of programs such as HOMER which determine regions of significant read density in comparison to the surrounding genomic regions.

Photoreceptors: Photoreceptor cells are a class of retinal neurons (consisting of rods and a variety of cones) that detect light that enters the eye, and generates a signal by altering the membrane potential in the cell.

Postnatal Days: When referring to mouse ages, days after birth (postnatal) are used, abbreviated as P.

Progenitor: In reference to the mammalian retina, all core retinal neurons and Müller glia are born from a multipotent progenitor, hereafter referred to as progenitors or retinal progenitor cells.

TSA: The histone deacetylase (HDAC) inhibitor trichostatin-A (TSA) broadly inhibits HDACs and contributes to major gains in histone acetylation and opening of chromatin.

Chapter 1

Introduction

Text and Figures Adapted from VandenBosch and Reh 2019

Loss of neurological sensory tissue through disease and damage affects the lives of many. These conditions can be lifelong disabilities, as the mammalian central nervous system (CNS) has almost no regenerative ability. In particular, damage to the mammalian retina causes permanent vision loss. In humans, some of the most common types of retinal diseases are Retinitis Pigmentosa and Macular Degeneration, which lead to death of photoreceptors (Al-Zamil and Yassin 2017; Ferrari et al. 2011). A considerable effort has gone into developing new approaches towards reducing neural degeneration, but few have been clinically successful. As a result, there is an urgent need for methods to stimulate repair of neuronal tissue. The approaches being taken to repair neuronal tissue can be grouped into 1) efforts to replace lost tissue through stimulation of endogenous regeneration, or 2) through in vitro generation of neuronal structures to be transplanted to the site of damage. These regenerative efforts could help millions of patients with neuronal degeneration and damage.

In the course of my dissertation work, I have focused on a regenerative approach to retinal repair. Current research in mammalian retinal regeneration takes cues from the regenerative teleost fish, which can regenerate the full neural retina using a combination of transcription factors expressed in the Müller glia (MG), many of which have also been used in the generation of new neurons in cultured somatic cells (Wan and Goldman 2016; Goldman 2014; Bermingham-McDonogh and Reh 2011). There is also increasing evidence that epigenomics play a crucial role in the restructuring of cells to alter cell fate in regeneration (VandenBosch and Reh 2019). It has become clear through studies of both regenerative and non-regenerative tissue that the epigenetic landscape is very important in proper tissue development, and in regulating cell fate transitions. Given the non-regenerative state of the murine retina, I compared the epigenomic landscapes of retinal cells with and without regenerative capacity, as well as observations of the regenerated retina, retinal neurons, and attempted to modulate the epigenomic landscape during regeneration. In this introduction, I will cover background material for these subjects in order to present context for my research.

Epigenetics

The epigenomic landscape has a strong influence on cell fate determination, cell differentiation and other developmental processes that are highly relevant to regeneration

(Ronan, Wu, and Crabtree 2013). The epigenome is generally characterized by chromatin states, or the interaction between DNA and histones or other packing proteins, often as facilitated by modifications to DNA or proteins (Jenuwein and Allis 2001; Kouzarides 2007). There are two basic states of chromatin: euchromatin associated with open transcription, and heterochromatin, associated with inactive transcription (Corso-Díaz et al. 2018). Histones are a major component of chromatin, packaged together into histone octets that interact with 147 bp fragments of DNA to create nucleosomes (Kouzarides 2007). The generation of dense heterochromatin, or decompaction into euchromatin are both associated with many posttranslational modifications to the N-terminal tail structures of the core nucleosome histones. In euchromatin, lysine acetylation of histones is associated with open and accessible chromatin, due to steric interactions (Kouzarides 2007). The best-characterized modifications are histone H3 lysine 27 acetylation (H3K27ac) and H3 lysine 4 acetylation (H3K4ac). Histone acetylation is catalyzed by Histone Acetyltransferases (HATs) and these acetyl groups are removed by Histone Deacetylases (HDACs) (Jenuwein and Allis 2001). Euchromatin is also associated with some methyl modifications, such as histone H3K4me3 and H3K36me3 (Kouzarides 2007). Condensed heterochromatin is almost exclusively associated with histone methylation. These modifications are added to histone tails by Histone Methyltransferases (HMTases), and removed by Histone Lysine Demethylases (KDMs). The most well-characterized heterochromatic histone methyl mark is H3 lysine trimethylation (H3K27me3), which is catalyzed by Polycomb Repressive Complex 2 (PRC2) (Jenuwein and Allis 2001). Also well studied is the repressive H3K9me2/3, though as with euchromatic histone modifications, many other modifications have been reported. However, heterochromatin and euchromatin markers can be found in combination with each other to create bivalent chromatin, which is frequently found around genes that may be rapidly activated during cell fate decisions (Kouzarides 2007; Corso-Díaz et al. 2018). The combinations of all of these histone modifications, with some redundancy, help determine the chromatin state. In addition to epigenetic regulation through histone modifications, DNA methylation can also affect gene expression. There are many varieties of DNA methylation, but the best studied is 5-methylcytosine at C-phosphate-G (CpG) islands (Corso-Díaz et al. 2018). This type of DNA modification is most associated with heterochromatin and reduced expression. However, when found within the gene body, it is more associated with enhanced transcription. DNA methylation was previously considered to be a more permanent epigenetic modification, but has been shown to change with development and cellular activities (Jenuwein and Allis 2001). These modifications are added by DNA methyltransferases (DNMTs) and removed by Tet-eleven translocation enzymes, APOBECs, and AID (Corso-Díaz et al. 2018).

Methods in Epigenetics

There are many ways in which the epigenome can be assayed, depending on the level of detail desired. For the purpose of this study, I will focus on methods of assaying chromatin state only. To observe specific chromatin modifications, the most common assay method is Chromatin Immunoprecipitation (ChIP) sequencing (DeAngelis, Farrington, and Tollefsbol 2008). This assay requires chromatin fragments from cells of interest, commonly formaldehyde fixed to preserve protein-DNA interactions. Antibodies against the modification or transcription factor of interest are incubated with chromatin to isolate genomic regions of interest. These DNA fragments are eluted and sequenced by Next-Generation Sequencing (NGS), and reads can be aligned to generate a dataset of regions of interest for analysis. Historically, this protocol has required large numbers of cells, making analysis on rare populations difficult. However, in recent years, new methods are being developed for low—down to single cell—numbers (Dahl and Gilfillan 2018). Alternatively, for DNA methylation states, there are a multitude of methods for analysis (Kurdyukov and Bullock 2016). The most common of these is bisulfite sequencing, which converts cytosine to uracil dependent on methylation status, allowing the methylome to be read dependent on specific conversions (Kurdyukov and Bullock 2016).

However, as the epigenome can be highly redundant in terms of modifications to condense and decompact chromatin, it can also be highly effective to assay overall chromatin states to get a general picture of what genomic regions are accessible for expression and cis-regulation. One of the original methods for assaying chromatin is DNase-seq, which utilizes the inherent bias in Dnases for cleaving regions of more open chromatin, and leaving heterochromatic regions uncut (John et al. 2013; Wilken et al. 2015). This yields short DNA fragments from euchromatic regions that can be sequenced to generate regions of interest for analysis (John et al. 2013; Wilken et al. 2015). Given that this technique requires millions of cells, however, it is a non-ideal assay for small populations. This eventually led to the generation of an Assay for Transposase-Accessible Chromatin (ATAC-seq), which employs a similar technique using a Tn5 transposase, but is efficient with a few thousand cells (Buenrostro, Wu, Chang, et al. 2015). This assay allows for isolation of small numbers of cells and assaying of open chromatin regions for analysis of cis-regulatory binding motifs and gene ontology, as well as cross-data comparisons with transcriptomics and other chromatin assay data. Most recently, the need for extremely low cell number assays as well as assays on heterogeneous populations has pushed these methods forward, with the generation of single-cell ATAC sequencing, which present new challenges in data analysis, but far more potential for analyzing mixed cell populations (X. Chen et al. 2018; Buenrostro, Wu, Litzenburger, et al. 2015; Packer and Trapnell 2018).

Glial Response to Injury

Although most regions of the central nervous system and most sensory organs of mammals do not regenerate new neurons or sensory receptor cells, other vertebrates have a range of regenerative abilities in these same structures. Neuronal regeneration is robust in the brain and retina of teleost fish species, and new neurons are regenerated primarily from fate changes of radial and astroglial cells in these regions (Alunni and Bally-Cuif 2016). In fish, damage instigates a glial response which can be beneficial to regeneration through mitotic cell proliferation and initiation of neurogenesis (Alunni and Bally-Cuif 2016). After damage, glia upregulate common neurogenesis genes like Ascl1, Brn2, Myt1l, Sox2, and NeuroD4 (Alunni and Bally-Cuif 2016). Regulatory factors such as Notch and Shh are also expressed in regenerating fish glia, though their roles are more complex (Alunni and Bally-Cuif 2016; Gemberling et al. 2013). By contrast, in mammals, the glial response to injury—called "reactive gliosis"—is quite different, and leads instead to the formation of a "scar" that prevents migration and potentially inhibits neurogenesis and differentiation (Alunni and Bally-Cuif 2016). Why is there such a difference between species in the response to injury? Gene expression differences in glia across species may partly explain the differences in response to injury. For example, radial glia are the primary progenitor for neurons in the brain (Noctor et al. 2001) and gene expression analyses have shown that progenitors and some types of glia, like radial glia in the brain, or Müller glia in the retina, are highly related (Nelson et al. 2011). In regenerative species, a low-level of constitutive expression of neurogenesis-related genes may predispose these animals to regeneration throughout their lives due to continuous growth (Alunni and Bally-Cuif 2016). Indeed, injury to the developing nervous system in mammals can lead to much more robust regenerative responses than what is seen in adults. However, another potential answer to this question may lie in critical differences in the epigenome. In the following sections I will review the evidence that epigenomic differences across species may underlie the limits to regeneration observed in mammalian neural tissue.

Pioneering factors

The generation of induced neurons (iN) from somatic cells in culture mimics some of the changes that occur in in vivo regeneration, and much can be learned from this method of neurogenesis. Many of these studies have been carried out in fibroblasts, where viral over-expression of Brn2, Ascl1, and Myt1l (collectively known as the BAM factors) in fibroblasts induces the cells to generate functional neurons (Vierbuchen et al. 2010; Wapinski et al. 2017). In fish and amphibians, CNS neuronal regeneration occurs via the neuronal glia, which reprogram their fate and act as progenitors in regenerative species. Several groups have shown that mammalian glia can also be reprogramed by infecting mature astrocytes or NG2+ cell with similar transcription factors, including Ascl1, Pax6, Brn2, Neurog2, and Sox2 to generate functional neurons (Gascón et al. 2017). The combination of Neurog2 and Ascl1 has been proven to generate functional neurons not only in cultured astrocytes, but additionally in the endogenous neural progenitor cells (NG2+ cells) of the injured spinal cord (Ohori et al. 2006). Furthermore, downstream targets of these factors, NeuroD4 and Insm1, can independently be used to reprogram astrocytes into mature neurons. Gotz and colleagues have monitored the chromatin state changes during the reprogramming process and find that there is an increase in the repressive histone modification H4K20me3 around the Neurod4 gene, in astrocytes as they mature in culture, and this modification appears to be downstream of binding by the transcriptional co-repressor, REST (Giacomo Masserdotti et al. 2015). Whether this mechanism can be generalized to other neural and neural progenitor genes in astrocytes awaits additional investigation. In general, the addition of one to two transcription factors to glia can be sufficient to reprogram the cells to a neural or neural progenitor state. These factors act as “pioneering” factors, capable of restructuring the epigenome to induce widespread expression changes. The core transcription factor used for neural reprogramming discussed in this dissertation is Ascl1. This transcription factor contains a basic-Helix-Loop-Helix (bHLH) DNA-binding domain, which binds to E-box motifs in the genome (Bertrand, Castro, and Guillemot 2002; Casey et al.

2018). Ascl1 is a pioneering factor, as it restructures chromatin to a more neural-like state and without the addition of other factors, is capable of neurogenic reprogramming in fibroblasts (Dennis, Han, and Schuurmans 2019; Bertrand, Castro, and Guillemot 2002; Iwafuchi-Doi and Zaret 2014). As seen in fibroblast studies with Ascl1, addition of the pioneering factor does in fact cause widespread chromatin accessibility changes within 2–5 days of induced expression (Wapinski et al. 2017). The addition of HDAC inhibitors to a pioneering factor-based iN system can additionally exploit these chromatin changes and have been shown to improve the genesis of new mature neurons. This pioneering activity is one key to understanding fate changes in glial cells as they are induced to regenerate neuronal tissues.

Retinal Development

The retina has proven to be an excellent system for studying neural development, due to the well-characterized cell types and its relative isolation from the rest of the CNS (Brzezinski and Reh 2015). The retina is composed of five basic types of neurons, with many sub-types of each of these, and one intrinsic type of glia, the Müller glia (MG). The generation of each of these neurons from the common progenitor requires a complex combination of regulatory transcription factors, commonly including basic Helix-loop-helix (bHLH), homebox, and forkhead domain-containing transcription factors (Ohsawa and Kageyama 2008). In addition to the neurons and intrinsic glia, there are astrocytes that migrate into the retina from the optic nerve, microglia, and endothelial cells. The different classes of neurons and the Müller glia are all generated during development from a common progenitor, and each class of neuron arises at a specific time during development. Ganglion cells, horizontal cells and cone photoreceptors are all generated early in development, while amacrine cells, rod photoreceptors and bipolar cells are generated somewhat later in the process. The two waves of retinal neurogenesis has led to many studies into the functional difference between early and late progenitors which have demonstrated sequential changes in gene expression and cis-regulation that contribute to differing neurogenic potential (Zibetti et al. 2019; Clark et al. 2019; Brzezinski et al. 2011; La Torre, Georgi, and Reh 2013).

While some transcription factors such as the bHLH factor Ascl1 and the homeobox factor Otx2 are commonly expressed in multiple neuron classes, these cells all retain distinct functions and their development is carefully regulated (Ohsawa and Kageyama 2008; Brzezinski and Reh 2015). In the development of photoreceptors and bipolar cells, Ascl1 and Otx2 are both necessary. Otx2 regulates both the expression of Bipolar genes such as Chx10 and photoreceptor genes (Brzezinski et al. 2013; Kim, Matsuda, and Cepko 2008), though the level of expression of Otx2 varies by cell fate, with higher Otx2 expression found in bipolar cells (Wang et al. 2014). Similarly, the expression of Nuclear Factor I (NFI) transcription factors directs late born cell fates, with the presence of NFI factors inducing MG and Bipolar cell fates (Clark et al. 2019). For development of photoreceptors, a concert of factors are necessary (Brzezinski and Reh 2015), however specific factors, such as Prdm1 are necessary for specifying the photoreceptor cell fate (Brzezinski et al. 2010). Prdm1 in particular acts to restrict cell competence to direct fate determination between photoreceptor and bipolar cells (Brzezinski et al. 2013). This distinct method of specification illustrates the ways in which complex combinations of transcription factors can overlap but direct distinct fates in development.

There have been several studies characterizing the epigenome of developing retina and evaluating the effects of experimentally altering histone modifications on neurogenesis in this system. Dnase mapping of the developing mammalian retina demonstrated that widespread changes in chromatin accessibility correlate with changes in gene expression (Wilken et al. 2015). Early postnatal enhancer accessibility at key neurogenic genes is replaced over the course of one week by accessibility in differentiated neuronal enhancer regions, thus identifying thousands of putative regulatory regions during key stages of mouse retinal development (Wilken et al. 2015). A similar analysis of human development, identified approximately 300k Dnase-accessible regions that correlate with gene expression changes (Hoshino et al. 2017). This accessibility is in part informed by highly dynamic histone modifications (Aldiri et al. 2017). Histone modifications, especially H3K27me3, are highly correlated with gene expression (Aldiri et al. 2017). This is especially true of differentiation genes, which see many changes in the chromatin landscape during development (Aldiri et al. 2017).

In addition to global changes in the retinal epigenome with development, some studies have shown cell-type specific regulation, particularly for photoreceptors: rods and cones. In mice, many chromatin changes occur within the immature photoreceptor cells as they mature. For example, CCCTC-binding factor (CTCF) chromatin looping motifs show large changes with cone maturation, correlating with significant gene expression changes (Daum et al. 2017). The epigenome of rods has a unique chromatin structure. Rather than having multiple heterochromatin foci, rod nuclei contain one extremely dense center with a unique epigenetic composition (Hughes et al. 2017; Mo et al. 2016). DNA methylation is in this case associated with inaccessible and inactive regions (Singh et al. 2017). Cone photoreceptors have more open chromatin than rods and less methylation than rods (Mo et al. 2016); there is evidence that the differential pattern of methylation between these cells may contribute to the unique rod chromatin architecture, since conditional deletion of three DNA methyltrasferases (DNMTs) disrupts the unique rod chromatin and thus leads to dysregulation of phototransduction genes and retinal thinning (Singh et al. 2017).

Functional studies of chromatin modifying enzymes have shown the importance of these modifications to retinal development. For example, Lysine 27 methylation is crucial for proliferation and maintaining progenitor status in development (Fujimura et al. 2018; Ueno et al. 2017; Yan et al. 2016; Popova et al. 2012; Zhang et al. 2015). Conditional deletion of Ezh2—the enzymatic component of Polycomb Repressive Complex 2 (PRC2)—in retinal progenitor cells reduces proliferation and disrupts normal development of retinal lamination and differentiation (Fujimura et al. 2018). RNAseq analysis of knockout retinas shows increases in genes known to be repressed by H3K27me3 in other systems, including targets that account for changes to proliferation (Fujimura et al. 2018; Zhang et al. 2015). Additionally, deletion of the H3K27 demethlase Jmjd3 leads to increases in proliferation and loss of Vsx1+ bipolar cells (Raeisossadati et al. 2018). H3K27me3 associated with bivalency is also well characterized in the mammalian retina (Fujimura et al. 2018; Popova et al. 2012; Bernstein et al. 2006). Bivalent regions tend to have the most changes in expression during development, and K27me3 contributes significantly to amacrine cell gene bivalency (Fujimura et al. 2018; Popova et al. 2012). H3K27me3 has been shown to change inversely in development with active H3K4me2 (Popova et al. 2012). Both modifications are most highly associated in the retina with gene rich areas, and genes that are highly expressed at some point in the generation of the neural retina (recently on or off), while non-retinal genes are less likely to be modified at all (Fujimura et al. 2018; Popova et al. 2012). However, while loss of Ezh2 function leads to substantial changes in photoreceptor development and maturation, this does not significantly affect retinal ganglion cell development (Daum et al. 2017; Cheng et al. 2018; Davis and Rebay 2017), suggesting that all cell types are not equally reliant on this form of regulation. Nevertheless, it is clear that H3K27me3 and its related enzymes are important regulators of retinal development.

In addition to histone methylation, acetylation is also important for retinal development and normal function. HDAC1 in particular plays key roles in late postnatal development and maturation, as it regulates proliferation, and later the transition in photoreceptor development from young immature neurons to mature ones in the first postnatal week (Ferreira et al. 2017). In development, HDAC1 binds to Pax6 and pairs its catalytic activity to Pax6 binding sites and targets such as Ascl1, Atoh7, and NeuroD1 (de Melo et al. 2003). Histone acetylation also binds Brd2 and Setd5 complexes to regulate semaphorins (Villain et al. 2018). The role of acetylation on development becomes a common theme throughout many neuronal systems. Overall, it is clear that many different epigenetic modifications contribute to retinal development, and the combinatorial nature of this system makes it difficult to parse the relative contributions of these modifications to specific developmental processes. Even so, knowledge of the developmental epigenetic landscape provides an important context for related changes that occur after injury.

Retinal Regeneration

Although the adult mammalian retina has little ability to regenerate new neurons after their loss from injury or disease, retinal regeneration has been shown to occur in non-mammalian vertebrates (Lamba, Karl, and Reh 2008). Amphibians, particularly urodeles, have the capacity to completely regenerate an entirely new retina from the retinal pigmented epithelium (RPE). If the retina is removed (or even separated from the adjacent RPE cells, the cells lose their pigmentation, reduce their expression of genes characteristic of this cell fate, and express genes of embryonic retinal progenitor cells (Lamba, Karl, and Reh 2008). The RPE-derived retinal progenitors undergo multiple rounds of cell division and produce a new, laminated retina that can restore functional vision to the animals (Lamba, Karl, and Reh 2008). This remarkable feat is analogous to the capacity of these animals to regenerate other parts of their bodies, like their limbs, and it is very likely that the dramatic changes in gene expression and phenotypic plasticity require equally dramatic changes in the epigenome.

Teleost fish are also capable of regenerating all types of retinal neurons after injury or disease, though in this case, the source of the new neurons is not the RPE, but rather the radial-glial like Müller glia (Gemberling et al. 2013; Fausett and Goldman 2006). As noted above, the

Müller glial cell is the only glial cell generated during development by the retinal progenitor, and has a gene expression profile that is in many ways quite similar to that of the embryonic progenitor cell (Nelson et al. 2011; Blackshaw et al. 2004). A key difference between the embryonic progenitor cell and the Müller glia is the proneural transcription factor, Ascl1. This gene is expressed by retinal progenitors, but not by mature Müller glia. After injury, the Müller glia of fish express Ascl1 and this gene is necessary for the Müller glia to produce the progenitor-like cells needed for regenerating neurons. In addition to Ascl1, critical changes occur in Stat3 signaling, multiple growth factors and receptors, canonical Wnt signaling, and microRNAs, and these are all necessary for successful regeneration (Gemberling et al. 2013; Goldman 2014). Recent evidence also points towards a role for the Yap pathway in inducing MG cell-cycle re-entry after damage in both mouse and Xenopus (Hamon et al. 2017, 2019). After a variety of damage assaults, the Müller glia will utilize these expression changes to dedifferentiate to a neural progenitor-like state, migrate to the site of injury and repopulate the damaged neural tissue. This regeneration is efficient and cell-type specific to regenerate only the damaged areas of the fish retina.

The changes in cell signaling and transcription factors leading to regeneration of new neurons in this system is accompanied by critical changes in the epigenome. DNA methylation changes in particular have received the most detailed analysis (C Powell, Elsaeidi, and Goldman 2012; Curtis Powell et al. 2013; C Powell, Cornblath, and Goldman 2014). DNA methylation regulators are expressed at low levels in quiescent MG, but after injury they are upregulated within 4 days (C Powell, Elsaeidi, and Goldman 2012). These include genes important for demethylation of existing CpGs, e.g. Gadd45, Apobec2a/b, and Tet, as well as four Dnmts. While the vast majority of DNA methylation sites in MG are not changed after injury (~98%), the small percentage of sites that do change are potentially informative. Among the DMBs (differentially methylated bases), approximately equal numbers show increased and decreased methylation (Curtis Powell et al. 2013). Demethylated promoter-associated DMBs correlate with increased gene expression. Interestingly, the promoters of pluripotency factors and regeneration-associated genes have low levels of methylation at their promoters in MG from uninjured retinas (Curtis Powell et al. 2013). Thus, the expression of stem cell and progenitor genes appears not to be repressed via this mechanism. Nevertheless, functional studies indicate that changes in DNA methylation that occur after injury are important for the regeneration process in the fish, since knockdown of Apobec2a/ b inhibits regeneration while treatment with 5-dAza increases tuba1a:gfp expression, an indicator of MG reprogramming in response to injury (C Powell, Elsaeidi, and Goldman 2012; C Powell, Cornblath, and Goldman 2014). During this period of regeneration in the fish retina, regeneration associated genes are additionally regulated by HDACs (Mitra et al. 2018). HDACs are essential for regulating the regeneration process, and small molecule inhibition of their activity in the fish model reversibly blocks regeneration after damage (Mitra et al. 2018). Apart from these studies, there is little known about epigenetic changes in fish Müller glia during the regeneration process.

Changes in methylation in mammalian Müller glia have also been studied, though to a lesser extent than the fish. Similar to the zebrafish, the pluripotency factors' promoters are hypomethylated in I glia from uninjured fish retina. In the immediate response period 4–12 hours after retinal damage, the transcription factor Oct4 loses nearby DNA methylation in mouse Müller glia, but it is quickly re-silenced after 24 h (Reyes-Aguirre and Lamas 2016). The loss of methylation in mammalian I glia after injury is interesting, though it is not clear how widespread this phenomenon is or if it occurs at genes critical for regeneration. Future studies on DNA methylation in mammalian Müller glia could potentially shed light on this issue, given the importance of DNA methylation in developing retina.

Although fish have the capacity to regenerate neurons from MG, mammals lack this ability. Injury to the mammalian retina typically leads to MG hypertrophy and reactivity (Bringmann et al. 2009). Some effort has been made to stimulate the MG to generate new neurons, using growth factors and activating signaling pathways. Although some of these treatments lead to a re-entry of some MG into the mitotic cell cycle, evidence for regeneration of new neurons has been scant. Several years ago, Pollak et al. demonstrated that mouse MG could be maintained in dissociated cell culture, and they used this system to test the ability of proneural transcription factors to reprogram the MG into retinal progenitors (Pollak et al. 2013). Many progenitor genes were reactivated in the MG with Ascl1 over-expression, and a subset of the Ascl1-expressing MG generated new neurons in the cultures (Pollak et al. 2013).These cultured cells were shown to have a uniquely neuronal morphology, and were electrically active. Analysis with ChIP demonstrated that Ascl1 induced a reduction in the repressive H3K27me3 modification and an increase in H3K27ac at key progenitor and neural genes establishing that MG restructure their chromatin state to better match that of progenitors and young neurons, facilitating effective changes in fate (Pollak et al. 2013). These in vitro studies were later followed up in vivo; using transgenic mice to specifically direct Ascl1 expression to MG. Ueki et al found MG from two week old mice could not be reprogrammed to regenerate neurons in vivo with Ascl1 expression alone, as was seen in culture (Ueki et al. 2015). However, the addition of a damage model—NMDA excitotoxic damage, killing the ganglion cells—successfully induces neurogenesis, and the newly generated neurons had morphology resembling primarily bipolar cells and amacrine cells (Ueki et al. 2015). This capacity was lost as the animals matured, and the combination of Ascl1 and damage failed to generate new neurons of any variety in adult murine retinas. It was later discovered that intravitreal injections of the histone deacetylase inhibitor trichostatin-A (TSA) in a mouse treated with Ascl1 and NMDA, could stimulate further neurogenesis, and again produce a small population of primarily bipolar interneurons (Jorstad et al. 2017). This neurogenesis could be bolstered by inhibition of the STAT pathway (Jorstad et al. 2019). It is this trifecta of factors (Ascl1, NMDA, TSA) inducing neurogenesis that motivated my study into the epigenomic landscape of the adult MG and derived neurons, to be further discussed in Chapter 2. These studies combined demonstrate the feasibility of using *in vivo* reprogramming factors, in conjunction with epigenetic modification, to drive regeneration in a non-regenerating species.

In this particular body of work, I have pursued investigations into the broader chromatin landscape of the developing mammalian retina. Examinations of the overall chromatin landscape from neurogenic progenitors to adult MG were anticipated to reveal areas in which genome accessibility was lost with neurogenic potential, and to reveal regulatory regions that may be relevant for MG maturation from progenitors. Further, analysis into the epigenomic state of MG-derived neurons in the adult retina were hypothesized to demonstrate increases in neurogenic genome accessibility, and to identify regions opened in the case of successful regeneration and potentially elucidate a mechanism of successful neurogenesis from mature MG. Finally, small molecule inhibitors for epigenetic modifiers were employed to modulate the epigenome in combination with glial-specific Ascl1 expression in an attempt to alter neurogenesis and cell fate decisions in murine regeneration. Overall, this dissertation demonstrates the ways in which the murine MG epigenome is restructured through development and regeneration to generate a specific set of retinal neurons under our model of murine regeneration.

Chapter 2

Epigenomic Accessibility in the Regenerated Adult Retina

Figures modified from Jorstad et al. 2017

Introduction

Efforts within the Reh lab thus far have made major advancements in the production of new retinal neurons in mice. Using the principal of pioneering neurogenic transcription factors, mammalian Müller Glia (MG) have been shown to be capable of neuronal transdifferentiation (Pollak et al. 2013; Ueki et al. 2015; Jorstad et al. 2017, 2019). The factor Ascl1 has been commonly used in culture of somatic cells such as fibroblasts to generate induced Neurons (iNs) (Vierbuchen et al. 2010; Wapinski et al. 2017). Ascl1 has been used both on its own, and with a combination of other factors such as Brn2 and Myt1l (BAM factors) to successfully restructure the epigenome and transcriptome of cells to that of a more neuronal state (G Masserdotti et al. 2015; Wapinski et al. 2017). Similarly, former members of the Reh lab have shown that Ascl1 alone can produce retinal neurons in MG cultures, but in vivo reprogramming of adult tissues presents more of a challenge (Pollak et al. 2013; Ueki et al. 2015).

A previous graduate student in the lab discovered that the triple factor combination of Ascl1, NMDA damage, and Trichostatin-A histone deacetylase inhibition (ANT) allowed for adult neurogenesis *in vivo*. The study, published in Nature letters 2017, detailed this neurogenesis (Jorstad et al. 2017). In this regenerative paradigm, adult mice of the genotype Glast-CreER;Rosa-flox-stop-tTA;tetO-mAscl1-ires-GFP to induce expression of Ascl1 with a fluorescent reporter specifically in the MG as directed through the Glast promoter. Ascl1 expression in adult mice was induced through a series of five intraperitoneal injections of tamoxifen. On day 8, three days after the last tamoxifen injection, NMDA (N-methyl-D-aspartate) was injected intravitreally to induce excitotoxic damage to the retinal ganglion cells (Shen, Liu, and Yang 2006; Lebrun-Julien et al. 2009). At day 10, the histone deacetylase (HDAC) inhibitor Trichostatin-A (TSA) was injected intravitreally to open chromatin broadly in the retina. After the treatment is complete, retinas were collected 2-5 weeks after treatment for analysis (Figure 2.0A). This treatment yields transdifferentiation from MG to predominantly bipolar neurons in the inner nuclear layer (INL) (Figure 2.0 B,B’). MG-derived neurons in adult murine retinas are primarily bipolar or amacrine-like in morphology and gene expression and represent approximately 20% of the GFP+ Ascl1-overexpressing cells (Figure 2.0 C). These new retinal neurons have neuronal-like elecrophysiological responses and integrate into the retinal circuitry effectively (Figure 2.0 D-F’’).

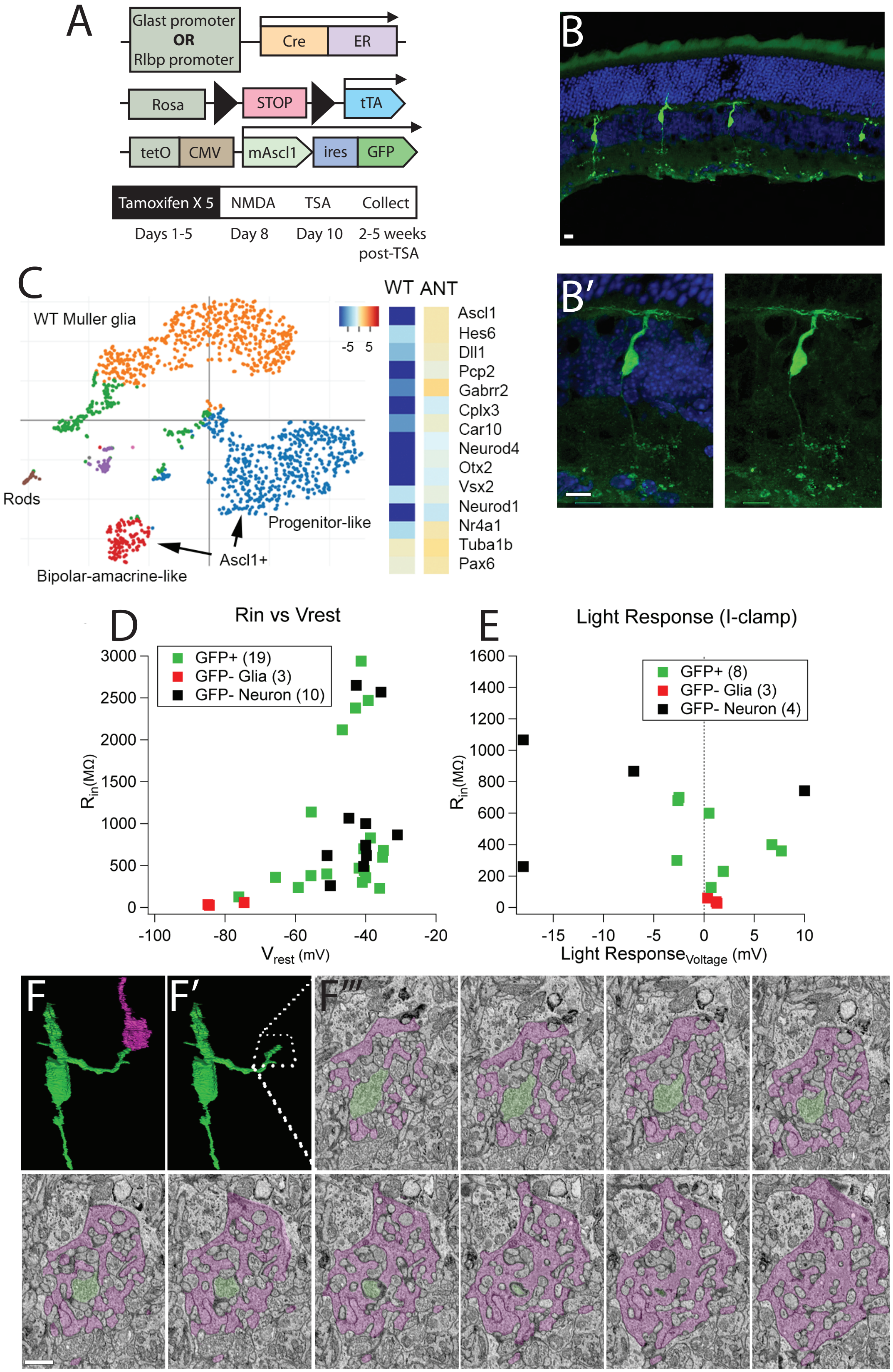
Based on these results, I sought to explore the epigenomic changes to the cells with combinations of these factors and potentially reveal a mechanism to effective neurogenesis. I hypothesized that partially-treated cells (Ascl1 alone or with only one of two additional factors) would be lacking in neurogenic-related accessibility and binding motifs. To answer this question, I performed ATAC-seq on fluorescence-activated cell sorting (FACS) purified control and treatment cells, as well as on bipolar cells, and H3K27ac ChIP-qPCR. Through analyses of these datasets and others, I found that partial treatments did increase neurogenic gene accessibility, but that the full ANT treatment had the most neurogenic-related accessibility. This accessibility profile also overlapped considerably with a bipolar cell accessibility profile, confirming the new cell fate identity.

Results

Partial treatments demonstrate partial neuronal accessibility

In order to first compare the epigenomes of treatments, I performed ATAC-seq on FACS purified MG and MG-derived neurons. I utilized wild-type adult MG (Rlbp-CreER;flox-stop-TdTomato) as well as four treatment groups (Glast-CreER;Rosa-flox-stop-tTA;tetO-mAscl1-ires-GFP): Ascl1 alone, Ascl1 + NMDA, Ascl1 + TSA, and the full ANT treatment. Two to four weeks after these treatments, or two days after TdTomato induction in WT, I collected the retinas, dissociated, and FACS purified the fluorescent cells for ATAC sequencing. Reads from ATAC sequencing were aligned with Bowtie2, and peaks were called with Homer findPeaks (Heinz et al. 2010; Langmead and Salzberg 2012). As each treatment group had differing depth of sequencing, I assumed each group should have fairly similar numbers of accessible regions, and used Samtools View (Li et al. 2009) to downsample each sample to the level of the lowest depth of sequencing such that the number of peaks for each group was approximately 40k.

When observing overall accessibility in each of these samples, I find a number of key differences between treatment groups. The wild type adult MG have high accessibility around anticipated glial genes such as Rlbp1, as well as retained accessibility around some progenitor-related genes, but there is little apparent neuronal-related accessibility as anticipated (Figure 2.1 A). In partial treatment groups—including Ascl1 alone—which do not form neuronal cells, glial accessibility is strikingly absent, though progenitor-like accessibility is retained. In two-factor treatment groups, some accessibility around neuronal genes is seen, despite the lack of neuronal


A
Glast promoter
OR
Rlbp promoter
Cre
ER
Rosa
STOP
tTA
tetO
CMV
mAscl1
ires
GFP
Tamoxifen X 5
NMDA
TSA
Collect
Days 1-5
Day 8
Day 10
2-5 weeks post-TSA
B
B′
C
WT Muller glia
Rods
Progenitor-like
Ascl1+
Bipolar-amacrine-like
WT ANT
-5 5
Ascl1
Hes6
Dll1
Pcp2
Gabrr2
Cplx3
Car10
Neurod4
Otx2
Vsx2
Neurod1
Nr4a1
Tuba1b
Pax6
D
Rin vs Vrest
GFP+ (19)
GFP- Glia (3)
GFP- Neuron (10)
3000
2500
2000
1500
1000
500
0
$R_{in}(M\Omega)$
-100
-80
-60
-40
-20
V_{rest} (mV)
E
Light Response (I-clamp)
GFP+ (8)
GFP- Glia (3)
GFP- Neuron (4)
1600
1400
1200
1000
800
600
400
200
0
$R_{in}(M\Omega)$
-15
-10
-5
0
5
10
Light Response$_{Voltage}$ (mV)
F
F′
F′′′

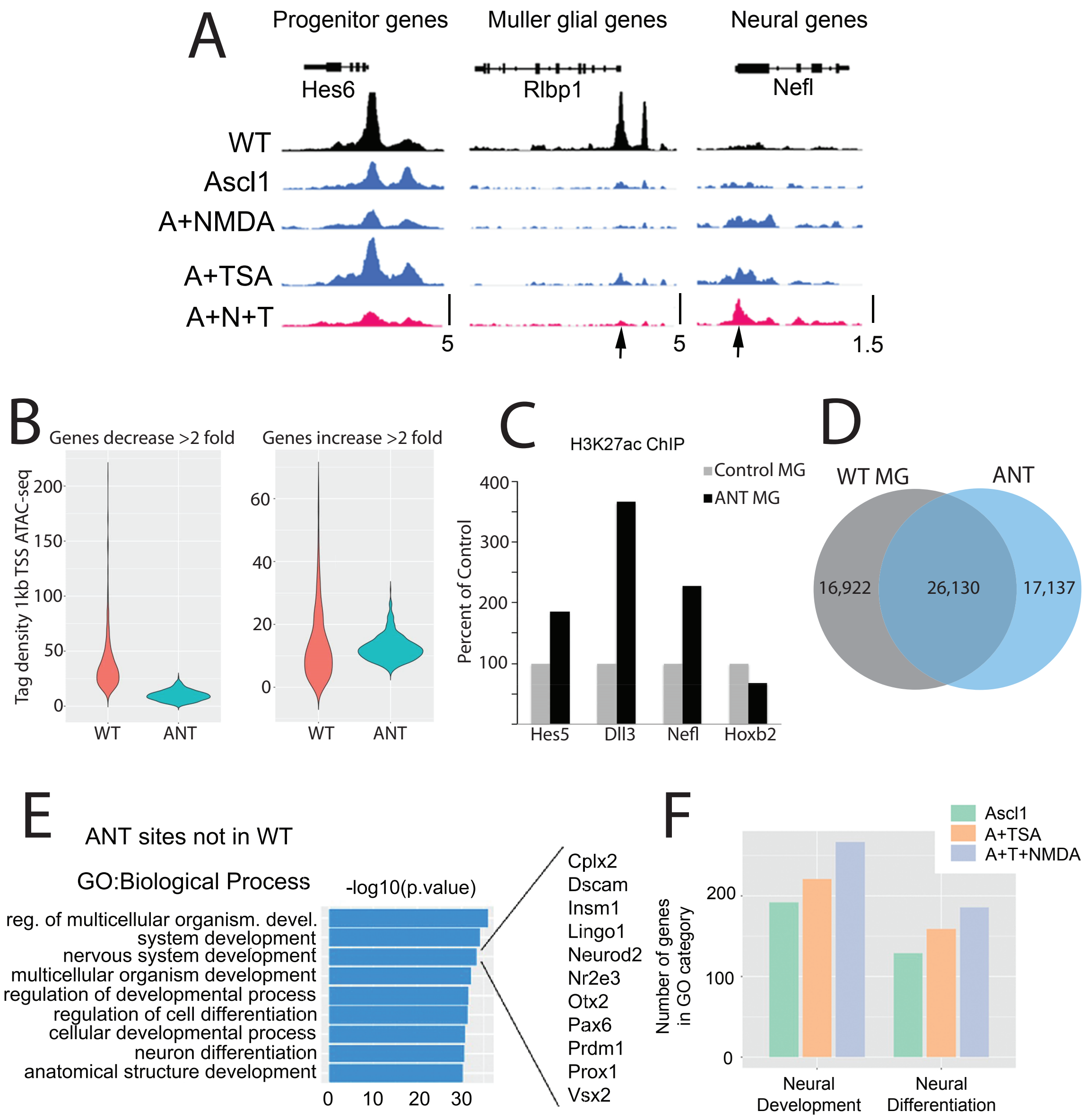
morphologies (Figure 2.1 A). For the full ANT treatment, neuronal-related accessibility was most pronounced in tracks, but also appears to have reduced progenitor-related accessibility, indicating a potential maturation of produced neuronal cell types (Figure 2.1 A). Reprogramming paradigms do appear to alter the chromatin profile, even with partial treatments that do not produce neurons.

ANT treated MG-derived neurons have increasingly neuronal chromatin

To compare the outright changes from WT MG and ANT populations with induced neurogenesis, I specifically compared the accessible domains and gene annotations of each dataset to find total changes. Using RNA-seq data from previous study of the ANT treatment, I compared transcription start site (TSS) tag density in both treatment groups to genes increasing and decreasing expression between MG and ANT. This analysis showed that glial-specific expressed genes lose TSS accessibility, while genes that increase expression with ANT demonstrate modest increases in ATAC tag density (Figure 2.1 B). Similarly, ChIP-PCR of H3K27ac around progenitor-like and neuronal gene promoters demonstrates increases in histone acetylation, indicative of increased progenitor and neuronal profiles as compared to WT MG (Figure 2.1 C). Observations of general chromatin accessibility establish a progression of accessibility from a glial, non-neuronal chromatin profile to increasingly neuronal gene-associated accessibility.

When comparing accessible domains between the two treatment groups by BEDOPs (Neph et al. 2012), I found 26,130 common peaks between WT MG and ANT treated cells. This leaves 17,137 regions which are unique to the ANT treated MG (Figure 2.1 D). Genome-wide analysis of these regions show Gene Ontology categories (enriched by Genomic Regions

Figure 2.0 Ascl1 + NMDA+ TSA Treatment in Adult Retinas Generates New Neurons
A. Mouse strains and experimental paradigm for overexpression of Ascl1 in MG with additional drug treatments. B Morphology of Ascl1 overexpressing MG treated with NMDA and TSA (ANT) with B'. higher magnification of bipolar morphology cell. Scale bars 10um. C. t-SNE plot of clustering from single cell RNA-seq of wild-type (WT) MG and ANT-treated cells. Heat map at right shows average expression of neural genes for all Ascl1+ cells compared to WT MG.D. Input resistance and resting membrane potential data from individual recordings on GFP- MG, MG-derived GFP+ cells, and GFP- neurons. E. Data for light responses recorded by current-clamp on cells as in (D). F-F''. Three dimensional reconstruction of ANT-derived cell by serial block face scanning electron microscopy (SBFSEM) demonstrating F. GFP+ cell in green penetrating cone terminal (magenta), and isolated GFP+ cell in F'. F''. SBFSEM image series of GFP+ cell process within a cone terminal. Scale bar 1um.



2.1 ANT Treatment Opens Chromatin Around Neural Development Genes

A. Paradigm for Ascl1 overexpression and Ascl1, NMDA, TSA (ANT) treatment timeline. B. ATAC-seq tracks normalized to reads per million for WT MG, and partial and full ANT treatment paradigms at genes for progenitors (Hes6), MG (Rlbp1), and Neurons (Nefl). Regions of altered accessibility are indicated with arrows. Scale in reads per million dictated at the bottom for each region. C. Violin plots of read density in regions 1kb upstream of genes that change in expression by twofold down (left) or up (right) for WT MG and ANT treatment. P Values for read density changes are 2.2e-16 for reduced expression, and 5.7e-05 for increase expression. D. qPCR quantification of H3K27ac ChIP on WT MG and ANT treatment. Data represented as percent of WT MG for neural genes which increase in expression, and control gene (Hoxb2) that does not change. E. Overlaps of ATAC-seq peaks by BEDOPS between WT MG and ANT treated cells. F. Gene Ontology (GO) enrichment by

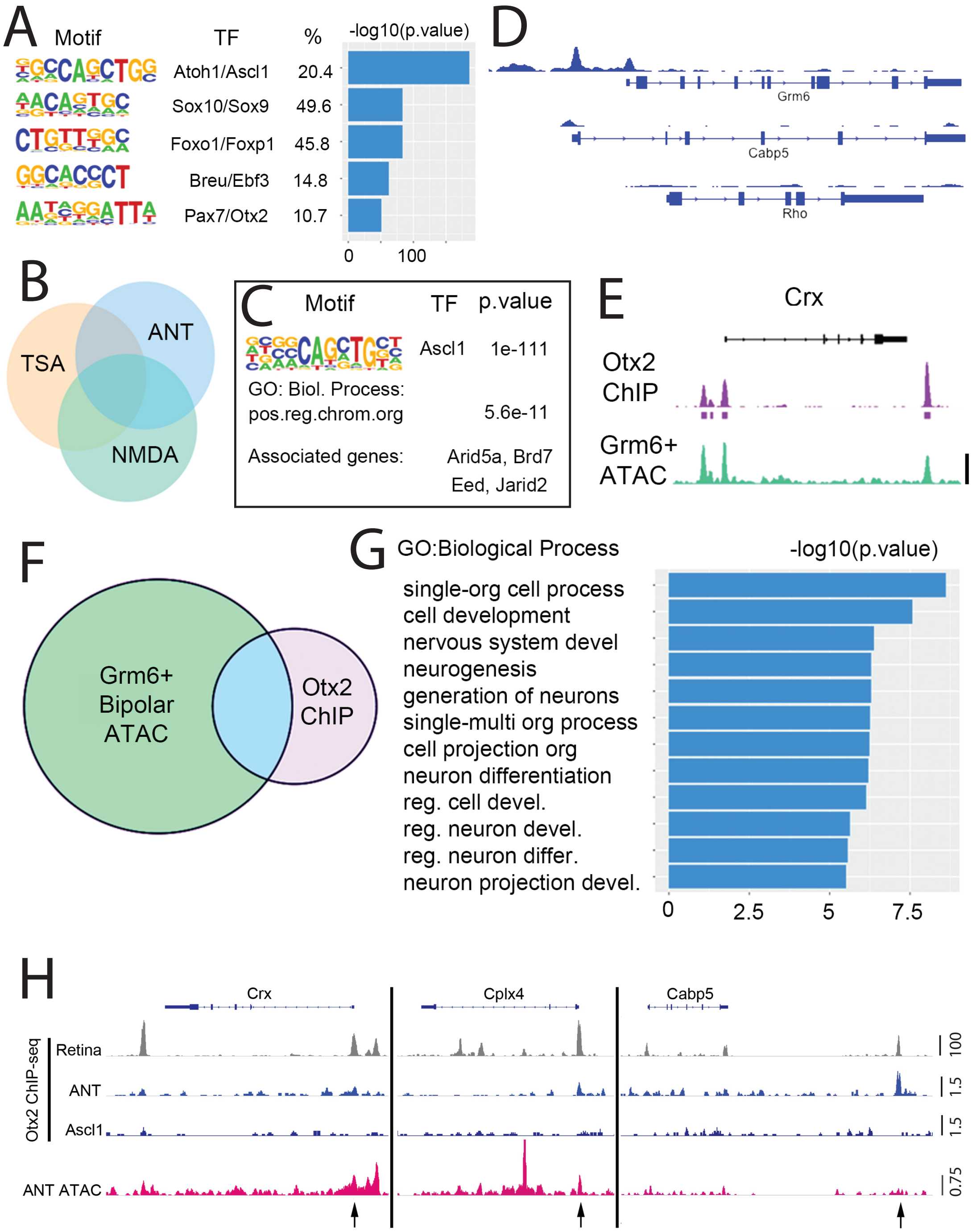
Enrichment of Annotations Tool [GREAT]) specific to organism and nervous system development(McLean et al. 2010). These genomic annotations include common retinal development genes such as Otx2, Vsx2, and Pax6 (Figure 2.1 E). Comparing the genes in Nervous System Development and Differentiation categories, I found that there is accessibility present in the partial treatment groups for neuronal GO categories as seen in the tracks (Figure 2.1 F). However, as the number of factors increased from Ascl1 alone to +TSA and to the full ANT treatment, the number of genes annotated in each category increases, potentially indicating a neuronal gene accessibility threshold necessary for appropriate neurogenesis (Figure 2.1 F). This analysis between WT and full ANT treatment shows that accessibility by ATAC reflects the induction of neurogenesis, and identifies neural development genes as a major category that changes in the process of generating new retinal neurons.

ANT treated MG-derived neurons resemble bipolar cells via Otx2 activity

Given that accessibility reflects neurogenesis, I wanted to explore potential regulators as demonstrated by binding motif accessibility. Again using the regions found in the ANT treatment and not WT MG, I used Homer's findMotifsGenome algorithm to identify binding motifs enriched in ANT treated cells. Among these regions, the top scoring motif was an E-box commonly associated with Ascl1 binding, indicating that the primary method of neurogenesis seen here is based on the overexpression of Ascl1 (Figure 2.2 A). The following top motifs are consistent with binding for Sox9, Foxo1, Ebf3, and Otx2 (Figure 2.2 A). I also compared the full ANT treatment with the two-factor partial treatments to find accessible domains unique to the full neurogenic treatment, which consists of 12,323 peaks (Figure 2.2 B). These regions were best associated with genes in chromatin remodeling GO categories such as Eed, Jarid2, and Brd7 (Figure 2.2C). Again for these regions, the top enriched motif is an E-box domain associated with Ascl1 binding (Figure 2.2 C). These results suggest that the combination of Ascl1 with retinal damage and TSA-induced HDAC inhibition may promote neurogenesis through the increased expression of chromatin remodelers and through the secondary action of downstream transcription factors such as Ascl1.

2.1 Continued.

GREAT for peaks that gain accessibility from WT MG to ANT treated cells. Examples of Nervous System Development genes shown on the right. G. The number of accessible genes in neural-related GO categories for cells from Acl1, Ascl1+ TSA, and ANT treatments.



2.2 MG-Derived Neurons have Bipolar-like Chromatin

A. Top predicted transcription factor motifs in peaks found in ANT treated cells and not WT MG, listing associated transcription factor, percent of peaks with the represented motif, and -log10 P value. B. Overlaps by BEDOPS of peaks from cells treated with Ascl1 + TSA, Ascl1 + NMDA, and full ANT treatment. C. Top GO categories and predicted motif and associated genes and transcription factor. D. ATAC-seq tracks for Grm6+ Bipolar cells at Bipolar genes (Grm6, Cabp5) and a control Rod gene (Rho). Track heights set to 6 reads per million. E. Otx2 ChIP-seq peaks from whole retina (Lamonerie) compared to Grm6+ Bipolar cell accessibility

Because Otx2 is a known transcription factor important for the development of both bipolar neurons and photoreceptors and was represented as a top binding motif in ANT-specific accessible domains, I further pursued its role in neurogenic regulation. To determine if this accessibility matches that of mature bipolar cells, I FACS purified bipolar cells from adult Grm6-TdTomato mice, and subjected them to ATAC-seq. I downsampled the reads from this ATAC sample using Samtools view to get a resulting 42,772 peaks using Homer findPeaks. This ATAC sample demonstrates apparent accessibility in known bipolar genes, and little to no accessibility around rod-related genes, confirming its purity (Figure 2.2 D). When the bipolar accessible domains are overlapped with the ANT ATAC data, I find 17,781 peaks common between the two datasets, indicating that the chromatin landscape of the MG derived neurons is highly similar to that of bipolar neurons. I additionally compared the bipolar ATAC data with an Otx2 ChIP-seq from whole adult retinas published by Samuel et al. on whole adult retinas, finding bipolar accessibility at known Otx2 binding sites (Figure 2.2 E). In the Grm6 bipolar cell ATAC data, there are 6,674 Otx2-binding peaks, 4,753 of which are common to the ANT chromatin landscape (Figure 2.2 F). For these bipolar and MG-derived neuron shared Otx2 binding sites, the top Biological Process GO categories of associated genes include neural development and differentiation (Figure 2.2 F). These neuronal enrichments are not seen in the WT MG, indicating that Otx2 transcription factor activity likely helps to drive the bipolar cell fate seen in ANT treated MG derived neurons. Otx2 ChIP on FACS purified ANT treated cells performed by Matt Wilken confirms that the ANT treatment allows Otx2 to specifically bind to neuronal cis-regulatory regions (Figure 2.2 G). This in part explains the mechanism of neurogenesis with the full ANT treatment in adult MG.

Figure 2.2 Continued

by ATAC. Scale for track height is 100 reads per million for Otx2 ChIP, 10 reads per million for Grm6+ ATAC. F. Overlaps by BEDOPS of peaks from ATAC-seq for Grm6+ bipolar cells and Otx2 ChIP-seq on whole retina (Lamonerie). G. Gene Ontology enrichment by GREAT for peaks overlapping between ANT treated cells and Bipolar cell Otx2 binding sites, ranked by -log10 P value. H. Tracks for Otx2 ChIP seq on whole retina (Lamonerie), ANT treated cells, and Ascl1 only treated cells, as well as ANT ATAC-seq at Bipolar cell genes (Crx, Cplx4, and Cabp5). Scale for tracks shown to the right in reads per million. Arrows mark regions of open chromatin that are bound by Otx2.

Conclusions

While zebrafish and posthatch chicks readily regenerate retinal neurons, this regeneration response is strikingly absent in mammals (Lamba, Karl, and Reh 2008; VandenBosch and Reh 2019). In regenerative species, damage stimulates a rapid upregulation of Ascl1 expression in the MG that is necessary to de-differentiate MG to a progenitor-like state and to generate new neurons (Fausett, Gumerson, and Goldman 2008; Gemberling et al. 2013). In the mouse, damage does not stimulate any expression of Ascl1 and instead instigates a gliosis response to form a scar (Alunni and Bally-Cuif 2016; Bringmann et al. 2009). The overexpression of Ascl1 in murine MG and cultured somatic cells has proven to be capable of generating neurons, but *in vivo* studies in the adult retina prove that Ascl1 alone is insufficient (Wapinski et al. 2013; Pollak et al. 2013; Ueki et al. 2015). The addition of NMDA damage and the histone deacetylase inhibitor TSA together with Ascl1 overexpression induce transdifferentiation to a bipolar cell fate in 20-30% of cells (Jorstad et al. 2017). The necessity for additional factors in Ascl1-based reprogramming as well as the production of a single neuronal class indicates a number of restrictions to mammalian regeneration, potentially epigenetic in nature.

This analysis of adult retinal regeneration profiled the chromatin of MG-derived cell populations with a variety of treatments in order to uncover the mechanism of reprogramming under our paradigm. I utilized FACS purification of MG and regeneration factor-treated cells for bulk ATAC-sequencing. Analysis of the full ANT treatment as well as partial treatments compared to WT MG demonstrates changes to the chromatin landscape. While Ascl1 alone or with a single additional factor does not produce neurons in adult retinas, these partial treatments demonstrate losses in glial-related accessibility, and gains in neurogenic gene accessibility. Two-factor treated cells additionally have more neurogenic gene accessibility than Ascl1 alone, showing that added reprogramming factors help to restructure the chromatin landscape towards neurogenesis, but in the case of adult regeneration, more than one factor is necessary.

When comparing the neurogenic ANT treatment to WT MG, it is clear that the ANT treated cells have a neuronal chromatin profile, thus matching gene expression profiles outlined by Jorstad et al. 2017. ANT-specific accessible domains are associated with genes in neurogenic gene ontology categories, and are enriched for neurogenic binding motifs. The primary enriched motif is an E-box consistent with Ascl1 binding, which is consistent with our mode of reprogramming. However, additional enriched motifs such as Foxo1 and Otx2 are indicative of transcriptional activity downstream of Ascl1.

I performed additional analysis on retinal bipolar cells to compare to the bipolar-like cells produced by ANT treatment. Bipolar ATAC-seq overlaps considerably with those from ANT treated cells, including at known Otx2 binding sites around neurogenic genes. My analysis demonstrates the consistency of ANT-treated MG-derived neurons with a bipolar cell profile. In particular, it appears that Ascl1 overexpression drives Otx2 downstream to produce a bipolar cell fate, though both of these factors are important in the development of not only bipolar cells but also photoreceptors. This mechanism demonstrates a bias in Ascl1 activity in the MG towards a bipolar fate that will be addressed in the following chapters.

Though this analysis does characterize neurogenic profiles and epigenetic mechanisms for reprogramming, bulk ATAC-seq has its limitations. In particular, the ANT treatment generates a mixed population of cell fates. Only recently, methods of single cell ATAC-sequencing have emerged, which also generate new hurdles in data analysis to be resolved as this methodology becomes more commonplace. Moving forward, in order to reveal more nuanced epigenomic changes in regeneration and neurogenesis, it may become necessary to perform single cell ATAC-seq analyses to isolate individual populations.

So what questions does this analysis open up for future work? While this analysis of adult regeneration demonstrates a lot of changes in chromatin structure once neurogenesis is complete, there are intermediate chromatin states not captured by the timepoint I have used. By capturing cells for RNA- and ATAC-seq within the first few days after reprogramming factors, one might potentially answer more mechanistic questions. For instance, by analyzing transcriptomics and chromatin immediately after damage, one could start to uncover the reasons why damage to the mammalian retina allows for increased regenerative responses, and discover what changes occur in the MG. Secondly, it is known that TSA opens chromatin broadly, but in this reprogramming paradigm, it is unclear the ways in which TSA is opening chromatin to allow regeneration. By assaying chromatin accessibility within 24 hours of TSA injections, as well as timepoints immediately following, one could follow the mechanism of how TSA changes the overall chromatin structure. This, paired with Ascl1 ChIP-seq would allow for the mechanism of Ascl1 pioneering and transcriptional activity to be elucidated.

In sum, my analysis of accessible chromatin in MG derived neurons details the mechanism of adult murine regeneration. As the number of necessary reprogramming factors are added, there are sequential increases in accessible chromatin around neuronal genes needed for neurogenesis. This reprogramming is primarily driven by Ascl1 binding activity around said neurogenic genes, but is aided by downstream factors such as Otx2. Otx2 preferentially acts on chromatin regions consistent with bipolar cell accessibility profiles to drive fate decisions towards a bipolar fate, as confirmed by analysis in Jorstad et al. 2017. Future work on adult regeneration can follow up on transition cell states with scATAC-sew, as well as on early timepoints after damage and TSA. In sum, with the current data I can conclude that increases in accessibility around neurogenic genes and around Ascl1 and related downstream factor binding motifs correlates with regeneration responses.

Chapter 3

Cis-Regulatory Accessibility Directs Müller Glial Development

Text and figures modified from VandenBosch et al. 2019

Introduction

Neuronal loss caused by disease and damage to the mammalian retina can lead to permanent vision loss. As discussed in Chapter 2, while some species are readily capable of regenerating lost neurons, mammalian retinas are not regenerative. In the mammalian retina, loss of neurons caused by direct damage to the retina leads to reactive gliosis of the Müller glia (MG), similar to that of astrocytes in the brain (Bringmann et al. 2009).

Teleost fish, by contrast, are capable of regenerating retinal neurons, including photoreceptors and ganglion cells, after damage. This regeneration is carried out by the MG, which respond to damage by generating progenitor-like cells, similar to those in the developing retina (Goldman 2014; Gemberling et al. 2013). This regeneration is accompanied by waves of gene expression and morphological changes to the MG, regulated by epigenomic changes directing regeneration (for review see VandenBosch and Reh 2019). The murine retina also undergoes epigenomic changes after damage, but neurogenic programs are not re-expressed, and neuronal regeneration does not occur (VandenBosch and Reh 2019).

A critical difference between the fish MG and the mammalian MG in their response to damage is their expression of the proneural transcription factor Ascl1. In fish, Ascl1 is quickly upregulated after damage, and is necessary for regeneration of new neurons (Ramachandran, Fausett, and Goldman 2010; Fausett, Gumerson, and Goldman 2008). In the murine retina, Ascl1 is expressed in retinal progenitors and necessary for development of rods and bipolar cells (Ohsawa and Kageyama 2008); however it is not expressed in mature MG and after damage or in disease models, mouse MG do not spontaneously upregulate Ascl1 (Bringmann et al. 2009; Ueki et al. 2015). We recently directed Ascl1 expression to mouse MG using a tetO transgenic approach to test whether Ascl1 expression is sufficient to induce regeneration. Expression of Ascl1 in the MG of mice over the age of 12 days post-natal (P) stimulated MG to generate new bipolar neurons after NMDA damage (Ueki et al. 2015). In adult mice, however, Ascl1 over-expression in the MG is no longer sufficient to induce neurogenic potential, even in the presence of damage (Jorstad et al. 2017). In mature mice, the addition of the histone deacetylase trichostatin-A (TSA), in combination with Ascl1 overexpression and NMDA damage is required for neurogenesis; up to 30% of the Ascl1-expressing MG produce functional bipolar- and amacrine-like interneurons, confirmed by single cell transcriptomics, electrophysiology, and electron microscopy (Jorstad et al. 2017).

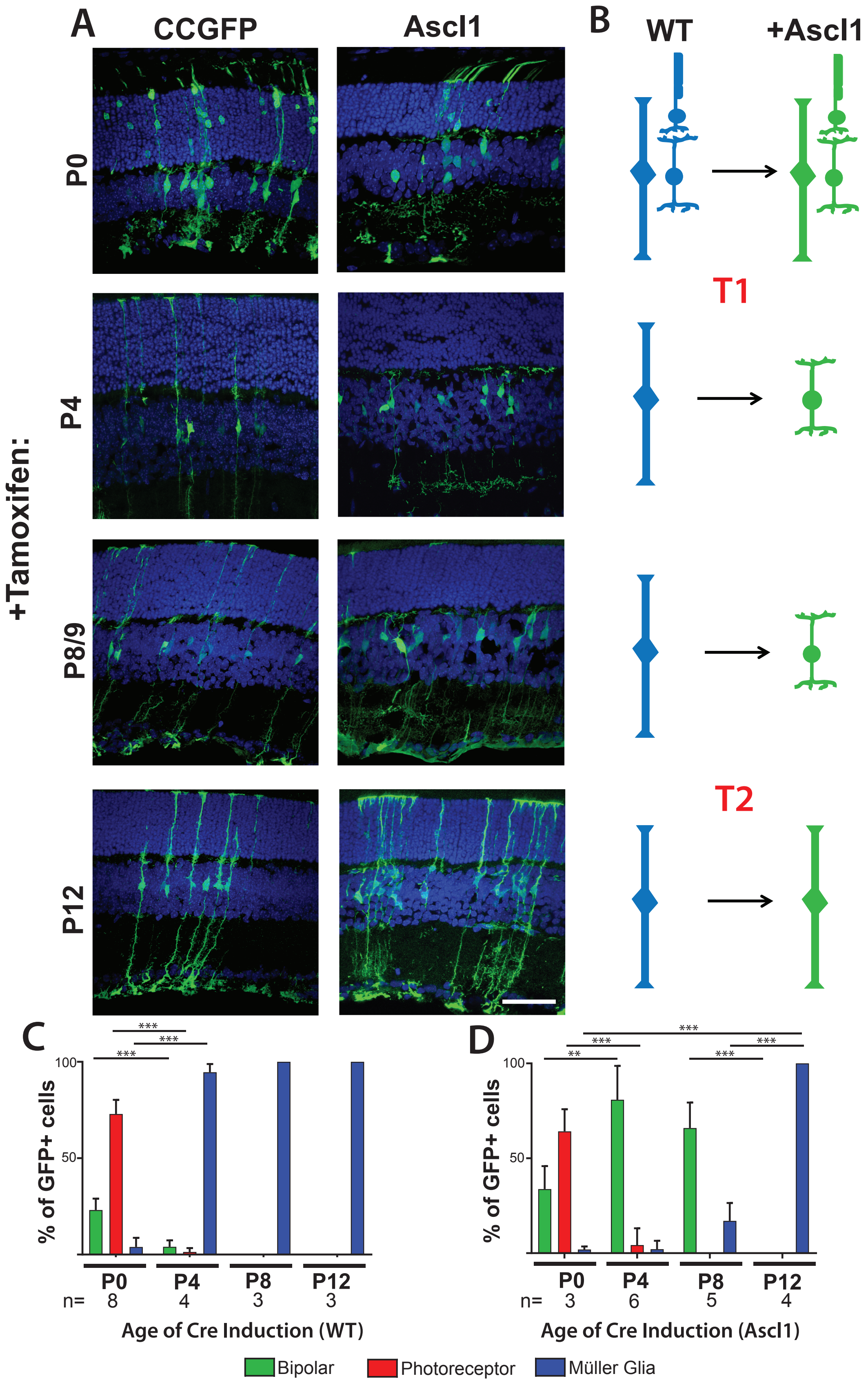
The fact that the neurogenic program can be activated in mature MG by Ascl1 only in combination with HDAC inhibition suggests that epigenetic mechanisms may limit regeneration from the MG. In addition, even with the addition of HDAC inhibitors, the Ascl1-expressing MG only generate a subset of the neurons in the retina, suggesting that epigenetic factors may also limit the types of neurons that can be regenerated from mammalian MG. Thus, the expression of Ascl1, along with HDAC inhibition, does not fully recapitulate the multipotent progenitor state present in developing retina. Therefore, we asked whether changes in the transcriptome or epigenetic landscape might account for the difference in neurogenic potential between MG and retinal progenitors.

Because retinal progenitors are highly neurogenic and because there is a clear timeline of restriction of neurogenic potential in MG as they develop, I wanted to characterize the epigenomic accessibility of MG from birth to adulthood. To address the question of what distinguishes mature MG from late progenitors, I performed a transcriptomic and epigenomic comparison of FACS-isolated postnatal progenitors, young MG (before eye-opening at P12), and adult MG. Analysis by ATAC and RNA sequencing demonstrated a clear trend in the loss of neurogenesis-related motif accessibility and expression. Immature MG were found to have an intermediate epigenomic and transcriptomic profile.

Results

Ascl1 Induces Neurogenesis in Young MG Until P12

In the mouse retina, progenitor cells generate rods, bipolar cells, some amacrine cells, and MG for the first four postnatal days. In order to trace the lineages of retinal progenitors and MG, Kristen Cox and Laura Chipman in the lab used a tamoxifen-inducible creER mouse driven by a progenitor/glial-specific promoters to activate a fluorescent reporter (Glast-CreER:flox-stop-CC-GFP). The cell fates of the progeny of retinal progenitors were traced in these mice by administration of an IP injection of Tamoxifen to initiate the recombination at P0 or P4. When cells were lineage traced at P0, the types of neurons described above were present in the progeny: 72.6% of GFP+ cells had a photoreceptor fate, 22.7% were bipolar cells, and the remaining cells were Müller Glia (MG) (Figure 3.1 A,C). These are similar to the ratios of these cells that are birth-dated at this age in mice (Young 1985). By P4, the percentage of GFP+ neurons significantly decreases with only 2.72% of cells having a bipolar cell fate, while the


A
CCGFP
Ascl1
B
WT
+Ascl1
+Tamoxifen:
P0
P4
P8/9
P12
T1
T2
C
D
% of GFP+ cells
100
50
***
**
P0
P4
P8
P12
n= 8 4 3 3
Age of Cre Induction (WT)
n= 3 6 5 4
Age of Cre Induction (Ascl1)
Bipolar
Photoreceptor
Müller Glia

remainder throughout the retina were MG. At the far retinal periphery however, some retinal progenitor cells are still present at P4 and so some rods and bipolar cells are observed (Data not shown). After neurogenesis is complete, the Glast-CreER:flox-stop-CC-GFP is expressed only in MG. As a result, when Tamoxifen injections are given at P8 or after, 100% of GFP+ cells were MG. These results are consistent with previous reports that neurogenesis in the retina ends after P4, with the exception of some neurogenesis at the periphery (Young 1985; Wohl, Hooper, and Reh 2019).

To demonstrate neurogenic potential through early retinal development, Ascl1 expression was driven in progenitors and young MG similar to cell tracing described above. Ascl1 expression driven as previously described (Ueki et al. 2015) by Glast-CreER;Flox-stop-LNL-tTA;TetOCMV-Ascl1-ires-GFP was induced by an injection of Tamoxifen. With the induction of Ascl1 expression at P0, ratios of GFP+ neurons are not significantly different from WT lineages, though there might be a trend towards reduced MG. This suggests that Ascl1 is expressed at sufficient levels in retinal progenitors to sustain neurogenesis, and that additional Ascl1 has little effect. At P4 and P8, however, over-expression of Ascl1 in immature MG has a dramatic effect on the cells: 75% and 65% of GFP+ cells, respectively, are now bipolar neurons, with few—if any—photoreceptor neurons. When of Ascl1 is delayed to P12, however, 100% of GFP+ cells are now MG, and at this age, expression of Ascl1 is no longer alone sufficient to reprogram the MG to neurons, similar to results previously reported (Figure 3.1 A,D). These results show there is a rapid change in the competence of cells to generate rods with Ascl1-overexpression summarized in Figure 3.1 B. Some major change (Transition [T]1) occurs in the progenitor cells as they become MG to restrict the ability of Ascl1 to generate rods. A second major change (Transition [T]2) appears to control the ability of MG to generate bipolar cells, and this occurs after P8. After this time, damage to the retina and inhibition of histone deacetylases is needed to induce neurogenesis from the MG.

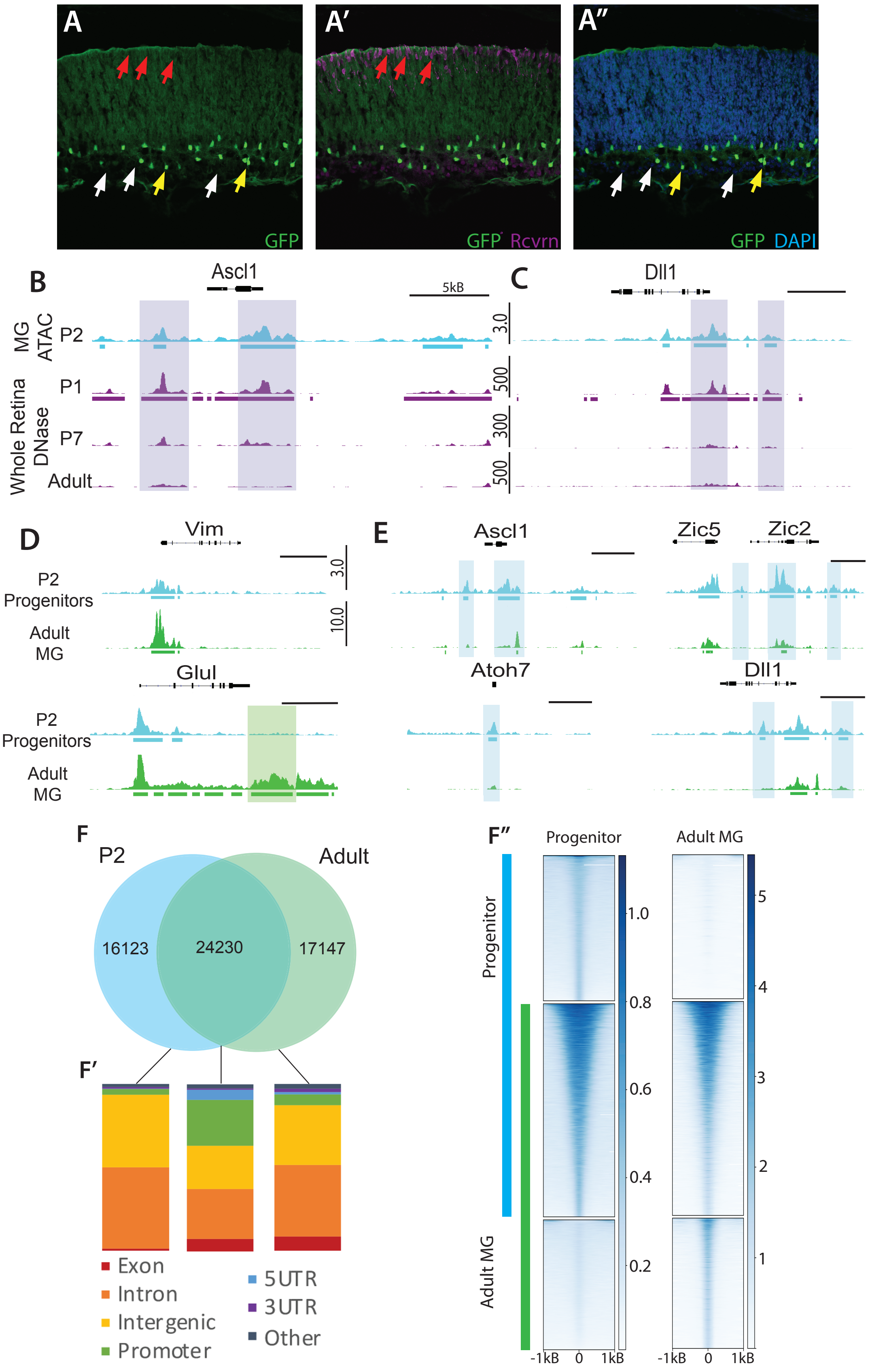
Figure 3.1 Ascl1 Overexpression in Young MG Induces Bipolar Neurogenesis
A. Cell tracing of progenitors and MG was induced with CC-GFP or Ascl1-ires-GFP on a Glast promoter at P0, P4, P8/9, and P12. All analyzed at P21. Scale bar 50 um B. Graphical summary of reprogramming observed with Ascl1. C. Cell counting quantification of GFP cell tracing in (A). D. Cell counting quantification of Ascl1 induction tracing in (A).

Chromatin Accessibility in Retinal Progenitors

To determine the differences in the epigenomic landscape of retinal progenitors and developing MG, I used an Assay for Transposase-Accessible Chromatin (ATAC) sequencing to probe for differences in their accessibility (Buenrostro, Wu, Chang, et al. 2015). To isolate retinal progenitor cells, I used a knock-in Sox2-GFP mouse line that transiently expresses GFP under control of the Sox2 promoter (Arnold et al. 2011). These GFP+ retinas were sorted by Fluorescence-Activated Cell Sorting (FACS) at P2, isolating the more strongly fluorescent amacrine cells from the weakly fluorescent progenitors that dominate the P2 retina (Figure 3.2 A-A''). Two biological replicates were carried out (first run had 121M reads and 101k peaks, with a replicate with 95M reads and 68k peaks). When overlapped by BEDOPS (Neph et al. 2012), there were 40k common peaks between the samples, which were used for the subsequent analysis.

Our lab previously characterized chromatin accessibility by Dnase I in whole retinas at P0, P7 and Adult (Wilken et al. 2015). I compared the progenitor ATAC-seq results with that from DnaseI-seq of the whole retina at the three ages. At P0, approximately 30% of the retinal cells are progenitors, while at P7 there are few progenitors remaining and none in the adult (Young 1985). Thus, I would anticipate the greatest overlap in accessible peaks to be between the progenitor ATAC-seq and the P0 retina. I found that progenitor-specific genes, such as Ascl1, show similar accessibility in the P0 retina and P2 FACS purified progenitor cells (Figure 3.2 B,C). Comparison of the progenitor ATAC-seq with older whole retina DNaseI-seq shows a reduction in accessibility at many regions near progenitor-specific genes, consistent with the loss of progenitor cells as the retina matures.

Figure 3.2 Progenitor and Müller Glial Accessibility is Generally Similar.
A-A''. Fluorescent imaging of Sox2-GFP with Recoverin (A') and DAPI (A''). White arrows identify DAPI(+) GFP(-) cells, Yellow arrows identify DAPI(+) amacrine cells with high GFP, red arrows identify Recoverin(+) GFP(-) cells. B. Genomic tracks of P2 progenitor ATAC and whole retina DNase(Wilken et al. 2015) around Ascl1 and C. Dll1. D. Tracks for progenitors and adult MG (Jorstad et al. 2017) ATAC-seq at glial genes and E. progenitor genes. F. BEDOPS overlaps of progenitor and adult MG peak calls. F'. Genomic region annotation of accessibility profiles. F''. Tag density in progenitor and adult MG ATAC at accessibility profiles.


A
A′
A″
GFP
GFP Rcvrn
GFP DAPI
B
Ascl1
5kB
MG ATAC
P2
Whole Retina DNase
P1
P7
Adult
C
Dll1
3.0
500
300
500
D
Vim
3.0
10.0
P2 Progenitors
Adult MG
Glul
P2 Progenitors
Adult MG
E
Ascl1
Zic5
Zic2
Atoh7
Dll1
F
P2
Adult
16123
24230
17147
F′
Exon
Intron
Intergenic
Promoter
5UTR
3UTR
Other
F″
Progenitor
Adult MG
Progenitor
Adult MG
1.0
0.8
0.6
0.4
0.2
5
4
3
2
1
-1kB
0
1kB
-1kB
0
1kB

Chromatin Landscapes in MG and Progenitors Have a High Level of Overlap

To determine if there are specific molecular differences in chromatin accessibility between the multipotent progenitor and the mature MG, I compared the progenitor accessible domains to the adult WT MG accessible domains from Chapter 2. The accessible chromatin in MG peaks has been assessed in both FACS purified MG from adult mouse retina, as well as from MG maintained in dissociated culture and assayed through DNAse-I hypersensitivity by Matt Wilken (Ueki et al. 2015). Not previously addressed is a small percentage of known rod contamination in the MG ATAC sample. When assaying adult MG chromatin, I found that a small amount of rod photoreceptor DNA and RNA is carried along with the adult MG during FACS (Jorstad et al. 2017); however, when MG are maintained in dissociated culture, very few rods (<0.1%) survive. Therefore, to reduce the effects of these contaminants during analysis, I overlapped MG-specific regions of accessibility with Dnase-seq from cultured P12 MG (Ueki et al. 2015). When I examine the peaks that are present in both the freshly isolated MG ATAC-seq and the peaks from the cultured MG, I find they largely overlap, yielding 39,771 peaks from the original 42,535.

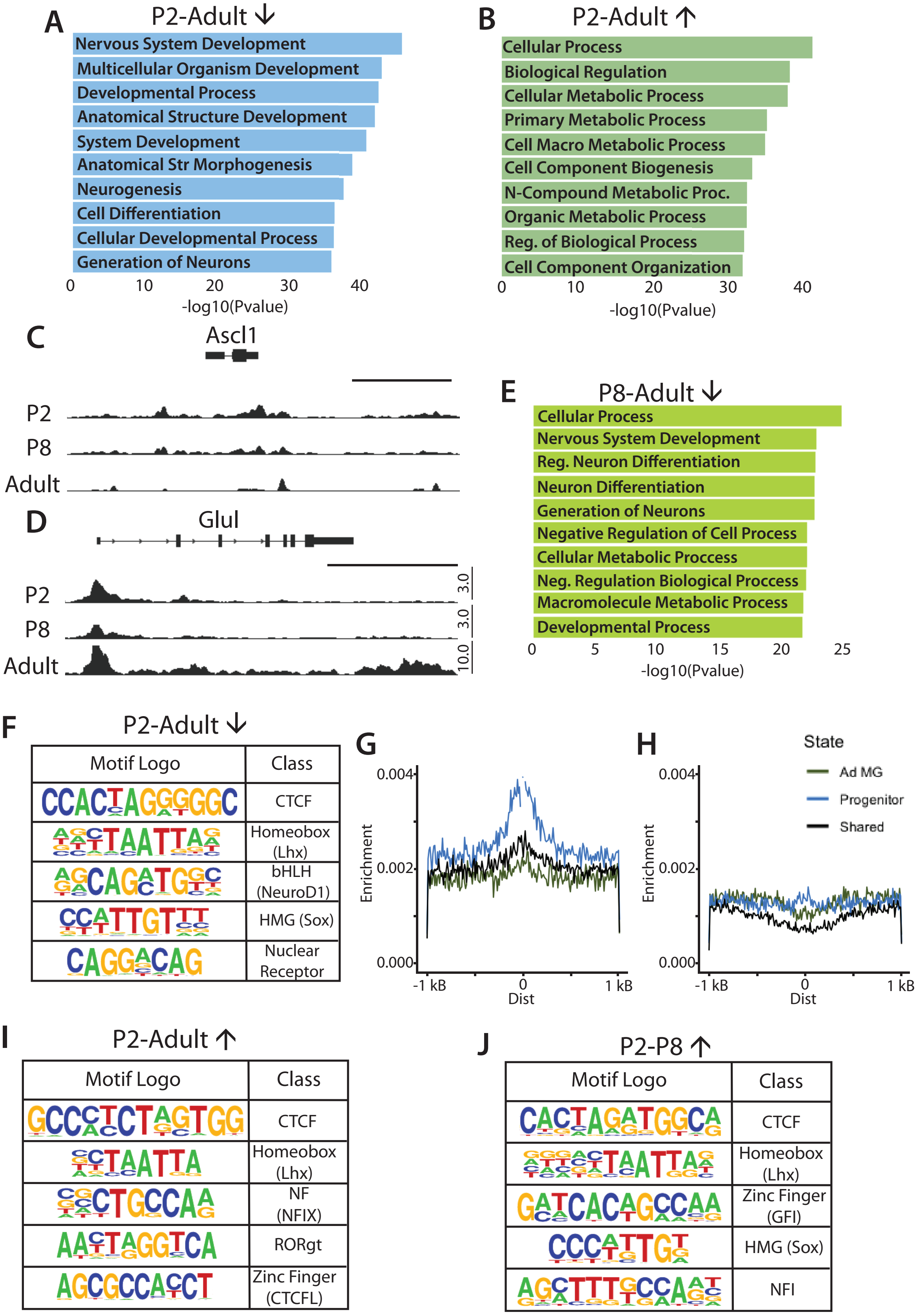
Comparing the MG accessible peaks with those of the progenitor cells, I find extensive overlap in overall accessibility. Analysis of accessible chromatin domains in either progenitor cells or MG allowed some general conclusions: (1) Both progenitor cells and MG have similar regions of accessible chromatin near genes that are expressed at high levels in MG; however, the MG show a greater signal at many of these peaks, and also had unique peaks near glial genes that were not present in the progenitor cells (Figure 3.2 D). (2) Somewhat surprisingly, genes that are important for progenitor cell function (e.g. Ascl1, Dll1) had many of the same accessible regions near promoters in both the MG and the progenitors, though in many cases the P2 progenitors had more regions of accessibility at these genes than the mature MG (Figure 3.2 E). The difference in accessibility between these cell types varies from reduced peak height to a complete loss of some peaks. (3) Comparison of peak calls demonstrated that approximately sixty percent of the progenitor peaks (24,230 peaks) were shared between progenitors and MG, while P2 progenitors had 16k exclusive peaks, and the adult MG have 17k unique peaks (Figure 3.2 F). Promoter regions were over-represented in regions of shared accessibility, while peaks that were unique to either MG or progenitor cells were predominantly found in intronic and intergenic regions (Figure 3.2 F’). Read density in these categories of accessibility showed that shared accessible regions had an overall higher read density in broader regions, whereas cell type specific accessible regions had narrow regions of read density (Figure 3.2 F''). These differences suggest that while promoter and major regulatory regions retain similar accessibility, many putative regulatory regions differed in accessibility between these cell types, likely reflecting the difference in their respective patterns of gene expression.

Cis-Regulatory Binding Sites Characterize Glial Development

In order to explore putative regulatory regions that are specific to progenitor and MG populations, I analyzed and annotated the regions of changing accessibility. I defined regions of changing accessibility as regions that were either unique peaks called by Homer and overlapped by BEDOPS from P2 progenitors to adult MG, or alternatively showed differences in tag density by a logFC (fold change) > 2 in the top 1000 regions of Differential Accessibility (DA). These two different pipelines gave similar results for gene ontology and binding motif annotation.

When I carried out Gene Ontology analysis for those regions that had greater accessibility in the progenitor cells than in the MG (Gain of Accessibility or GOA), I found these regions were associated with genes that were enriched for GO terms of Neural Development /Neurogenesis and Developmental Process/Cell Differentiation (Figure 3.3 A). By contrast, the peaks that are not accessible in retinal progenitors, but present in mature MG (ie. Gain of Accessibility or GOA), were associated with genes that were enriched for GO terms of more general cell function: e.g. metabolic and cell process genes (Figure 3.3 B). Those accessible regions present in both cell types are also enriched primarily in metabolic genes.

I next performed ATAC-seq on FACS purified immature P8 MG to better understand the dynamic changes in the accessible chromatin that occur as MG mature (Figure 3.3 C,D). For the P8 sample, there were 49.6M reads and 16k peaks by Homer findPeaks, of which 98.35% overlapped with the P7 DnaseI whole retina data set. Tracks of the P8 sample showed similar accessibility to the progenitors both at proneural sites (Figure 3.3 C), and at glial genes (Figure 3.3 D). I carried out DA analysis between the P8 and adult MG, and the progenitor cells and P8 MG. Interestingly, I found GO terms of Nervous System Development and Generation of Neurons as some of the top Biological Process terms in the P8 MG accessible regions that are lost or reduced in the mature MG cells (Figure 3.3 E). Thus, it appears that many of the putative


A
P2-Adult ↓
Nervous System Development
Multicellular Organism Development
Developmental Process
Anatomical Structure Development
System Development
Anatomical Str Morphogenesis
Neurogenesis
Cell Differentiation
Cellular Developmental Process
Generation of Neurons
0
10
20
30
40
-log10(Pvalue)
B
P2-Adult ↑
Cellular Process
Biological Regulation
Cellular Metabolic Process
Primary Metabolic Process
Cell Macro Metabolic Process
Cell Component Biogenesis
N-Compound Metabolic Proc.
Organic Metabolic Process
Reg. of Biological Process
Cell Component Organization
0
10
20
30
40
-log10(Pvalue)
C
Ascl1
P2
P8
Adult
D
Glul
P2
P8
Adult
3.0
3.0
10.0
E
P8-Adult ↓
Cellular Process
Nervous System Development
Reg. Neuron Differentiation
Neuron Differentiation
Generation of Neurons
Negative Regulation of Cell Process
Cellular Metabolic Proccess
Neg. Regulation Biological Proccess
Macromolecule Metabolic Process
Developmental Process
0
5
10
15
20
25
-log10(Pvalue)
F
P2-Adult ↓
Motif Logo
Class
CCACTAGGGGGC
CTCF
TAATTA
Homeobox (Lhx)
CAGATGG
bHLH (NeuroD1)
CCATTGT
HMG (Sox)
CAGGACAG
Nuclear Receptor
G
0.004
0.002
0.000
Enrichment
-1 kB
0
1 kB
Dist
H
State
Ad MG
Progenitor
Shared
0.004
0.002
0.000
Enrichment
-1 kB
0
1 kB
Dist
I
P2-Adult ↑
Motif Logo
Class
GCCCTCTAGTGG
CTCF
CCTAATTA
Homeobox (Lhx)
CTGCCAA
NF (NFIX)
AACTAGGTCA
RORgt
AGCGCCACCT
Zinc Finger (CTCFL)
J
P2-P8 ↑
Motif Logo
Class
CACTAGATGGCA
CTCF
TAATTA
Homeobox (Lhx)
GATCACAGCCAA
Zinc Finger (GFI)
CCCATTGT
HMG (Sox)
AGCTTTGCCAA
NFI

cis-regulatory regions near genes associated with neurogenesis are still accessible in the immature P8 MG, and suggests these cells may be more amenable to reprogramming to progenitor cells than mature MG.

The peaks of accessible chromatin near genes associated with neurogenesis that are unique to the progenitor cells (i.e. Not present or reduced in the mature MG) may be relevant to the differences between these cells in their ability to generate neurons. I analyzed these putative neurogenesis-related cis-regulatory elements for transcription factor binding motifs using Homer findMotifsGenome against randomly selected background regions according to the base settings by Homer. I found that the top five transcription factor motifs present in the peaks unique to progenitor cells (LOA) were – CTCF, Lhx (eg. Lhx2), bHLH (eg. Neurod1), HMG (eg. Sox2) and nuclear receptor (Figure 3.3 F). Progenitor cells are known to express several members of each of these transcription factor families; for example, the proneural bHLH factors Neurog2, Olig 2 and Ascl1 are all expressed in retinal progenitors at this stage (Brzezinski et al. 2011). The enriched nuclear receptor motif (CAGGRCAG) appears to be similar to an RORa motif (AGGTCA) as compared by Homer, but this relationship is not strong with a Homer score of 0.64, and an offset of -3, indicating that this may be due to background noise. When comparing E-boxes with specificity to Ascl1 and Neurog2, we found that Ascl1-specific E-boxes had far more distinct central enrichment, especially in regions of progenitor-specific accessibility, whereas Neurog2 E-boxes are centrally depleted (Figure 3.3 G,H). Factors such as Lhx and Sox are known to be expressed in both progenitors and glia, and thus their presence in progenitor-specific accessible domains is likely indicative of restructuring of regulatory regions during development.

Figure 3.3 Cis-regulatory Binding Motifs Differ During Müller Glial Development
A. Gene ontology associated with accessibility decreases from P2 progenitors to adult MG, and B. accessibility increases from P2 progenitors to adult MG. C, D. Tracks for P2 progenitors, P8, and adult MG comparing reads for Ascl1 (C) and Glutamine Synthetase (D). E. Gene ontology associated with accessibility decreases from P8 to adulthood in MG. F. Predicted motif enrichment for accessibility decreases (Bedops) from P2 progenitors to adult MG. G. Lineplot of predicted central enrichment by HOMER for Ascl1-specific motif CAGSTG and H. Neurog2-specific motif CAKATG in progenitor-specific, shared, and adult MG-specific accessible domains (Bedops). I. Predicted motif enrichment for accessibility increases from P2 progenitors to adult MG (Bedops) and J. increases from P8 to adult MG (DA).

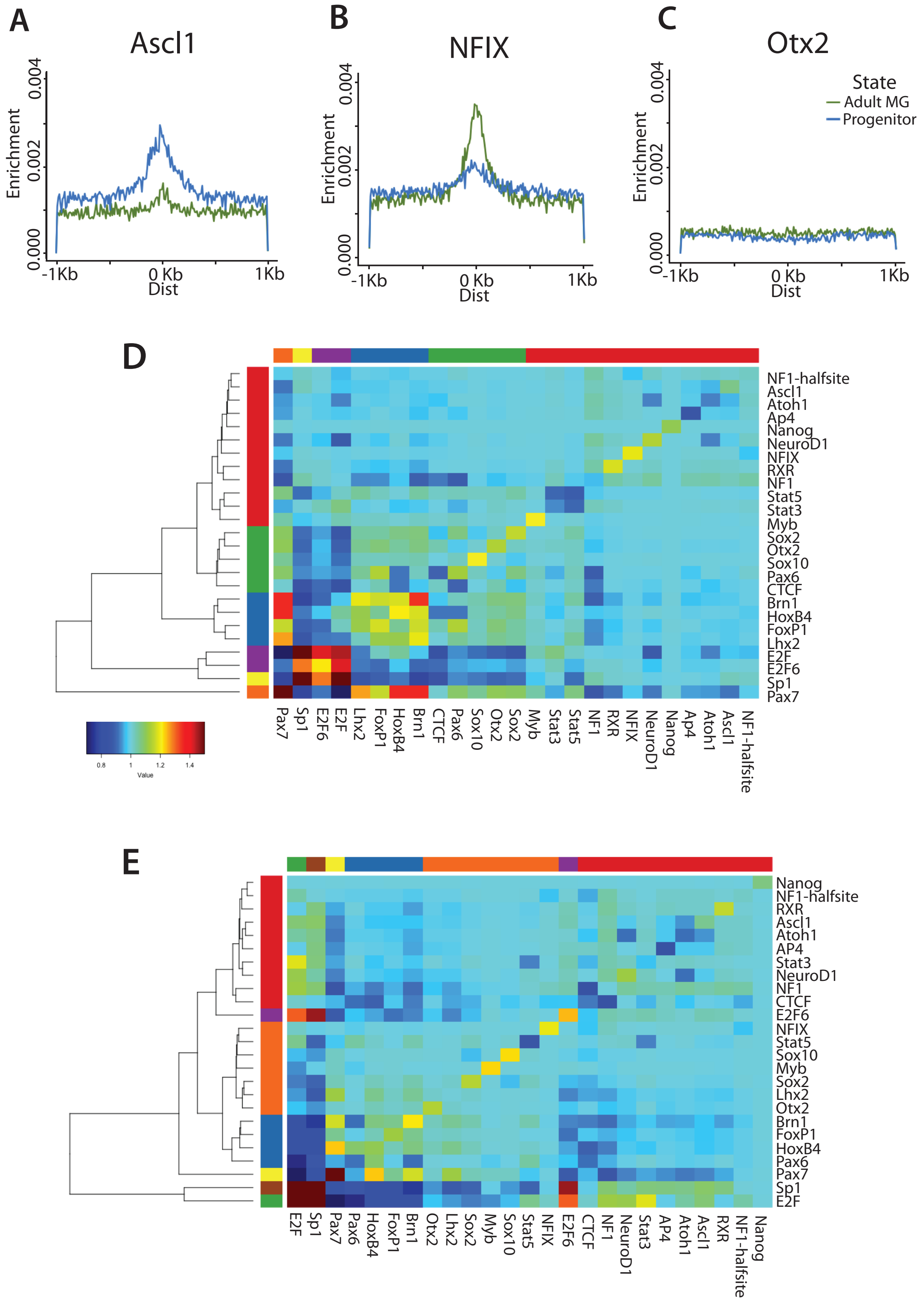
While the accessible chromatin regions specific to progenitors were enriched for proneural transcription factors, the accessible regions present in the MG, but not present in the progenitors (GOA), have a very different set of enriched transcription factor binding motifs. Although CTCF and Lhx motifs are still among the top 5, the NFI (eg. NFIx) binding motif is enriched in those accessible regions unique to the MG (Figure 3.3 I). This motif is also present in the accessible regions that differ between the immature P8 MG and the progenitors, making this an early marker of the unique MG epigenetic landscape (Figure 3.3 J). The motifs enriched in the MG-specific accessible chromatin regions may well reflect the importance of Lhx and NFI transcription factors in MG maturation(Hägglund, Dahl, and Carlsson 2011; de Melo, Clark, and Blackshaw 2016; Gordon et al. 2013; Shu et al. 2003)

Central enrichment modeling also highlighted the differences between progenitors and MG. For example, progenitor-specific accessible regions were centrally enriched for predicted Ascl1 motifs, with much lower accessibility in the adult MG at these sites (Figure 3.4 A). The opposite is true for the NFIx motif: adult MG-specific accessibility profile showed much higher enrichment for NFIx binding motifs within accessible domains than the progenitors (Figure 3.4 B). By contrast, homeobox domain transcription factors (e.g. Otx2) show no central enrichment for either progenitor- or MG-specific accessible domains (Figure 3.4 C). These motif enrichments indicate a potential role bHLH factors in maintaining the neurogenic potential in progenitor cells, and of NFI transcription factors in regulating MG fate.

Since many DNA-binding transcription factors may act in tandem with other transcription factors in a combinatorial manner to regulate gene expression, I analyzed predicted motifs for co-occurrence between the top predicted binding motifs for progenitor (Figure 3.4 D) and adult MG (Figure 3.4 E) accessible domains. Through this analysis, I found that Sp1 and E2F domains commonly co-occur in both progenitor- and MG-specific accessible regions. I also found some co-occurrence of Brn1, HoxB4, FoxP1, and Lhx2 motifs, particularly in progenitor-specific accessible domains. Overall, co-occurrence analysis does not indicate any particular co-regulatory group that may be defining progenitor (Ascl1) or glial (NFIx) cell fate.

Figure 3.4 Individual Motif Changes Do Not Advise Co-Occurrence

A-C. Central enrichment for predicted motifs for (A) Ascl1, (B) NFIX, and (C) Otx2. D. Co-occurrence analysis for top overall predicted motifs in P2 progenitor-specific accessible regions and E. Adult MG-specific accessible regions each clustered by co-occurrence ratios.

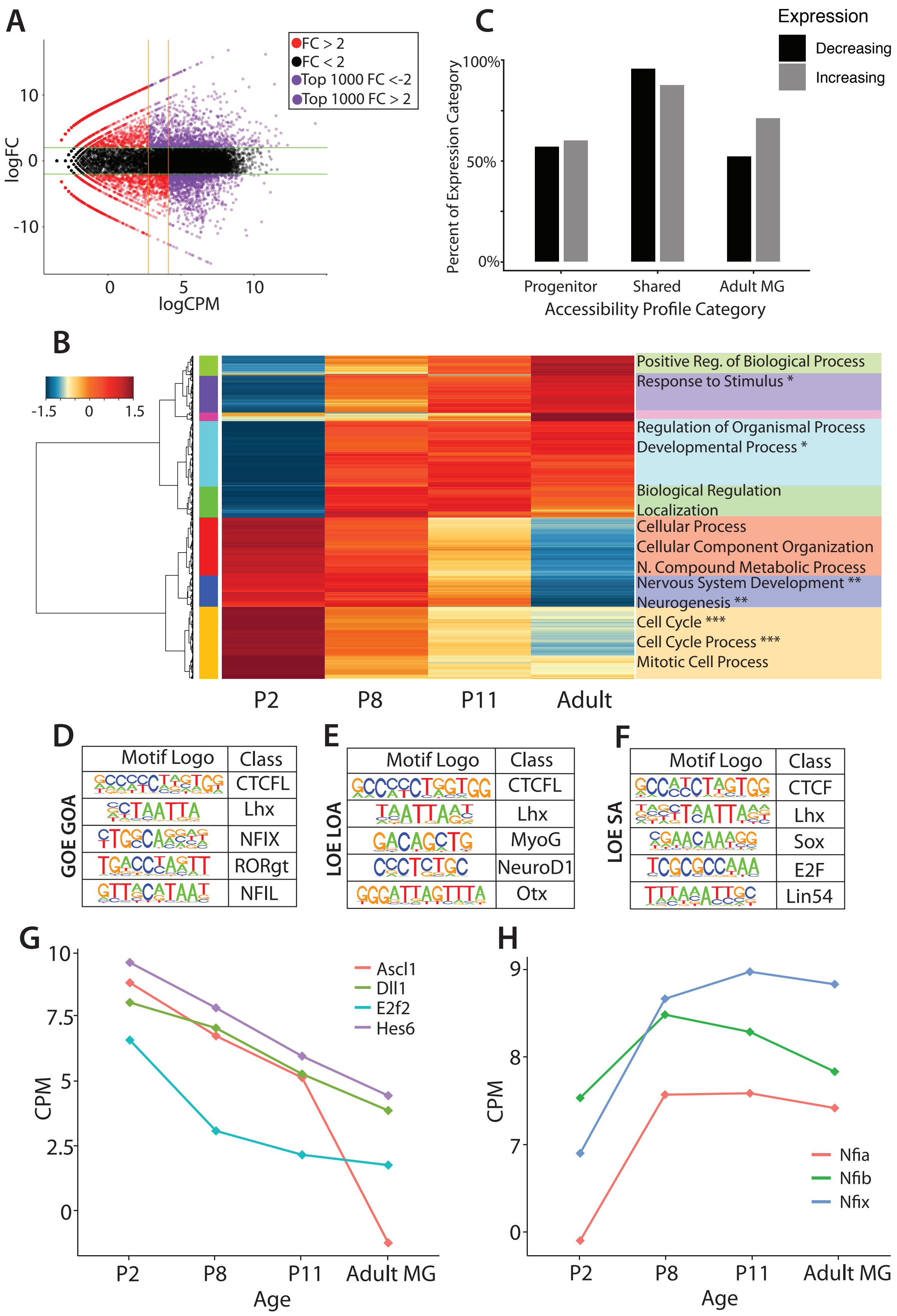

A
Ascl1
Enrichment
0.000
0.002
0.004
-1Kb
0 Kb
1Kb
Dist
B
NFIX
Enrichment
0.000
0.002
0.004
-1Kb
0 Kb
1Kb
Dist
C
Otx2
State
Adult MG
Progenitor
Enrichment
0.000
0.002
0.004
-1Kb
0 Kb
1Kb
Dist
D
NF1-halfsite
Ascl1
Atoh1
Ap4
Nanog
NeuroD1
NFIX
RXR
NF1
Stat5
Stat3
Myb
Sox2
Otx2
Sox10
Pax6
CTCF
Brn1
HoxB4
FoxP1
Lhx2
E2F
E2F6
Sp1
Pax7
Pax7
Sp1
E2F6
E2F
Lhx2
FoxP1
HoxB4
Brn1
CTCF
Pax6
Sox10
Otx2
Sox2
Myb
Stat3
Stat5
NF1
RXR
NFIX
NeuroD1
Nanog
Ap4
Atoh1
Ascl1
NF1-halfsite
0.8
1
1.2
1.4
Value
E
Nanog
NF1-halfsite
RXR
Ascl1
Atoh1
AP4
Stat3
NeuroD1
NF1
CTCF
E2F6
NFIX
Stat5
Sox10
Myb
Sox2
Lhx2
Otx2
Brn1
FoxP1
HoxB4
Pax6
Pax7
Sp1
E2F
E2F
Sp1
Pax7
Pax6
HoxB4
FoxP1
Brn1
Otx2
Lhx2
Sox2
Myb
Sox10
Stat5
NFIX
E2F6
CTCF
NF1
NeuroD1
Stat3
AP4
Atoh1
Ascl1
RXR
NF1-halfsite
Nanog

Immature MG Expression is Intermediate to Progenitors and Mature MG

To better understand the molecular basis for the difference in accessibility between the progenitors and the MG, I, along with postdoc Stefanie Wohl carried out RNA-seq on FACS purified progenitors and immature and mature MG. MG were sorted using Rlbp-creER:flox-stop-tdTomato reporter mice, and progenitors were sorted using Sox2-GFP mice as previously described. Differential expression analysis was performed to demonstrate overall differences between progenitors and mature MG. I selected the 1000 most highly expressed genes with logFC >2, which were differentially expressed in MG (Gain of Expression, GOE) or in progenitors (Loss Of Expression, LOE) by logCPM values (Figure 3.5 A). Genes were clustered via k-means clustering, and GO terms were associated with specific clusters. Genes that showed marked increases over the period from P2 to adult MG had Biological Process terms of Response to Stimulus, and Biological Regulation, while genes that were highly expressed in progenitors, but expressed at much lower levels in MG were associated with GO terms that included Cell Cycle, Nervous System Development and Neurogenesis (Figure 3.5 B).Thus, the changes in gene expression between progenitors and MG are very similar to the changes in DNA accessibility.

I also compared the gene expression in the immature MG at P8. The P8 and P11 immature MG express many of the genes that are expressed in the retinal progenitors, albeit at a lower level than the progenitors, but in addition they express genes more characteristic of mature MG (Figure 3.5 B). The heatmap shows that four of the five clusters of GOE genes have the greatest increase between P2 and P8. LOE genes that lose expression most rapidly, in the tan

Figure 3.5 Glial Expression and Accessibility Profiles Follow Developmental Trends
A. MA plot of expression from progenitors to adult MG demonstrating thresholds for filtering high fold change and counts for the top genes with changing expression. B. Euclidian hierarchical clustering by expression changes from P2 to Adult, heat map of top gene expression, and related gene ontology categories. Categories marked with a * indicates categories that overlap with ATAC MG increased accessibility. Those marked with a ** indicate where progenitor-specific accessibility overlaps, and *** indicates where shared accessibility overlaps. C. Overlaps of top gene expression changes with ATAC profiles. The percentage of genes that increase or decrease expression are represented on the y axis, split by ATAC categories (x axis). D. Motif enrichment for accessible regions with gained expression and accessibility (GOE GOA), E. lost expression and accessibility (LOE LOA), and F. lost expression and shared accessibility (LOE SA). G,H. Expression profiles of (G) progenitor genes of interest, and (H) NFI transcription factors


A
FC > 2
FC < 2
Top 1000 FC <-2
Top 1000 FC > 2
logFC
10
0
-10
0
5
10
logCPM
C
Expression
Decreasing
Increasing
Percent of Expression Category
100%
50%
0%
Progenitor
Shared
Adult MG
Accessibility Profile Category
B
-1.5
0
1.5
Positive Reg. of Biological Process
Response to Stimulus *
Regulation of Organismal Process
Developmental Process *
Biological Regulation
Localization
Cellular Process
Cellular Component Organization
N. Compound Metabolic Process
Nervous System Development **
Neurogenesis **
Cell Cycle ***
Cell Cycle Process ***
Mitotic Cell Process
P2
P8
P11
Adult
D
GOE GOA
Motif Logo
Class
CTCFL
Lhx
NFIX
RORgt
NFIL
E
LOE LOA
Motif Logo
Class
CTCFL
Lhx
MyoG
NeuroD1
Otx
F
LOE SA
Motif Logo
Class
CTCF
Lhx
Sox
E2F
Lin54
G
10
7.5
5
2.5
0
CPM
Ascl1
Dll1
E2f2
Hes6
P2
P8
P11
Adult MG
Age
H
9
8
7
0
CPM
Nfia
Nfib
Nfix
P2
P8
P11
Adult MG
Age

cluster are primarily associated with the cell cycle, whereas the cluster (blue) most closely associated with the GO terms of Neurogenesis and Nervous System Development decline over the first postnatal week more gradually, but have very low levels of expression in mature MG (Figure 3.5 B). Comparing expression up and down across MG maturation reveals an intermediate level of expression in young MG with progenitor gene expression retained at relatively high levels through P8.

Are the changes in gene expression between MG and retinal progenitors reflected in their chromatin accessibility? To answer this question, I compared the genes that change in their expression (GOE, LOE) with the developmental ATAC categories (GOA, LOA). I directly compared the GOE and LOE genes to accessibility categories (GOA, LOA, SA [Shared Accessibility]) using gene annotations by GREAT to neighboring genes, yielding a percent of total genes for GOE and LOE categories for each accessibility profile (Figure 3.5 C). Increasing and decreasing expression categories were represented broadly in accessibility profiles. Progenitor and shared categories of ATAC accessibility both overlap similarly with increasing and decreasing expression genes with a slight skew towards decreasing expression genes (Figure 3.5 C). Adult MG accessible regions are primarily associated with genes that increase in expression, though overall ATAC accessibility does not always reflect expression changes. The best correlation between changes in gene expression and changes in accessibility was found in the adult MG: GOA regions are primarily associated with genes that increased in expression (Figure 3.5 C, right hand bars). This correlation was not as clear in the other categories. Accessible chromatin regions that were shared between progenitors and MG were associated with genes that showed a small reduction in expression between progenitors and MG. However, accessible chromatin regions that were specific to progenitors were not necessarily associated with genes that were more highly expressed in progenitors than MG. Despite the lack of good correlation between accessibility and gene expression on a global level, analysis of specific genes and sets of genes are informative. For example, the regions of lost expression and accessibility (LOE/LOA) are best associated with Neurogenic GO categories, and this trend is continued in comparisons from P8 to Adult MG LOE/LOA (Figure 3.5 B).

By overlapping the ATAC-seq data with the gene expression results, I was able to better define some of the differences in transcription factor expression and putative binding that might be regulating the difference in neurogenic competence between these two cell types. For example, LOE LOA regions are enriched for binding motifs for proneural bHLH factors, whereas those genes that gain accessibility and expression (GOE GOA) are enriched for binding motifs for NFI and ROR (Figure 3.5 D,E). These results are in line with those obtained from the analysis of the ATAC seq results (described above), indicating that the binding motifs that differ between these cells are associated with and may regulate those genes that similarly change in expression. Transcription factors that bind to these motifs are also found to change expression in accordance with changes in motif accessibility (Figure 3.5 G,H). Interestingly, those putative cis-regulatory elements that are accessible in both MG and progenitors, but that lose expression in mature MG (LOE SA) are associated primarily with genes for cell cycle, and are enriched for the binding motif for the cell-cycle regulator E2F transcription factor (Figure 3.5 F,G). This suggests that the differences between progenitor cells and MG in their cell proliferation are unlikely to be regulated by changes in chromatin accessibility.

Conclusions

In this chapter, I have explored the epigenetic profile of retinal progenitors and Müller glia alongside the expression profile throughout glial maturation. I have demonstrated epigenetic evidence of putative cis-regulatory elements that change in accessibility through postnatal development and potentially regulate the maturation and neurogenic potential of the MG. Furthermore, I have shown that the intermediate profile of immature MG allows for improved reprogramming to bipolar neurons without additional factors, thus characterizing the mechanism of transitions states in the neurogenic potential of immature mammalian glia.

Cell tracing and Ascl1 induction in young postnatal retinas characterizes two distinct transition periods in retinal development. The first—T1—being a restriction in the ability of MG to generate rod photoreceptors. The second developmental transition period—T2—would appear to be a loss of neurogenic capabilities with limited factors around P12, as mice begin to open their eyes. From this point on, the addition of factors such as the histone deacetylase inhibitor Trichostatin-A in combination with damage and Ascl1 can again induce generation of bipolar neurons, suggesting that the further restrictions on reprogramming by changes in the epigenome are likely responsible for this second transition as well.

Retinal progenitor genes such as Ascl1 are necessary for the normal development of rod photoreceptors and bipolar cells (Ohsawa and Kageyama 2008), but the addition of these factors does not reproduce rod neurogenesis, only bipolar interneurons (Jorstad et al. 2017; Ueki et al. 2015). This restriction of cell fates with Ascl1 overexpression indicates an inability of ectopic Ascl1 to reproduce development. Another group has previously shown potential development of new photoreceptors from adult MG using rod-specific transcription factors and beta-catenin (Yao et al. 2018). However, their methods raise questions about viral specificity and do not answer questions of why mammalian MG do not follow a traditional regeneration paradigm. The generation of rods uses rod TFs with beta catenin, and traces rod neurogenesis using a rho-tdtomato virus that may not be completely MG-specific. Further analysis with a transgenic reporter line would be necessary to confirm these results.

In order to explore the potential epigenetic mechanisms regenerative capacity and losses therein in the mammalian MG, I performed ATAC-seq on postnatal neurogenic retinal progenitors as well as on developing MG and compared the epigenomic profiles of neurogenic and non-neurogenic cells in the retina, in combination with an expression analysis of the developing MG from progenitors to mature MG. My analysis indicates that the epigenomic landscape of progenitors and mature MG are very similar, with an approximate 60% of progenitor accessible domains overlapping with those of mature MG, consistent with similarities in expression between progenitors and MG (Jadhav, Roesch, and Cepko 2009; Nelson et al. 2011). These shared regions are associated with many genes expressed in glia, and were particularly enriched around promoter regions. Cell-type specific accessible regions were enriched in intronic and intergenic regions of the genome, consistent with previous evidence that enhancers may be more dynamic during development (Aldiri et al. 2017).

Accessible regions that are enriched in the retinal progenitors are specifically associated with developmental and neurogenic genes. This is reinforced by expression analysis, demonstrating that progenitor-specific genes, enriched in GO categories associated with early development and neurogenesis, showed more than 2 logFC compared with MG. Many regions of accessibility that are reduced or lost as the MG mature are associated with these same genes that lose expression during postnatal development, reinforcing that accessibility correlates with expression. Motif enrichment analysis of the progenitor-specific accessible domains reveals specific enrichment in bHLH binding domains. This class of transcription factor is well-characterized as part of the retinal developmental process, though there are many variations of E-box motifs in the genome that are relatively specific to different bHLH domains. Aydin et. Al described variations of E-box specificity (CAGSTG for Ascl1 and CAKATG for Neurog2) (Aydin et al. 2019); we found a nonspecific E-box enriched by HOMER, consistent with progenitor cell potential to generate multiple types of neurons (both excitatory and inhibitory), with the fourth position in the motif being equally probable to be A or C. However, specific assaying for specific E-box motifs demonstrates a preference for the Ascl1 motif throughout. The loss seen in bHLH binding motifs in the transition from progenitors to MG confirms that these transcription factors reflects the decline in expression of Ascl1 and related bHLH TFs as progenitors transition to MG.

As MG mature, the cells have an increase in both accessibility and expression associated with genes important for glial function. There was a correlation between those genes that show increases in nearby accessible chromatin and genes that increase in expression during glial maturation. In fact, while areas of decreasing accessibility have some correlation with decreasing expression, areas of increasing accessibility are much more strongly enriched for associations with genes of increasing expression. This could indicate two things: firstly, that progenitors demonstrate some putative priming of mature glial genes in anticipation of a future fate, or secondly, that once MG differentiate, progenitor genes are more thoroughly shut down via epigenetic regulation to prevent dedifferentiation. Young MG in particular have a unique intermediate profile. Glial genes that are highly increased in expression in adult MG gain accessibility before P8, but this is not accompanied by a similar drop in progenitor gene expression. The top 1000 LOE genes retain a relatively high level of expression in P8 immature MG, accompanied by retention of accessibility around neurogenic genes, which is lost in adulthood. This intermediate expression and accessibility profile explains mechanistically why younger MG have increased neurogenic potential that is lost by P12.

Cis-regulatory accessibility continues to change and develop during MG maturation. While many enriched motif classes are common between decreasing and increasing accessibility, the NFI binding motif is uniquely enriched in the developing glia. NFI domains are similarly enriched surrounding genes that increase expression and gain accessibility, indicating a putative role in the development and maturation of the glial fate. These NFI domains can be seen in regions of accessibility even in the younger P8 MG, consistent with evidence for an intermediate profile of immature MG. NFI transcription factors are relevant to the developing glia as they are well known for their role in CNS glial development and regulation of proliferation (Shu et al.

2003; Heng et al. 2015; Deneen et al. 2006). In addition, NFIa/b/x are expressed in retinal progenitors and MG, and conditional knockouts of these transcription factors in the developing retina are associated with defects in gliogenesis and the production of bipolar neurons (Clark et al. 2019). As progenitors develop, there is an acquired association of NFI motifs with Lhx2 binding sites, which may be related to guiding the neurogenic potency of late retinal progenitors (Zibetti et al. 2019). Combined, this suggests a role for NFI to direct late born neuronal cell fates, and the increased presence of NFI binding motifs in maturing glia may predispose glia towards a bipolar neuron fate. The lack of diverse neuronal cell types driven by Ascl1 overexpression in MG may be explained by the activity of NFI even in young MG specifically directing cell fates. The restriction of NFI activity in MG in combination with Ascl1 overexpression thus could direct neurogenesis to alternative cell fates.

Though RNA-seq and ATAC data demonstrate epigenomic changes that co-occur with changes in gene expression, not all gene expression changes are associated with changes in accessibility. This is best demonstrated with cell cycle and proliferation-related genes. These regions are associated with E2F binding motifs, consistent with their roles in regulating mitotic proliferation(Wu et al. 2001; Stevaux and Dyson 2002). Expression of genes associated with the mitotic cell cycle declined as progenitors withdrew from the cell cycle, with reduced expression even in young non-proliferating MG. However, regions of accessibility associated with these genes do not show much, if any changes in accessibility between progenitors and MG. This suggests that the loss of proliferative capacity in maturing MG may not be limited by epigenetic regulation.

In sum, I have characterized the ways in which cis-regulatory elements change in the development of MG from neuropotent progenitors. Developing MG lose expression of neurogenic genes and accessibility of related cis-regulatory elements, and gain accessibility of glial-defining NFI binding sites. However, young MG demonstrate intermediate profiles. By P8, MG demonstrate early gains in NFI binding sites, while retaining progenitor-like expression and accessibility. This intermediate profile translates into neurogenic potential, as before P12 MG, overexpression of Ascl1 alone is sufficient to induce neurogenesis, forming bipolar neurons. Overall, my results show that accessibility to regulatory binding motifs helps to direct the development of MG and its ability to generate new neurons with Ascl1.

Chapter 4

Epigenetic Barriers Exist to a Diverse Regenerative Response

Some figures and text modified from VandenBosch et al. 2019

Introduction

Induced neurogenesis is effective in many systems, both in vitro and in vivo. The expression of neurogenesis factors (such as the BAM factors: Brn2, Ascl1, and Myt1l) in somatic cells in culture effectively generates new neurons and restructures the epigenome appropriately (Wapinski et al. 2013, 2017; Gascón et al. 2017). Within the Reh lab, there is an extensive body of evidence demonstrating the capabilities of Ascl1 alone in generating new retinal neurons *in vivo* (Ueki et al. 2015; Jorstad et al. 2017; Pollak et al. 2013). These new neurons successfully integrate into existing retinal circuitry and are electrically active. However, regardless of current treatment methods and supplementary factors, the predominant retinal neuron generated from Ascl1 expression is the bipolar cell (Ueki et al. 2015; Jorstad et al. 2017).

This restriction to one cell fate in induced mammalian regeneration presents several problems. Firstly, in the generation of therapeutics for cases of retinal neuron losses through damage and disease, there are many types of cells in need of replacement. Diseases of the eye causing blindness are extremely common, with approximately 36 million individuals worldwide experiencing blindness, and another 216.6 with moderate-to-severe visual impairment (Bourne et al. 2017). Retinal disorders leading to blindness are extremely common in people experiencing vision loss: macular degeneration affects 10% of people over 65 years old with 8-10 million individuals moderately to severely affected, and retinitis pigmentosa affects 1 in 3,000-7,000 individuals (Al-Zamil and Yassin 2017; Ferrari et al. 2011). These are all conditions in which rod and cone photoreceptors are lost, resulting in permanent vision loss. Conditions such as glaucoma affect 3.54% of the global population 40-80 years old (Tham et al. 2014), causing death of the retinal ganglion cells (Weinreb, Aung, and Medeiros 2014). Ischemic strokes such as central retinal artery occlusion, by comparison, are relatively rare affecting approximately 1 in 100,000 people, but are an example of cases wherein the bipolar cells may be lost (Varma et al. 2013). Thus, when generating therapeutics, the generation of a diverse population of retinal neurons is important, and current techniques do not sufficiently generate all types of neurons.

Similarly, current regenerative techniques do not effectively spur regeneration on the same scale as regenerative species. Though teleost retinal regeneration has been mimicked through the use of transcription factors necessary for regeneration, there is a distinct lack of a robust regenerative response. It is known that Ascl1 is important for the proliferation and dedifferentiation of MG after damage in regenerative species, and that it is necessary for the normal development of bipolar cells and photoreceptors. While this overexpression of Ascl1 can induce some MG to re-enter the mitotic cell cycle, particularly when additional factors such as EGF of STAT inhibitors are added (Jorstad et al. 2017, 2019), even in the best cases, the amount of proliferation is less than that observed in fish (Lamba, Karl, and Reh 2008). In addition, Ascl1 expression in mouse MG yields a limited diversity of retinal neurons (Ueki et al. 2015; Jorstad et al. 2017, 2019). The predominant cell type produced from Ascl1 in murine retinas in all currently published conditions is the bipolar neuron, despite the above evidence for Ascl1's neurogenic potential. I hypothesize that this restriction in cell fate is due to bias in the activity of Ascl1 potentially because of epigenetic restrictions in the MG.

These epigenetic barriers to fate change decisions and regeneration have been indicated in my previous analyses of development and regeneration in the murine MG. Analysis of adult regeneration in Chapter 2 revealed that increases in accessibility surrounding neurogenic genes accompanies improved neuronal regeneration. Regions of increased accessibility are closely associated with Ascl1 binding domains as well as some downstream factors, and closely matches bipolar cell accessibility. These increases in neuronal-related accessibility associated with improved neurogenesis in mice is also closely matched with the intermediate accessibility and regenerative capabilities of younger glia. In Chapter 3, I characterized the losses and gains in accessibility as MG develop from birth. I found that MG between P4 and P12, which are more easily reprogrammed with Ascl1 alone, retain progenitor-like accessibility and expression around neurogenic genes. Regardless, in all cases of regeneration, neurogenesis fates are restricted.

This fate restriction may be in part due to increases in glial-specific epigenome accessibility. I found that the transcription factor class of NFI is enriched in regions of increased accessibility as early as P8. NFI transcription factors are vital to the development of glia throughout the central nervous system (Heng et al. 2015; Shu et al. 2003; Glasgow et al. 2017; Clark et al. 2019). In retinas, NFI factors increase in expression over postnatal development. Altering expression of NFI factors in triplicate thus affects ratios of cell development. Increased NFI expression increases production of MG and bipolar cells, while loss of NFI function reduces the number of these same cells (Clark et al. 2019). Interestingly, losses in NFI function also increase proliferation and delay the development of MG development. In my data (Chapter 2), I find that not only does NFI expression increase early in young MG, but also binding motifs for

NFI factors increase in accessibility. The activity of NFI factors in MG thus may be directing fate during regeneration.

Given the evidence for epigenetic restrictions of neurogenic fate during regeneration, I wanted to discover the ways in which MG are epigenetically restricted. I approached this question through a number of methods. Firstly, I took existing ATAC-seq data of my own as well as from other publications, and compared both retinal progenitors and mature MG to retinal neurons. I hypothesized that given progenitor cells' heightened neurogenic potential, they would have more similarities to rod photoreceptors. Instead, I found that the overall accessibility profile overlaps of progenitors with retinal neurons is remarkably similar to the same comparison with adult MG and neurons. What I did find was that bipolar-specific accessible domains are enriched in predicted E-box domains. Further probing with Ascl1 overexpression ChIP-seq data confirmed that Ascl1 preferentially binds to bipolar-specific accessible domains, indicating a bias in Ascl1 activity to direct fate.

Additionally, I attempted to modulate the epigenomic landscape by using small molecule inhibitors to histone modulator enzymes in combination with Ascl1 in explanted tissues. I hypothesized that the disruption of the epigenomic landscape in combination with reprogramming factors would alter the fate decisions made by the MG. I found that in young retinas, while most drug treatments did not appear to change fate choices, broad disruptions of histone modifications with Trichostatin-A (TSA) or BIX01294 increased the number of MG-derived neurons in the outer nuclear layer where photoreceptors reside. These results did not replicate in vivo, thus raising questions about the roles of damage in the reprogramming process. From these results I conclude that epigenetic barriers exist to fate decisions in reprogramming that bias activity of Ascl1.

Results

Retinal Neuron Chromatin Overlaps Similarly with Progenitor and MG

I first addressed changes in neural competence through comparing epigenomic profiles. I hypothesized that the changes in neurogenic competence that occur as cells transition from progenitor cells to MG even with forced Ascl1 expression are due to changes in their epigenome. To determine if DNA accessibility might underlie these differences in (1) neurogenic competence, and (2) fate restriction to predominantly bipolar cell neurogenesis, I compared

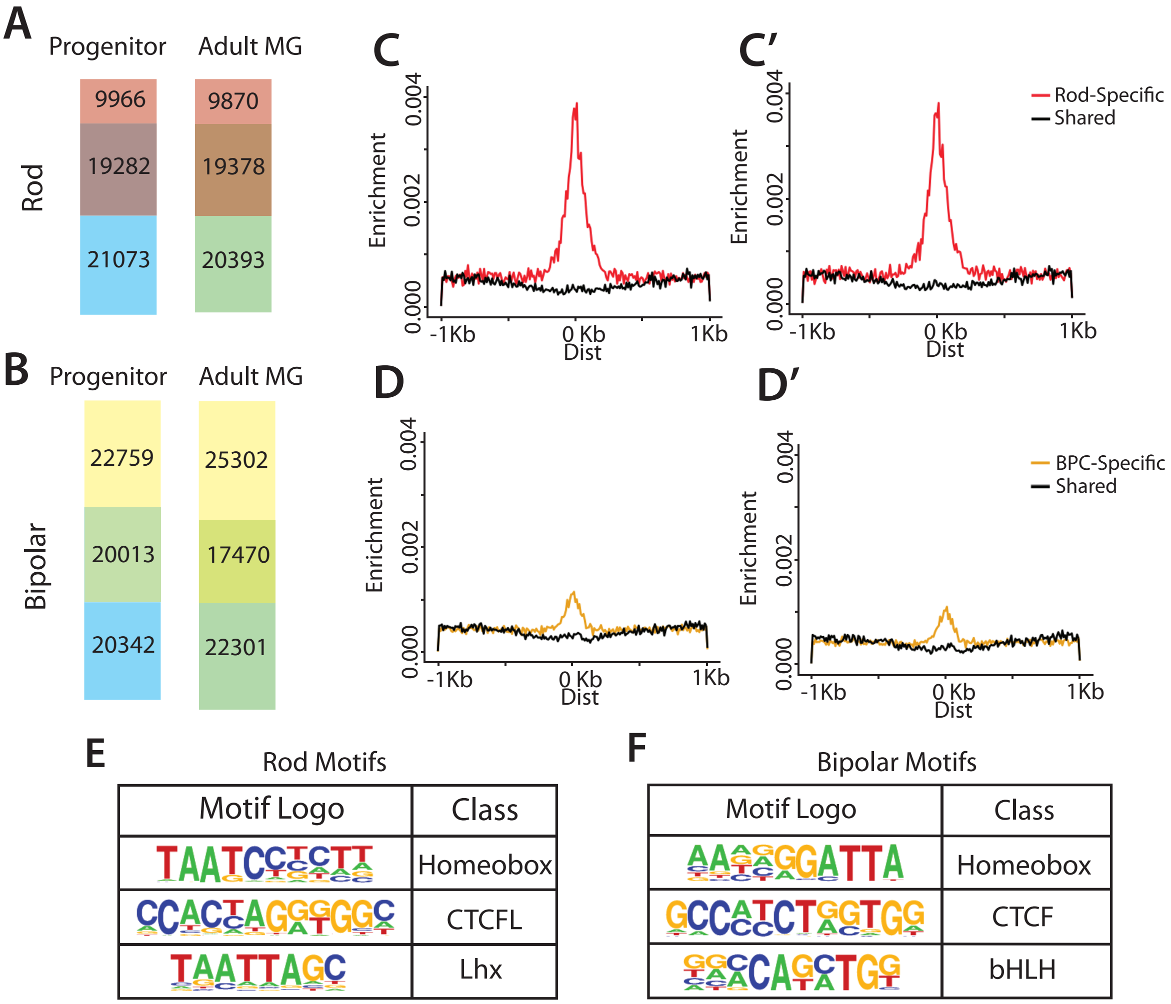


Figure 4.1 Progenitors and Müller Glia Overlap with Neurons Similarly

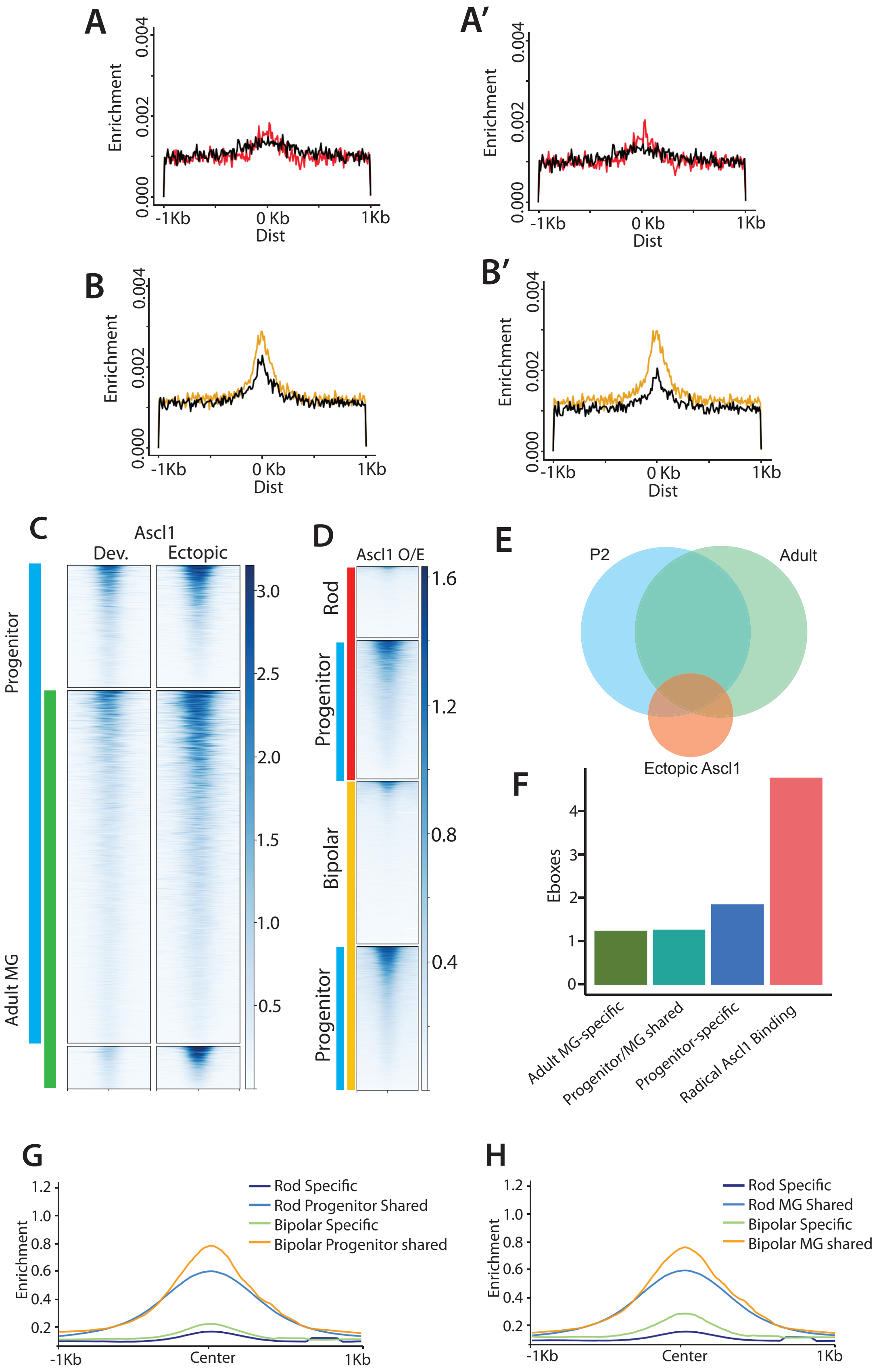
A. Rod chromatin accessibility (Hughes) overlaps by bedops with both progenitors (left) and MG (right). Numbers of peaks in category overlaps listed. B. Grm6+ bipolar chromatin accessibility (Hughes) overlaps by bedops with both progenitors (left) and MG (right). Numbers of peaks in category overlaps listed. C. Otx2 predicted central enrichment in rod-specific accessible domains and those shared with progenitors and C'. adult MG. D Otx2 predicted central enrichment in bipolar-specific accessible domains and those shared with progenitors and D'. adult MG. E, F. Predicted motifs for rod-specific (E) and Bipolar-specific (F) accessible regions.

ATAC-seq data from progenitor cells, MG, rod photoreceptors (Hughes et al. 2017), and bipolar cells (Chapter 2). I found that both progenitor cells and adult MG accessible regions overlapped with rods by approximately 19k peaks or 47.5% of glial/progenitor-accessible domains (Figure 4.1 A). A similar analysis of bipolar cells and retinal progenitors or MG gave very similar results: bipolar cell accessibility overlapped with progenitor accessible regions by approximately 20k peaks (50% of progenitor domains), and with MG accessible regions by approximately 17k peaks (42.7% of glial domains) (Figure 4.1 B). For all overlaps, GO categories were similar between comparisons to progenitors or MG (Table S8). As Otx2 is involved downstream of Ascl1 in the generation of both cell types (Omori et al. 2011), I looked for central enrichment of this motif in both neuronal-specific and MG-shared accessible regions. For rods, the rod-specific accessible regions (ie. Not present in the MG or the progenitor cells) are strongly centrally-enriched for Otx2 binding sites (Figure 4.1C,C'). I did not see a similar central enrichment for Otx2 in the accessible regions shared between rods and progenitor cells or rods and MG (Figure 4.1 C,C'). A similar analysis of the overlapping and uniquely accessible regions for bipolar cells and either MG or retinal progenitors gave strikingly similar results: the accessible regions that are unique to bipolar cells and not shared with progenitor cells or MG show strong central enrichment for the Otx2 binding motif; however, regions shared among these cell types do not have this signature (Figure 4.1 D,D'). Thus, the ability of Ascl1 to promote the bipolar fate is not due to MG having an epigenome that is more similar to bipolar cells than rod photoreceptor cells.

Overall motif enrichment in rod and bipolar neurons are additionally quite similar. Rod-specific regions—when comparing to either MG or progenitors—are enriched for homeobox, and CTCF domains (Figure 4.1 E, Table S2). Shared accessible regions in both comparisons

Figure 4.2 Ascl1 differentiates Bipolar fates from Rods

A. Ascl1 predicted central enrichment in rod-specific accessible domains and those shared with progenitors and A'. adult MG. B. Ascl1 predicted central enrichment in bipolar-specific accessible domains and those shared with progenitors and B'. adult MG. C. Heatmap of Ascl1 ChIP-seq read density across ATAC peak overlaps with developmental (P2) Ascl1 ChIP on the left and induced overexpression of Ascl1 in cultured MG ChIP on the right. D. Ascl1 overexpression ChIP-seq read density in Rod, Bipolar, and Progenitor accessible regions. E. Representation of ATAC overlaps with Ascl1 ChIP peaks. F. Average numbers of Ascl1-related E-boxes (Casey et al. 2018). predicted in each of the Ascl1-binding overlap categories represented in the Ascl1 bubble in (E). G, H. Central enrichment for Ascl1 binding in rod, bipolar, and progenitor (G) or adult MG (H) accessible regions.


A
Enrichment
0.000
0.002
0.004
-1Kb
0 Kb
1Kb
Dist
A′
Enrichment
0.000
0.002
0.004
-1Kb
0 Kb
1Kb
Dist
B
Enrichment
0.000
0.002
0.004
-1Kb
0 Kb
1Kb
Dist
B′
Enrichment
0.000
0.002
0.004
-1Kb
0 Kb
1Kb
Dist
C
Ascl1
Dev.
Ectopic
Progenitor
Adult MG
3.0
2.5
2.0
1.5
1.0
0.5
D
Ascl1 O/E
Rod
Progenitor
Bipolar
Progenitor
1.6
1.2
0.8
0.4
E
P2
Adult
Ectopic Ascl1
F
Eboxes
0
1
2
3
4
Adult MG-specific
Progenitor/MG shared
Progenitor-specific
Radical Ascl1 Binding
G
Enrichment
0.2
0.4
0.6
0.8
1.0
1.2
-1Kb
Center
1Kb
Rod Specific
Rod Progenitor Shared
Bipolar Specific
Bipolar Progenitor shared
H
Enrichment
0.2
0.4
0.6
0.8
1.0
1.2
-1Kb
Center
1Kb
Rod Specific
Rod MG Shared
Bipolar Specific
Bipolar MG shared

show enrichment for CTCF, Klf, and ETS binding motifs. Bipolar cell accessible regions that are shared with both progenitor cells and MG also showed enrichment for these TF binding motifs. Interestingly, in both comparisons to MG and to retinal progenitors, bipolar cell-specific accessibility demonstrated enrichment not only for homeobox domains, but also a notably increased presence of predicted proneural bHLH domains (Figure 4.1 F). The presence of bHLH accessibility in bipolar cells may underlie the fact that MG primarily generate bipolar cells with the overexpression of Ascl1, as the transcription factor may preferentially bind to E-box motifs that specifically regulate bipolar cell fate.

Ascl1 Binding Sites are Enriched in Bipolar Regions

These results imply that bHLH transcription factors such as Ascl1 may play a specific role in directing glia to a bipolar interneuron fate, regardless of age. I next looked at predicted central enrichment of Ascl1 in these neuronal populations (Figure 4.2 A-B'). Rod-specific accessible regions demonstrate some central enrichment of predicted Ascl1 binding motifs, though the overall enrichment does not appear to be notably elevated from regions with accessibility shared with progenitors or MG (Figure 4.2 A, A'). By contrast, bipolar cell accessible regions have distinct central enrichment of predicted Ascl1 binding sites, which is also found in shared accessible regions, though to a lesser extent (Figure 4.2 B, B').

Because the role of Ascl1 appears to be a key difference between Bipolar cell and Rod photoreceptor cells, I took a closer look at its binding in accessible and inaccessible domains. In addition to previous predictive analysis, I compared accessibility domains using Ascl1 ChIP-seq from both P0 retinas as well as from Ascl1 overexpression in cultured MG as performed by my colleague Matt Wilken (Figure 4.2 C, D). While developmental Ascl1 largely binds in shared and progenitor accessibility domains as well as some non-glial sites, ectopic Ascl1 has a strong distinct niche in Adult MG and in so-called "radical" binding sites (Figure 4.2 E). Casey et al 2018 recently demonstrated that ES cell reprogramming with Ascl1 revealed a particular class of accessible regions with repeated E-box motifs (Casey et al. 2018). Similar to that study, I find that those regions of the DNA that are inaccessible in MG or Progenitors, but bind Ascl1 when over-expressed (pioneered sites) had on average 4.7 E-boxes per peak, while other Ascl1 bound sites in MG have fewer than 2 E-boxes on average (Figure 4.2 F). I additionally assayed E-box number in Rod and Bipolar accessible domains, but found no difference in average number of E-boxes per peak (Data not shown).

I found that Ascl1 also binds more strongly to bipolar accessible domains as compared to rod accessible domains (Figure 4.2 D). Read density was highest for progenitor and glial-shared accessible regions, but there was distinct central enrichment for Ascl1 binding in bipolar-specific accessible domains (Figure 4.2 G, H). Ascl1 presence in bipolar-specific accessible domains substantiates the hypothesis that preferential binding of overexpressed Ascl1 in MG may direct fate decisions. Not only is there an enrichment of predicted E-boxes in bipolar cell accessible domains, but also, overexpression of Ascl1 in MG does preferentially bind to bipolar-specific sites, thus potentially directing fate decisions.

Screening Epigenetic Modulators for Reprogramming Effects

The changes in the epigenome as progenitor cells mature into MH parallel the restriction of the ability of MG to be reprogrammed into retinal neurons, especially of diverse fates. Therefore, I attempted a small molecule screen to attempt to modulate the native epigenomic landscape to potentially expand the neurogenic potential of the MG. I acquired a library of small molecules specifically targeting histone modifying enzymes (Table 4.T). In particular, many

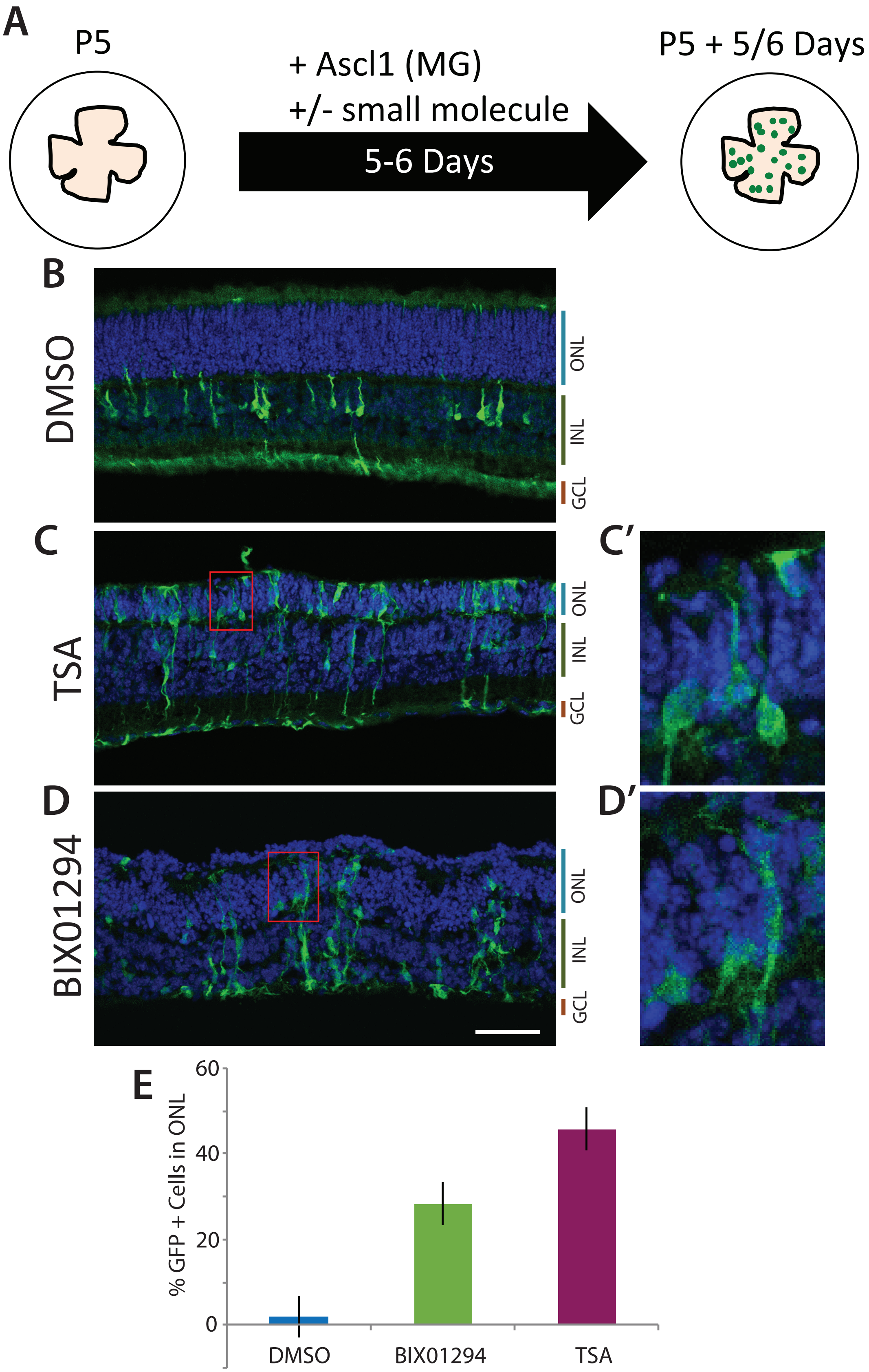
Table 4.T Drugs Screened

Drug Name	**Target**	**Histone Modification**	**Regeneration Effect**
A196	SUV420	H4K20me3	None
A366	G9a	H3K9me2/3	None
AMI-1	Prmt1	H4R3me1/2c	None
BIX-01294	G9a	H3K9me2/3	GFP+ ONL cells
GSK343	EZH2	H3K27me3	None
JIB04	Jumonji domains	H3K27me3 +	Toxic
KDM5-C49	Jarid1	H3K4me2/3	None
LP99	BRD7/9	H3K9ac +	None
RN1	LSD1	H3K4/9me1/2	None
R-PFI	SetD7	H3K4me1	None
TSA	HDACs class I/II	Histone Acetylation	GFP+ ONL cells
UNC0379	SetD8	H4K20me1	None

small molecules inhibited the addition of repressive modifications such as well characterized histone methylation marks. In order to screen these drugs, I cultured explanted mouse retinas of the Glast-CreER;Flox-stop-LNL-tTA;TetOCMV-Ascl1-ires-GFP lineage at P5 on filters on a gas-liquid interface. Within the culture medium, I added small molecule inhibitors with 4-hydroxy tamoxifen to induce Ascl1 overexpression in the MG. These retinas were cultured for 5-6 days before fixation and preparation for histology (Figure 4.3 A). Explant sections were analyzed for morphology, positioning in retinal layers, and the presence of common neuronal markers.

I found in the analysis of the small molecule screen, that most drugs did not significantly alter neurogenesis. However, in the case of the previously characterized drug TSA (See Chapter 2), which broadly opens chromatin (Yoshida, Horinouchi, and Beppu 1995), young explanted retinas show a change in cell localization within the retina. While Ascl1 alone in control retinas yields bipolar neurons in the inner nuclear layer (INL), the addition of TSA ex vivo results in a stark increase of cells found in the outer nuclear layer (ONL), among photoreceptors (Figure 4.3 B, C, C', E). The presence of MG-derived cells in this layer could be due to increased proliferation, as this layer is where MG cell bodies descend to replicate. However, there is no evidence of EDU positivity in GFP labeled MG (Data not shown). In addition to the effects seen from TSA, I additionally found that the G9a inhibitor BIX 01294 (Chang et al. 2009) similarly perturbed GFP+ cell body localization. Similar to the effects seen by TSA, MG-derived cell bodies migrated from the INL to the ONL and were Otx2 positive, and EDU negative (Figure 4.3 D, D', E). Combination of the two drugs ex vivo did not increase ONL cell presence (Data not shown). Thus, broad epigenomic modulator changes can sufficiently alter cell localization with Ascl1 ex vivo.

Figure 4.3 Epigenetic Inhibitor Drugs Alter Cell Localization with Ascl1 Ex Vivo
A. Experimental paradigm for small molecule screening on explanted P5 NakaMash retinas. B-D. Ascl1+/GFP+ cells in (B) DMSO treated retinas, (C) TSA-treated retinas, and (D) BIX01294-treated retinas. C' and D' are expanded panels from their respective origins. Ganglion cell layer, inner nuclear layer, and outer nuclear layer are labeled to the right. Scale bar is 50 um for B, C, D. E. Cell counts of GFP+ cells present in the ONL. Error bars represent standard deviation.

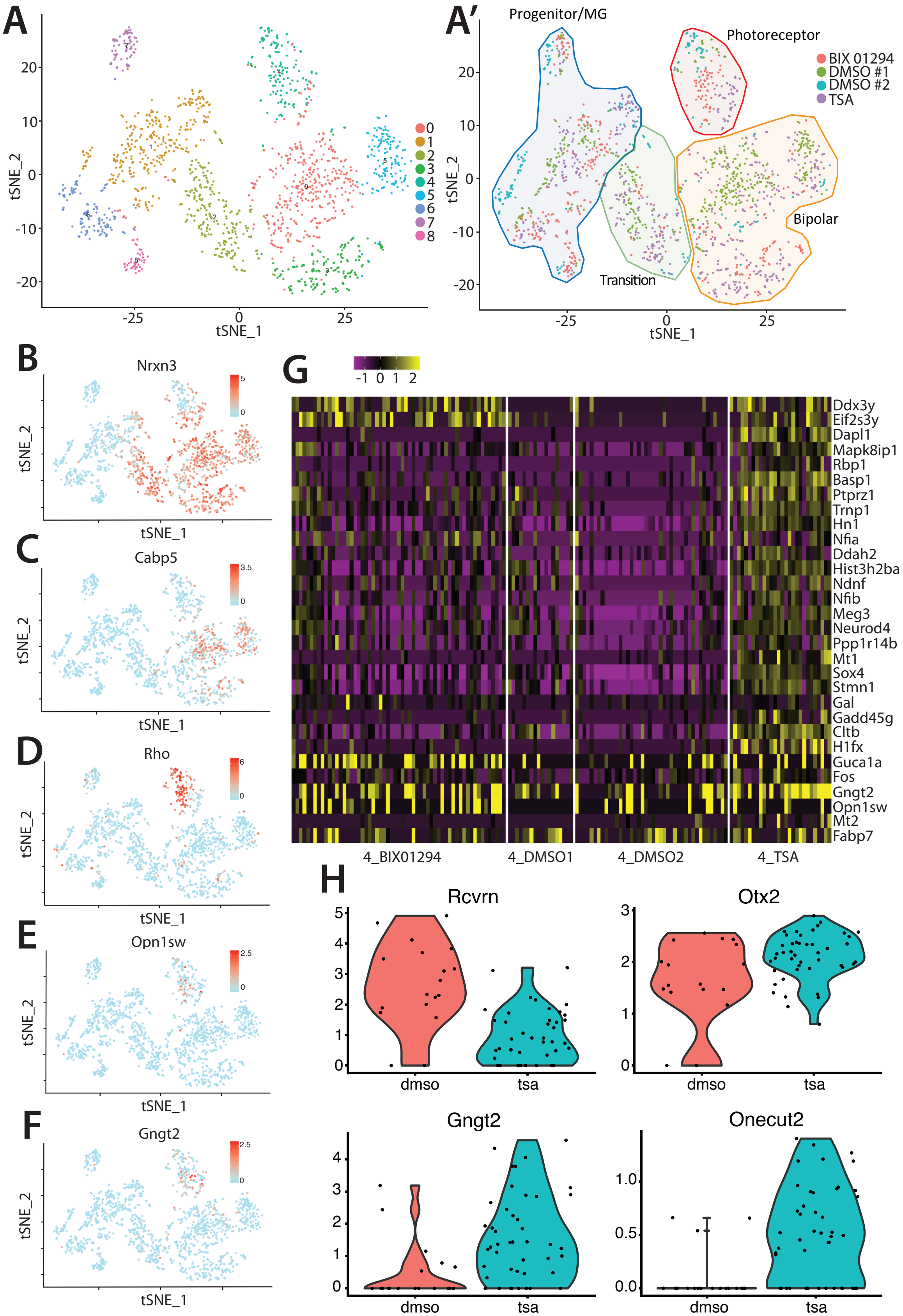

A
P5
+ Ascl1 (MG)
+/- small molecule
5-6 Days
P5 + 5/6 Days
B
DMSO
ONL
INL
GCL
C
TSA
ONL
INL
GCL
C′
D
BIX01294
ONL
INL
GCL
D′
E
% GFP + Cells in ONL
60
40
20
0
DMSO
BIX01294
TSA

Single Cell Analysis of Ex Vivo Reprogramming

In order to analyze potential fate changes in depth, I performed single cell RNA-seq analysis using the 10x platform. Retinas were explanted as described above, and FACS purified for GFP positive cells before sending to the Trapnell lab for single cell processing. Due to concerns about photoreceptor contaminants during FACS purification that may not be MG-derived, I filtered all cells for a low level of Glul expression, which is present across all clusters. Analysis of the resulting cell sequencing in Seurat (Butler et al. 2018) demonstrated a distribution of four distinct cell classes: MG/progenitor-like cells, bipolar interneurons, bipolar- and progenitor-like transition cells, and photoreceptor-like cells (Figure 4.4 A, A'). Clustering of filtered cells after filtration yielded 9 clusters via Seurat (Figure 4.4 A). Clusters 1, 6, 7, and 8 are all consistent with MG/progenitor-like fates. Cluster 7 demonstrated a presence of cell cycle markers. Cluster 2 demonstrated expression of both glial and neuronal markers, making it an apparent cluster of transition cells. Clusters 0, 3, and 5 are consistent with bipolar cell markers, though cluster 3 is biased for small molecule-treated cells (both BIX01294 and TSA) (Figure 4.4 B). Finally, cluster 4 contains markers for photoreceptor cells, though still contains a large number of cells from the control Ascl1-only cells (Figure 4.4 B, C).

Characterization of the photoreceptor cluster revealed a unique cone-like transcriptome in drug-treated cells. When comparing cluster 4 to all other clusters in Seurat, these cells have characteristically high expression of common phototransduction genes such as Rhodopsin, Recoverin, and Nr2e3 (Figure 4.4 B). When comparing treatment groups within cluster 4, I found higher levels of expression for NFI transcription factors and NeuroD4, as well as higher expression of cone genes (ie. Gngt2 and Opn1sw) in both drug treatments (Figure 4.4 C, D).

Figure 4.4 Single Cell Analysis of Drug Treated Retinas Reveals Bipolar and Photoreceptor Cell Identities
A. Seurat-generated t-SNE plots of DMSO- and drug-treated Ascl1+ MG single cell RNA-seq represented by canonical correspondence analysis-directed clustering (clusters in A), and (A') experimental treatments. B. Plot showing expression levels of neuronal marker Nrxn3 enriched in main neuron clusters. C. Plot of bipolar gene cabp5 expression enrichment in bipolar clusters. D-F Plots showing photoreceptor-specific gene expression in cluster 4. Rod gene expression (D), and cone-specific genes (E, F) demonstrated. G. Heat map of top 30 genes differentially expressed between treatments within cluster 4, separated by drug treatment. H. Violin plots of gene expression in cluster 4 between DMSO and TSA treatments of key differentially expressed genes Recoverin, Otx2, Gngt2, and Onecut2.


A
tSNE_2
tSNE_1
20
10
0
-10
-20
-25
0
25
0
1
2
3
4
5
6
7
8
A’
Progenitor/MG
Photoreceptor
Transition
Bipolar
BIX 01294
DMSO #1
DMSO #2
TSA
B
Nrxn3
C
Cabp5
3.5
D
Rho
6
E
Opn1sw
2.5
F
Gngt2
2.5
G
-1 0 1 2
Ddx3y
Eif2s3y
Dapl1
Mapk8ip1
Rbp1
Basp1
Ptprz1
Trnp1
Hn1
Nfia
Ddah2
Hist3h2ba
Ndnf
Nfib
Meg3
Neurod4
Ppp1r14b
Mt1
Sox4
Stmn1
Gal
Gadd45g
Cltb
H1fx
Guca1a
Fos
Gngt2
Opn1sw
Mt2
Fabp7
4_BIX01294
4_DMSO1
4_DMSO2
4_TSA
H
Rcvrn
Otx2
Gngt2
Onecut2
dmso
tsa
0.0
0.5
1.0

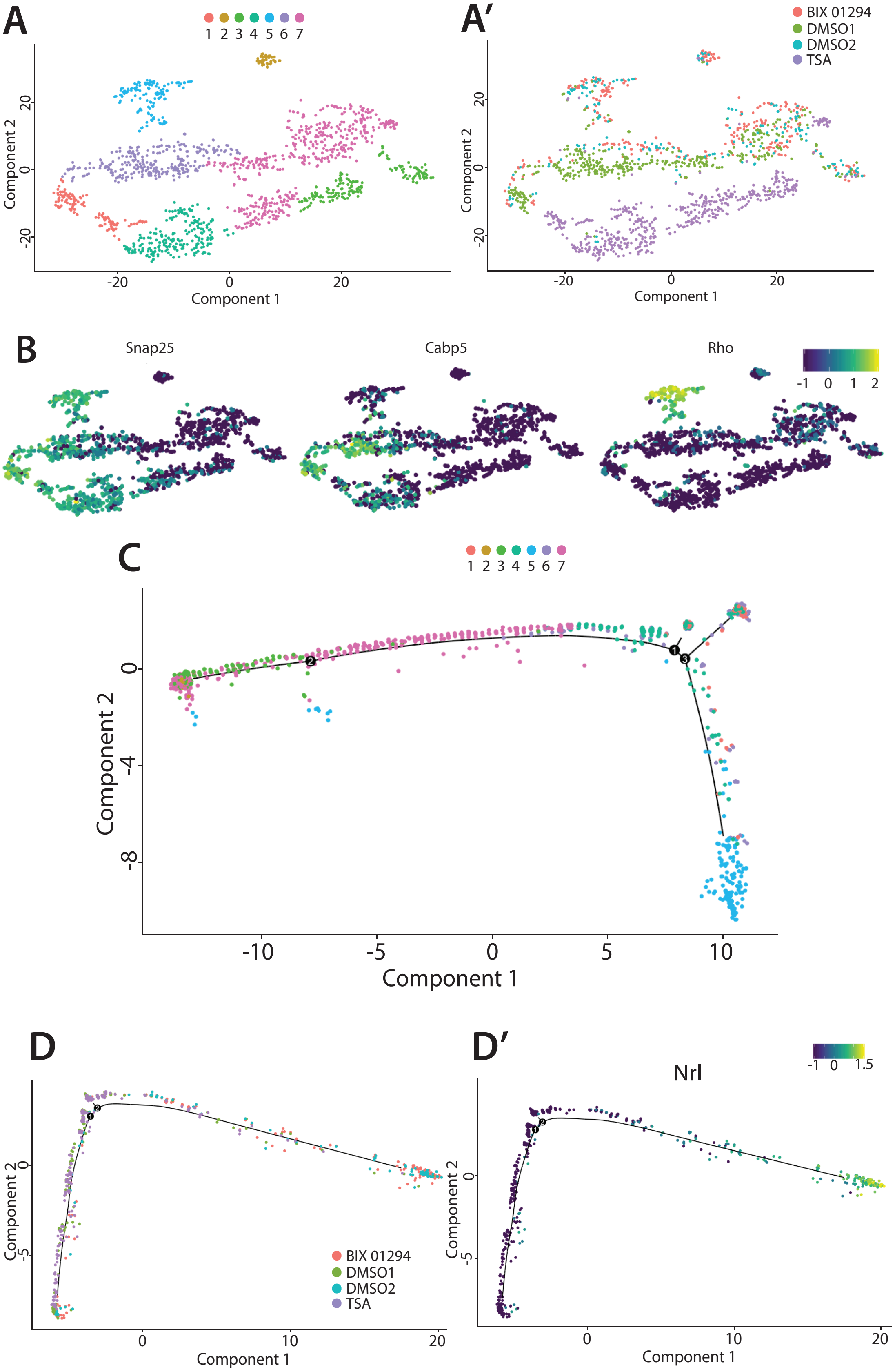
Specifically for TSA treated photoreceptor-like cells, I found that cells were reduced in expression of rod genes such as Rcvrn, and higher in Gngt2, with similar levels of Otx2 as control cells. Interestingly, Onecut2 was also higher in TSA treated cluster 4 cells (Figure 4.4 D). Transcriptomic analysis indicates that potentially MG-derived photoreceptors from TSA treatment are more characteristically cone-like, with reduced rod-related expression.

Reclustering these same cells with Monocle 2 (Qiu et al. 2017) generates the same four general types of cells with similar markers, though with less resolution for transition cell types (Figure 4.5 A-B). The generation of a pseudotime trajectory of these cells demonstrates a transition from MG to neuronal populations with MG at the earliest pseudotime, and neurons, particularly photoreceptors, at the latest time (Figure 4.5 C). I subsequently isolated out neuronal and transition-like clusters and performed additional trajectory analysis. In this isolated trajectory, the bipolar- and photoreceptor-like clusters were specifically isolated, with transition-like cells at the central branch point (Figure 4.5 D, D'). Overall, this analysis through Monocle demonstrates the robustness of the analysis for cell types. In addition, the trajectory analysis confirms the cell fate transition from MG to neurons, with transition-like cells as a bridge between bipolar and photoreceptor-like MG-derived neurons.

Conclusions

This chapter explores the ways in which the epigenomic profile of MG may direct cell fate decisions. It is apparent that glial accessibility profiles do not significantly change in their similarity to mature retinal neurons during the differentiation from progenitors to adult MG. However, Ascl1 may demonstrate preferential binding to bipolar accessible domains, thus directing fate decisions when reprogramming with this transcription factor. Additionally, *in vitro* modulation of the epigenomic landscape in young retinas using small molecule inhibitors alters cell localization and potentially alters fate decisions. This analysis characterizes the importance

Figure 4.5 Pseudotime Trajectories Demonstrate Neuronal Derivation
A.Monocle-generated plots DMSO and drug-treated Ascl1+ MG single cell RNA-seq represented by principal component analysis-generated clusters (A) and (A') experimental treatments. B. Plots showing expression levels of neuron-specific gene Snap25 (left), bipolar-specific Cabp5 (middle), and rod-specific Rho (right). C. Monocle generated trajectory of cells in (A) colored by cluster. Pseudotime 0 on left branch. D. Monocle generated pseudotime trajectory of only neuronal clusters from (A): 1, 4, 5, and 6, colored by drug treatment. D'. Expression level of Nrl along trajectory in (D).


A
1 2 3 4 5 6 7
Component 2
20
0
-20
-20
0
20
Component 1
A’
BIX 01294
DMSO1
DMSO2
TSA
Component 2
20
0
-20
-20
0
20
Component 1
B
Snap25
Cabp5
Rho
-1 0 1 2
C
1 2 3 4 5 6 7
Component 2
0
-4
-8
-10
-5
0
5
10
Component 1
1
2
3
D
Component 2
0
-5
0
10
20
Component 1
BIX 01294
DMSO1
DMSO2
TSA
1
2
D’
Nrl
-1 0 1.5
Component 2
0
-5
0
10
20
Component 1
1
2

of epigenomic landscapes in directing expression regulation during transdifferentiation.
In order to explore the restriction in neurogenic fate capabilities of MG to produce bipolar interneurons over rod photoreceptors, I compared progenitor and MG accessibility profiles to existing data for retinal neurons. This intriguingly does not show any major differences in the overall degree to which MG and progenitor accessible chromatin overlap with mature retinal neurons. Thus, the developmental differences in open chromatin between MG and progenitors as compared to retinal neurons does not explain the differences in neurogenic potential between the two cell types, nor does the degree to which the accessible chromatin of MG overlaps with rods versus bipolar cells explain the propensity of MG for generating bipolar cells over rods. However, in comparing bipolar-specific and rod-specific domains to each other, it is apparent that bipolar cells have a higher enrichment and affinity for bHLH binding, demonstrating more enrichment for predicted E-box domains. Aydin et al. previously described bHLH transcription factor specific E-box motifs CAGSTG and CAKATG which preferentially bind Ascl1 and Neurog2 respectively (Aydin et al. 2019). When analyzing enriched E-box motifs in retinal neurons here, we found no distinction in E-box specificity as previously described. Indeed, overexpression of Ascl1 in culture demonstrates a higher affinity of Ascl1 binding to bipolar cell specific accessible domains as compared to rods. Similar enrichment for E-box accessibility in a variety of bipolar cells was also shown by Murphy et al (Murphy et al. 2019). This may help to explain why the overexpression of Ascl1 in MG preferentially generates bipolar cells in vivo, though the factor is necessary for the generation of both bipolar cells and photoreceptors.

To alter the epigenomic landscape in conjunction with Ascl1-based reprogramming, I screened small molecule inhibitors targeting epigenetic modifiers on young explanted retinas overexpressing Ascl1 in the MG. Most small molecules did not generate a noticeable change in cell fate decisions as most GFP+ cells appear bipolar or MG in morphology. However, with more broadly acting drugs (Chang et al. 2009; Yoshida, Horinouchi, and Beppu 1995), the progeny of MG appear to change their migratory patterns. With both the HDAC inhibitor TSA as well as the G9a inhibitor BIX 01294 in ex vivo retinal explants, Ascl1-overexpressing cells migrate to the ONL. Single cell RNA-seq analysis of these cells in culture helped to characterize distinct cell populations, specifically bipolar cells, transition cells, and photoreceptors. Photoreceptors derived from TSA and BIX treatment had cone-like gene expression. Though it is promising to see photoreceptor-like cells in single cell analysis, there are concerns regarding neuronal contamination and doublets, especially as photoreceptor cells are present in the DMSO control conditions. Regardless, this analysis demonstrates the ways in which epigenomic modulation can alter cell fate decisions with Ascl1 overexpression.

Given the *ex vivo* results of epigenetic modification with Ascl1 reprogramming, I also attempted Ascl1 + TSA reprogramming *in vivo*. Ascl1 overexpression was induced at P6, and TSA was injected intravitreally at P8 to replicate prior TSA treatments. However, the *ex vivo* results did not replicate, and all GFP+ cells localized to the INL with bipolar cell morphology (Data not shown). This failure to replicate changes to fate decisions with Ascl1indicates that this reprogramming still requires additional factors. Given that explantation inherently acts as a damage model via severing the optic nerve, it is likely that damage would restore GFP+ cells to the ONL. However, traditional models of retinal damage are not effective on mouse retinas under P12. This would mean that in order to test the hypothesis that damage would alter cell fate decisions at this age, I would need to explore additional models. The microtubule destabilizer colchicine would be effective at inducing a damage response at a young age (Fischer, Morgan, and Stell 1999; Fischer and Reh 2002). This model of reprogramming would be a good future direction for this project. Alternatively, an exploration into the role of damage and its effects on the epigenomic landscape would be useful in illuminating the ways in which mammalian glia respond to damage and how that could affect neurogenesis responses. This would be a particularly interesting analysis in comparison to regenerative species such as the teleost fish.

In sum, the analysis in this chapter demonstrated the importance of the epigenomic landscape in directing transdifferentiation, and the ways in which the epigenome is not fully predictive. As seen in Chapter 3, native accessibility can direct transcription factor binding, as progenitors maintain open chromatin around E-box domains that direct neurogenesis. However, in this analysis, I found that while progenitors are more capable of generating photoreceptors, they do not have a significantly more overlap with a variety of mature retinal neurons. Instead, it is apparent that the pioneering activity of Ascl1 has an inherent preference for binding at bipolar cell domains. Despite this lack of inherent similarity to retinal neuron fates, I also found that broad changes to the epigenome can still direct fate decisions. Future explorations will be necessary to determine the role of damage on the epigenome and the ways in which this can direct fate.

Chapter 5

Discussion

Mammalian Regeneration Implies Epigenomic Differences

The focus of this dissertation centers around the roles of epigenomic changes in Müller Glia (MG) development, specifically as it pertains to retinal regeneration. Though mammalian retinal progenitor cells are known to have distinct similarities to MG, they have much more neurogenic potential. Progenitors are actively differentiating into retinal neurons, while MG maintain a consistent cell fate and are not known to generate new neurons without external stimulation in the mammalian system. Because of these changes to neurogenic potential, I sought to explore the epigenomic landscape to discover mechanisms of gene regulation in the mammalian retinal glia.

While mammalian retinas are non-neurogenic, other species are highly regenerative, such as the teleost fish. After damage, fish retinas undergo regeneration within three days (Lamba, Karl, and Reh 2008; Thummel et al. 2008). This regeneration occurs via the MG, which dedifferentiate into progenitor-like cells. This regeneration process is facilitated by a cascade of expression changes (Gemberling et al. 2013). Within the first 6 hours, the neurogenic bHLH transcription factor Ascl1 is highly upregulated in the MG (Ramachandran, Fausett, and Goldman 2010). Ascl1 expression in fish MG has been found to be essential for cell proliferation and regeneration as a whole (Fausett, Gumerson, and Goldman 2008).

By contrast, mammalian retinas are not inherently regenerative. When damaged, mammalian MG instead undergo a gliosis response to form a scar (Alunni and Bally-Cuif 2016). These cells do not express Ascl1 on their own in the case of damage or disease, though it is expressed in retinal progenitors and is necessary for the production of retinal neurons. Previous research has instead shown that the induction of expression of Ascl1 in MG can instead induce neurogenesis. Cultured P12 MG overexpressing Ascl1 will express both progenitor and neuronal genes, and take on a neuronal morphology (Pollak et al. 2013). *In vivo,* Ascl1 induces MG to transdifferentiate into primarily bipolar cells (Ueki et al. 2015; Jorstad et al. 2017). Over postnatal development, this neurogenesis is additionally limited, requiring further factors in order to induce MG transdifferentiation into bipolar cells (Jorstad et al. 2017). In all cases of induced regeneration in mammalian retinas, the primary neuron generated from MG with Ascl1 overexpression is the bipolar interneuron (Ueki et al. 2015; Jorstad et al. 2017). The current efforts at inducing mammalian regeneration indicates several restrictions to regeneration. Firstly, the ability for MG to become neurons is increasingly restricted as the MG mature. Secondly, primarily bipolar cells are generated regardless of mode of regeneration, demonstrating a lack in cell fate diversity.

There is a clear restriction in the ability of MG to generate a variety of retinal neurons with reprogramming factors. Ascl1-based regeneration in murine retinas consistently generates a population of neurons that predominantly consists of bipolar cells (Ueki et al. 2015; Jorstad et al. 2017). This is somewhat surprising considering that as late as P2 or P3, progenitor cells are differentiating into both bipolar and rod photoreceptor cells (Young 1985; Ohsawa and Kageyama 2008; Brzezinski and Reh 2015). The generation of both of these neurons is also facilitated by the activity of Ascl1, which indicates certain restrictions in the capability of MG to generate photoreceptors (Ohsawa and Kageyama 2008). Other groups have demonstrated potential neurogenesis from adult MG to rod photoreceptors using viral induction of expression of beta-catenin, followed by a triple factor cocktail of photoreceptor development genes Otx2, Crx, and Nrl (Yao et al. 2018). However, there are concerns regarding the cell specificity of the fluorescent marker used to label MG-derived rods. The use of a rhodopsin promoter to label new rods is concerning, given that if the reporter virus is not completely specific to MG, it will label existing rods in the retina. Additionally, the use of rod-related factors does not answer the question of why the MG are restricted in their ability to generate a variety of neurons with common regeneration factors such as Ascl1. In regenerative species, Ascl1 is a necessary initial factor to induce retinal regeneration, but the mammalian retina is clearly restricted.

There are several implications to the known developmental changes in MG-based neurogenesis that informed the directions of this dissertation. Given that progenitors and MG are known to be transcriptomically similar, but perform different functions in terms of retinal support and developmental neurogenesis (Jadhav, Roesch, and Cepko 2009; Nelson et al. 2011; Ohsawa and Kageyama 2008), it is clear that there are inherent differences in gene regulation during development. However, these changes do not appear to develop immediately upon MG birth from the common retinal progenitor, as young MG have a much higher potential for regeneration with minimal factors (Chapter 3). Though it is possible to generate neurons from all ages of MG given the right condition of factors, there are clear restrictions to cell fate decisions. With Ascl1-driven reprogramming, we have been unable to reliably generate rod photoreceptor cells. Postnatal progenitors are capable of generating both rod photoreceptors and bipolar cells (Young 1985; Ohsawa and Kageyama 2008), but after P4 photoreceptors are not generated given any combination of factors with Ascl1. Consequently, I anticipated that all MG would have inherent blocks to generation of photoreceptors that may be apparent in the accessible epigenome. These hypotheses also imply that changes to the epigenomic landscape may alter regenerative capacity.

For my analysis of mammalian glial epigenomic landscapes, I utilized FACS purification of progenitors, MG, and MG-derived neurons for analysis of the epigenome by ATAC-sequencing. I found that as MG develop from multipotent progenitors, they lose accessibility at neurogenesis-associated regulatory regions, and gain glial-associated regulatory accessibility, all of which correlates with expression changes. These changes occur sequentially over MG development, with young MG presenting with an intermediate epigenomic and transcriptomic profile, which may explain losses in regenerative capacity. In the case of adult MG regeneration with ANT treatment, accessibility around neurogenic genes increases, primarily around Ascl1 E-box domains. The fate decisions made during induced regeneration appear to be directed by the preference of Ascl1 for binding bipolar cell domains, but can be altered through broad changes to the epigenome with small molecule drugs.

Relevance of Epigenomics in Neuronal Regeneration

My results also implicate histone modifications in the restriction of young MG to generate neurons after Ascl1 overexpression. Inhibition of histone deacetylases (HDACs) in young retinas leads to an increase in the types of neurons that are generated from the MG, resembling bipolar cells and cone photoreceptors (Chapter 4). Histone acetylation is a key regulator of development and regeneration in neural tissues (VandenBosch and Reh 2019). Acetylation is associated with open chromatin and more active transcription, and as a result, changes in chromatin acetylation should precede the changes in gene expression that are necessary for regeneration (Kouzarides 2007). Studies in neural systems that spontaneously regenerate after injury support the hypothesis that histone acetylation is necessary for regeneration; however, an important caveat is that inhibitors of HDACs may have effects on cell proliferation that are independent of their effects on chromatin accessibility and transcriptional activation (Seto and Yoshida 2014; VandenBosch and Reh 2019). In addition to the caveat that HDACs act on proteins other than histones, a second caveat is that not all HDACs are the same, and that they participate in many different complexes depending on the other proteins present in the cell. Broad methods to modify the epigenome, such as small molecule HDAC inhibitors, affect many different HDACs and many different complexes, and it is difficult to know which of these are critical in any given context (Seto and Yoshida 2014). Thus, future studies focused on specific HDAC and HAT family members will undoubtedly provide a more nuanced view of the roles of acetylation. This conclusion is highlighted by the results on peripheral nerve regeneration and HDAC3 and HDAC5, where nuclear cytoplasmic shuttling provides an additional level of control (VandenBosch and Reh 2019). Despite these caveats, it is clear from even this limited number of examples that cellular reprogramming can be facilitated by manipulations in histone acetylation and thus will likely be important to consider in the development of regenerative therapies in sensory systems and CNS.

In addition to histone acetylation, methylation of histone tails also has clear roles in neuronal development. H3K27 trimethylation is historically the best-studied histone methyl mark and appears to broadly regulate transcription. Repressive histone modifications such as this correlate especially to the shutdown of early development and neurogenesis gene programs (VandenBosch and Reh 2019). In the retina, conditional deletion of Ezh2 (necessary for the repressive H3K27me3 modification) leads to a reduction in proliferation of the progenitors, likely via an increase in the cyclin dependent kinase p21cip (Zhang et al. 2015). Re-entry of glia into the mitotic cycle after injury may require Ezh2 to repress this inhibitory factor, and supplementing Ezh2 to glia could facilitate regenerative proliferation. These types of modifications also can contribute strongly to epigenetic memory and therefore can affect fate changes in regeneration in many tissue types, thus targeted rewriting of repressive histone modifications could be beneficial in future attempts to reprogram cells in efforts to regenerate neuronal tissue. However, inhibition of Ezh2 in MG did not increase the range of neuronal fates generated by the MG, suggesting that this form of epigenetic regulation is not as critical in the retina (Chapter 4).

DNA methylation acts as a much more stable and consistent repressor of gene expression as compared to histone modifications and thus may be more of a barrier to regeneration. During development, prosensory and proneural gene programs, while partially regulated by histone modifications, also appear to be repressed via DNA methylation (Singh et al. 2017). Importantly, DNA methylation appears to change in response to damage (C Powell, Elsaeidi, and Goldman 2012; Curtis Powell et al. 2013; C Powell, Cornblath, and Goldman 2014). Changes in DNA methylation accompany the spontaneous regeneration that occurs after injury to the fish retina, and functional studies support a role for demethylation in retinal regeneration in this species. However, it is not clear which genes needed for regeneration are repressed by this method, since the pluripotency and regeneration-associated gene promoters are not among those demethylated after injury. Thus, in non-regenerative systems, the lack of appropriate changes in DNA methylation may prevent expression of regeneration-associated genes. To date there have been relatively few studies that test this hypothesis, though the availability of small molecule DNMT inhibitors should facilitate in vivo testing.

In addition to the general regulators of the epigenome discussed above, the role of transcription factors in regulating gene expression changes cannot be overstated. While many studies have demonstrated that repressive chromatin can prevent gene expression, transcription factors can engage the genome and under the proper circumstances, reactivate genes in regions of heterochromatin (C Powell, Elsaeidi, and Goldman 2012; Wan and Goldman 2016; Reyes-Aguirre and Lamas 2016). During fate changes and regeneration, the most dynamic changes to the epigenome appear to be in enhancer regions where transcription factors are binding (Curtis Powell et al. 2013). Thus, transcription factor activity regulates fate changes beyond simple expression via changes to the epigenome. Some transcription factors that are particularly effective at stimulating reprogramming, such as Sox2 and Ascl1 are particularly effective in inducing neuronal fate changes because they induce major chromatin remodeling (Wapinski et al. 2017). These so-called pioneering transcription factors likely interact with chromatin remodelers to regulate the accessibility of their gene targets, and as a result the epigenetic state changes can be driven by transcription factors.

While individually, many components discussed here can change cellular fate and be beneficial in attempts for regeneration, a combinatorial approach is most likely to be effective in creating the most effective regeneration program for any given neuronal tissue (Vierbuchen et al. 2010; Ohori et al. 2006). By using neurogenic pioneering transcription factors, somatic cells may take advantage of native developmental pathways that act in targeted manners to change transcriptional programs and effect chromatin remodeling changes; however, the manipulation of chromatin remodelers to increase accessibility at regeneration-associated genes can alter and improve regeneration both in vitro and in vivo (Wapinski et al. 2017; Jorstad et al. 2017). Thus, a combination of neurogenic transcription factor expression with targeted epigenetic remodeling may produce the best results in generating new neurons in diseased and damaged tissue.

Müller Glial Development is Characterized by Changes to Regulatory Region Accessibility

As anticipated by prior characterization demonstrating transcriptomic similarities between progenitors and MG (Jadhav, Roesch, and Cepko 2009; Nelson et al. 2011), the two cell types have additionally similar open chromatin regions. Regions surrounding cell cycle genes have similar accessibility in both postnatal progenitors as well as in mature MG, and progenitors demonstrate early accessibility near glial-related genes. Despite these differences, regions of accessibility that change in development reveal the ways in which the epigenome changes around putative regulatory elements. Lost accessibility was found to be enriched around Nervous System Development genes, in line with the neurogenic potential of postnatal progenitors. These areas of decreased accessibility are additionally enriched for predicted E-boxes, which bind neurogenic bHLH transcription factors in development. This neurogenic accessibility in progenitors is reflected by high neuronal gene expression in progenitors that is lost by adulthood. Overall, both transcriptomics and chromatin accessibility point to a loss in neurogenic potential including around regulatory regions.

In exchange, as glia develop there are similar gains in accessibility. Adult MG-enriched accessible domains are predicted to contain binding domains for NFI transcription factors. This enrichment is consistent with correlating changes in expression, indicating that these NFI domains likely regulate glial gene expression. This is consistent with evidence from other groups which shows that NFI transcription factors are necessary for the development of glial cells such as astrocytes in the central nervous system (Shu et al. 2003; Heng et al. 2015; Deneen et al. 2006). Similarly in the retina, changes to expression of NFI transcription factors in triplicate alters the differentiation of late born retinal cells, including both MG and bipolar cells (Clark et al. 2019). The changes in accessibility for NFI binding sites as MG develop could point towards a regulatory mechanism that may direct regeneration to similar late born cells, or otherwise limit regenerative capabilities. Future experiments will explore regeneration in the context of NFI knockdowns in an attempt to alter fate decisions or otherwise improve regeneration.

Young Müller Glia Retain Progenitor-like Accessibility and Expression

The distinct losses in regenerative capacity as MG age spurred an investigation into the epigenome of younger MG with more neurogenic potential. Analysis by both RNA- and ATAC-seq reveals a clear intermediate state of younger MG. Expression analysis demonstrated that while MG-specific genes increase in expression early, Progenitor-specific genes retain expression as well. In particular, genes related to the generation of neurons continue to be expressed at P8 and decrease slowly as MG develop into adulthood. This is of note as P8 MG are not inherently neurogenic, though they do have increased neurogenic potential with induced overexpression of Ascl1. Correspondingly, chromatin accessibility analysis reveals a chromatin profile that is similar to both progenitors and MG. Accessible domains enriched in young MG as compared to adult MG are found nearby neuronal genes indicating a retention of accessibility that could inform neurogenic potential. There is some presence of E-Box binding domains, as well as early occurrences of NFI binding sites. The changes to immature P8 MG correlate with higher neurogenic potential seen with Ascl1-based reprogramming, and as neurogenic accessibility and expression decreases, induced regeneration becomes harder to achieve.

Adult Reprogramming Reopens Neural Chromatin

Beyond normal development of MG, I also explored the changes of chromatin landscape with reprogramming of the adult MG. The ANT treatment consists of Ascl1 in tandem with DMA damage and TSA HDAC inhibition, which induces neurogenesis to create bipolar cells (Jorstad et al. 2017). Partial combinations of these treatments do not induce neurogenesis as shown by histological analysis. In my ATAC analysis of complete and partial treatments, I found that all treatments are associated with increases in accessibility around neural genes. These neural associations in the accessible epigenome increased with additional factors, with the full ANT treatment having the largest number of genes in Neural Development and Neural Differentiation GO categories, implying a potential threshold of neural accessibility that allows for neurogenesis. This theory for regeneration also complements my finding that younger MG with more neurogenic potential have more accessibility around neural genes.

I also compared the results of adult reprogramming to bipolar cells, as this is the closest retinal neuron class to what our regeneration paradigm generates. I did find that epigenetically, ANT treated cells overlap well with Grm6+ bipolar cells, specifically around neuronal genes.

This overlap also includes retinal-specific Otx2 binding sites. This, in conjunction with the single cell RNA-seq data from Nik Jorstad (Jorstad et al. 2017), confirms that the neurons generated from the ANT regeneration paradigm are bipolar in phenotype.

However, these analyses presented some challenges that will require novel solutions. Primarily, I was limited by the bulk nature of ATAC used at the time this study originated. Though with the developmental study I was able to work with pure populations of a single cell type, after reprogramming factors have been added, sorted cells contain multiple cell fates. Single cell RNA-seq analysis demonstrates two specific populations of progenitor-like MG, and bipolar-like cells. However, when analyzed in bulk, the analysis loses the distinction between different cell fates, as well as potential transition cell states, if analysis were to be performed before final differentiation. More recently, a number of methods have come to the forefront that allow for single cell analysis of chromatin accessibility (Packer and Trapnell 2018). Future analyses of mixed populations such as regeneration paradigms from MG should include single cell techniques in order to form this distinction and determine the precise epigenomic landscape generated through reprogramming paradigms.

Direction of Fate in Regeneration is Complex

As all examples of induced mammalian regeneration using Ascl1 result in the generation of bipolar cells, I explored potential explanations for bias in fate decisions by MG. While postnatal progenitors are capable of generating both bipolar cells and rod photoreceptors utilizing the expression of Ascl1 (Young 1985; Ohsawa and Kageyama 2008), Ascl1 overexpression in all MG generates only one cell type (Chapter 3, Ueki et al. 2015; Jorstad et al. 2017). Comparisons of progenitors and MG to mature retinal neurons revealed that both postnatal progenitors and mature MG overlap retinal neurons very similarly. The lack of distinct differences between cells over development as they relate to neurons implies that the development of the total epigenome does not significantly change in a way that changes fate determination. However, this does not make comparisons to neuron precursors, and thus further analysis should be done. Instead, I found that Ascl1 when overexpressed, has an inherent bias towards binding to bipolar-specific accessible domain, indicating a bias in fate decisions based on Ascl1 pioneering activity.

However, though broad comparisons between glia and neuron epigenomes was not revealing of extensive developmental changes that inform fate decisions, inhibition of epigenetic modulators was successful in altering reprogramming outcomes. While most small molecules used did not appear to alter fates in young explanted retinas, the HDAC inhibitor Trichostatin-A, and the G9a inhibitor Bix-01294 encourage cell localization to the outer nuclear layer among photoreceptors. Both of these small molecule drugs act broadly on the epigenome (Chang et al. 2009; Yoshida, Horinouchi, and Beppu 1995), suggesting that nonspecific, broad changes to the chromatin landscape are more permissive of alternate fate decisions. However, these results could not be replicated *in vivo* using only Ascl1 overexpression and intravitreally injected small molecules. This may be due to the fact that explantation inherently behaves like a damage model, attesting to the apparent role of damage in inducing regeneration.

Future Directions

In view of the results and limitations of the studies presented in this dissertation, there are several directions this project could take in the future. In order to pursue further directions from the results found, it would be beneficial to begin probing transcription factors based on those found to be differentially accessible. In particular, factors such as the NFI class of transcription factors appear to play a role in fate determination, and thus exploring its roles in induced regeneration. Knockouts by CRISPR combined with Ascl1 overexpression may alter regeneration responses. Some current results also point toward altered regeneration in young MG, but do not replicate *in vivo*. Because of this, it would be of interest to pursue replication. To directly attempt to replicate *ex vivo* results with small molecule drugs, it will likely be important to explore modes of damage, as *ex vivo* studies of regeneration inherently behave as a damage model. Previous *in vivo* data with Ascl1-based reprogramming have established that damage contributes greatly to induction of regenerative phenotypes (Ueki et al. 2015; Jorstad et al. 2017). Unfortunately, traditionally utilized modes of damage in the mouse retina are only effective in older retinas. Thus, to test epigenetic inhibitors in young mice, it will be necessary to use alternative methods such as microtubule destabilization by colchicine which work well in younger retinas (Fischer, Morgan, and Stell 1999; Fischer and Reh 2002). Beyond this exploration, it would be germane to explore the ways in which damage models affect the glia with and without Ascl1 overexpression. Collectively, these proposed directions may uncover more of the mechanism of fate restriction and induced regeneration.

Additionally, future analysis of chromatin accessibility within the mammalian retina must explore more advanced techniques. In particular, when inducing regenerative responses, A variety of cell fates result. Even when utilizing FACS purification of cells, the resulting purified population is heterogeneous. When analyzing transcriptomics, this problem has been thoroughly solved through the many well-vetted methods of single cell RNA-seq and subsequent analysis pipelines. However, single cell chromatin analysis through ATAC and ChIP-seq is still new (Packer and Trapnell 2018). Single cell ATAC-seq is now available through the 10x platform, and research groups such as the Shendure and Ren labs have made many advances in analysis software and pipelines for scATAC techniques (H. Chen et al. 2019). Taking into account these advances, it would be of great value to utilize single cell ATAC-sequencing to analyze chromatin accessibility in heterogeneous populations and combine this data with related scRNA-seq. Aside from regenerative scenarios, this technique could easily be applied to retinal development. Chromatin analysis at a single cell level up until P4 would allow for discernment of transition states and neuronal precursors for further developmental analysis. Utilization of cutting-edge techniques on regeneration and development would allow for a fuller analysis of mechanism and gene regulation.

Conclusions

This dissertation has focused primarily on explorations into epigenetic states and the ways it changes with development and stimulated regeneration. I have explored this subject from three angles: development, adult regeneration, and biases in fate determination. Developmental analysis directly demonstrated epigenetic and transcriptomic correlative changes as MG develop. Accessibility around neuronal gene categories decreased over development, but was retained briefly in young MG. Similarly, upon differentiation, MG gained accessibility at binding sites for transcription factors that regulate late born cell fates. Both of these details point to an intermediate state in young MG that may allow for easier reprogramming in young retinas. Similarly, in adult reprogramming, regeneration factors contributed to the opening of chromatin around neural—and particularly bipolar-associated—genes and related regulatory elements. This role of neurogenic accessibility conferring regenerative capabilities in MG is reflected in the finding that broad chromatin restructuring drugs appear to be capable of redirecting cell localization *ex vivo*. However, analyses of progenitors and MG in comparison to mature retinal neurons does not expose an epigenomic bias in progenitors for generating a variety of retinal neurons. Resulting questions from this analysis lead us towards potential analyses into the roles of damage on MG and to single cell chromatin analyses of induced regeneration and early postnatal development. From this dissertation, I conclude that the epigenomic accessibility of MG aids in the direction of regenerative capability, but more work is necessary to understand fate direction in the context of regeneration and development.

Methods

Mice

All mice were housed at the University of Washington and treated with protocols approved by the University of Washington's Institutional Animal Care and Use Committee. Mice expressed Cre-recombinase under the *Glast* promoter from Jackson Labs with *Rosa-flox-stop-tTA* (Jackson labs) in combination with either *tetO-mAscl1-ires-GFP* (M. Nakafuku University of Cincinnati) or CCGFP. Mice having EGFP knocked-into the Sox2 open reading frame were obtained from Jackson Laboratories (Stock: 017592) and bred to generate P2 litters(Arnold et al. 2011). *Rlbp1CreERT2* mice were crossed to *R26-stop-flox-CAG-tdTomato* mice (Jackson Labs, also known as Ai14; 129SvJ background). Mice of both sexes were used for this study and analyzed together in their respective treatment groups.

Injections

Intraperitoneal injections of 50 µl of tamoxifen (Sigma) at 100 mg/kg in corn oil were given to induce expression of Ascl1 and GFP. Tamoxifen was administered once for P0 or P4 induction, for 2 consecutive days in mice aged P6-P9, or 4 consecutive days for older ages.

IHC and cell counts

For lineage tracing with GFP and Ascl1 induction, animals were euthanized and the eyes removed for dissection and removal of the cornea and lens. Eyes were then fixed in 4% PFA in PBS for 1 h before being transferred to a 30% sucrose in PBS solution and kept overnight at 4 °C. Eyes were then frozen at −80 °C in O.C.T. (Sakura Finetek) and sectioned to 14µm by cryostat (Leica). Slides were incubated at room temperature in blocking solution (10% normal horse serum, 0.5% Triton X-100, in PBS) for 1 h. Slides were then incubated overnight at 4 °C in primary antibody diluted in blocking solution. Slides were then washed three times in PBS for 15 minutes each before a 1 h room temperature incubation in secondary antibodies (Life Technologies) in PBS. Slides were washed once before being incubated 5-10 minutes with 1:10,000 DAPI (Sigma) in the dark. At this point, slides were washed three times in PBS and coverslipped with Fluoromount-G (SouthernBiotech). Primary antibodies: goat anti-Sox2 (Sanda Cruz, 1:500), mouse anti-HuC/D (Invitrogen, 1:100), chicken anti-GFP (Abcam, 1:500), goat anti-Otx2 (R&D Systems, 1:100), rabbit anti-Recoverin (Millipore, 1:1000).

Section imaging was performed using an Olympus Fluoview confocal microscope, and random fields throughout the retina were sampled for cell counts. Cell types were identified and counted by localization within the retina, cell morphology, and marker co-staining. Significance values between treatments were determined by one-way ANOVA with a post-hoc tukey test or by t-test.

Explant Culturing

Whole dissected P5 retinas were cut halfway to the center in order to lay flat. Flattened retinas were cultured in 6-well tissue culture plates on top of 0.4 um Millicell cell culture inserts (Millipore PICM03050). Culture inserts were place in 1.5 ml Neurobasal media with 1% FBS (Clontech), 1 mM L-glutamine (Invitrogen), N2 supplement (Invitrogen), 1% Penicillin-Streptomycin (invitrogen), with 1.5 ul DMSO or 1000x drug in DMSO. Explanted retinas were cultured for 5-6 days before fixing for 30 minutes in 4% Paraformaldehyde and rehydrating overnight in 30% sucrose. Fixed explanted retinas were embedded in OCT (Tissue-Tek) for cryosectioning and IHC.

FACS

FACS protocol according to (Wohl and Reh 2016)

Retinas were isolated via dissection away from surrounding tissues and then washed in PBS. Fluorescence was confirmed via live imaging under an inverted fluorescent microscope (Zeiss). Retinas were then incubated on a nutator in Papain and DNase I for 10 min at 37 °C. Retinas were then triturated to generate a single-cell suspension and transferred to a tube containing an equal volume of Ovomucoid. The suspension was then spun down at 300*g* for 10 min at 4 °C. Cells were resuspended in a solution of 100:1:1 Neurobasal:DNase:Ovomucoid, and passed through a 35 μm filter before sorting using a BD FACSAria III Cell Sorter (BD Biosciences). Gating was performed to isolate intact cells from debris and to isolate positive fluorescent glial or progenitor cells. Progenitor cells were isolated from Sox2+ Amacrine cells by removing the higher fluorescent neuronal population. Positive fractions containing fluorescently labelled MG were then spun down at 300*g* and resuspended for the appropriate assay.

ATAC

Purified cells from live-cell FACS were input into a 15 µl transposase reaction with an input of 100k cells in a protocol modified from the Greenleaf lab (Buenrostro, Wu, Chang, et al. 2015). Transposition was carried out with reagents from the Nextera DNA Sample prep kit: 7.5 µl 2X TD Buffer, 0.75 µl Tn5 Transposases, and nuclease-free water to 15 µl. The reaction was mixed and incubated at 37 °C for one hour before being purified with the Qiagen Reaction Cleanup Minelute kit and eluted into 10 µl. Libraries were prepared through subsequent PCR using Illumina Nextera kit (Cat. No. FC-121-1030) using a test qPCR output to estimate the number of cycles necessary to properly amplify the library. Amplified libraries were purified with the Qiagen PCR cleanup minelute kit and eluted into 20 µl. Library QC was performed using gel electrophoresis, and quantitated on a Qubit 3.0 Fluorometer with the dsDNA HS Assay kit and A260/280 and A260/230 checked by nanodrop before sending for Illumina NextGen sequencing on a Next Seq 500 in rapid mode employing a paired-end 50 base read length sequencing strategy (Seattle Genomics). Adapter and barcode sequence were removed from the reads and low-quality sequences (Phred score <33) were removed using Trim Galore (Krueger 2015). Remaining reads were mapped using Bowtie2 (Langmead and Salzberg 2012), marking duplicate reads with Picard (http://broadinstitute.github.io/picard/), and removing reads using Samtools (Li et al. 2009). Alignment data was normalized for coverage using a custom R script (https://rpubs.com/achitsaz/98857) and visualized using the Integrated Genomics Viewer(J. T. Robinson et al. 2011)

Peak calling and comparison

Peaks were called using HOMER (Heinz et al. 2010) findPeaks dnase style with a minimum distance of 415 and size of 150. Bedops -e 1 and -n 1 functions were used to compare peak files for binary peak differences (Neph et al. 2012). For differential accessibility comparisons, the R package EdgeR (M. D. Robinson, McCarthy, and Smyth 2010) was used to compare all peak regions between two ATAC samples against the reads of each file as previously described. The RSubread (Liao, Smyth, and Shi 2019)function featureCounts was used to generate a matrix of counts per million across all peaks. The counts matrix was filtered against low-reads rows and in the case of any sample that is more deeply sequenced than another, the EdgeR (M. D. Robinson, McCarthy, and Smyth 2010) function thincounts was used to thin one sample randomly to the level of the lower depth of sequencing. Dispersion, fitting and differential signal testing were performed using negative binomial generalized linear models as specified in the edgeR guide. Cumulative differences in accessibility at each gene were calculated as the sum of the fold differences of all peaks nearest to each gene. Peaks of interest were identified by selecting for those with a log2FC above 2, and the top 1k peaks up and down were selected by log2CPM. The peakIDs for each of these regions were used to generate new peak files and perform further analysis.

Ascl1 Chromatin Immunoprecipitation-Sequencing (ChIP-Seq):

P0 retinas or cultured, post-natal day 12, Müller glia (+/- Ascl1 overexpression, rtTA germline:tetO-Ascl1-ires-GFP mice ± doxycycline) were digested with papain/DNase to single cells and fixed with 0.75 % formaldehyde for 10 minutes at room temperature. Sonication was performed with a probe sonicator (Fisher Scientific): 12 pulses, 100 J/pulse, Amplitude: 45, 45 seconds cooling at 4 ºC between pulses. Immunoprecipitation performed with 40 μL anti-mouse IgG magnetic beads (Invitrogen Cat: 110.31) and 4 μg mouse anti-MASH1 antibody (BD Pharmingen Cat: 556604) or 4 μg mouse IgG against chromatin from 5 million cells per condition according to Diagenode LowCell Number Kit using IP and Wash buffers as described in (Castro et al. 2011). Libraries were prepared with standard Illumina adaptors and sequenced to an approximate depth of 36 million reads each. Sequence reads (36 bp) were mapped to the mouse mm9 genome using bwa (v 0.7.12-r1039). Merging and sorting of sequencing reads from different lanes was performed with SAMtools (v1.2). The HOMER software suite was used to determine and score peak calls (‘findPeaks’ function, v4.7) as well as motif enrichment (‘findMotifs’ function, v4.7, using repeat mask). For STATi and control Ascl1 ChIP-seq, reads were aligned to the mm10 genome using Bowtie2. The .sam files were converted to sorted .bam files using SAMtools. MACS2 was used to call peaks with default settings using the broad peaks annotation. Peak overlap analyses were performed using Bedops. The control Ascl1 ChIP-seq .bam file was downsampled by a factor of 0.69 to normalize the number of mapped reads over the common peaks found between treatment and control samples. This downsampled .bam file was used for all analyses. Differential accessibility analysis in Ascl1 ChIP-seq peaks was determined using edgeR as detailed in the edgeR user guide.

Bulk RNA seq

For RNA-Seq, FAC-sorted cells were resuspended into Qiazol and RNA was extracted using the Qiagen RNeasy MinElute kit. Samples were tested for QC using nanodrop, and sent for sequencing. 500 ng per sample (50 ng μl^{-1}) was sequenced on an Illumina HiSeq and reads that passed Illumina's base call quality filter were mapped to mm10 using TopHat v2.0.12. To generate counts for each gene using htSeq-count v0.6.1p1, in "intersection-strict" overlap mode, genes with zero counts across all samples were removed, and data normalized using edgeR v3.12.0. Further analysis was done using Bioconductor and R (version 3.2.3).

EdgeR was used to compare reads across RNA-seq samples according to the published manual guide for exactTest(M. D. Robinson, McCarthy, and Smyth 2010). Prior to testing, samples with higher reads were thinned to the level of other samples using thinCounts. Dispersion, fitting and differential signal testing were performed using negative binomial generalized linear models as specified in the edgeR guide. Differential expression was filtered to generate the top 1k genes up and down across development by selecting for genes with a logFC greater than two, and top genes of this category were selected by highest log2CPM. The top genes were clustered using hclust to perform ward D2 agglomeration with Euclidian distances. Genes were filtered against annotated ATAC peaks by dplyr.

Single Cell RNA-seq

Single cell RNA-seq was performed according to Jorstad et al.

Explanted Glast-CreER;LNL-tTa;CC-GFP;Ascl1-IRES-GFP retinas were dissociated with papain and DNase and were processed for FACS purification of GFP+ cells. Live cells were pelleted at 300g for 10 minutes at 4 °C before resuspension in 0.04% BSA in Phosphate Buffered Saline at an approximate concentration of 2,000 cells per ul. Cell suspension was loaded onto the Single Cell 3' Chip with a targeted cell recovery of 4,000 cells. GEM generation and barcoding, RT, cleanup, cDNA amplification, and library construction were performed according to the Chromium Single Cell 3′ Reagent Kits User Guide. Library QC was determined by TapeStation (Agilent Technologies). Single-cell libraries were sequenced on the Illumina NextSeq 500/550 v2 kit. Reads were processed in Cell Ranger (10x Genomics) and aligned to mm10. Filtered cell outputs were analyzed with Seurat v2.2.1 and Monocle 2 according to published tutorials for cell mapping, clustering, and differential expression analysis.

Homer annotatePeaks.pl

For basic annotation of ATAC peaks, the Homer function annotatePeaks.pl was used (Heinz et al. 2010). While in most cases, peak annotation to genes was accomplished with the online tool GREAT (McLean et al. 2010), annotating each region for closest gene, the basic usage was used:

```
annotatePeaks.pl <peak file> mm9 > output.txt
```

For stats on genome location:

```
annotatePeaks.pl <peak file> mm9 -annStats > output_stats.txt
```

For annotation of specific motifs, a position weight matrix was either taken from the homer database, from the findmotifs function, or was generated using seq2profile.pl in Homer, and was used in annotatePeaks as such:

```
annotatePeaks.pl <peak file> mm9 -m pwm.motif > output_motif.txt
```

However, for the generation of lineplots, a table of 10bp bins for motif enrichment were generated for graphing with ggplot2:

```
annotatePeaks.pl <peak file> mm9 -m pwm.motif -size 2000 -hist 10 > output_plot.txt
```

For motif co-occurrence:

```
annotatepeaks.pl <peak file> mm9 -size 2000 -hist 20 -m <motifs of interest> -cpu 10 -matrix fileout > fileout.motif.freq
```

Homer Motif discovery

To discover enrichment of predicted DNA binding motifs for further analysis, we employed the Homer function findMotifsGenome.pl using suggested basic usage settings. From there, we identified top motifs from the homerResults output

Gene Ontology

For annotation of gene ontology (GO) categories, we used the Bioconductor package GOstats (Falcon and Gentleman 2007). Gene lists from ATAC and RNAseq were input to a hyperGTest with a p value cutoff of 0.001 for Biological Process ontology categories. The top 20 GO terms were plotted in ggplot2. To acquire genes of interest from specific GO categories, we found annotations of the category ID on the Jax Mouse Genome Informatics database and subset our original genelist based on the genes in each GO category.

Heat maps and plots (deeptools)

To generate heat maps and lineplots of ATAC and ChIP read enrichment, we used deeptools (Ramírez et al. 2016) computeMatrix reference-point to calculate enrichment scores by region along a bed file:

```
computeMatrix reference-point -S <bigwig files> -R <bed files> --referencePoint center -a 1000
-b 1000 –skipZeros – MAT_file.tab.gz
```

This is then plotted with the command:

```
plotheatmap -m MAT_file.tab.gz -out HM_file.png –colorMap Blues –missingdatacolor 1.0
```

Or, to plot the lineplots only:

```
plotProfile -m MAT_file.tab.gz -out Plot_file.png –yMax 3.0
```

References

Al-Zamil, Waseem M, and Sanaa A Yassin. 2017. “Recent Developments in Age-Related Macular Degeneration: A Review.” *Clinical Interventions in Aging* 12: 1313–30. https://doi.org/10.2147/CIA.S143508.

Aldiri, Issam, Beisi Xu, Lu Wang, Xiang Chen, Daniel Hiler, Lyra Griffiths, Marc Valentine, et al. 2017. “The Dynamic Epigenetic Landscape of the Retina During Development, Reprogramming, and Tumorigenesis.” *Neuron* 94 (3): 550-568.e10. https://doi.org/10.1016/j.neuron.2017.04.022.

Alunni, Alessandro, and Laure Bally-Cuif. 2016. “A Comparative View of Regenerative Neurogenesis in Vertebrates.” *Development (Cambridge, England)* 143 (5): 741–53. https://doi.org/10.1242/dev.122796.

Arnold, Katrin, Abby Sarkar, Mary Anna Yram, Jose M Polo, Rod Bronson, Sumitra Sengupta, Marco Seandel, Niels Geijsen, and Konrad Hochedlinger. 2011. “Sox2(+) Adult Stem and Progenitor Cells Are Important for Tissue Regeneration and Survival of Mice.” *Cell Stem Cell* 9 (4): 317–29. https://doi.org/10.1016/j.stem.2011.09.001.

Aydin, Begüm, Akshay Kakumanu, Mary Rossillo, Mireia Moreno-Estellés, Görkem Garipler, Niels Ringstad, Nuria Flames, Shaun Mahony, and Esteban O. Mazzoni. 2019. “Proneural Factors Ascl1 and Neurog2 Contribute to Neuronal Subtype Identities by Establishing Distinct Chromatin Landscapes.” *Nature Neuroscience* 22 (6): 897–908. https://doi.org/10.1038/s41593-019-0399-y.

Bermingham-McDonogh, Olivia, and Thomas A Reh. 2011. “Regulated Reprogramming in the Regeneration of Sensory Receptor Cells.” *Neuron* 71 (3): 389–405. https://doi.org/10.1016/j.neuron.2011.07.015.

Bernstein, Bradley E., Tarjei S. Mikkelsen, Xiaohui Xie, Michael Kamal, Dana J. Huebert, James Cuff, Ben Fry, et al. 2006. “A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells.” *Cell* 125 (2): 315–26. https://doi.org/10.1016/J.CELL.2006.02.041.

Bertrand, Nicolas, Diogo S. Castro, and François Guillemot. 2002. “Proneural Genes and the Specification of Neural Cell Types.” *Nature Reviews Neuroscience* 3 (7): 517–30. https://doi.org/10.1038/nrn874.

Blackshaw, S, S Harpavat, J Trimarchi, L Cai, H Huang, W P Kuo, G Weber, et al. 2004.

"Genomic Analysis of Mouse Retinal Development." *PLoS Biol* 2 (9): E247. https://doi.org/10.1371/journal.pbio.0020247.

Bourne, Rupert R A, Seth R Flaxman, Tasanee Braithwaite, Maria V Cicinelli, Aditi Das, Jost B Jonas, Jill Keeffe, et al. 2017. "Magnitude, Temporal Trends, and Projections of the Global Prevalence of Blindness and Distance and near Vision Impairment: A Systematic Review and Meta-Analysis." *The Lancet Global Health* 5 (9): e888–97. https://doi.org/10.1016/S2214-109X(17)30293-0.

Bringmann, A, I Iandiev, T Pannicke, A Wurm, M Hollborn, P Wiedemann, N N Osborne, and A Reichenbach. 2009. "Cellular Signaling and Factors Involved in Muller Cell Gliosis: Neuroprotective and Detrimental Effects." *Prog Retin Eye Res* 28 (6): 423–51. https://doi.org/10.1016/j.preteyeres.2009.07.001.

Brzezinski, Joseph A, Euiseok J Kim, Jane E Johnson, Thomas A Reh, and Thomas A. Reh. 2011. "Ascl1 Expression Defines a Subpopulation of Lineage-Restricted Progenitors in the Mammalian Retina." *Development (Cambridge, England)* 138 (16): 3519–31. https://doi.org/10.1242/dev.064006.

Brzezinski, Joseph A, Deepak A Lamba, Thomas A Reh, and Thomas A. Reh. 2010. "Blimp1 Controls Photoreceptor versus Bipolar Cell Fate Choice during Retinal Development." *Development (Cambridge, England)* 137 (4): 619–29. https://doi.org/10.1242/dev.043968.

Brzezinski, Joseph A, and Thomas A Reh. 2015. "Photoreceptor Cell Fate Specification in Vertebrates." *Development (Cambridge, England)* 142 (19): 3263–73. https://doi.org/10.1242/dev.127043.

Brzezinski, Joseph A, Ko Uoon Park, Thomas A Reh, and Thomas A. Reh. 2013. "Blimp1 (Prdm1) Prevents Re-Specification of Photoreceptors into Retinal Bipolar Cells by Restricting Competence." *Developmental Biology* 384 (2): 194–204. https://doi.org/10.1016/j.ydbio.2013.10.006.

Buenrostro, Jason D., Beijing Wu, Ulrike M. Litzenburger, Dave Ruff, Michael L. Gonzales, Michael P. Snyder, Howard Y. Chang, and William J. Greenleaf. 2015. "Single-Cell Chromatin Accessibility Reveals Principles of Regulatory Variation." *Nature* 523 (7561): 486–90. https://doi.org/10.1038/nature14590.

Buenrostro, Jason D, Beijing Wu, Howard Y Chang, and William J Greenleaf. 2015. "ATAC-Seq: A Method for Assaying Chromatin Accessibility Genome-Wide." *Current Protocols in*

Molecular Biology 109 (January): 21.29.1-9. https://doi.org/10.1002/0471142727.mb2129s109.

Butler, Andrew, Paul Hoffman, Peter Smibert, Efthymia Papalexi, and Rahul Satija. 2018. “Integrating Single-Cell Transcriptomic Data across Different Conditions, Technologies, and Species.” *Nature Biotechnology 2018 36:5* 36 (5): 411–20. https://doi.org/10.1038/nbt.4096.

Casey, Bradford H, Rahul K Kollipara, Karine Pozo, and Jane E Johnson. 2018. “Intrinsic DNA Binding Properties Demonstrated for Lineage-Specifying Basic Helix-Loop-Helix Transcription Factors.” *Genome Research* 28 (4): 484–96. https://doi.org/10.1101/gr.224360.117.

Castro, Diogo S, Ben Martynoga, Carlos Parras, Vidya Ramesh, Emilie Pacary, Caroline Johnston, Daniela Drechsel, et al. 2011. “A Novel Function of the Proneural Factor Ascl1 in Progenitor Proliferation Identified by Genome-Wide Characterization of Its Targets.” *Genes & Development* 25 (9): 930–45. https://doi.org/10.1101/gad.627811.

Chang, Yanqi, Xing Zhang, John R Horton, Anup K Upadhyay, Astrid Spannhoff, Jin Liu, James P Snyder, Mark T Bedford, and Xiaodong Cheng. 2009. “Structural Basis for G9a-like Protein Lysine Methyltransferase Inhibition by BIX-01294.” *Nature Structural & Molecular Biology* 16 (3): 312–17. https://doi.org/10.1038/nsmb.1560.

Chen, Huidong, Caleb Lareau, Tommaso Andreani, Michael E. Vinyard, Sara P. Garcia, Kendell Clement, Miguel A Andrade-Navarro, Jason D. Buenrostro, and Luca Pinello. 2019. “Assessment of Computational Methods for the Analysis of Single-Cell ATAC-Seq Data.” *BioRxiv*, August, 739011. https://doi.org/10.1101/739011.

Chen, Xi, Ricardo J. Miragaia, Kedar Nath Natarajan, and Sarah A. Teichmann. 2018. “A Rapid and Robust Method for Single Cell Chromatin Accessibility Profiling.” *Nature Communications* 9 (1): 5345. https://doi.org/10.1038/s41467-018-07771-0.

Cheng, Lin, Lucy J. Wong, Naihong Yan, Richard C. Han, Honghua Yu, Chenying Guo, Khulan Batsuuri, et al. 2018. “Ezh2 Does Not Mediate Retinal Ganglion Cell Homeostasis or Their Susceptibility to Injury.” Edited by Tudor C. Badea. *PLOS ONE* 13 (2): e0191853. https://doi.org/10.1371/journal.pone.0191853.

Clark, Brian S., Genevieve L. Stein-O’Brien, Fion Shiau, Gabrielle H. Cannon, Emily Davis-Marcisak, Thomas Sherman, Clayton P. Santiago, et al. 2019. “Single-Cell RNA-Seq

Analysis of Retinal Development Identifies NFI Factors as Regulating Mitotic Exit and Late-Born Cell Specification.” *Neuron* 102 (6): 1111-1126.e5. https://doi.org/10.1016/J.NEURON.2019.04.010.

Corso-Díaz, Ximena, Catherine Jaeger, Vijender Chaitankar, and Anand Swaroop. 2018. “Epigenetic Control of Gene Regulation during Development and Disease: A View from the Retina.” *Progress in Retinal and Eye Research* 65 (July): 1–27. https://doi.org/10.1016/J.PRETEYERES.2018.03.002.

Dahl, John Arne, and Gregor D Gilfillan. 2018. “How Low Can You Go? Pushing the Limits of Low-Input ChIP-Seq.” *Briefings in Functional Genomics* 17 (2): 89–95. https://doi.org/10.1093/bfgp/elx037.

Daum, Janine M, Özkan Keles, Sjoerd JB Holwerda, Hubertus Kohler, Filippo M Rijli, Michael Stadler, and Botond Roska. 2017. “The Formation of the Light-Sensing Compartment of Cone Photoreceptors Coincides with a Transcriptional Switch.” *ELife* 6 (November). https://doi.org/10.7554/eLife.31437.

Davis, Trevor L, and Ilaria Rebay. 2017. “Antagonistic Regulation of the Second Mitotic Wave by Eyes Absent-Sine Oculis and Combgap Coordinates Proliferation and Specification in the Drosophila Retina.” *Development (Cambridge, England)* 144 (14): 2640–51. https://doi.org/10.1242/dev.147231.

DeAngelis, J Tyson, Woodrow J Farrington, and Trygve O Tollefsbol. 2008. “An Overview of Epigenetic Assays.” *Molecular Biotechnology* 38 (2): 179–83. https://doi.org/10.1007/s12033-007-9010-y.

Deneen, Benjamin, Ritchie Ho, Agnes Lukaszewicz, Christian J. Hochstim, Richard M. Gronostajski, and David J. Anderson. 2006. “The Transcription Factor NFIA Controls the Onset of Gliogenesis in the Developing Spinal Cord.” *Neuron* 52 (6): 953–68. https://doi.org/10.1016/J.NEURON.2006.11.019.

Dennis, Daniel J., Sisu Han, and Carol Schuurmans. 2019. “BHLH Transcription Factors in Neural Development, Disease, and Reprogramming.” *Brain Research* 1705 (February): 48–65. https://doi.org/10.1016/J.BRAINRES.2018.03.013.

Falcon, S., and R. Gentleman. 2007. “Using GOstats to Test Gene Lists for GO Term Association.” *Bioinformatics* 23 (2): 257–58. https://doi.org/10.1093/bioinformatics/btl567.

Fausett, Blake V, and Daniel Goldman. 2006. “A Role for Alpha1 Tubulin-Expressing Müller

Glia in Regeneration of the Injured Zebrafish Retina." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 26 (23): 6303–13. https://doi.org/10.1523/JNEUROSCI.0332-06.2006.

Fausett, Blake V, Jessica D Gumerson, and Daniel Goldman. 2008. "The Proneural Basic Helix-Loop-Helix Gene Ascl1a Is Required for Retina Regeneration." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 28 (5): 1109–17. https://doi.org/10.1523/JNEUROSCI.4853-07.2008.

Ferrari, Stefano, Enzo Di Iorio, Vanessa Barbaro, Diego Ponzin, Francesco S Sorrentino, and Francesco Parmeggiani. 2011. "Retinitis Pigmentosa: Genes and Disease Mechanisms." *Current Genomics* 12 (4): 238–49. https://doi.org/10.2174/138920211795860107.

Ferreira, Renata C, Evgenya Y Popova, Jessica James, Marcelo R S Briones, Samuel S Zhang, and Colin J Barnstable. 2017. "Histone Deacetylase 1 Is Essential for Rod Photoreceptor Differentiation by Regulating Acetylation at Histone H3 Lysine 9 and Histone H4 Lysine 12 in the Mouse Retina." *The Journal of Biological Chemistry* 292 (6): 2422–40. https://doi.org/10.1074/jbc.M116.756643.

Fischer, Andy J., and Thomas A. Reh. 2002. "Exogenous Growth Factors Stimulate the Regeneration of Ganglion Cells in the Chicken Retina." *Developmental Biology* 251 (2): 367–79. https://doi.org/10.1006/DBIO.2002.0813.

Fischer, Andy J, Ian G Morgan, and William K Stell. 1999. "Colchicine Causes Excessive Ocular Growth and Myopia in Chicks." *Vision Research* 39 (4): 685–97. https://doi.org/10.1016/S0042-6989(98)00178-3.

Fujimura, Naoko, Andrea Kuzelova, Anja Ebert, Hynek Strnad, Jitka Lachova, Ondrej Machon, Meinrad Busslinger, and Zbynek Kozmik. 2018. "Polycomb Repression Complex 2 Is Required for the Maintenance of Retinal Progenitor Cells and Balanced Retinal Differentiation." *Developmental Biology* 433 (1): 47–60. https://doi.org/10.1016/J.YDBIO.2017.11.004.

Gascón, Sergio, Giacomo Masserdotti, Gianluca Luigi Russo, and Magdalena Götz. 2017. "Direct Neuronal Reprogramming: Achievements, Hurdles, and New Roads to Success." *Cell Stem Cell* 21 (1): 18–34. https://doi.org/10.1016/j.stem.2017.06.011.

Gemberling, Matthew, Travis J. Bailey, David R. Hyde, and Kenneth D. Poss. 2013. "The Zebrafish as a Model for Complex Tissue Regeneration." *Trends in Genetics* 29 (11): 611–

20. https://doi.org/10.1016/J.TIG.2013.07.003.

Glasgow, Stacey M, Jeffrey C Carlson, Wenyi Zhu, Lesley S Chaboub, Peng Kang, Hyun Kyoung Lee, Yoanne M Clovis, et al. 2017. "Glia-Specific Enhancers and Chromatin Structure Regulate NFIA Expression and Glioma Tumorigenesis." *Nature Neuroscience* 20 (11): 1520–28. https://doi.org/10.1038/nn.4638.

Goldman, Daniel. 2014. "Müller Glial Cell Reprogramming and Retina Regeneration." *Nature Reviews. Neuroscience* 15 (7): 431–42. https://doi.org/10.1038/nrn3723.

Gordon, Patrick J, Sanghee Yun, Anna M Clark, Edwin S Monuki, L Charles Murtaugh, and Edward M Levine. 2013. "Lhx2 Balances Progenitor Maintenance with Neurogenic Output and Promotes Competence State Progression in the Developing Retina." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 33 (30): 12197–207. https://doi.org/10.1523/JNEUROSCI.1494-13.2013.

Hägglund, Anna-Carin, Lina Dahl, and Leif Carlsson. 2011. "Lhx2 Is Required for Patterning and Expansion of a Distinct Progenitor Cell Population Committed to Eye Development." *PloS One* 6 (8): e23387. https://doi.org/10.1371/journal.pone.0023387.

Hamon, Annaïg, Diana García-García, Divya Ail, Juliette Bitard, Albert Chesneau, Deniz Dalkara, Morgane Locker, Jérôme E. Roger, and Muriel Perron. 2019. "Linking YAP to Müller Glia Quiescence Exit in the Degenerative Retina." *Cell Reports* 27 (6): 1712-1725.e6. https://doi.org/10.1016/J.CELREP.2019.04.045.

Hamon, Annaïg, Christel Masson, Juliette Bitard, Linn Gieser, Jérôme E Roger, and Muriel Perron. 2017. "Retinal Degeneration Triggers the Activation of YAP/TEAD in Reactive Müller Cells." *Investigative Ophthalmology & Visual Science* 58 (4): 1941–53. https://doi.org/10.1167/iovs.16-21366.

Heinz, Sven, Christopher Benner, Nathanael Spann, Eric Bertolino, Yin C Lin, Peter Laslo, Jason X Cheng, Cornelis Murre, Harinder Singh, and Christopher K Glass. 2010. "Simple Combinations of Lineage-Determining Transcription Factors Prime Cis-Regulatory Elements Required for Macrophage and B Cell Identities." *Molecular Cell* 38 (4): 576–89. https://doi.org/10.1016/j.molcel.2010.05.004.

Heng, Yee Hsieh Evelyn, Bo Zhou, Lachlan Harris, Tracey Harvey, Aaron Smith, Elise Horne, Ben Martynoga, et al. 2015. "NFIX Regulates Proliferation and Migration Within the Murine SVZ Neurogenic Niche." *Cerebral Cortex (New York, N.Y. : 1991)* 25 (10): 3758–

78. https://doi.org/10.1093/cercor/bhu253.

Hoshino, Akina, Rinki Ratnapriya, Matthew J Brooks, Vijender Chaitankar, Matthew S Wilken, Chi Zhang, Margaret R Starostik, et al. 2017. “Molecular Anatomy of the Developing Human Retina.” *Developmental Cell* 43 (6): 763-779.e4. https://doi.org/10.1016/j.devcel.2017.10.029.

Hughes, Andrew E. O., Jennifer M. Enright, Connie A. Myers, Susan Q. Shen, and Joseph C. Corbo. 2017. “Cell Type-Specific Epigenomic Analysis Reveals a Uniquely Closed Chromatin Architecture in Mouse Rod Photoreceptors.” *Scientific Reports* 7 (1): 43184. https://doi.org/10.1038/srep43184.

Iwafuchi-Doi, Makiko, and Kenneth S Zaret. 2014. “Pioneer Transcription Factors in Cell Reprogramming.” *Genes & Development* 28 (24): 2679–92. https://doi.org/10.1101/gad.253443.114.

Jadhav, Ashutosh P., Karin Roesch, and Constance L. Cepko. 2009. “Development and Neurogenic Potential of Müller Glial Cells in the Vertebrate Retina.” *Progress in Retinal and Eye Research* 28 (4): 249–62. https://doi.org/10.1016/J.PRETEYERES.2009.05.002.

Jenuwein, T, and C D Allis. 2001. “Translating the Histone Code.” *Science (New York, N.Y.)* 293 (5532): 1074–80. https://doi.org/10.1126/science.1063127.

John, Sam, Peter J Sabo, Theresa K Canfield, Kristen Lee, Shinny Vong, Molly Weaver, Hao Wang, et al. 2013. “Genome-Scale Mapping of DNase I Hypersensitivity.” *Current Protocols in Molecular Biology* Chapter 27 (July): Unit 21.27. https://doi.org/10.1002/0471142727.mb2127s103.

Jorstad, Nikolas L., Matthew S. Wilken, William N. Grimes, Stefanie G. Wohl, Leah S. VandenBosch, Takeshi Yoshimatsu, Rachel O. Wong, Fred Rieke, and Thomas A. Reh. 2017. “Stimulation of Functional Neuronal Regeneration from Müller Glia in Adult Mice.” *Nature* 548 (7665): 103–7. https://doi.org/10.1038/nature23283.

Jorstad, Nikolas L., Matthew S. Wilken, Levi Todd, Paul Nakamura, Nick Radulovich, Marcus J. Hooper, Alex Chitsazan, Brent A. Wilkerson, Fred Rieke, and Thomas A. Reh. 2019. “STAT Pathway Activation Limits the Ascl1-Mediated Chromatin Remodeling Required for Neural Regeneration from Müller Glia in Adult Mouse Retina.” *BioRxiv*, September, 753483. https://doi.org/10.1101/753483.

Kim, Douglas S, Takahiko Matsuda, and Constance L Cepko. 2008. “A Core Paired-Type and

POU Homeodomain-Containing Transcription Factor Program Drives Retinal Bipolar Cell Gene Expression." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 28 (31): 7748–64. https://doi.org/10.1523/JNEUROSCI.0397-08.2008.

Kouzarides, Tony. 2007. "Chromatin Modifications and Their Function." *Cell* 128 (4): 693–705. https://doi.org/10.1016/J.CELL.2007.02.005.

Krueger, Felix. 2015. "Trim Galore. A Wrapper Tool around Cutadapt and FastQC to Consistently Apply Quality and Adapter Trimming to FastQ Files." 2015. https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/.

Kurdyukov, Sergey, and Martyn Bullock. 2016. "DNA Methylation Analysis: Choosing the Right Method." *Biology* 5 (1). https://doi.org/10.3390/biology5010003.

Lamba, Deepak, Mike Karl, and Thomas Reh. 2008. "Neural Regeneration and Cell Replacement: A View from the Eye." *Cell Stem Cell* 2 (6): 538–49. https://doi.org/10.1016/j.stem.2008.05.002.

Langmead, Ben, and Steven L Salzberg. 2012. "Fast Gapped-Read Alignment with Bowtie 2." *Nature Methods* 9 (4): 357–59. https://doi.org/10.1038/nmeth.1923.

Lebrun-Julien, Frédéric, Laure Duplan, Vincent Pernet, Ingrid Osswald, Przemyslaw Sapieha, Philippe Bourgeois, Kathleen Dickson, Derek Bowie, Philip A Barker, and Adriana Di Polo. 2009. "Excitotoxic Death of Retinal Neurons in Vivo Occurs via a Non-Cell-Autonomous Mechanism." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 29 (17): 5536–45. https://doi.org/10.1523/JNEUROSCI.0831-09.2009.

Li, Heng, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, Richard Durbin, and 1000 Genome Project Data Processing 1000 Genome Project Data Processing Subgroup. 2009. "The Sequence Alignment/Map Format and SAMtools." *Bioinformatics (Oxford, England)* 25 (16): 2078–79. https://doi.org/10.1093/bioinformatics/btp352.

Liao, Yang, Gordon K Smyth, and Wei Shi. 2019. "The R Package Rsubread Is Easier, Faster, Cheaper and Better for Alignment and Quantification of RNA Sequencing Reads." *Nucleic Acids Research* 47 (8): e47. https://doi.org/10.1093/nar/gkz114.

Masserdotti, G, S Gillotin, B Sutor, D Drechsel, M Irmler, H F Jorgensen, S Sass, et al. 2015. "Transcriptional Mechanisms of Proneural Factors and REST in Regulating Neuronal

Reprogramming of Astrocytes." *Cell Stem Cell* 17 (1): 74–88. https://doi.org/10.1016/j.stem.2015.05.014.

Masserdotti, Giacomo, Sébastien Gillotin, Bernd Sutor, Daniela Drechsel, Martin Irmler, Helle F. Jørgensen, Steffen Sass, et al. 2015. "Transcriptional Mechanisms of Proneural Factors and REST in Regulating Neuronal Reprogramming of Astrocytes." *Cell Stem Cell*, June. https://doi.org/10.1016/j.stem.2015.05.014.

McLean, Cory Y, Dave Bristor, Michael Hiller, Shoa L Clarke, Bruce T Schaar, Craig B Lowe, Aaron M Wenger, and Gill Bejerano. 2010. "GREAT Improves Functional Interpretation of Cis-Regulatory Regions." *Nature Biotechnology* 28 (5): 495–501. https://doi.org/10.1038/nbt.1630.

Melo, Jimmy de, Brian S Clark, and Seth Blackshaw. 2016. "Multiple Intrinsic Factors Act in Concert with Lhx2 to Direct Retinal Gliogenesis." *Scientific Reports* 6: 32757. https://doi.org/10.1038/srep32757.

Melo, Jimmy de, Xiangguo Qiu, Guoyan Du, Leah Cristante, and David D. Eisenstat. 2003. "Dlx1,Dlx2,Pax6,Brn3b, AndChx10 Homeobox Gene Expression Defines the Retinal Ganglion and Inner Nuclear Layers of the Developing and Adult Mouse Retina." *The Journal of Comparative Neurology* 461 (2): 187–204. https://doi.org/10.1002/cne.10674.

Mitra, Soumitra, Poonam Sharma, Simran Kaur, Mohammad Anwar Khursheed, Shivangi Gupta, Riya Ahuja, Akshai J Kurup, Mansi Chaudhary, and Rajesh Ramachandran. 2018. "Histone Deacetylase-Mediated Müller Glia Reprogramming through Her4.1-Lin28a Axis Is Essential for Retina Regeneration in Zebrafish." *IScience* 7 (September): 68–84. https://doi.org/10.1016/j.isci.2018.08.008.

Mo, Alisa, Chongyuan Luo, Fred P Davis, Eran A Mukamel, Gilbert L Henry, Joseph R Nery, Mark A Urich, et al. 2016. "Epigenomic Landscapes of Retinal Rods and Cones." *ELife* 5 (March). https://doi.org/10.7554/eLife.11613.

Murphy, Daniel, Andrew. E.O. Hughes, Karen A. Lawrence, Connie A. Myers, and Joseph C. Corbo. 2019. "Cis-Regulatory Basis of Sister Cell Type Divergence in the Vertebrate Retina." *BioRxiv*, May, 648824. https://doi.org/10.1101/648824.

Nelson, Branden R, Yumi Ueki, Sara Reardon, Mike O Karl, Sean Georgi, Byron H Hartman, Deepak A Lamba, and Thomas A Reh. 2011. "Genome-Wide Analysis of Müller Glial Differentiation Reveals a Requirement for Notch Signaling in Postmitotic Cells to Maintain the Glial Fate." *PloS One* 6 (8): e22817. https://doi.org/10.1371/journal.pone.0022817.

Neph, Shane, M. Scott Kuehn, Alex P. Reynolds, Eric Haugen, Robert E. Thurman, Audra K. Johnson, Eric Rynes, et al. 2012. "BEDOPS: High-Performance Genomic Feature Operations." *Bioinformatics* 28 (14): 1919. https://doi.org/10.1093/BIOINFORMATICS/BTS277.

Noctor, S C, A C Flint, T A Weissman, R S Dammerman, and A R Kriegstein. 2001. "Neurons Derived from Radial Glial Cells Establish Radial Units in Neocortex." *Nature* 409 (6821): 714–20. https://doi.org/10.1038/35055553.

Ohori, Y, S Yamamoto, M Nagao, M Sugimori, N Yamamoto, K Nakamura, and M Nakafuku. 2006. "Growth Factor Treatment and Genetic Manipulation Stimulate Neurogenesis and Oligodendrogenesis by Endogenous Neural Progenitors in the Injured Adult Spinal Cord." *J Neurosci* 26 (46): 11948–60. https://doi.org/10.1523/JNEUROSCI.3127-06.2006.

Ohsawa, Ryosuke, and Ryoichiro Kageyama. 2008. "Regulation of Retinal Cell Fate Specification by Multiple Transcription Factors." *Brain Research* 1192 (February): 90–98. https://doi.org/10.1016/J.BRAINRES.2007.04.014.

Omori, Yoshihiro, Kimiko Katoh, Shigeru Sato, Yuki Muranishi, Taro Chaya, Akishi Onishi, Takashi Minami, Takashi Fujikado, and Takahisa Furukawa. 2011. "Analysis of Transcriptional Regulatory Pathways of Photoreceptor Genes by Expression Profiling of the Otx2-Deficient Retina." *PloS One* 6 (5): e19685. https://doi.org/10.1371/journal.pone.0019685.

Packer, Jonathan, and Cole Trapnell. 2018. "Single-Cell Multi-Omics: An Engine for New Quantitative Models of Gene Regulation." *Trends in Genetics* 34 (9): 653–65. https://doi.org/10.1016/J.TIG.2018.06.001.

Pollak, Julia, Matthew S. Wilken, Yumi Ueki, Kristen E. Cox, Jane M. Sullivan, Russell J. Taylor, Edward M. Levine, and Thomas A. Reh. 2013. "ASCL1 Reprograms Mouse Müller Glia into Neurogenic Retinal Progenitors." *Development* 140 (12): 2619–31. https://doi.org/10.1242/DEV.091355.

Popova, Evgenya Y., Xuming Xu, Andrew T. DeWan, Anna C. Salzberg, Arthur Berg, Josephine Hoh, Samuel S. Zhang, and Colin J. Barnstable. 2012. "Stage and Gene Specific Signatures Defined by Histones H3K4me2 and H3K27me3 Accompany Mammalian Retina Maturation In Vivo." Edited by Christoph Englert. *PLoS ONE* 7 (10): e46867.

https://doi.org/10.1371/journal.pone.0046867.

Powell, C, E Cornblath, and D Goldman. 2014. “Zinc-Binding Domain-Dependent, Deaminase-Independent Actions of Apolipoprotein B MRNA-Editing Enzyme, Catalytic Polypeptide 2 (Apobec2), Mediate Its Effect on Zebrafish Retina Regeneration.” *J Biol Chem* 289 (42): 28924–41. https://doi.org/10.1074/jbc.M114.603043.

Powell, C, F Elsaeidi, and D Goldman. 2012. “Injury-Dependent Muller Glia and Ganglion Cell Reprogramming during Tissue Regeneration Requires Apobec2a and Apobec2b.” *J Neurosci* 32 (3): 1096–1109. https://doi.org/10.1523/JNEUROSCI.5603-11.2012.

Powell, Curtis, Ana R Grant, Eli Cornblath, and Daniel Goldman. 2013. “Analysis of DNA Methylation Reveals a Partial Reprogramming of the Müller Glia Genome during Retina Regeneration.” *Proceedings of the National Academy of Sciences of the United States of America* 110 (49): 19814–19. https://doi.org/10.1073/pnas.1312009110.

Qiu, Xiaojie, Qi Mao, Ying Tang, Li Wang, Raghav Chawla, Hannah Pliner, and Cole Trapnell. 2017. “Reversed Graph Embedding Resolves Complex Single-Cell Developmental Trajectories.” *BioRxiv*, February, 110668. https://doi.org/10.1101/110668.

Raeisossadati, Reza, Marília Inês Móvio, Lais Takata Walter, Silvia Honda Takada, Carolina Beltrame Del Debbio, and Alexandre Hiroaki Kihara. 2018. “Small Molecule GSK-J1 Affects Differentiation of Specific Neuronal Subtypes in Developing Rat Retina.” *Molecular Neurobiology*, July, 1–12. https://doi.org/10.1007/s12035-018-1197-3.

Ramachandran, Rajesh, Blake V Fausett, and Daniel Goldman. 2010. “Ascl1a Regulates Müller Glia Dedifferentiation and Retinal Regeneration through a Lin-28-Dependent, Let-7 MicroRNA Signalling Pathway.” *Nature Cell Biology* 12 (11): 1101–7. https://doi.org/10.1038/ncb2115.

Ramírez, Fidel, Devon P Ryan, Björn Grüning, Vivek Bhardwaj, Fabian Kilpert, Andreas S Richter, Steffen Heyne, Friederike Dündar, and Thomas Manke. 2016. “DeepTools2: A next Generation Web Server for Deep-Sequencing Data Analysis.” *Nucleic Acids Research* 44 (W1): W160-5. https://doi.org/10.1093/nar/gkw257.

Reyes-Aguirre, Luis I., and Monica Lamas. 2016. “Oct4 Methylation-Mediated Silencing As an Epigenetic Barrier Preventing Müller Glia Dedifferentiation in a Murine Model of Retinal Injury.” *Frontiers in Neuroscience* 10 (November): 523. https://doi.org/10.3389/fnins.2016.00523.

Robinson, James T, Helga Thorvaldsdóttir, Wendy Winckler, Mitchell Guttman, Eric S Lander, Gad Getz, and Jill P Mesirov. 2011. "Integrative Genomics Viewer." *Nature Biotechnology* 29 (1): 24–26. https://doi.org/10.1038/nbt.1754.

Robinson, Mark D, Davis J McCarthy, and Gordon K Smyth. 2010. "EdgeR: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data." *Bioinformatics (Oxford, England)* 26 (1): 139–40. https://doi.org/10.1093/bioinformatics/btp616.

Ronan, J L, W Wu, and G R Crabtree. 2013. "From Neural Development to Cognition: Unexpected Roles for Chromatin." *Nat Rev Genet* 14 (5): 347–59. https://doi.org/10.1038/nrg3413.

Seto, Edward, and Minoru Yoshida. 2014. "Erasers of Histone Acetylation: The Histone Deacetylase Enzymes." *Cold Spring Harbor Perspectives in Biology* 6 (4): a018713. https://doi.org/10.1101/cshperspect.a018713.

Shen, Yin, Xiao-Ling Liu, and Xiong-Li Yang. 2006. "N-Methyl-D-Aspartate Receptors in the Retina." *Molecular Neurobiology* 34 (3): 163–80. https://doi.org/10.1385/MN:34:3:163.

Shu, Tianzhi, Kenneth G Butz, Celine Plachez, Richard M Gronostajski, and Linda J Richards. 2003. "Abnormal Development of Forebrain Midline Glia and Commissural Projections in Nfia Knock-out Mice." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 23 (1): 203–12. https://doi.org/10.1523/JNEUROSCI.23-01-00203.2003.

Singh, Ratnesh K., Ramya K. Mallela, Abigail Hayes, Nicholas R. Dunham, Morgan E. Hedden, Raymond A. Enke, Robert N. Fariss, Hal Sternberg, Michael D. West, and Igor O. Nasonkin. 2017. "Dnmt1, Dnmt3a and Dnmt3b Cooperate in Photoreceptor and Outer Plexiform Layer Development in the Mammalian Retina." *Experimental Eye Research* 159 (June): 132–46. https://doi.org/10.1016/J.EXER.2016.11.014.

Stevaux, Olivier, and Nicholas J Dyson. 2002. "A Revised Picture of the E2F Transcriptional Network and RB Function." *Current Opinion in Cell Biology* 14 (6): 684–91. https://doi.org/10.1016/S0955-0674(02)00388-5.

Tham, Yih-Chung, Xiang Li, Tien Y. Wong, Harry A. Quigley, Tin Aung, and Ching-Yu Cheng. 2014. "Global Prevalence of Glaucoma and Projections of Glaucoma Burden through 2040: A Systematic Review and Meta-Analysis." *Ophthalmology* 121 (11): 2081–90. https://doi.org/10.1016/J.OPHTHA.2014.05.013.

Thummel, Ryan, Sean C Kassen, Jacob E Montgomery, Jennifer M Enright, and David R Hyde. 2008. "Inhibition of Müller Glial Cell Division Blocks Regeneration of the Light-Damaged Zebrafish Retina." *Developmental Neurobiology* 68 (3): 392–408. https://doi.org/10.1002/dneu.20596.

Torre, Anna La, Sean Georgi, and Thomas A Reh. 2013. "Conserved MicroRNA Pathway Regulates Developmental Timing of Retinal Neurogenesis." *Proceedings of the National Academy of Sciences of the United States of America* 110 (26): E2362-70. https://doi.org/10.1073/pnas.1301837110.

Ueki, Yumi, Matthew S. Wilken, Kristen E. Cox, Laura Chipman, Nikolas Jorstad, Kristen Sternhagen, Milesa Simic, Kristy Ullom, Masato Nakafuku, and Thomas A. Reh. 2015. "Transgenic Expression of the Proneural Transcription Factor Ascl1 in Müller Glia Stimulates Retinal Regeneration in Young Mice." *Proceedings of the National Academy of Sciences* 112 (44): 13717–22. https://doi.org/10.1073/PNAS.1510595112.

Ueno, Kazuko, Toshiro Iwagawa, Genki Ochiai, Hideto Koso, Hiromitsu Nakauchi, Masao Nagasaki, Yutaka Suzuki, and Sumiko Watanabe. 2017. "Analysis of Müller Glia Specific Genes and Their Histone Modification Using Hes1-Promoter Driven EGFP Expressing Mouse." *Scientific Reports* 7 (1): 3578. https://doi.org/10.1038/s41598-017-03874-8.

VandenBosch, Leah S, and Thomas A Reh. 2019. "Epigenetics in Neuronal Regeneration." *Seminars in Cell & Developmental Biology*, April. https://doi.org/10.1016/j.semcdb.2019.04.001.

Varma, D D, S Cugati, A W Lee, and C S Chen. 2013. "A Review of Central Retinal Artery Occlusion: Clinical Presentation and Management." *Eye* 27 (6): 688. https://doi.org/10.1038/EYE.2013.25.

Vierbuchen, T, A Ostermeier, Z P Pang, Y Kokubu, T C Sudhof, and M Wernig. 2010. "Direct Conversion of Fibroblasts to Functional Neurons by Defined Factors." *Nature* 463 (7284): 1035–41. https://doi.org/10.1038/nature08797.

Villain, Gaëlle, Loïc Poissonnier, Baraa Noueihed, Gaëlle Bonfils, Jose Carlos Rivera, Sylvain Chemtob, Fabrice Soncin, and Virginie Mattot. 2018. "MiR-126-5p Promotes Retinal Endothelial Cell Survival through SetD5 Regulation in Neurons." *Development (Cambridge, England)* 145 (1): dev156232. https://doi.org/10.1242/dev.156232.

Wan, Jin, and Daniel Goldman. 2016. "Retina Regeneration in Zebrafish." *Current Opinion in*

Genetics & Development 40 (October): 41–47. https://doi.org/10.1016/J.GDE.2016.05.009.

Wang, Sui, Cem Sengel, Mark M Emerson, and Constance L Cepko. 2014. "A Gene Regulatory Network Controls the Binary Fate Decision of Rod and Bipolar Cells in the Vertebrate Retina." *Developmental Cell* 30 (5): 513–27. https://doi.org/10.1016/j.devcel.2014.07.018.

Wapinski, Orly L., Thomas Vierbuchen, Kun Qu, Qian Yi Lee, Soham Chanda, Daniel R. Fuentes, Paul G. Giresi, et al. 2013. "Hierarchical Mechanisms for Direct Reprogramming of Fibroblasts to Neurons." *Cell* 155 (3): 621–35. https://doi.org/10.1016/J.CELL.2013.09.028.

Wapinski, Orly L., Qian Yi Lee, Albert C. Chen, Rui Li, M. Ryan Corces, Cheen Euong Ang, Barbara Treutlein, et al. 2017. "Rapid Chromatin Switch in the Direct Reprogramming of Fibroblasts to Neurons." *Cell Reports* 20 (13): 3236–47. https://doi.org/10.1016/J.CELREP.2017.09.011.

Weinreb, Robert N, Tin Aung, and Felipe A Medeiros. 2014. "The Pathophysiology and Treatment of Glaucoma: A Review." *JAMA* 311 (18): 1901–11. https://doi.org/10.1001/jama.2014.3192.

Wilken, Matthew S, Joseph A Brzezinski, Anna La Torre, Kyle Siebenthall, Robert Thurman, Peter Sabo, Richard S Sandstrom, et al. 2015. "DNase I Hypersensitivity Analysis of the Mouse Brain and Retina Identifies Region-Specific Regulatory Elements." *Epigenetics & Chromatin* 8 (1): 8. https://doi.org/10.1186/1756-8935-8-8.

Wohl, Stefanie G, Marcus J Hooper, and Thomas A Reh. 2019. "MicroRNAs MiR-25, Let-7 and MiR-124 Regulate the Neurogenic Potential of Müller Glia in Mice." *Development (Cambridge, England)* 146 (17): dev179556. https://doi.org/10.1242/dev.179556.

Wohl, Stefanie G, and Thomas A Reh. 2016. "The MicroRNA Expression Profile of Mouse Müller Glia in Vivo and in Vitro." *Scientific Reports* 6: 35423. https://doi.org/10.1038/srep35423.

Wu, Lizhao, Cynthia Timmers, Baidehi Maiti, Harold I. Saavedra, Ling Sang, Gabriel T. Chong, Faison Nuckolls, et al. 2001. "The E2F1–3 Transcription Factors Are Essential for Cellular Proliferation." *Nature* 414 (6862): 457–62. https://doi.org/10.1038/35106593.

Yan, Naihong, Lin Cheng, Kinsang Cho, Muhammad Taimur A. Malik, Lirong Xiao, Chenying Guo, Honghua Yu, Ruilin Zhu, Rajesh C. Rao, and Dong Feng Chen. 2016. "Postnatal Onset of Retinal Degeneration by Loss of Embryonic Ezh2 Repression of Six1." *Scientific*

Reports 6 (1): 33887. https://doi.org/10.1038/srep33887.

Yao, Kai, Suo Qiu, Yanbin V Wang, Silvia J H Park, Ethan J Mohns, Bhupesh Mehta, Xinran Liu, et al. 2018. "Restoration of Vision after de Novo Genesis of Rod Photoreceptors in Mammalian Retinas." *Nature* 560 (7719): 484–88. https://doi.org/10.1038/s41586-018-0425-3.

Yoshida, Minoru, Sueharu Horinouchi, and Teruhiko Beppu. 1995. "Trichostatin A and Trapoxin: Novel Chemical Probes for the Role of Histone Acetylation in Chromatin Structure and Function." *BioEssays* 17 (5): 423–30. https://doi.org/10.1002/bies.950170510.

Young, Richard W. 1985. "Cell Differentiation in the Retina of the Mouse." *The Anatomical Record* 212 (2): 199–205. https://doi.org/10.1002/ar.1092120215.

Zhang, Jianmin, Russell J Taylor, Anna La Torre, Matthew S Wilken, Kristen E Cox, Thomas A Reh, and Monica L Vetter. 2015. "Ezh2 Maintains Retinal Progenitor Proliferation, Transcriptional Integrity, and the Timing of Late Differentiation." *Developmental Biology* 403 (2): 128–38. https://doi.org/10.1016/j.ydbio.2015.05.010.

Zibetti, Cristina, Sheng Liu, Jun Wan, Jiang Qian, and Seth Blackshaw. 2019. "Epigenomic Profiling of Retinal Progenitors Reveals LHX2 Is Required for Developmental Regulation of Open Chromatin." *Communications Biology* 2: 142. https://doi.org/10.1038/s42003-019-0375-9.