Elucidating the cis-regulatory landscape of retinal development and regeneration

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Abstract

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The mammalian retina is a heterogeneous mix of neurons and one glial cell type that mediate photo-sensation. The mechanisms of its development and the generation of cell type diversity is an area of on-going investigation. Unlike some lower vertebrates, mammalian retinas are largely incapable of regenerating lost neurons after retinal injury. In this work, I describe my efforts to map the *cis*-regulatory landscape of retinal development by employing DNase-hypersensitivity sequencing of retina at several important stages. The resulting data was categorized based on behavior to generate lists of putative *cis*-regulatory elements and transcription factors that regulate specific stages of retinal development. In addition, several putative enhancers were discovered for two key transcription factors, *Otx2* and *Ascl1*. Next, we used this strategy to characterize the differences in *cis*-regulatory elements between retinal progenitors and cultured Muller glial cells. We found that the pro-neural transcription factor *Ascl1*, which is critical for retinal regeneration in the zebrafish, is able to partially reprogram mouse Muller glia into retinal neurons. Specifically, many retinal progenitor and neuronal genes

were activated and the local chromatin environment was remodeled at ASCL1 binding sites. However, we found that ASCL1 binding within Muller glia only partially recapitulates the developmentally appropriate binding pattern found in retinal progenitors. Further, ASCL1 is able to bind to non-hypersensitive regions of chromatin in Muller glia. By comparing the accessible DNA of retinal progenitors and Muller glia, we were able to identify another factor, *Zic1*, which augments reprogramming. Finally, we show that perturbation of the epigenome during reprogramming, through the use of histone de-acetylase inhibitors, enhances reprogramming towards the photoreceptor cell fate. These results indicate that understanding the epigenetic state of cells can lead to insights into development and reprogramming with implications for regenerative medicine.

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Chapter 1:

Introduction

Introduction to the Retina

The vertebrate retina is composed of a thin layer (approximately one half millimeter) of neuronal tissue lining the back of eye. The tissue is a heterogenous mix of neurons and Muller glia with a minor component of astrocytes and endothelial cells constituting the retinal vasculature. There are 6 primary neuron sub-types that participate in light sensation and electrochemical signaling to the brain: rod and cone photoreceptors bipolar, amacrine, and horizontal cell interneurons, and ganglion cells. The Muller glia are the resident glial cell type of the retina and span the retinal tissue, providing structural support as well homeostatic support for the retinal neurons. Additionally, a layer of retinal pigment epithelial cells (RPE) line the back of the photoreceptor layer, making contact with the light sensing outer segments and are critical for photoreceptor survival.

The purpose of the retina is to sense light and transmit the light stimulus to the brain. Light sensation is performed by the photoreceptor cells lining the outer layer of the neural retina, with rod-photoreceptors mediating vision in low light conditions and cone photoreceptors mediating color and high acuity vision. Photoreceptors relay a light stimulated signal to the bipolar cells in the INL (inner nuclear layer) through a layer of synapses comprising the OPL (outer plexiform layer). Bipolars, in turn, relay the signal to RGCs through the IPL. Then, ganglion cells relay the signal through the optic nerve to the visual cortex of the brain. Each synaptic layer (IPL and OPL) also contains the processes of amacrine and horizontal cells, respectively, which reside in the INL and modulate the synaptic transmission of light stimuli at these synaptic layers. (Figure 1B; from Ohsawa and Kageyama, 2008)

Various diseases and injuries cause death and degeneration of retinal neurons directly or indirectly. Photoreceptors are sensitive to light of excessive intensity or duration and can result

in photoreceptor cell death directly. There are also several retinal diseases caused by genetic factors (Rattnet et al., 1999; den Hollander et al., 2010). For example, age related macular degeneration (AMD) causes loss of the RPE cells, leading to photoreceptor death. Retinitis Pigmentosa causes death of PRs directly, often due to a mutation in components of the phototransduction machinery or photoreceptor metabolism. Glaucoma, caused by excessive intraocular pressure, leads to death of ganglion cells, breaking the circuit of light transmission from photoreceptors and interneurons to the brain. These are just a few major examples, and many more conditions exist which result in retinal cell death and blindness. Unfortunately, mammals do not possess an intrinsic regenerative capacity to replace lost neurons, a feature present in several other species (see later sections).

Retinal Development from the Neuroepithelium

The initial deterministic event in the formation of the vertebrate retina is the induction of the eye field in the anterior neural plate of the developing embryo. The transcription factor Otx2, expressed throughout the anterior neural plate, cooperates with the 'eye-field transcription factors': *Rax, Pax6*, *Lhx2*, and *Six3* to form a self-reinforcing, auto-regulatory loop that specifies the formation of the optic cups (Acampora et al., 1995; Zuber et al., 2003). These outgrowths from the neural plate form a double-layered invagination, the outer layer of which becomes the RPE cells, the inner layer eventually giving rise to the neural retina. Interestingly, the eye field transcription factors have been demonstrated to be both necessary for eye formation and sufficient to induce ectopic eyes when over-expressed in inappropriate tissue (Chow et al., 1999; Loosli et al., 1999).

Retinal neurons are born from retinal progenitors in the neural layer in an ordered, but highly overlapping fashion (Figure 1A; from Ohsawa and Kageyama, 2008). Early retinal progenitors were demonstrated to be multipotent and capable of generating all retinal cell types through retroviral lineage tracing (Turner and Cepko, 1987; Turner et al., 1990). 3H-Thymidine radio-labeled birthdating studies determined the temporal order of their production (Young, 1985). Ganglion cells are the first cell type to be born, starting at embryonic day 11 (E11) along with the other early cell types: horizontal cells and cone photoreceptors. A second wave of late cell types generates amacrines, bipolars, rod photoreceptors, and Muller glia, the last cell type to be born at approximately post-natal day 6 (P6) in the retinal periphery.

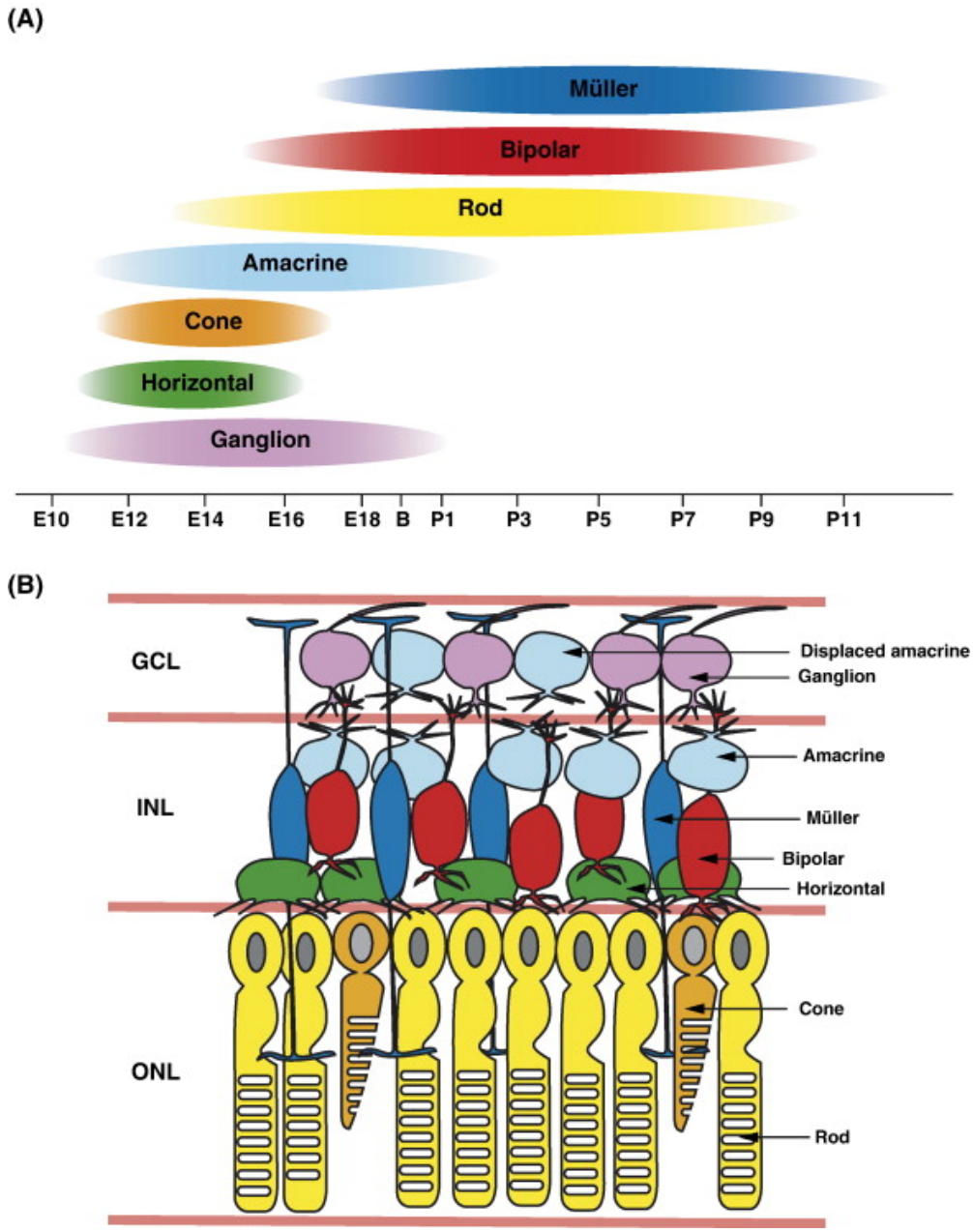


Figure 1.1: Retinal cell birth order and tissue structure from Ohsawa and Kageyama, 2008. (A) Diagram of the developmental age that the indicated retinal cell types are born, based on 3H-thymidine radio-labeling. X-axis indicates age: E: embryonic day; P: post-natal day. (B) Mature tissue structure of the retina; GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

Mechanisms of Neurogenesis in the Retina

Several proneural bHLH transcription factors have an instructive role in the production of retinal neurons: Atoh7, Neurog2, Neurod4, Neurod1, and Ascl1. (Reviewed by Hatakeyama and Kageyama, 2004). The proneural factor Atoh7 is required for ganglion cell production (Brown et al., 2001) and initiates the transcriptional cascade leading to Pou4f2 expression and ganglion cell maturation (Liu et al., 2001). Loss of function experiments with Atoh7 result under-production of ganglion cells and over-production of amacrine cells (Brown et al., 2001). Over-expression of Ascl1 in the developing retina results in the over-production of rod photoreceptors and bipolar cells (when combined with Neurod4) at the expense of Muller glia (Hatakeyama et al., 2001; Akagi et al., 2004). Loss of function of either Ascl1 or Neurod4 has a minimal effect on bipolar cell production, however, in the Ascl1-null, proliferation was decreased by 50% and the resulting retinas were relatively small (Tomita et al., 2000; Brzezinksi et al., 2011). In many cases, it has been observed that single loss of function experiments with these proneural bHLH factors does not result in gross abnormality or loss of specific cell types; double or triple knock-down of the proneural factors is often required to observe elimination of neuron subtypes (Morrow et al., 1999; Tomita et al., 2000; Inoue et al., 2002; Hatakeyama et al., 2001; Akagi et al., 2004). For example, triple knockout mice lacking Neurod1, Neurod4, and Neurog2 have a severe horizontal cell deficit and an increase in ganglion cells. Whereas triple knock-outs of Neurod1, Neurod4, and Ascl1 lack photoreceptors, but possess increased ganglion cell numbers. Finally, the retinas of mice with double knockouts of just Neurod1 and Neurod4 completely lack amacrine cells. Together, these studies indicate that the specification of retinal neurons by proneural bHLH factors is complex and there is some degree of functional redundancy in production and specification neuron sub-types.

In addition to the proneural factors, retinal neurons are also specified by homeodomain transcription factors, including: *Otx2, Chx10, Pax6*, *Onecut1/2* and *Prox1*, among others (Reviewed by Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008). For example, Pax6 is present in ganglion, amacrine, and horizontal cells. Misexpression of Pax6 along with different proneural bHLH factors changes the cell type that is over-produced: Viral over-expression of Pax6 and Neurod4 in retinal progenitors causes an increase in horizontal cells, while over-expression of Pax6 and Neurod1 induces extra amacrines; however, over-expression of Neurod4 or Neurod1, without Pax6, did not over-produce either cell type (Inoue et al., 2002). Horizontal cell generation requires the homeodomain factor Prox1; null mice fail to generate horizontal cells and misexpression of Prox1 promotes horizontal cell genesis (Dyer et al., 2003). In addition, knock-out of *Onecut1/2* results in failure to generate mature horizontal cells. Interestingly, these genes are downstream of *Pax6* and although horizontal cells are specified, they are not maintained in *Onecut*-null retinas (Wu et al., 2013; Klimova et al., 2015).

Another critical homeodomain transcription factor in retinal development is Otx2. Initially expressed in the eye field and subsequently down-regulated in retinal progenitors, it is re-expressed in nascent bipolars and photoreceptors along with the homeodomain factor, Crx. Loss of function of either Otx2 or Crx converts developing photoreceptors into amacrine cells (Chen et al., 1997; Furukawa et al., 1999; Nishida et al., 2003). In nascent bipolar cells, Otx2 cooperates with the transcription factor Chx10/Vsx2, which is necessary to stabilize the bipolar fate (Burmeister et al., 1996). In rod photoreceptors, Otx2 activates transcription of Crx and Nrl, which is also required for rod photoreceptor development. (Mears et al., 2001). Interestingly, Otx2, Crx, and Nrl bind each others' promoters, forming an auto-regulatory network that initiates

and sustains the photoreceptor gene expression state (Chen et al., 1997; Furukawa et al., 1997; Mears et al., 2001; Corbo et al., 2010; Hao et al., 2012; Samuel et al., 2014).

Mechanisms of Gliogenesis in the Retina

Notch signaling is necessary for the maintenance of retinal progenitors (Hatakeyama et al., 2004) and eventually formation of Muller glia by repression of the proneural bHLH transcription factors. Mis-expression of Hesr2, Hes1, Hes5, or Notch1 in the developing retina promoted the production of Muller glia at the expense of neurons and Hes1/5 deficient retinas under-produced Muller glia (Hojo et al., 2000; Furukawa et al., 2000; Satow et al., 2001; Yaron et al., 2006; Jadhav et al., 2006). Notch signaling is transmitted through the Notch Intracellular Domain (NICD) and activates expression of Hes1 and Hes5, inhibitory bHLH factors. Hes1 and Hes5 then directly repress expression of proneural factors such as Ascl1 and Neurod4, thereby preventing neuronal differentiation. Even as late in development as P12, after the Muller glia are born, Notch signaling is sustained and inhibition leads to re-expression of Ascl1 and loss of some glial markers (Nelson et al., 2011). Other signaling pathways that stimulate generation of Muller glia include CNTF/LIF, epidermal growth factor (EGF), and bone morphogenetic protein (BMP) (Bonni et al., 1997; Lillien, 1995; Ueki and Reh, 2013; Ueki et al., 2015). These studies indicate that several signaling pathways with are important for gliogenesis, with Notch signaling particularly important for repressing the neural fate.

The cis-Regulatory Landscape of the Retina

Enhancer Mapping in the Retina

While much work has been done characterizing the transcription factors present in retinal progenitors and differentiated neurons, relatively few studies have examined the cis-regulatory landscape of the vertebrate retina. Those studies mostly include analysis of transgenic animals to identify enhancers for specific genes. In a typical experiment, the region several kilobases upstream of the transcription start site (TSS) is cloned into a reporter construct that is used to make a transgenic animal by random insertion. Alternatively, these constructs are electroporated directly in the tissue of interest and expression of the reporter gene is examined. Once a region that drives reporter gene expression is observed in the correct cell/tissue type at the developmental stage of interest, that regulatory region is partitioned and sub-cloned as smaller pieces into a new reporter construct. This process is iterated until the minimal sequence unit that drives reporter gene expression is determined. Enhancers for several important retinal genes have been discovered by this method, including photoreceptor genes: Rhodopsin (Woodford et al., 1994; Nie et al., 1996), Irbp (Fong et al., 1999), Rhodopsin Kinase (Young et al., 2005), Prmd1 (Wang et al., 2014); and bipolar genes: Grm6, Cabp5, and Chx10 (Kim et al., 2008); among others. This technique can be modified to search through a larger genomic space through the use of bacterial artificial chromosomes (BAC) transgenics. For example, 6 enhancers of a critical regulator of retinal development, Otx2, have been discovered by this method up to 153kb away from the TSS (Kurokawa et al., 2014). These enhancers drive reporter expression in a spatial and temporally dynamic pattern across the developing nervous system. At least two identified regions drive expression in the developing retina in addition to forebrain (Kurokawa et al., 2004; Muranishi et al., 2011), one of which was regulated by antagonism between Notch-Hes signaling and the eye-field transcription factor, Rax. These studies indicate that retinal enhancers can be discovered through the process of systematically partitioning broad stretches of

DNA sequence, however, this process is laborious, expensive, and limited to enhancers sufficient to drive transcription in isolation.

Importantly, the cis-regulatory landscapes of many retinal transcription factors have not been determined in the retina. In the case of Ascl1, despite extensive transgenic analysis in the brain and spinal cord, the identified enhancers did not show activity in the retina, highlighting the need for an unbiased, high-throughput assay to discover regulatory elements. An understanding of the enhancers and upstream regulators of this transcription factor has implications for reprogramming of Muller glia and regeneration of the retina (see previous and later sections).

High-Throughput Analysis of Retinal cis-Regulatory Landscape

Recently, high-throughput sequencing technology has enabled the detection of cis-regulatory features on a genome-wide scale. One such technology, chromatin immuno-precipitation coupled to high-throughput sequencing (ChIP-seq), has been used to map the binding sites of 3 transcription factors that are critical for bipolar and rod photoreceptor development: Otx2, Crx, and Nrl (Corbo et al., 2010; Hao et al., 2012; Samuel et al., 2014). Each of these studies has revealed thousands of downstream target regions that are potentially regulated by these transcription factors. By integrating these data, regions of cooperativity between transcription factors can be determined, and network connectivity information can be accumulated (Andzelm et al., 2015; Yang et al., 2015). This approach has already yielded clinically valuable data by using the Crx ChIP-seq based transcriptional network to identify candidate regulated genes. Combined with exome sequencing, these data were to identify new

mutations in MAK that are causally linked to Retinitis Pigmentosa (Ozgül et al., 2011). However, each transcription factor ChIP-seq experiment is necessarily limited to single factors and their downstream binding sites. Therefore, this method alone is not sufficient to elucidate the cis-regulatory landscape in an unbiased manner.

One method to expand the discovery rate of cis-regulatory regions is using ChIP-seq against histone modifications. This was demonstrated recently when the histone 3 lysine 27 trimethylation (H3K27me3) and H3K4me2 landscape was determined in the retina at several developmental ages (Popova et al., 2012; Popova et al., 2014). Integration of these data sets demonstrated that particular categories of genes display different histone modification patterns. Known rod photoreceptor specific genes are characterized by a lack of H3K27me3 at any age, but an accumulation of H3K4me2 at P7, an age corresponding to the onset of photoreceptor maturation. Based on this pattern, a group of genes not previously implicated in rod development were predicated to be rod specific due to the observation of this histone pattern in their promoter regions, however this remains to be validated. Another conclusion from these experiments is that cell type specific genes in the retina (except for photoreceptor genes) display a high H3K37me3 level and a low but distinct H3K4me2 peak. This possibly indicates that the activation associated histone modification is present at the cell type specific gene in the corresponding minority cell type, but the inhibitory histone modification is present in the majority of cells. Subsequent studies have nullified the function of the enzymes responsible for these histone modifications and observed profound changes in cell type specific gene regulation (Popova et al., 2015; Zhang et al., 2015; Iida et al., 2015), indicating an instructive role for these histone modifications in retinal development.

Although histone modification ChIP-seq has been useful (see above), no high-throughput studies of the developing retina have use a more comprehensive assay of active cis-regulatory elements. One method that has the potential to reveal most, if not all (promoters, enhancers, insulators, silencers, and locus control regions), types of active regulatory regions of the genome is DNase I hypersensitivity high-throughput sequencing (DNase-seq) (Thurman et al., 2012). The use of DNase I to discover regions of DNA depleted of nucleosomes dates back almost 40 years (Weintraub and Groudine, 1976). In theory, changes to local chromatin structure by the repositioning of nucleosomes (due to DNA-binding factors, histone modifications, or other mechanisms) renders the underlying DNA sequence hypersensitive to nuclease digestion, relative to compact, nucleosomal DNA (Gross and Garrard, 1988; Felsenfeld and Groudine, 2003). It was only recently that treatment with this enzyme was coupled to high-throughput sequencing to reveal regions of active regulation on a genome-wide scale (John et al., 2013). Remarkably, this technique can be used to detect the presence of DNA-binding proteins within hypersensitive regions, known as footprinting (Galas et al., 1978;), and subsequently elucidate transcription factor networks on a genome-wide scale (Neph et al., 2012; Stergachis et al 2014). Part of the aim of this dissertation is to use DNaseI-hypersensitivity sequencing to elucidate the cis-regulatory landscape of the developing mouse retina and CNS.

Cell Fate Reprogramming with Transcription Factors

The first demonstration of transcription factor mediated reprogramming occurred when Davis and colleagues transfected cDNA for MyoD into fibroblasts and observed differentiation into muscle cells (Davis et al., 1987). This process of transdifferentiation, whereby cell types are interconverted without passing through a pluripotent stage, has been demonstrated between a

variety of cell types (Reviewed by Pereira et al., 2012). However, only reprogramming into neurons will be relevant to this thesis.

Transcription Factor Mediated Neural Reprogramming of Fibroblasts

The first demonstration of reprogramming to the neural fate (from across cells of a different embryonic germ layer) used cultured fibroblasts that were transduced with transcription factors: Ascl1, Pou3f2, and Myt1l. These 3 factors, narrowed down from an initial candidate pool of 19 and then combined, converted mouse fibroblasts into neurons with 20% efficiency (Vierbuchen et al., 2010). The reprogrammed cells adopted a neuronal morphology, expressed neuronal markers such as Tuj1, displayed action potentials, and may even have formed synapses with neighboring cells. Interestingly, Ascl1 alone was sufficient for neural reprogramming, albeit at a lower efficiency and the induced neuronal morphology was less complex. When fibroblasts where transduced with these reprogramming factors *in vitro* and transplanted into the mouse brain, these cells differentiated into neurons *in vivo* (Torper et al., 2013). Subsequent to the original protocol, mouse and human fibroblasts have been directed to several specific types of neurons, including: dopaminergic (Caiazzo et al., 2011; Pfisterer et al., 2011), spinal motor (Son et al., 2011), GABAergic (Ambasudhan et al., 2011), glutamatergic neurons (Vierbuchen and Wernig, 2012), and tripotent neural progenitors (Lujan et al., 2012). These protocols employ different transcription factors, including: Zic1, Neurod1, Neurog2, and Sox2, among other sub-type specific factors. Interestingly, the majority of neural directed reprogramming protocols include the transcription factor, Ascl1 (Reviewed by Vierbuchen and Wernig, 2012).

The prevalence of Ascl1 among neural reprogramming paradigms may be due to this factor's ability to bind to heterochromatin and direct the binding pattern of its partnering

transcription factors (Wapinski et al., 2013). Ascl1 was observed to bind to regions of relative nucleosome enrichment by formaldehyde-assisted isolation of regulatory elements (FAIRE-seq), thus Ascl1 qualifies as a 'pioneer factor' (Iwafuchi-Doi and Zaret, 2014). Additionally, when both Ascl1 and the homeodomain transcription factor, Pou3f2 were expressed in fibroblasts, Pou4f2 was directed to sites where Ascl1 was present; in addition, Pou3f2 was only able to stably bind to target sites and enhance neuronal maturation when co-bound with Ascl1 (Wapinski et al., 2013).

Only a select set of criterion has been consistently examined in most reprogramming studies, which include a general neuronal morphology, pan-neuronal or neural-progenitor markers (most consistently Tuj1 and Map2), and in some cases electrophysiological activity. Electrophysiological studies are generally consistent with a neuronal-like phenotype, but are typically reduced in extent when compared to developmentally derived neurons or are often not directly compared to bona fide neurons within the report (Vierbuchen et al., 2010; Son et al., 2011; Torper et al., 2013). At this stage of research, the most relevant features of transdifferentiation to examine are not known. For example, a subset of reprogramming studies perform genome wide gene expression analysis on reprogrammed cells and the desired neuronal cell type and there is typically large overlap, but still differences between the two. Statistical measures of the similarity between global gene expression states have not been widely employed, although new techniques are becoming available to do so (Cahan et al., 2015). However, with the exception of a subset of genes described in the neurodevelopmental literature, it is not known what genes are required for transdifferentiation to be considered complete or even sufficient (i.e. – How do we decide what is complete-reprogramming?). Long-term follow-up of the behavior of neurons is lacking, although induced neurons in vivo have been shown to

survive for up to 12-weeks (Torper et al., 2013). These induced neurons displayed qualitatively more mature neuronal morphology, but represented only 4% conversion efficiency (Torper et al., 2013). Particular interest should be paid to assessing the fidelity of neuronal function (i.e. – action potential and synaptic characteristics, cell survival) of reprogrammed cells that maintain expression of the exogenous transcription factors. It may be the case that prolonged expression of progenitor genes within neurons, or higher than physiological levels of neuronal transcription factors, will negatively affect long-term neuronal function. This would limit the usefulness of reprogramming therapies in humans where neurons would need to be stably maintained for years or decades.

Neural Reprogramming of Glial Cells in vitro and in vivo

The first transdifferentiation of glial cells into neurons was demonstrated *in vitro* by transducing the transcription factor Pax6 into post-natal cortical astrocytes, which subsequently up-regulated the neuronal markers, Tuj1 and NeuN (Heins et al., 2002). Later, post-natal astrocytes transduced with the proneural bHLH factor Neurog2 transdifferentiated into cells with a neuronal morphology, synaptic protein staining, and capable of action potentials *in vitro* (Berninger et al., 2007). Further work demonstrated the efficacy of Neurog2 mediated reprogramming *in vitro*. Electrophysiological examination by patch clamping showed that action potential firing matured slowly between 6 and 10 days post induction of Neurog2. However, these properties were limited compared with control neurons in culture. Similar deficiencies were observed upon examination of synaptic functional properties. In addition, the cells were unable to form autapses (self-synpases), unlike control neurons (Berninger et al., 2007). However, these cells are directed towards the glutamatergic identity and can form pre and post-

synaptic networks with neighboring cells (Blum et al 2011). Interestingly, adult astrocytes could be reprogrammed with Neurog2 if isolated after cortical injury and passaged as neurospheres (Heinrich et al., 2010). Ascl1 is similarly able to reprogram post-natal astrocytes *in vitro* (Berninger et al., 2007). When co-transduced with the transcription factors Lmx1 and Nurr1, the astrocytes transdifferentiated into dopaminergic neurons relatively efficiently (18%) and displayed appropriate gene expression and electrophysiological characteristics (Addis et al., 2011). However, similar to the fibroblast reprogramming studies described above, the aforementioned studies only examined a small subset of genes that were pre-selected for testing. These cells displayed characteristic behaviors of dopaminergic neurons, such as action potential firing and release of dopamine, however, this was not compared to control dopaminergic neurons.

When Ascl1 reprogrammed human astrocytes are transplanted into mouse brain, they display a complex neuronal morphology, GABAergic markers, and synaptic markers (although this process required transduction of pluripotency factors prior to Ascl1) (Corti et al., 2012). Again, the molecular characterization of transplanted astrocytes was limited to staining for the proteins: Map2, Tuj1, synapsin, and GAD67 for GABA synthesis. Therefore, it is unclear how complete the reprogramming process is. Thus, incomplete characterization of the reprogramming of gene expression state is common in recent reports, although those that do demonstrate only a partial reprogramming. Extensive examination of the cell behaviors, especially over the course of months or years will be required to determine whether these cells can be considered fully reprogrammed or be clinically useful.

More recent work has employed viral vectors to reprogram astrocytes *in situ* in the rodent brain. When the neural progenitor associated transcription factor, Sox2, is transduced by this

method, adult mouse astrocytes transdifferentiate into calretinin-labeled interneurons capable of synaptic connections and action potentials (Niu et al., 2015). However, the efficiency of this transdifferentiation was not reported. Interestingly, this process is augmented by simultaneous stimulation with brain-derived neurotrophic factor (BNDF) and valproic-acid (VPA), an HDAC-inhibitor. Consistent with previous reprogramming paradigms, this process of Sox2-mediated reprogramming required subsequent expression of Ascl1, at least transiently (Niu et al., 2015).

These studies indicate that the phenotypic state of differentiated cells, and especially glial cells, is remarkably plastic and amenable to transcription factor mediated reprogramming to a neuronal state. It is tempting to speculate that the Muller glia cells of the retina will be a similarly reprogrammable source of cells for generation of new neurons due to their known expression of several progenitor genes and capacity to react to injury (Berninger et al., 2012).

Endogenous Retinal Regeneration Among Species

Retinal Regeneration in lower vertebrates

It has been known for over 100 years that amphibians are capable of completely regenerating the neural retina after surgical removal (Araki, 2007; Barbosa-Sabanero et al., 2012; Chiba, 2014). The source of new retinal cells is the retinal pigment epithelium (RPE), which upon sensing the loss of photoreceptor contact, undergo dedifferentiation, proliferation, and differentiation into all retinal cell types, and restore the full tissue, including the neural and original pigmented layers.

Similar to amphibians, teleost fist (including zebrafish), are capable of regenerating all neural retina cell types. However, unlike the amphibian, the source of the regenerated cells is not the pigmented epithelium, but instead the Muller glia, the resident glial population of the

retina. This was demonstrated through transgenic lineage tracing after retinal damage (Fausett and Goldman, 2006; Bernardos et al., 2007; Thummel et al., 2008). Remarkably, the zebrafish Muller glia respond to various retinal injuries (physical damage, light excitotoxicity, and chemical neurotoxic agents) by the process of de-differentiation, proliferation, and neuro- and glio-genesis to restore retinal structure and function. The process of dedifferentiation begin when the Muller glia contract their extensive processes that span the retinal layers and down-regulate glial specific gene expression and reinstate progenitor gene expression. These cells then re-enter the cell cycle undergo multiple rounds of division as retinal progenitors. Subsequently, these cells re-differentiate into new retinal neurons of all sub-types and Muller glia to restore the lost cell types (Stenkamp, 2011). The newly generated neurons migrate into the appropriate retinal layer based on their specific cell type, form synaptic connections in the two synaptic layers, and integrate into the circuitry of the undamaged structure of the retina (or forming appropriate *de novo* circuitry if no structure remains). Thus, retinal structure and function is regenerated and vision is restored (Lindsey and Powers, 2007; Sherpa et al., 2007; Mensinger et al., 2007).

Signaling Molecules and Growth Factors mediating regeneration in zebrafish

Several recent studies have investigate the role of signaling molecules and growth factors that are important for the initial stimulation of Muller glia and the subsequent regeneration. Tumor necrosis factor α (TNFα) was identified as a cytokine that is released from dying neurons in the injured retina and activates Muller glia, which subsequently release TNFα themselves to further stimulate proliferation and the regenerative response (Nelson et al., 2007; Nelson et al., 2008). TNFα signaling stimulated the expression of transcription factors Stat3 and Ascl1a,

which were then observed to mutually activate each others' expression in a feedback loop that presumably stabilizes the stimulated gene expression state (Nelson et al., 2013). Inhibition of TGFβ signaling in the retina is required for Muller glia to re-enter the cell cycle after injury (Lenkowski et al., 2013). Recently, heparin binding EGF (HB-EGF) was identified as an activator of the MAP-Kinase pathway that is necessary and sufficient for formation of Muller glia derived progenitors (Wan et al., 2012). Down stream targets of HB-EGF include the transcription factors involved in the regenerative response: *ascl1a*, *pax6b*, and *c-myc*. Through *ascl1a*, HB-EGF also up-regulates components of the Notch and Wnt signaling pathways, which are also important for regeneration. Canonical Wnt/β-Catenin signaling necessary and sufficient for activation of Muller glia after damage and forms a feedback loop with *ascl1a* whereby Wnt activates *ascl1a*, which inhibits expression of the Wnt signaling inhibitor, Dkk (Meyers et al., 2012; Ramachandran et al., 2011). Notch signaling, which is activated by the HB-EGF/ *ascl1a* pathway, forms a feedback loop by inhibiting HB-EGF and *ascl1a* expression, possibly to dampen and restrict the activation of Muller glia to the zone of injury (Wan et al., 2012; Conner et al., 2014). Interestingly, recent evidence indicates that several of the aforementioned signaling pathways form an extensive cross-regulatory network, whereby activation of one signaling pathways activates the others (Wan et al., 2014).

Transcriptional events in the regenerative response

One critical early event in the activation of Muller glia cell is the expression of the transcription factor, *ascl1a*, which occurs within 6-hours after retinal injury (Fausett et al., 2008). Morpholino knockdown of Ascl1a in the retina demonstrated that this gene is necessary for the regenerative response. As mentioned above, *ascl1a* may serve as a central node for signaling

events in activated Muller glia (Wan et al., 2012). Additionally, several genes important for regeneration have been identified as downstream targets of *ascl1a*. Ramachandran et al., (2010) demonstrated that *ascl1a* activates expression of the RNA-binding protein, *lin28*, which suppresses the microRNA, *let-7*, leading to proliferation and dedifferentiation. One of the targets of *let-7* is *hsdp1*, which along with *mps1*, are both up-regulated in activated Muller glia and are required for proliferation (Qin et al., 2009). Another important target of *ascl1a* is the transcriptional repressor, *insm1a*. The role of *insm1a* in the Muller glial during regeneration is complex: initially it is required for stimulation of Wnt signaling and subsequent dedifferentiation, but later *insm1a* represses expression of HB-EGF and cell cycle genes to allow differentiation of the Muller glia derived progenitor into retinal neurons (Ramachandran et al., 2012). Lastly, *ascl1a* activates the expression of cytidine deaminases, *apobec2a/b*, which enhance the proliferation of Muller glia following retinal injury (Powell et al., 2012). It is clear that *ascl1a* is a critical node in the translation of injury induced signaling and the downstream transcriptional response in dedifferentiating Muller glia.

Reactive Gliosis in Response to Injury

In contrast to teleost fish and amphibians, it was believed that the mammalian retina was incapable of regeneration after the loss of retinal neurons. Instead of repair and regeneration, mammalian Muller glia undergo a process called reactive gliosis in response to retinal injury and retinopathies (Dyer and Cepko 2000, Bringmann et al., 2009b). Several features characterize reactive gliosis. First, reactive glia increase in cell size and change their morphology to become hypertrophic. Reactive glia up-regulate certain ‘intermediate filament’ genes including: Vimentin, Nestin, and Glial Fibrillary Acidic Protein (GFAP).

In the short term, reactive gliosis has a neuro-protective effect through the release of antioxidants, neurotrophic and growth factors that aid neuron survival, in addition to the re-uptake of excess intercellular glutamate, which has an excitotoxic effect on neurons (Bringmann et al., 2006; Bringmann et al., 2009a). However, reactive gliosis accompanied by re-entry into the cell cycle and proliferation results in the formation of a glial scar that can ultimately prevent regeneration and negatively impact neuronal functions (Bringmann et al., 2009b). This process of proliferative gliosis results in the release of pro-inflammatory cytokines, such as TNFα, that have a cytotoxic effect on neurons. In addition, processes involved in the support of neuron homeostasis are impaired, including neurotransmitter recycling, potassium level homeostasis, and carbon dioxide removal. The formation of a fibrotic glial scar may also directly impede formation of new synapses and neurite outgrowths (Bringmann et al., 2009b; Profyris et al., 2004).

Despite the deleterious effects of reactive gliosis on retinal repair and homeostasis, the fact that Muller glia are able to sense the injury or death of neurons and activate an endogenous response suggests that reactive Muller glia may be amenable to retinal regeneration if exogenously guided by the right signaling molecules or transcription factors.

Regeneration of Neurons in the Mammalian Retina

Until the last 15 years, it was thought that mammalian Muller glia possessed no intrinsic regenerative capacity. However, a close examination of the mouse and rat retina after injury or in response to exogenous signaling molecules demonstrated that a limited regenerative response occurs in certain circumstances. The following is a select review of important studies that have contributed to our knowledge of retinal regeneration in mammals.

The question of whether or not adult rodent Muller glia cells are capable of proliferation and neurogenesis *in vivo* was addressed by the injection of various neurotoxic agents into the vitreous cavity and subsequently observing the behavior of the Muller glia. Ooto et al., (2004) found that NMDA induced neurotoxic damage induced Muller glia to re-renter the cell cycle, as indicated by BrdU-labeling, and some BrdU+ cells were observed in the outer nuclear layer (ONL) expressing rod and bipolar specific genes after 2-weeks post injury (Ooto et al., 2004). Similarly, damage to the photoreceptors by MNU or Sodium Iodate injection resulted in Rhodopsin expression in BrdU+ cells 15 days after injury (Wan et al., 2008; Jian et al., 2015). Interestingly, sub-toxic doses of glutamate or direct stimulation of the Muller glia by alpha-aminoadipate induced proliferation of the Muller glia and Rhodopsin expression in BrdU+ cells after 21 days post injury (Takeda et al., 2008). However, it is difficult to determine the efficiency of photoreceptor generation in these studies due to the lack of quantification and the impression is that the BrdU-labeled photoreceptors are rare. Together, these studies demonstrate that rodent Muller glia are capable of a limited proliferative and neurogenic response to retinal damage. In addition, the markers of bipolar cells or rod photoreceptors (rhodopsin) used in these studies are expressed on the cell surface, and due to the very high density of cells in the retina, without confocal analysis or nuclear markers, it is difficult to co-localize the nuclear BrdU and the cell surface markers.

The zebrafish responds to retinal injury by expression of various signaling molecules and growth factors that are necessary and/or sufficient for Muller glial regeneration (Hamon et al., 2015). Therefore, several groups have tested the hypothesis that treatment of the injured rodent retina with exogenous signaling molecules will enhance the regenerative response of Muller glia. Karl et al., (2008) demonstrated that treatment of the NMDA-injured adult mouse retina with

either EGF or FGF and Insulin, resulted in a large increase in Muller glia proliferation. Transgenic lineage tracing confirmed that the Muller glia were the source of the BrdU-labeled cells, 4% of which later differentiated into amacrine interneurons (Karl et al., 2008). FGF2 and Insulin had the similar effect of enhancing proliferation in the 2-week post-natal rat retina damaged by NMDA, although in this case, rods and bipolar were generated in small numbers (Das et al., 2006).

It is interesting to note that the effect of mitogen stimulation is dependent on the age of the animal. Close et al., (2006) found that EGF stimulation of the post-natal day-10 (P10) rat retina induced Muller glia proliferation, but this effect was strikingly reduced if the treatment occurred at P14. This correlated with a down-regulation of the EGF-receptor. However, light damage caused an up-regulation of EGF-receptor and induced proliferation if combined with exogenous EGF (Close et al., 2006). However, adult mice injured with NMDA and stimulated with exogenous EGF did not demonstrate Muller glia proliferation (Karl el al., 2008). A similar age-dependent of decline in Muller glia proliferation was observed in mouse retinas that were damaged by culture *ex vivo* and treated with EGF (Loffler et al., 2015). The strong proliferative effect of damage and EGF treatment at P8 was markedly reduced when damage began at P12. Sub-populations of the proliferative Muller glia in these studies were observed up-regulating progenitor and neuron specific genes, but this effect declined with animal age as well.

Several other signaling pathways have been stimulated to enhance the regenerative response of Muller glia. Canonical Wnt/β-Catenin signaling, important in the zebrafish regenerative response, promoted proliferation of Muller glia and differentiation into Rhodopsin and Nrl expressing photoreceptors after damage (Osakada et al., 2007; Del Debbio et al., 2010; Suga et al., 2014). Stimulation of Sonic Hedgehog signaling also promoted proliferation in the

MNU injured adult rat retina and Rhodopsin-labeled cells were observed 21-days post injury. Additional signaling molecules that have enhanced the proliferative and neurogenic response of Muller glia include: Retinoic Acid, DAPT (Notch signaling inhibitor), BDNF, and VPA, among others (Wan et al., 2007; Osakada et al., 2007; Das et al., 2006; Del Debbio et al., 2010). However, many of these experiments do not test these signaling molecules in isolation; thus it is difficult to determine their individual contributions to regeneration.

Recent evidence has complicated the interpretation of some of the previous studies reporting proliferation and neuronal differentiation of Muller glia in the outer nuclear layer of the injured retina. Joly et al, found that light damage to the photoreceptors of adult mice resulted in Muller glia that up-regulate the progenitor marker, Pax6, and migrate up to the inner ONL. These cells up-regulated their expression of key cell cycle genes, including CyclinD1 and Phospho-Histone-3, but crucially did not incorporate BrdU-labeling during the observation period from 6-hours to 3-days post light damage. However, the BrdU-labeled cells that appeared in the ONL did not express the cell cycle marker Ki67, but did co-localize with DNA LigaseIV, a component of the DNA damage repair response. This suggests that the BrdU-labeled cells in the ONL of previous studies may not be derived from proliferating Muller glia, but instead are photoreceptors incorporating BrdU through DNA-repair mechanisms (Joly et al., 2011). Only those studies that demonstrate co-labeling between BrdU and Muller glia specific markers, or those that use lineage tracing, will be able to distinguish between these possibilities.

Another factor that complicates the interpretation of retinal regeneration experiments in rodents is differences in the inherent regenerative capacity of mouse strains. Suga et al. observed differences in the proliferative capacity of Muller glia between mouse strains: C57BL/6 (BL6), 129x1/SvJ (129), and BDF1 (Suga et al., 2014). In response to damage of adult retinas by ex

vivo culture, BL/6 Muller glia proliferation was rare, however proliferation was increased 30-fold in strain 129 and also included co-labeling of Muller glia with the cell cycle marker, Ki67. A third strain, BDF-1, showed intermediate levels of proliferation. Interestingly, strain 129 responded to an exogenous Wnt signaling activator by increasing Muller glia proliferation a further 5-fold, whereas no significant was observed in BL6. There were also differences in the induction of progenitor genes between strains. After damage, 129 mice up-regulated MAP-Kinase pathway genes and a chromatin remodeler associated with neuronal progenitor proliferation, whereas BL6 mice up-regulated genes associated with inhibition of proliferation (Suga et al., 2014). This report suggests that different rodent strains and species can differ in their inherent capacity to respond to signaling molecules, re-enter the cell cycle, and/or re-express progenitor genes. Thus, these data complicate the interpretation of the regenerative response when comparing between studies that use different mouse strains, and especially between mouse and rat experiments.

An intriguing development with implications for retinal regeneration in humans is the demonstration that human Muller glial cells may be a viable source of retinal neurons. Muller glia can be grown from adult human retinas in dissociated culture and a subpopulation of these cells undergoes spontaneous immortalization (Lawrence et al., 2007). Additionally, they can be induced to form Nestin-labeled neurospheres. Human Muller glia can be directed to a ganglion cell fate, expressing the ganglion cell specific factor BRN3B, through treatment with FGF2 and the Notch signaling inhibitor, DAPT (Singhal et al., 2012; Bhatia et al., 2011). When transplanted into NMDA-damaged rat retinas, these cells correlated with an improvement in retinal function, as determined by negative scotopic threshold response of electroretinogram (nSTR ERG), a measure of ganglion cell function. However, through treatment with FGF,

taurine, retinoic acid, and IGF1 (FTRI), these cells can be directed to a photoreceptor cell fate, expressing rod specific genes: Rhodopsin and NR2E3. When transplanted into photoreceptor degenerating rat retinas (Rd-rats), these cells were observed migrating into the ONL and correlate with an improvement A-wave response to light stimulation, a measure of rod function (Lawrence et al., 2007; Jayaram et al., 2014). FTRI treatment increases canonical Wnt/β-Catenin signaling. Interestingly, TGFβ treatment or Wnt signaling inhibition can block the differentiation of human Muller glia to rod photoreceptors (Angbohang et al., 2016). Giannelli et al., (2011) observed strikingly higher generation of RCVN-positive photoreceptors from human Muller glia in culture, increasing in effect with taurine treatment. However, the aforementioned studies of human Muller glia suffer from a lack of quantification of conversion efficiency and Muller glia specific tracing to demonstrate that the ganglion cells and photoreceptors generated are derived from the Muller glia and not surviving neurons.

Summary of Dissertation

In this dissertation, I set out to describe the changes in the cis-regulatory landscape during retinal development and use this to discover new regulatory elements in chapter 1. In chapter 2, I describe our efforts to reprogram Muller glia cells into new retinal neurons via misexpression of the transcription factor, Ascl1. In chapter 3, describe the interaction between Ascl1 and the cis-regulatory landscape of Muller glia and attempt to use this knowledge to inform the reprogramming process.

Chapter 2:

DNase I hypersensitivity analysis of the mouse brain and retina identifies region-specific regulatory elements.

(text and figures modified from Wilken et al., 2015)

INTRODUCTION

The human central nervous system (CNS, brain, spinal cord and neural retina of the eye) contains billions of neurons, with hundreds of distinct types. Studies of neuronal morphology, neurotransmitter response and single-cell electrophysiological analyses have traditionally been used to define neuronal diversity and led to estimates of different types of neurons ranging in the thousands. Recent large-scale molecular mapping studies, however, have shown an even greater complexity than that previously appreciated (Bernard et al., 2012; Hawrylycz et al., 2012; Shen et al., 2012). The enormous diversity in gene expression and connectivity in the brain presents a challenge for traditional approaches to define regulatory networks and identify cis-regulatory elements active in this complex organ.

Several previous studies have used comparative genomics approaches to identify cis-regulatory modules (CRMs) in both developing and mature CNS, based on the fact that these are frequently conserved across species (Blow et al., 2010; Hardison and Taylor, 2012; Taylor et al., 2006; Visel et al., 2008). More recently, epigenetic approaches have used stereotyped patterns of histone modifications and the occupancy of DNA binding proteins to identify various types of CRMs (Arnold et al., 2013; de Laat and Duboule, 2013; Lee et al., 2011; May et al., 2012; Visel et al., 2009; Zinzen et al., 2009). Combinations of these approaches have also been effective, particularly in identifying gene promoters. Promoters are typically found within 100 base pairs of the transcription start site, associate with RNA polymerase II and frequently contain distinct sequence motifs such as the TATA box. Other types of CRMs, such as enhancers and insulators, have been somewhat more difficult to identify; however, the former are often bound by the transcriptional co-activator p300, and the latter by the zinc-finger transcription factor CTCF. In

addition, characteristic histone modifications, such as H3K4me1 (enhancers) and H3K4me3 (promoters) are also good predictors of specific types of CRMs (Shen et al., 2012).

While these advances have generated large numbers of potential CRMs, there are many reasons to suspect that this list of candidates is not yet comprehensive. Although many CRMs show substantial sequence conservation among species, recent estimates suggest that nearly 40% of CRMs active in the mouse are not active in humans, despite their conserved sequence, highlighting the limitation of inferring function from comparative sequence methods alone (Nord et al., 2013; Vierstra et al., 2014). However, it may be that some of these CRMs are active, but not examined for activity in the right cell type. In addition, certain patterns of histone modifications and DNA binding protein occupancy are well correlated with active enhancers and insulators, however CRMs utilizing alternate molecular mechanisms will be missed by approaches relying solely on these patterns.

To generate a more comprehensive view of CRMs, the use of DNase I hypersensitivity mapping at the genome scale (DNase-seq) has emerged as a powerful approach (Birney et al., 2007; Hesselberth et al., 2009; John et al., 2013; John et al., 2011; Sabo et al., 2006; Thurman et al., 2012). DNase I hypersensitive sites (DHSs) are sensitive markers of all of the main types of CRMs, and recent genome-wide mapping of DHSs in diverse human and mouse cell lines and tissues has generated fundamental insights into gene regulation and its evolution (John et al., 2013).

We therefore undertook a genome-scale, high-resolution mapping of accessible chromatin using DNase-seq to identify CRMs utilized *in vivo* in the developing and mature mouse brain, and three specific regions, the cerebral cortex, the cerebellum and the neural retina. Despite the overall complexity of the CNS, we were able to find DNase I hypersensitive regions

at the promoters of genes expressed in less than 1% of the total cells of the retina (e.g. specific ganglion and cone photoreceptor subtypes). By comparing CNS DHSs with DHSs active in other mouse cell lines and tissues, we were able to delineate a core "regulome" for the CNS. We were also able to carry out an analysis of transcription factor binding motifs in CRMs active within the developing and mature retina to identify stage-specific transcriptional regulators, and confirmed that a number of these potential CRMs display enhancer activity *in vitro* and *in vivo*. Overall, our results demonstrate the power of genome-wide DNase I mapping to provide answers to questions of neuronal diversity, brain evolution and the cis-regulation that underlies these processes.

RESULTS

Broad features of the regulatory landscape of mouse CNS

We carried out DNase-seq (according to ENCODE standard protocols; see methods and (Mercer et al., 2013; Thurman et al., 2012) for mature mouse whole brain, and dissected cerebral cortex, cerebellum, and neural retina (age: 8-week adult), as well as specific ages of developing brain and retina. Treatment of retinal nuclei with DNase I, library preparation and sequencing, and Hotspot calling was carried out in collaboration with the Stamatoyannopoulos lab. Brain samples were contributed by the Bender lab. Samples were prepared in duplicate, except where noted. We identified regions of increased DNaseI-hypersensitivity called hotspots (see Methods) with a false discovery rate of less than 1%, and specific 150 bp peaks within these regions. Approximately 100,000-250,000 DHS peaks were mapped in each sample.

The brain and retina DHSs overlapped with many previously identified cis-regulatory regions of the genome (Shen et al., 2012). This is shown in Figure 1A for the neurofilament gene, *Nefl*, a gene expressed throughout the nervous system. In the embryonic day 14.5 (E14.5)

mouse brain, DNase I hypersensitive regions align with ChIP-seq peaks from the Encode project (Rosenbloom et al., 2013) for marks of promoters (H3K4me3), enhancers (H3K4me1, H3K27ac) and poised or negatively regulated regions (H3K27me3) (Fig. 1A). When we compared the overlap of DHSs with different epigenetic modifications across the genome, we found that their overlap ranged from 72% (H3K27me3) to 99% (H3, depending on the particular mark (Fig 1B). The overlap of brain DHSs with genomic features is shown in Figure 1C (Fig. 2A for cerebral cortex, retina and the cerebellum). Overall, the distribution of brain DHSs across the genome is similar to that of mouse DHSs present in other tissues (Vierstra et al., 2014), with the majority of DHSs in intronic regions (54%) or distal intergenic regions (31%).

To define DHSs unique to the CNS, we compared DHSs from the mature whole brain and mature retina with those from other mouse cell and tissue types (collectively including 1,323,372 distinct DHSs (Vierstra et al., 2014). This comparison identified 4,465 DHSs unique to the CNS ("CNS-core"), thus defining a core "regulome" of CRMs that drive gene expression in the CNS. The distribution of CNS-core DHSs relative to genomic features is similar to that of all brain DHSs, though with a decrease in promoter proximity (Fig. 1C, right). We next asked (using GREAT (McLean et al., 2010)) whether there was specific enrichment of CNS-core DHSs near genes relevant to nervous system function. This gene ontology analysis showed highly significant enrichment near synaptic and axonal genes (cellular component) neurotransmitter regulation (biological process), and voltage-gated ion channels (molecular function; Fig. 2B). With respect to specific genes, CNS-core DHSs are located near many genes highly expressed in the nervous system, including those known to be involved in synapse formation and specificity (eg. Dscam, Dscaml1, complexins, contactins (Yamagata and Sanes, 2012; Zipursky and Grueber, 2013)) and other neuronal processes (Fig. 2C).

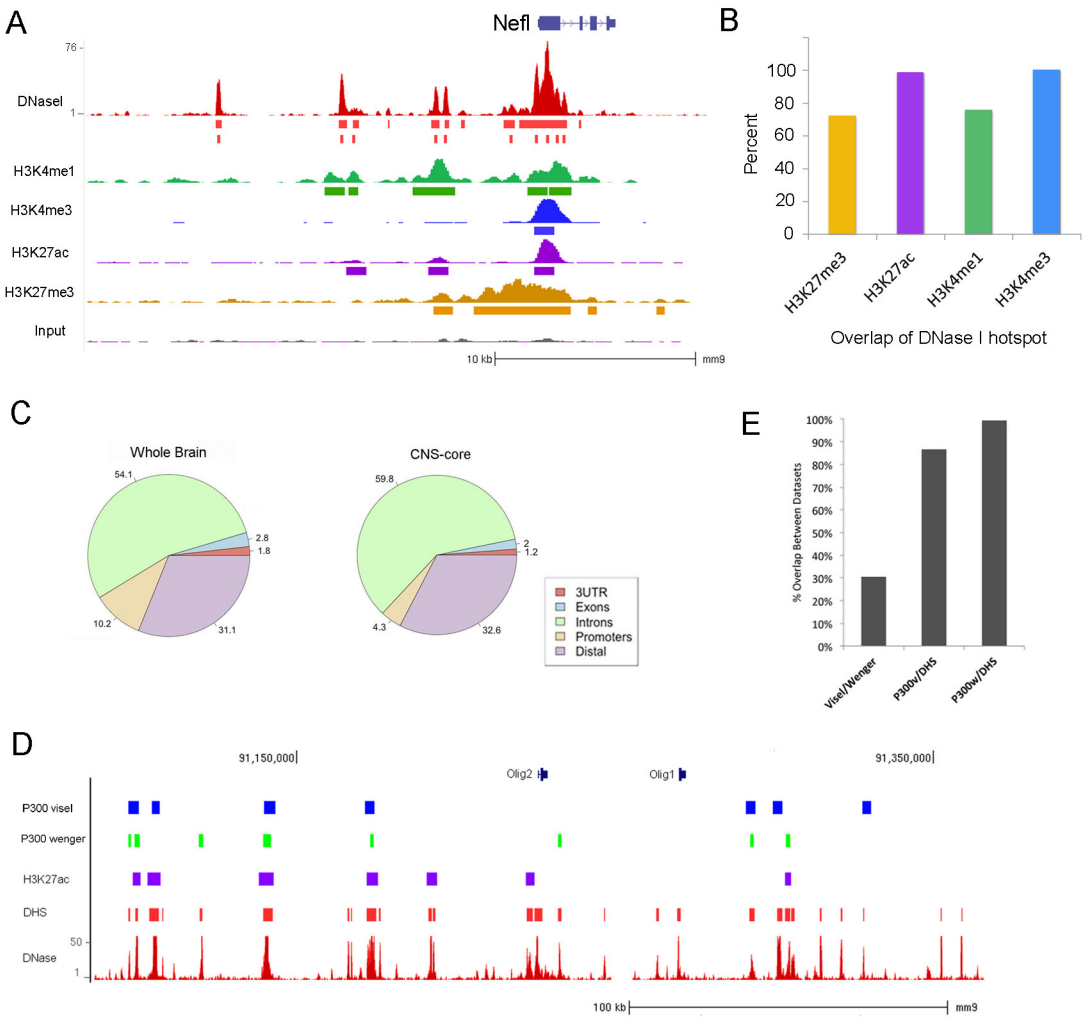


Figure 2.1: Global analysis of the DHS landscape of mouse CNS. (A) Comparison of DHSs to other epigenetic marks at the *Nefl* locus. DNase I cleavage patterns are shown for the E14.5 mouse brain (red); ChIP-seq for H3K4me1 (green), H3K4me3 (blue), H3K27ac (purple), and H3K27me3 (yellow). Solid bars under ChIP-seq signal represent peak calls. Top row of solid bars under DNaseI signal represent hotspot calls (see methods), bottom row represents DHS peak calls. (B) The percentage of ChIP-seq peaks of different histone modifications that overlap with DHSs in E14.5 brain. DHSs from the E14.5 mouse brain to the H3K27ac ChIP-seq data at the same age shows close to 100% (99.6%) of the H3K27ac sites overlap with those identified by a DHS. (C) Distribution of DHSs present in adult brain and in CNS-core DHSs relative to genomic features. (D,E) Comparison of DNase I hypersensitivity in E14.5 embryonic mouse brain to recent P300 or H3K27ac ChIP-seq (Nord et al., 2013; Visel et al., 2013; Wenger et al., 2013) studies of developing mouse cerebral cortex. (D) Correspondence between DNase I hypersensitivity, H3K27ac ChIP-seq, and two P300 ChIP-seq experiments. Peak calls for these three related studies near the Olig1 and Olig2 genes, along with the DNaseI hotspots for E14.5 brain. P300 Visel: peak calls for P300 ChIP-seq performed by (Visel et al., 2013); P300 Wenger: peak calls for P300 ChIP-seq performed by (Wenger et al., 2013); H3K27ac: peak cells for H3K27ac ChIP-seq (Nord et al., 2013); DHS: DNaseI Hotspots; DNase: DNaseI signal track. (E) Genome-wide comparisons for correspondence of the P300 ChIP-seq and DNaseI Hotspots

showing >80% of the P300 peaks overlap with DNaseI hotspots. Bar values indicate the percent of overlap between the indicated datasets.

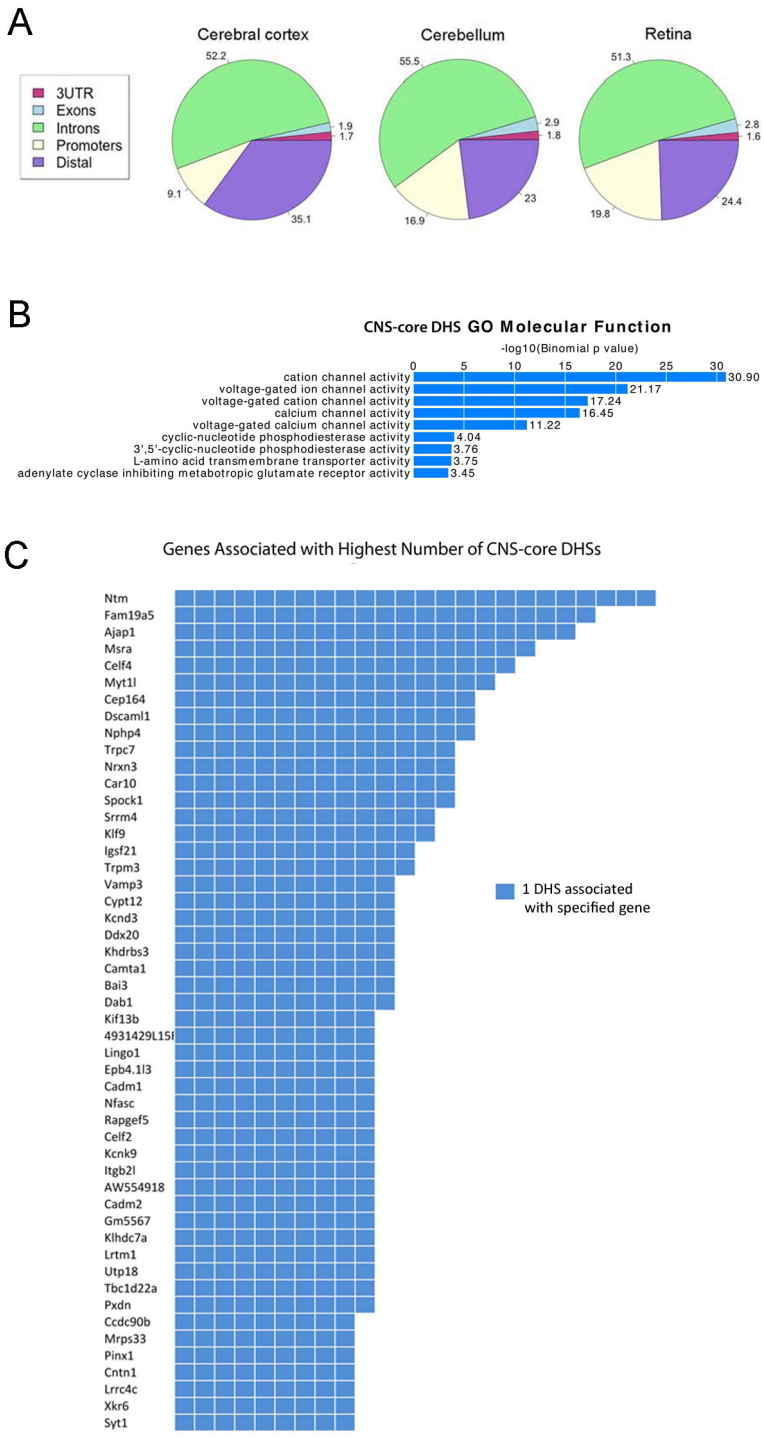


Figure 2.2: Genomic Partition and Gene Ontology Analysis. (A) Genomic partition of cortex, cerebellum, and retina DHSs. Distribution of DHSs present in mature cerebral cortex and cerebellum brain regions and mature retina relative to genomic features. (B) Gene Ontology Molecular Function enrichment of CNS-core DHSs. (C) Gene Ontology analysis from GREAT,

Molecular Function category, of the CNS-core set of DHSs showing enrichment near neuronal genes.

Characterization of CNS DHSs

The vast majority of DHSs in the CNS, CNS-core, and all CNS sub-regions are located either in introns or distal to gene transcription start sites (TSSs) and may be acting as remote enhancers (Mercer et al., 2013). To determine how the DNase I identification of putative cis-regulatory elements compares with other predictive epigenetic marks, we carried out a more detailed analysis of the developing brain. Several recent studies have characterized enhancers in embryonic mouse brain using either P300 ChIP or H3K27ac ChIP (Nord et al., 2013; Visel et al., 2013; Wenger et al., 2013). We compared the DNase I hypersensitivity data to these other chromatin signatures of enhancers for the E14.5 mouse brain, and the results are shown in Figure 1D. The peak calls for these three related studies are shown in Figure 1D near the Olig1 and Olig2 genes, along with the DNase I signal and hotspots for E14.5 brain. There is a good correspondence between the H3K27ac, the P300 and the DNase I hypersensitivity. However, there are also some regions where one P300 ChIP study shows a peak that corresponds with a DNase I hotspot which is not present in the other P300 study. There are other regions with DNase I hypersensitivity that are also identified in both the P300 ChIP studies, but not with the H3K27ac ChIP-seq. Thus, there appears to be good agreement with our DNase data and other well-characterized marks of enhancers, but the DNase I signal encompasses a wider range of potential regulatory elements.

Overall, comparing brain DHSs from E14.5 mouse to the H3K27ac ChIP-seq data at the same age (Fig. 1B), we find that close to 100% (99.6%) of the H3K27ac sites overlap with those identified by DNase I hypersensitivity. Genome-wide comparisons for correspondence of the P300 ChIP-seq and DNase I analysis, shows that although the two different P300 ChIP studies

identify somewhat different enhancers (Fig. 1E), the regions identified by either P300 ChIP-seq study fall largely (87% - 94%) within the sites of DNase I hypersensitivity in the E14.5 brain (Fig. 1E).

To further validate the effectiveness of the DNase I approach for identifying brain enhancers, we tested whether this method could identify brain enhancers that have already been tested in transgenic mice using the Vista Enhancer Browser Program (Visel et al., 2007). The Vista project has tested 435 elements from the mouse genome chosen for their high degree of sequence conservation across species and/or ChIP-seq evidence for putative enhancer marks. Of these, 94 show expression in embryonic brain. The H3K27ac ChIP-seq peaks successfully identified 58/94 of these elements, while a similar number (52/94) of these putative enhancers were identified by DNase I hotspots. Three examples are shown in Fig. 4. The mm871 enhancer (tan shaded) shows overlap with the DNase I peak, the P300 ChIP-seq peaks, and the H3K27ac peak, whereas the other two enhancers show a DNase I hotspot and two of the three other marks.

We also tested whether DNase I would be more effective as a discriminator than H3K27ac ChIP-seq for predicting whether a putative enhancer will *fail to* be expressed in the brain. Of the 435 mouse elements tested, 178 failed to be expressed in any tissue and 63/178 of these non-expressed elements had H3K27ac peaks. Nearly all of the elements with H3K27ac peaks that failed to show expression in the transgenic embryo also had DHSs in the E14.5 brain (56/63; 89%). Thus, while DHSs provide an effective method for identifying putative enhancers, they are no better than H3K27ac ChIP-seq at discriminating those elements that are not confirmed by transgenic analysis.

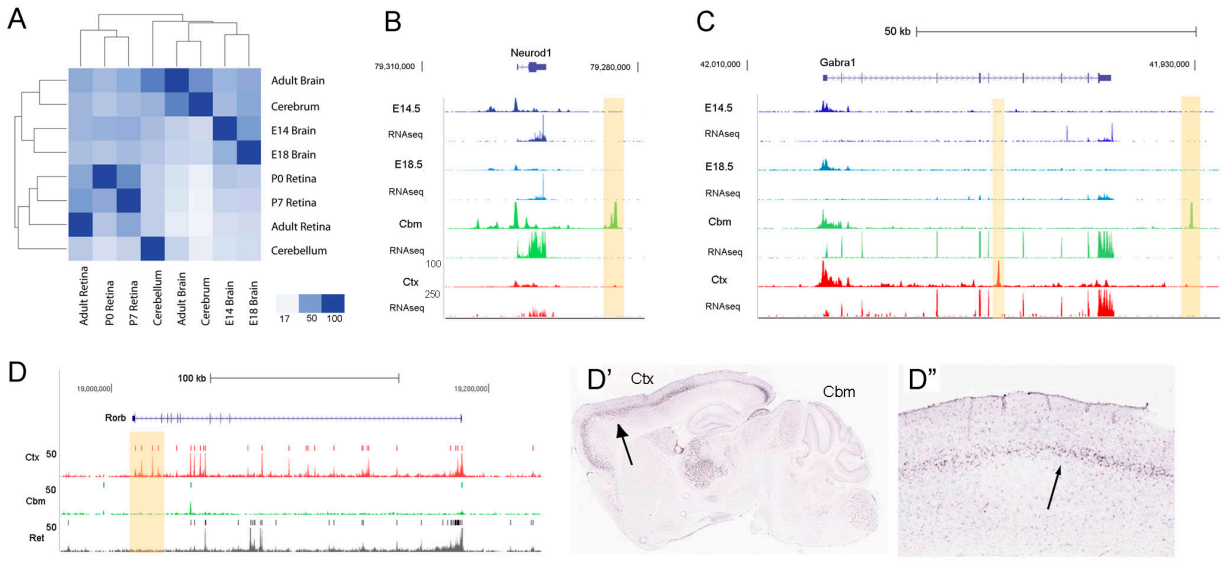


Figure 2.3: Brain region specific regulatory elements identified by DHSs. (A) Heatmap of cluster analysis of the DHSs present in CNS samples clustered according to the percent of overlapping DHS peaks in each tissue, indicated by color intensity. (B-D) Examples of region specific DNase I hypersensitivity landscapes with matching, previously generated RNA-seq data (Rosenbloom et al., 2013). (B) DHS in cerebellum associated with the *Neurod1* locus that is not present in the cerebral cortex (tan shading). (C) DHS associated with the *Gabra1* gene with examples of region-specific DHSs (tan shading). (D) Extensive differences in DNaseI landscape (and DHS peaks, indicated as blocks over signal tracks) at the *Rorb* locus. (D', D'') *Rorb* is expressed in cerebral cortex (Ctx; higher magnification in D'') but not cerebellum (Cbm), shown by *in situ* hybridization (Allen Brain Atlas); there are specific DHSs associated with this gene in cerebellum and cerebral cortex (tan shaded regions). E14.5: embryonic day 14.5 whole brain; E18.5: embryonic day 18.5 whole brain; Cbm: 8-week adult cerebellum; Ctx: 8-week adult cerebral cortex.

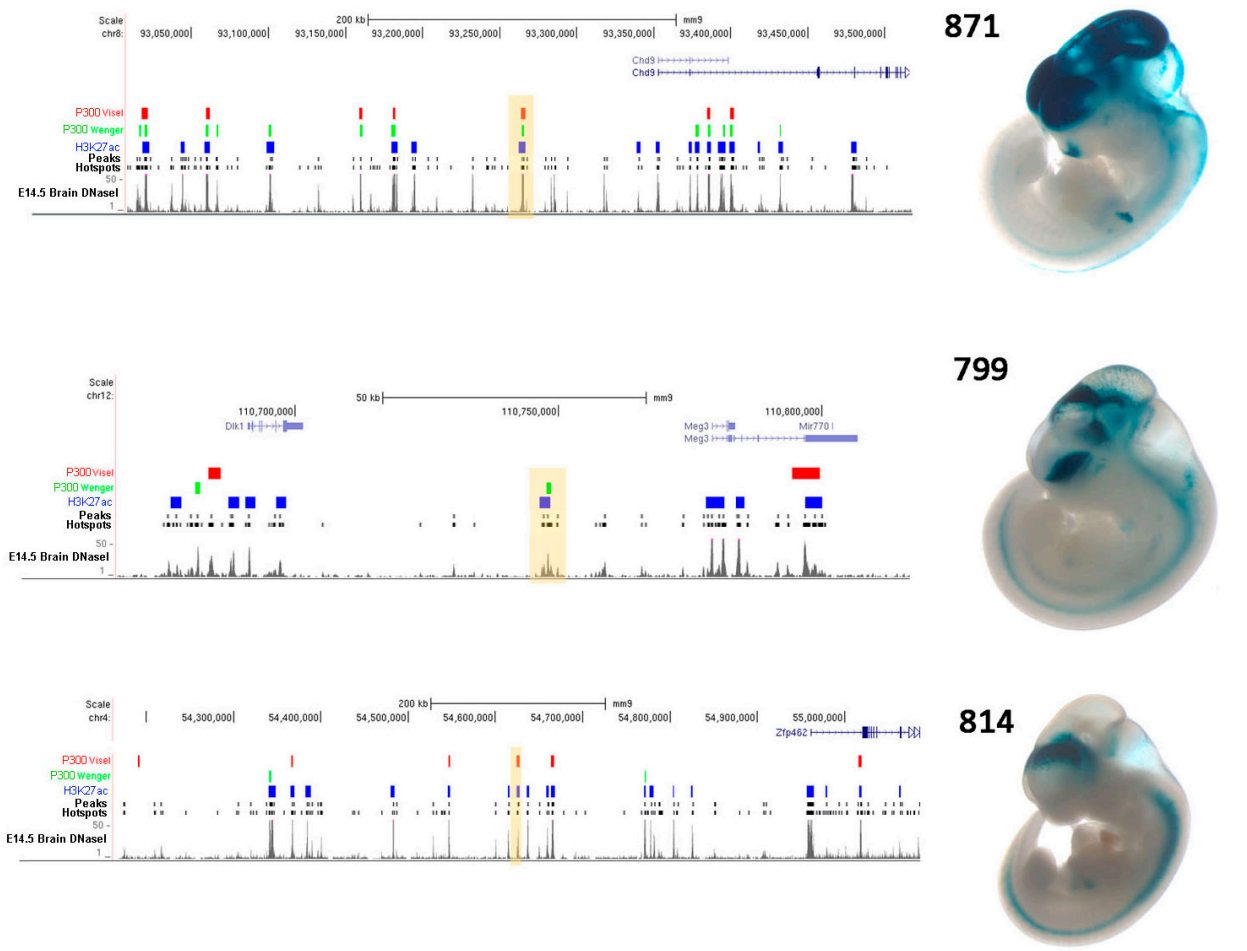


Figure 2.4: DNase I hypersensitivity corresponds to enhancer regions identified in previous studies and confirmed in transgenic mice for the Vista Browser project (Visel et al., 2007). Three different enhancers with their expression patterns are shown at right in transgenic mice, and as a tan shaded region in the UCSC browser tracks. The P300 and H3K27ac ChIP-seq peaks from previous studies are labeled as in Figure 1C.

Region and cell-type specific regulatory elements identified by DHSs

The DHS dataset is potentially useful to identify regulatory regions specific to particular CNS regions. Since many of the genes are shared between neurons, regardless of their location in the CNS, and many housekeeping genes are also likely to be shared across brain regions, comparing DHSs from different brain regions represents a potentially powerful approach to identifying neuronal sub-type CRMs. To compare DHS activity between different CNS regions and developmental time points, we performed a hierarchical clustering analysis (based on the

percentage of overlapping DHS peaks pairwise between each tissue) of developing and mature CNS samples (Fig. 3A). As expected the mature brain regions cluster and the developing brain samples cluster, but there are many unique DHSs between any particular CNS regions. These region-specific DHSs are present in many genes relevant to neural development and mature function. For example, there is a DHS present in cerebral cortex associated with the *Neurod1* locus that is not present in the cerebellum (Fig. 3B) even though the gene is expressed in both regions (Aprea et al., 2014). The *Gabra1* gene also shows clear examples of region-specific DHSs (Fig. 3C), and again is expressed throughout the adult brain (Bosman et al., 2005). Since approximately 50% of the DHSs are potential enhancers for associated genes based on the above analysis, these region-specific DHSs provide a large list of candidate region-specific enhancers for future exploration.

We hypothesized that the transcription factors regulating neuronal and glial gene expression might differ between these region-specific DHSs and reflect the specific complement of transcriptional regulators in these different brain regions. To test for enrichment of consensus binding sites in the region-specific DHSs, we created sets of DHS peaks that are (1) present in retina, but not cerebellum or cortex (2) present in cortex but not retina or cerebellum, and (3) present in cerebellum, but not retina or cortex. These sets of DHSs were analyzed with the MEME suite (DREME and CentriMo; (Bailey et al., 2011; Bailey et al., 2009; Machanick and Bailey, 2011)) and we found a distinct pattern of enrichment for transcription factor motifs in the different sets (Fig 6A). For example, in the retina-specific DHSs, Otx2 and Crx consensus sites were highly enriched, while in the cortex, Egr1 sites and E-box transcription factor sites predominated. Further analysis of the cortical DHSs with Centrimo, focusing on the Egr1 sites and the bHLH consensus sites, show very different sets of transcription factor enrichment and

gene category associations (Fig. 6B). CREB related signaling pathways and glutamate receptors were associated with the Egr1 DHSs, while the bHLH binding peaks were associated with ion transport and exocytosis genes. Overall, the comparison of DHSs in different brain regions provides a powerful approach to identify new potential enhancers for neuronal and glial gene expression and the transcription factors that regulate them.

Although genome-wide DHS mapping can potentially identify all CRMs active in the CNS, because of the wide diversity of neurons, most neuronal cell types represent a relatively small fraction of the total population in any given region. Therefore, we asked whether this technique has the sensitivity to detect active regulatory elements associated with genes that are only expressed in a small percentage of cells in the CNS. We used two different approaches to address this question. First, we chose several genes that are known to be expressed in relatively sparse cell populations in the CNS and examined their promoters for the presence of DHSs. Because these genes are active in only a small number of cells, and given the strong correlation between promoter hypersensitivity and gene expression (Thurman et al., 2012), this would provide a good method to evaluate the sensitivity of the DNase-seq. We queried the Allen Brain Atlas (Shen et al., 2012) for genes with laminar-specific expression, since the cortical laminae represent on average $1/6^{th}$ of the neurons in the cortex. We found peaks of DNase I hypersensitivity at the promoters of three laminar-specific genes *Rorb*, *Kcnn2* and *Etv1* in cerebral cortex (Fig. 3D; Fig. 7). *Rorb* is also highly expressed in the retina (Jia et al., 2009), but not the cerebellum, and so the region-specific DHSs are also apparent in this example. Thus, there are clearly identifiable cortex-specific DHSs even near genes expressed in only a small percentage of the cortical neurons. However, we cannot discount the possibility that DHSs at

laminar-specific genes are hypersensitive in a broader range of cell types, despite lack of mRNA expression.

To further determine whether DNase I hypersensitivity mapping can discover regulatory elements associated with gene expression in rare cell types of the CNS, we focused on an analysis of the neural retina, for which there has been extensive characterization of cell types and gene expression (Blackshaw et al., 2004; Siegert et al., 2012). Some retinal cell types, such as ganglion cells and cone photoreceptors, represent only a small fraction of the total cells in the mouse retina (~1-2%) (Jeon et al., 1998). As with laminar-specific genes, genes expressed in sub-populations of retinal cell types contain clear DHS signals near transcription start sites and nearby enhancers, as shown for two example genes (Fig. 5A). Specific DHSs were present for *Pou4f2*, a transcription factor expressed only in a subset of retinal ganglion cells (~ 1% total retinal cells). Similarly, *Opn1sw*, a gene present exclusively in short wave-length cone photoreceptors (comprising ~1% of total retinal cells in mice) displays a DHS at the transcription start site that is present at all 3 stages of retinal development. RNA-seq data (Roger et al., 2014) confirms expression of these two genes at early post-natal (P2) and mature (P21) stages of retinal development (Fig. 5A). The detection of DHSs at the TSSs of cell type specific genes of minority cell populations highlights the usefulness of DNase I hypersensitivity mapping for identifying regulatory elements in complex tissues comprised of many cell types. If these DHSs can be observed only in purified rare cell populations, it would suggest that DHSs can be detected from minority cell types in complex tissues even when they comprise ~1% of the total cell population. We cannot exclude the possibility that these DHSs associated with rare cell type specific genes are present in a broader range of cell types.

The results of the hierarchical clustering analysis we carried out suggested that the comparison of DHSs active in CNS regions to one another identifies region-specific regulators of genes. There were 49,383 DHSs common to brain and retina, and 51,187 DHSs in mature retina that were not in the brain; these latter DHSs were highly enriched near genes that are involved with photoreceptor and retinal phenotypes. To extend this analysis systematically, a recent gene expression characterization for specific retinal cell types purified from fluorescent reporter mice has provided "barcodes" for the basic retinal cell types and many subtypes (Siegert et al., 2012). When we analyzed genes specific to each retinal cell type we found retina-specific DHSs near these genes (Fig. 5B) as determined by GREAT analysis ("basal plus extension" association rules (Siegert et al., 2012)). We further defined a set of retina-specific DHSs (rsDHSs) by subtracting DHSs active in all other tissues and cell types in the ENCODE set (38 cells/tissues) from those active in the retina. This set showed an even greater enrichment near genes known to be expressed in retina, specifically those involved in photoreceptor function or related to retinal disease in human and mouse phenotype (Fig. 5C; Fig. 8). Since many retinal-specific DHSs would likely be associated with genes expressed in a unique retinal cell type, the photoreceptor, we compared DHSs active in the retina with the binding sites of Crx and Nrl, two transcription factors essential for photoreceptor development and maintenance (Furukawa et al., 1997; Yoshida et al., 2004). Using previously published ChIP-seq data (Corbo et al., 2010; Hao et al., 2012), we found that 97.4% of 5,724 CRX peaks and 76.7% of 7,411 NRL peaks overlap with a DHS in the mature retina (Fig. 5D). The overlaps for DHS hotspots were even greater than for the peaks: 99.9% for Crx and 80.6% for Nrl. Furthermore, a substantial fraction of retina-specific DHSs coincide with Crx or Nrl binding sites or are co-bound by both factors (33%, 30% and 24%, respectively; Fig. 5E). Together, these results demonstrate that DNase-seq is a highly

sensitive approach for identifying potential cis-regulatory elements that regulate CNS-region specific gene expression.

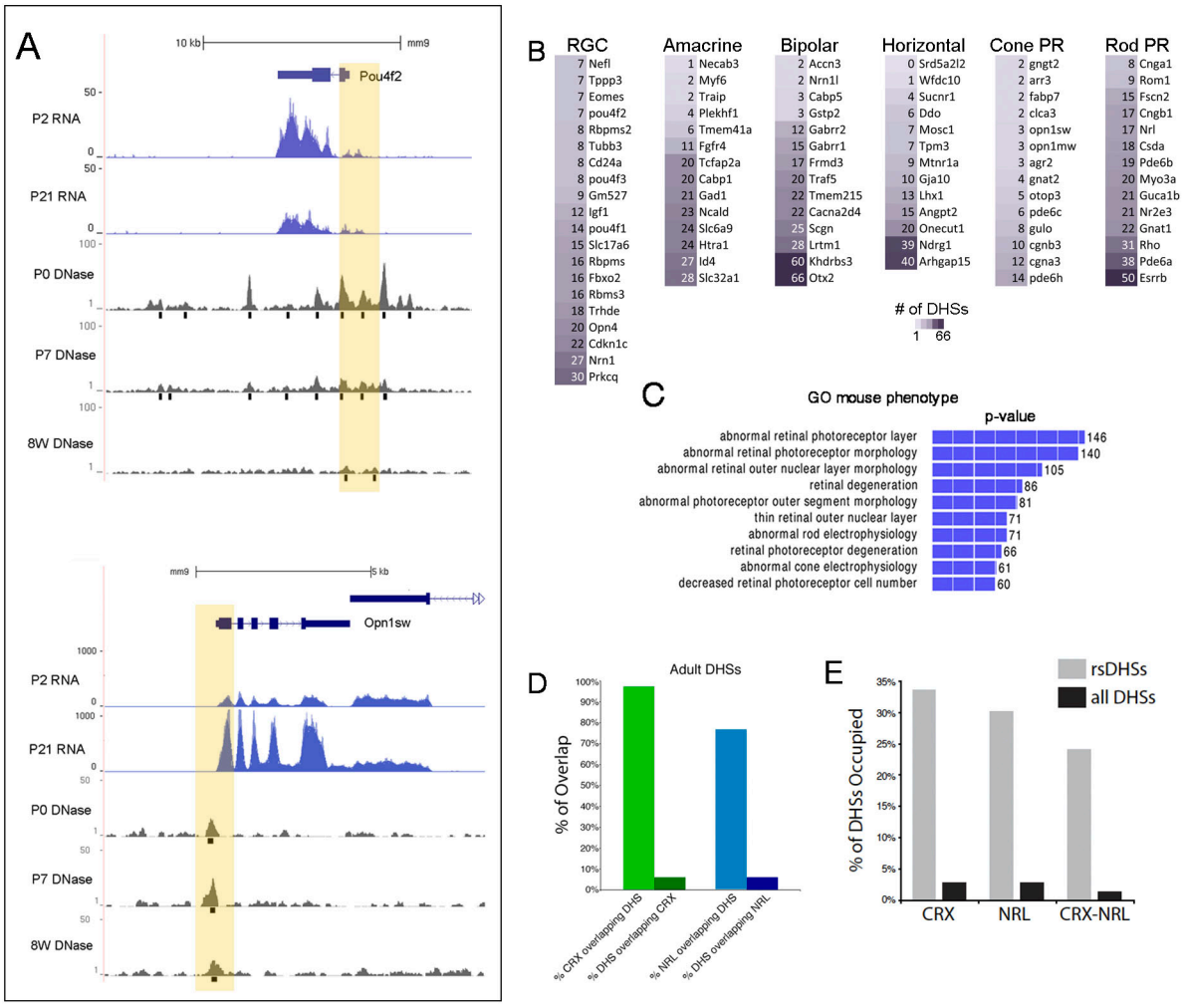


Figure 2.5: DNase I hypersensitivity identifies (rare) cell-type specific regulatory elements. (A) DNase I (P0, P7, and 8-week adult retina) and RNA-seq (P2 and P21 retina;(Roger et al., 2014) landscape near genes expressed exclusively in minority cell populations in the retina: ganglion cells (*Pou4f2)* and cone photoreceptors (*Opn1sw*). Black bars below DNaseI signal tracks indicate DNaseI peaks. Yellow boxes indicate DHS within promoter regions. (B) The number of DHSs near genes expressed specifically in indicated retinal cell-types, sorted by column. Color intensity increases with DHS number. (C) Gene Ontology - Mouse Phenotype enriched terms for retinal, but not brain, DHSs as determined by GREAT analysis. Numbers indicate the number of genes associated with each term. (D) Overlap between ChIP-seq peaks for two key photoreceptor-specific transcription factors, CRX and NRL. Although nearly all of the ChIP-seq peaks for these TFs overlap a DHS in the retina, there are still many retinal DHSs that are associated with genes expressed in other retinal cell types. (E) Overlap between CRX and NRL ChIP-seq peaks (including co-binding regions) with retinal-specific DHSs.

A

Cerebral cortex		
motif	E-value	total_sites
GTGGGNR	2.40E-156	14320
CAKMTGK	5.60E-145	14341
MA0472.1 EGR2	5.70E-138	16565
UP00007_1 Egr1_primary	7.70E-132	14078
MA0162.2 EGR1	9.80E-129	17137
UP00002_1 Sp4_primary	1.30E-106	12690
MA0161.1 NFIC	7.60E-105	42361
UP00046_2 Tcfe2a_secondary	1.70E-103	24761
MA0521.1 Tcf12	6.40E-103	11843
MA0522.1 Tcf3	1.30E-93	13560

Cerebellum		
motif	E-value	total_sites
CCATMTGB	6.70E-245	6326
TGCCAR	4.80E-225	14658
1	1.20E-221	9710
MA0161.1 NFIC	1.40E-211	31441
GCCARR	9.30E-182	20017
SYTGGCW	9.30E-179	15962
MA0091.1 TAL1::TCF3	1.10E-170	10856
UP00046_2 Tcfe2a_secondary	7.80E-162	20424
MA0119.1 TLX1::NFIC	1.10E-154	3998
MA0461.1 Atoh1	5.60E-150	7352

Retina		
motif	E-value	total_sites
RGATTA	7.80E-112	8568
MA0467.1 Crx	2.80E-95	13694
UP00176_1 Crx_3485.1	6.40E-90	9310
UP00265_1 Pitx3_3497.2	1.30E-86	8736
UP00111_1 Dmbx1_2277.1	8.70E-81	12014
UP00109_1 Obox6_3440.2	3.00E-77	9711
UP00112_1 Gsc_2327.3	1.50E-74	10230
UP00153_1 Pitx1_2312.1	2.30E-69	11506
UP00160_1 Obox3_3439.1	6.20E-62	9971
UP00267_1 Otx2_3441.1	2.40E-58	11808

B

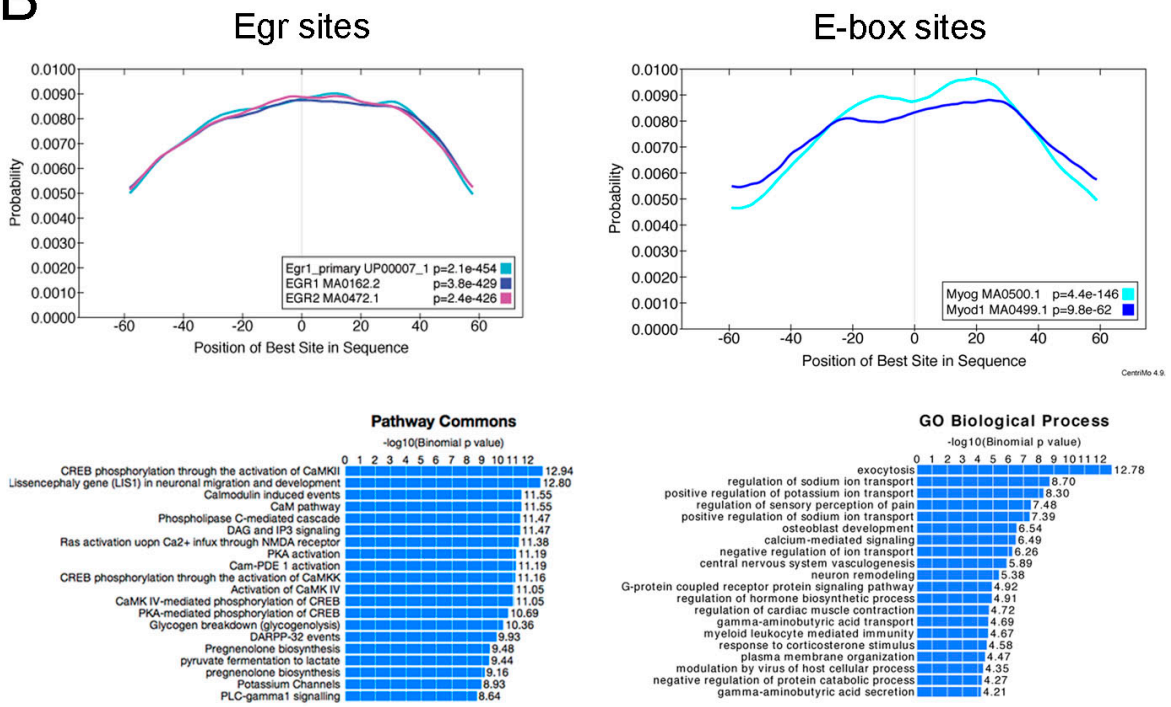


Figure 2.6: Sets of enriched DHSs between CNS tissues and CentriMo analysis. (A) The sets of DHSs enriched for cerebral cortex, cerebellum, or retina were analyzed with the MEME suite (DREME and CentriMo) and we found a distinct pattern of enrichment for transcription factor motifs in the different sets. Egr1 sites and bHLH transcription factor sites were highly enriched in cerebral cortex, whereas Crx sites were over-represented in DHSs from retina. (B) CentriMo analysis for Egr1 and E-box sites in cerebral cortical DHSs shows enrichment near the central regions of these DHSs for the over-represented transcription factor motifs, consistent with their role as enhancers. Below: GO enrichment terms associated with Egr and E-box sites in cortex.

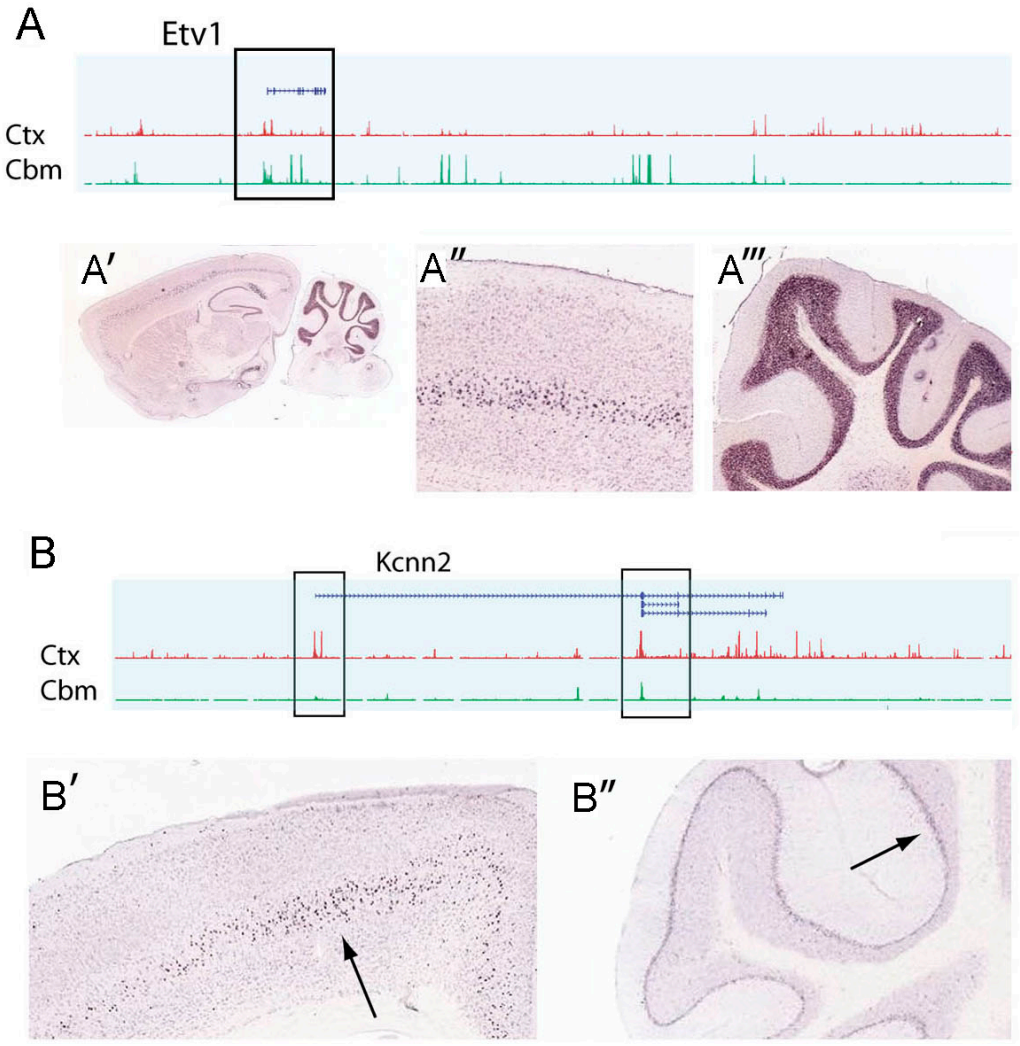


Figure 2.7: DNase-hypersensitivity at the promoters of cell-type specific genes. DNase I landscape (cerebral cortex, Ctx (red); cerebellum, Cbm (green); retina, Ret (black)) surrounding the gene bodies of *Etv1 and Kcnn2*. In situ data from 2014 Allen Institute for Brain Science. Available from: http://mouse.brain-map.org/; (Lein et al., 2007).

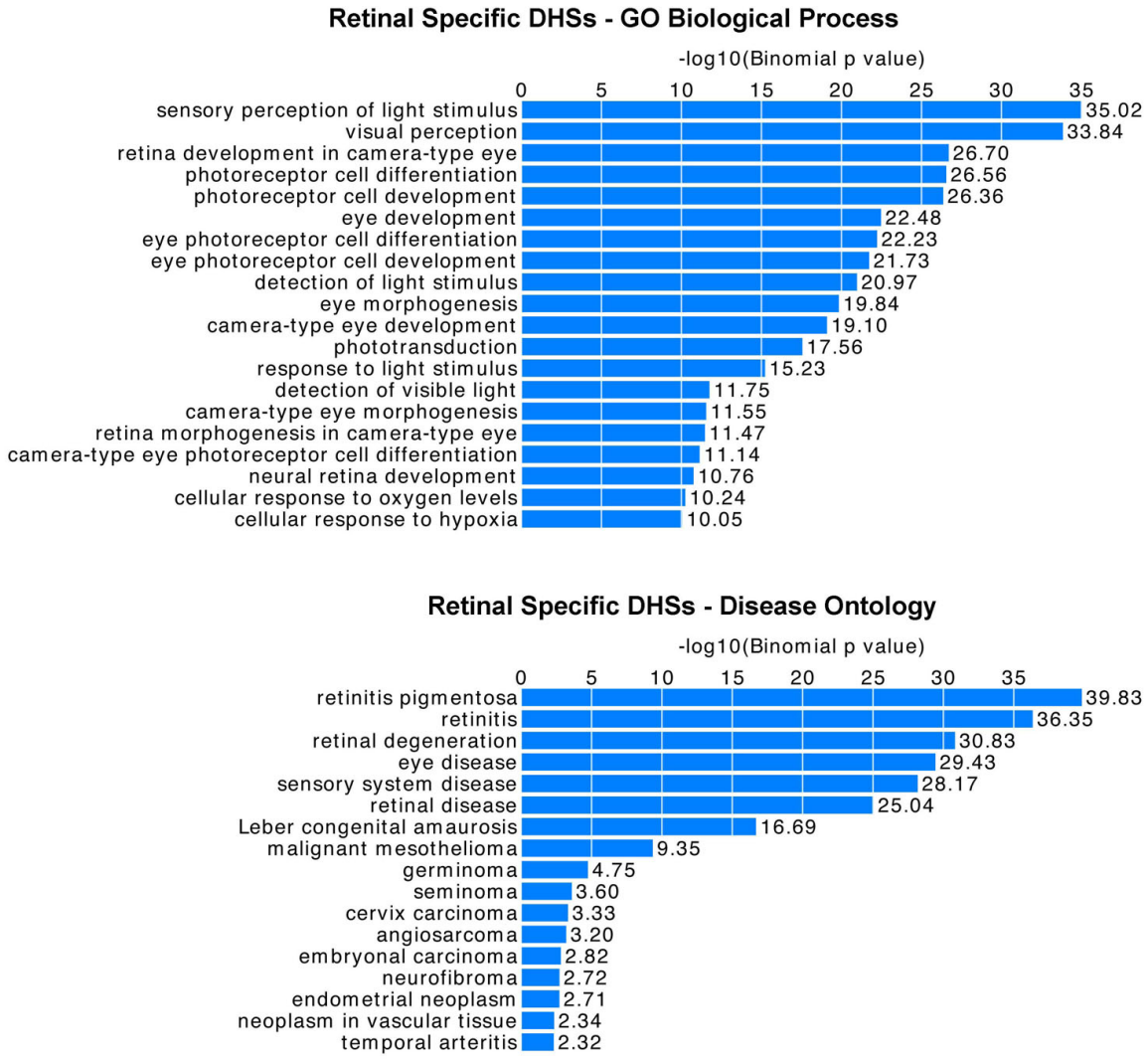


Figure 2.8: Gene ontology enrichment of retinal specific DHSs. Gene ontology ((A) biological process and (B) disease ontology categories for genes associated with retina-specific DHSs as determined by GREAT analysis.

Temporally dynamic regulatory elements

CNS development involves the processes of neurogenesis, differentiation, axon growth and pathfinding, target selection and synaptogenesis. These processes occur primarily over the last week of fetal development and the first week of postnatal development. Overall, our hierarchical clustering analysis demonstrated that fetal brain and neonatal retina are more closely related than mature regions of the CNS. However, different brain regions have markedly

different developmental stages at the same fetal age. To better analyze these processes in a sequential manner, we focused on a single CNS region (the retina) where the developmental processes are more synchronous and greater expertise is possessed by our laboratory. We observed clear developmentally dynamic patterns of chromatin accessibility surrounding two key developmental genes and two genes highly expressed in mature retina (Fig. 9A). *Neurog2*, and *Olig2*, two transcription factors expressed in retinal progenitors, and necessary for neurogenesis (Brzezinski et al., 2011), display prominent peaks of DNase I cleavage at the transcription start site and have several additional peaks in the surrounding intergenic space in P0 retina, but decrease during progression to P7 and adult retina. This corresponds to their expression patterns as demonstrated by RNA-seq (Fig. 10; (Roger et al., 2014)). The reverse pattern is observed for *Rho, Guca1a* and *Guca1b*, genes expressed specifically in developing and mature photoreceptors (Blackshaw et al., 2004; Hsiau et al., 2007; Rao et al., 2011): their promoters and neighboring DHSs show substantially increasing accessibility from P0 to adult stages (Fig. 4B). Again, the DHS dynamics surrounding these genes corresponds to gene expression (Fig. 10; (Roger et al., 2014)).

To more systematically evaluate the stage-specific dynamics of regulatory elements, we used k-means clustering to group all DHSs in the retina based on shared patterns of accessibility across P0, P7 and adult stages (Fig. 9C). This clustering analysis was carried out in collaboration with Kyle Siebenthall. Cluster groups designated by E (early), M (mid) and L (late) contain DHSs of peak intensity at P0, P7 and adult stages, respectively. Most clusters contain ~10,000 to 15,000 DHSs with the exception of the constitutively accessible group, which contains 35,000 DHSs (Fig. 9D). Furthermore, 5%-15% of DHSs in each cluster are located within gene promoters, with the exception of the constitutive group (35%; Fig. 9D). Gene

Ontology analysis showed that temporally patterned DHSs are highly enriched near genes of specific classes, commensurate with the developmental functions of those genes. Early clusters 1, 2 and 3 are generally associated with genes involved in stem cell maintenance, neuron generation, and gliogenesis (Fig. 12), reflecting the fact that neuronal production peaks in the retina at birth. Cluster E4 DHSs are primarily active at P0, but have some activity at P7, and are enriched near genes associated with synaptogenesis (i.e. "dendritic spine development"). Mid-stage clusters M1 and M2 are generally associated with many of the same genes in the early clusters, but also include the later processes of "axon extension" (Fig. 12), in agreement with the extensive neuronal differentiation and synapse formation that occurs at P7 (Mumm et al., 2005). Late clusters 1, 2 and 4 are generally associated with perception of light and photoreceptor maintenance (Fig. 12), reflecting the fact that photoreceptors comprise approximately ~80% of the adult mouse retina. This analysis suggests that the changing DHS landscapes are reflective of the stage-specific biological processes that are taking place during development. Therefore, further examination of their sequence content will likely reveal insights into upstream regulation of these DHSs.

As discussed in the previous section, Crx and Nrl are two transcription factors that play a role in photoreceptor differentiation, and their binding sites coincide extensively with retina-specific DHSs. Using the CRX and NRL ChIP-seq data (see above), we find the majority of CRX and NRL binding occurs in DHSs active in late-enriched stages of retinal development (66% and 60%, respectively; clusters L1-4, Fig. 9D). This corresponds to known patterns of expression for these genes ((Furukawa et al., 1997; Yoshida et al., 2004); Fig. 11). However, there are some Crx and Nrl binding sites present in DHSs active at all ages (30% and 34%, respectively). This suggests that some potential regulatory elements for photoreceptors are

accessible even early in their development (P0) while others become accessible as the cells mature; however, it is also important to note that the number of rods doubles between P0 and P7, and so this pattern might also reflect this change in cell number.

Given the enrichment of temporally patterned retina DHSs near specific classes of genes, we next sought to identify which transcription factors (TFs) could be controlling these patterns by analyzing TF binding motifs within the our k-means clustered DHSs in collaboration with Kyle Siebenthall. The full list of factors (Fig. 13) reveals significant enrichment ($P < 0.01$) of motifs for various TFs known to be involved in retinal development and neurogenesis. The cluster enrichment of a selected subgroup of TFs important for retinal development is displayed in Figure 4E. Importantly, the early clusters (especially E1 and E2) are enriched for motifs of TFs that are active in the developing retina (e.g. Lhx2, Pou3f2) (Kim et al., 2008; Tetreault et al., 2009), whereas the late clusters (L1, L2 and L4) are enriched for motifs of factors vital for mature retinal function (e.g. Otx2, Crx) (Roger et al., 2014). When we instead analyzed retina-specific DHSs, the motifs of 22 TFs are significantly enriched ($P < 0.01$), some of which have important functions in the retina (e.g. Rax, Otx2, Crx). Interestingly, many motifs enriched in temporally dynamic as well as retina-specific DHSs are recognized by TFs with as yet unexplored roles in retinal development.

Overall, these results show that temporal dynamics of specific developmental processes are reflected in temporal changes in chromatin accessibility surrounding genes involved in these processes. Furthermore, examination of TF motifs within temporally patterned DHSs can be used to identify the transcription factors that regulate these processes.

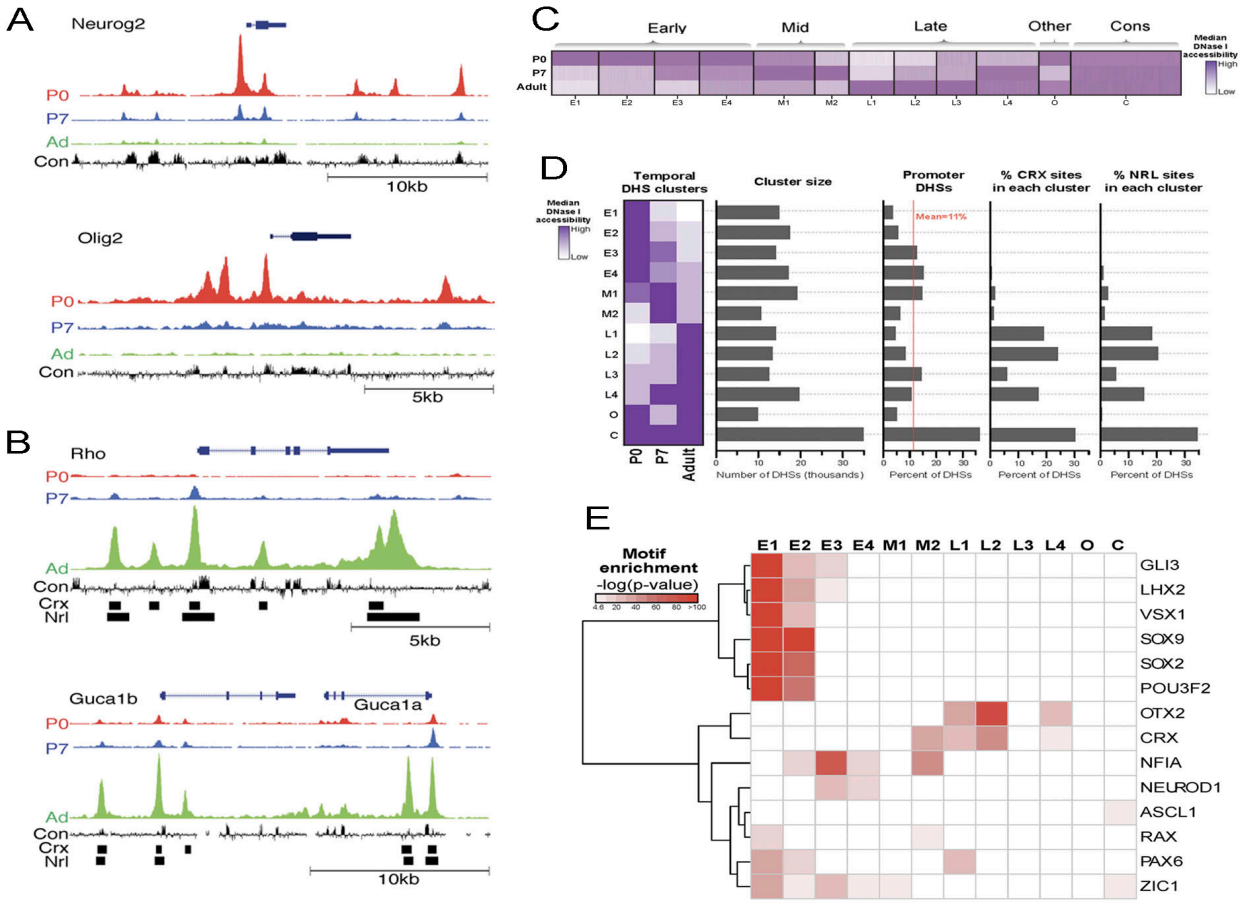


Figure 2.9: Examination of the developmentally dynamic DNase I landscape reveals stage specific cis-regulatory elements and transcription factors. DNase I landscape at representative gene loci near genes expressed in (A) developing (*Neurog2*, *Olig2*) or (B) mature (*Rho*, *Guca1b*) retina at P0 (red), P7 (blue) and Adult (Ad, green) stages, along with mammalian sequence conservation (Con, black). ChIP-seq peak locations for CRX and NRL are indicated by black boxes (Corbo et al., 2010; Hao et al., 2012). (C) Heatmap profile of accessibility at each DHS (columns) across developmental stages (rows), grouped by k-means clustering. Color intensity indicates the normalized DNase I accessibility according to the included scale. (D) Condensed heatmap of k-means-clustered DHSs (rows) in the retina between P0, P7 and adult stages (columns). Color intensity indicates median DNaseI accessibility of DHSs in each cluster. Also shown are the number of DHSs contained in each cluster, the percentage of DHSs within each cluster that overlap a gene promoter (1kb upstream of the transcription start site) and the percentage of total CRX or NRL ChIP-seq binding sites that overlap a DHS contained within each cluster. E, early; M, mid; L, late; O, other; C, constitutive. (E) Transcription factor binding motif enrichment analysis of all cluster groups shown for a selected group of transcription factors. Motif enrichment (-log10(p-value)) indicated as color intensity for each transcription factor (rows) within each temporal cluster group (columns).

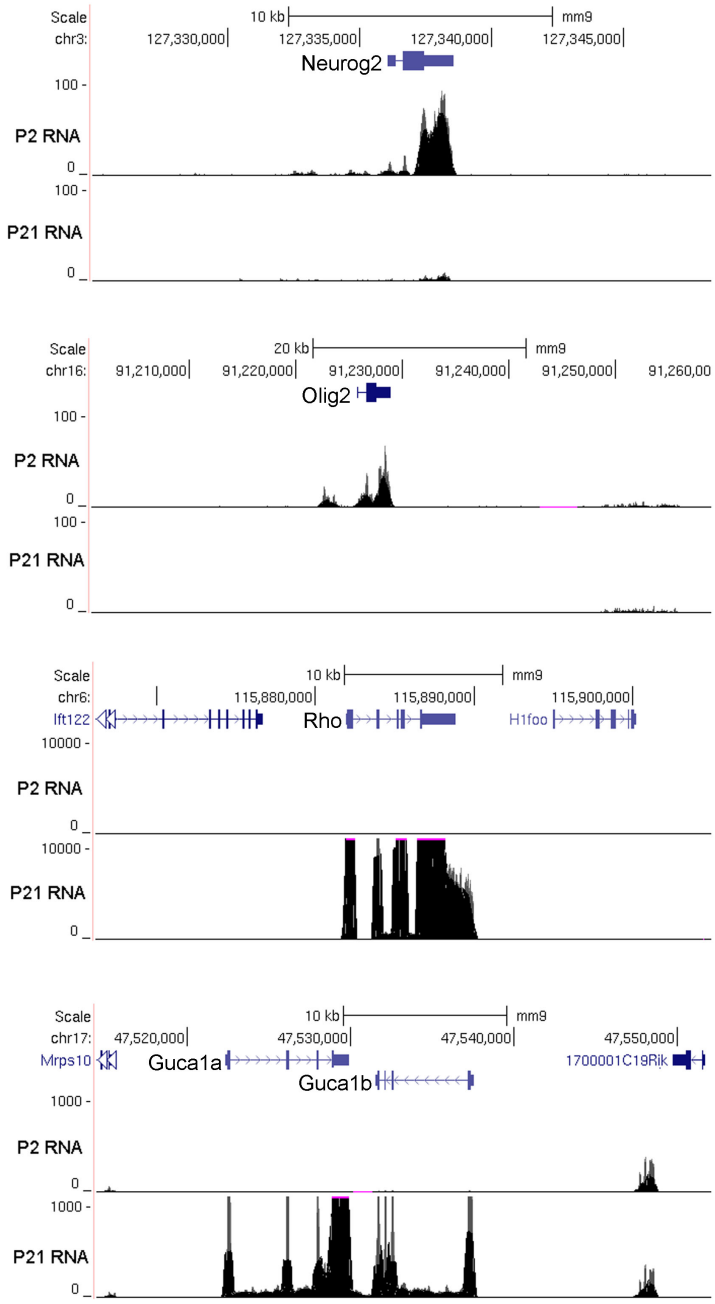


Figure 2.10: (Related to figure 2.9A,B) RNA-seq landscape for P2 and P21 retina surrounding two progenitor genes expressed in the early retina: *Neurog2* and *Olig2*; and three photoreceptor genes expressed in the mature retina: *Rho* and *Guca1a*/*b*. RNA-seq data previously generated by (Roger et al., 2014).

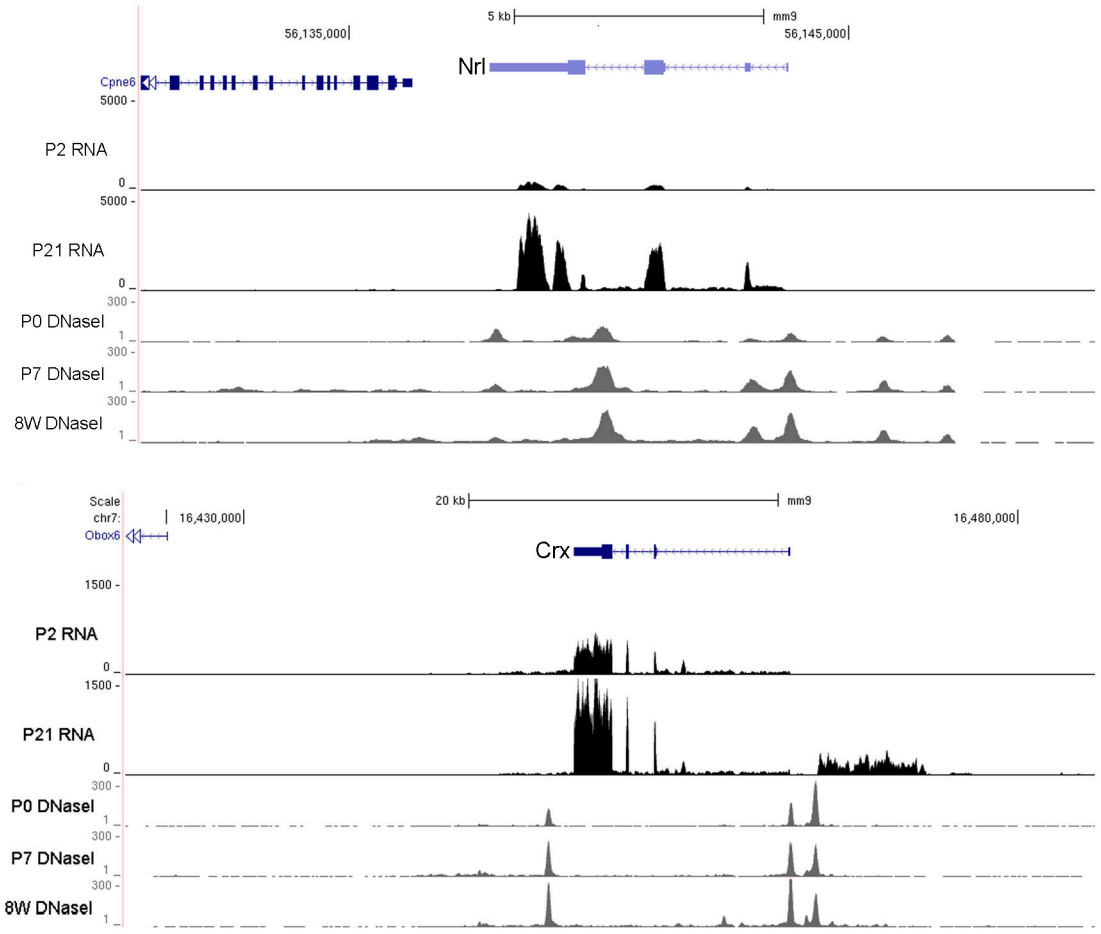


Figure 2.11: (Related to figure 2.9D,E) The RNA-seq landscape for P2 and P21 retina and the DNase I landscape for P0, P7, and 8-week adult (8w) retina surrounding three retinal development and differentiation genes: *Nrl*, and *Crx*. RNA-seq data previously generated by (Roger et al., 2014).

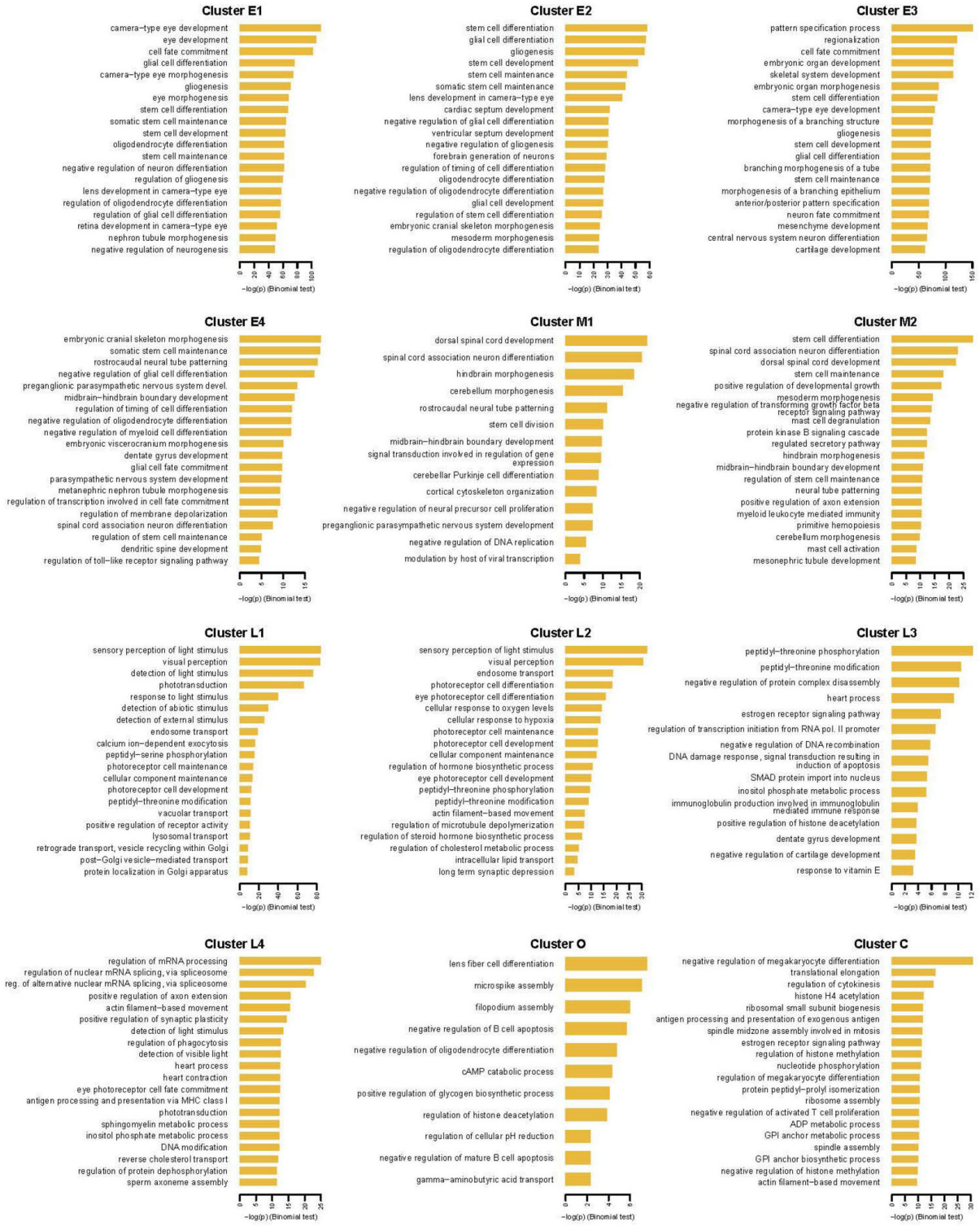
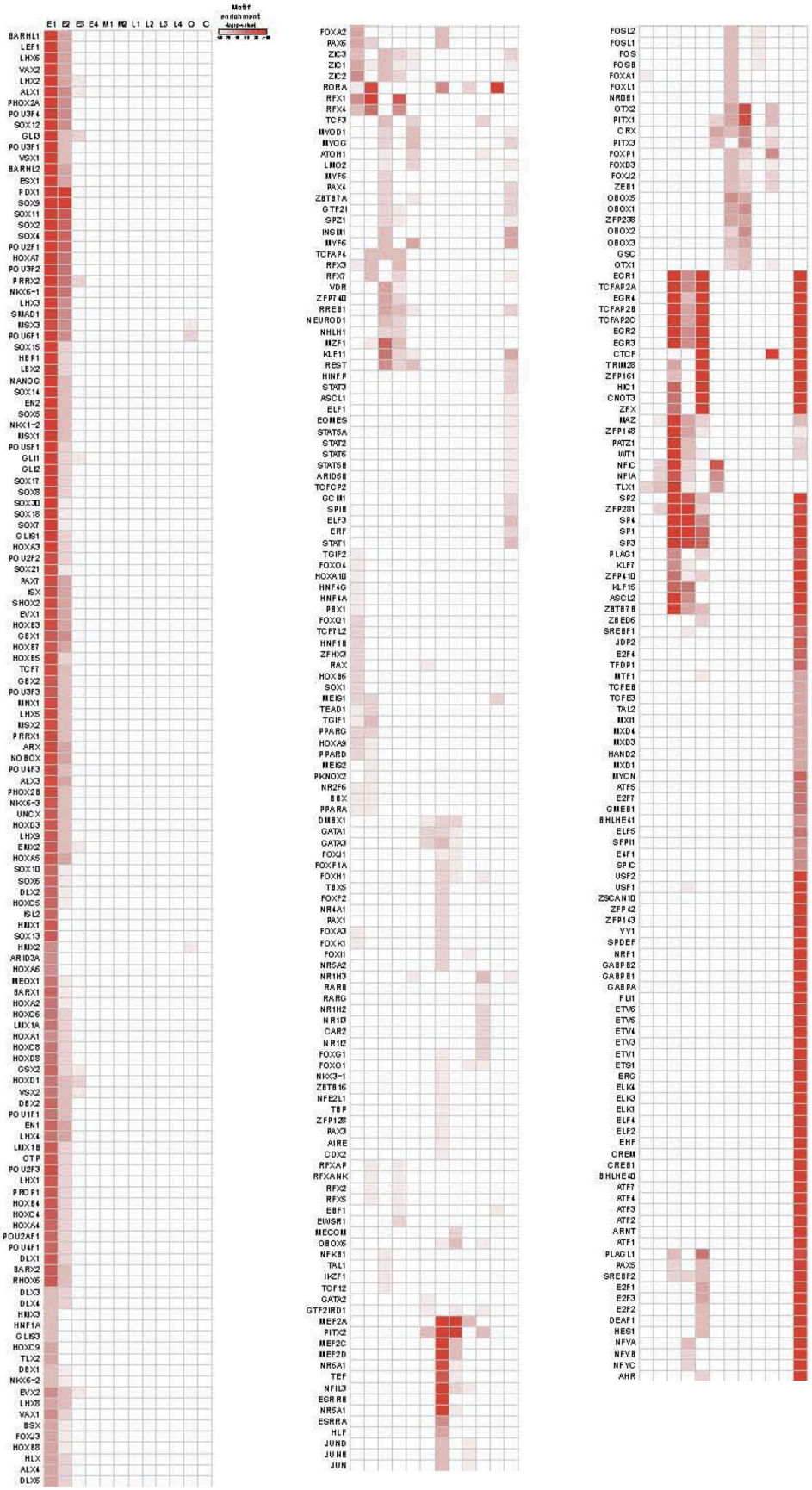


Figure 2.12: **Gene ontology analysis of genes near k-means-clustered DHSs from retina tissue.** Gene ontology (biological process) categories for genes associated with DHSs within each k-means temporal cluster of retinal DHSs (P0, P7, and adult retina) as determined by GREAT analysis. E, early clusters; M, mid clusters; L, late clusters; O, other cluster group; C, constitutive cluster group.


E1 E2 E3 E4 M1 M2 L1 L2 L3 L4 O C
Motif enrichment
BARHL1
LEF1
LHX6
VAX2
LHX2
ALX1
PHOX2A
POU3F4
SOX12
GLI3
POU3F1
VSX1
BARHL2
ESX1
PDX1
SOX9
SOX11
SOX2
SOX4
POU2F1
HOXA7
POU3F2
PRRX2
NKX6-1
LHX3
SMAD1
MSX3
POU6F1
SOX15
HBP1
LBX2
NANOG
SOX14
EN2
SOX5
NKX1-2
MSX1
POU5F1
GLI1
GLI2
SOX17
SOX8
SOX30
SOX18
SOX7
GLIS1
HOXA3
POU2F2
SOX21
RAX?
ISX
SHOX2
EVX1
HOXB3
GBX1
HOXB7
HOXB5
TCF7
GBX2
POU3F3
MNX1
LHX5
MSX2
PRRX1
ARX
NOBOX
POU4F3
ALX3
PHOX2B
NKX6-3
UNCX
HOXD3
LHX9
EMX2
HOXA5
SOX10
SOX5
DLX2
HOXC5
ISL2
HMX1
SOX13
HMX2
ARID3A
HOXA6
MEOX1
BARX1
HOXA2
HOXC6
LMX1A
HOXA1
HOXC8
HOXD8
GSX2
HOXD1
VSX2
DBX2
POU1F1
EN1
LHX4
LMX1B
OTP
POU2F3
LHX1
PROP1
HOXB4
HOXC4
HOXA4
POU2AF1
POU4F1
DLX1
BARX2
RHOX6
DLX3
DLX4
HMX3
HNF1A
GLIS3
HOXC9
TLX2
DBX1
NKX6-2
EVX2
LHX8
VAX1
ESX
FOXJ3
HOXB8
HLX
ALX4
DLX5
FOXA2
RAX6
ZIC3
ZIC1
ZIC2
RORA
RFX1
RFX4
TCF3
MYOD1
MYOG
ATOH1
LMO2
MYF5
PAX4
ZBTB7A
GTF2I
SPZ1
INSM1
MYF6
TCFAP4
RFX3
RFX7
VDR
ZFPT4D
RREB1
NEUROD1
NHLH1
MZF1
KLF11
REST
HINFP
STAT3
ASCL1
ELF1
EOMES
STAT5A
STAT2
STAT6
STAT5B
ARID5B
TCFCP2
GCM1
SPIB
ELF3
ERF
STAT1
TGIF2
FOXO4
HOXA10
HNF4G
HNF4A
PBX1
FOXQ1
TCFL2
HNF1B
ZFHX3
RAX
HOXB6
SOX1
MEIS1
TEAD1
TGIF1
PPARG
HOXA9
PPARD
MEIS2
PKNOX2
NR2F6
BBX
PPARA
DMBX1
GATA1
GATA3
FOXJ1
FOXF1A
FOXH1
TBX5
FOXF2
NR4A1
RAX1
FOXA3
FOXK1
FOXI1
NR5A2
NR1H3
RARB
RARG
NR1H2
NR1D1
CAR2
NR1I2
FOXG1
FOXO1
NKX3-1
ZBTB16
NFE2L1
TBP
ZFP128
RAX3
AIRE
CDX2
RFXAP
RFXANK
RFX2
RFX5
EBF1
EWSR1
MECOM
OBOX5
NFKB1
TAL1
IKZF1
TCF12
GATA2
GTF2IRD1
MEF2A
PITX2
MEF2C
MEF2D
NR6A1
TEF
NFIL3
ESRRB
NR5A1
ESRRA
HLF
JUND
JUNB
JUN
FOSL2
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FOSB
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FOXL1
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OTX2
PITX1
CRX
PITX3
FOXP1
FOXD3
FOXJ2
ZEB1
OBOX5
OBOX1
ZFP238
OBOX2
OBOX3
GSC
OTX1
EGR1
TCFAP2A
EGR4
TCFAP2B
TCFAP2C
EGR2
EGR3
CTCF
TRIM28
ZFP161
HIC1
CNOT3
ZFX
MAZ
ZFP148
PATZ1
WT1
NFIC
NFIA
TLX1
SP2
ZFP281
SP4
SP1
SP3
PLAG1
KLF7
ZFP410
KLF15
ASCL2
ZBTB7B
ZBED6
SREBF1
JDP2
E2F4
TFDP1
MTF1
TCFEB
TCFE3
TAL2
MXI1
MXD4
MXD3
HAND2
MXD1
MYCN
ATF5
E2F7
GMEB1
BHLHE41
ELF5
SPI1
E4F1
SPIC
USF2
USF1
ZSCAN10
ZFP42
ZFP143
YY1
SPDEF
NRF1
GABPB2
GABPB1
GABPA
FLI1
ETV6
ETV5
ETV4
ETV3
ETV1
ETS1
ERG
ELK4
ELK3
ELK1
ELF4
ELF2
EHF
CREM
CREB1
BHLHE40
ATF7
ATF4
ATF3
ATF2
ARNT
ATF1
PLAG1
RAX5
SREBF2
E2F1
E2F3
E2F2
DEAF1
HES1
NFYA
NFYB
NFYC
AHR

Figure 2.13: **Transcription factor binding motif enrichment within temporal clusters of total retinal DHSs.** Transcription factor binding motif enrichment (-log(p-value)) indicated as color intensity for each transcription factor (rows) within each temporal cluster group (columns) from P0, P7, and adult stages of mouse retina. E, early clusters; M, mid clusters; L, late clusters; O, other cluster group; C, constitutive cluster group.

Functional analysis of temporally dynamic DHSs surrounding Otx2

We chose to further examine the *Otx2* locus due to the critical role for this gene in retinal development and the enrichment of its binding motif in retinal-specific DHSs (Nishida et al., 2003). The *Otx2* locus contains many developmentally dynamic DHSs, which show either an increase or decrease in accessibility across stages and were assigned to early or late clusters in our k-means analysis. The DHS map (Fig. 14A) reveals a previously identified distal *Otx2* enhancer (FM1 (Kurokawa et al., 2004)) and other cis-regulatory elements (Emerson and Cepko, 2011), in addition to several potentially novel cis-regulatory elements. We selected 17 regions on the basis of DNase I accessibility and/or evolutionary sequence conservation for further study. Most striking are *Otx2* DHS-4 (~53 kb downstream of *Otx2*), which is highly active in P0 retina, but has decreased activity in the P7 and adult retina; and *Otx2* DHS-15 (~79kb upstream of *Otx2*), which shows the opposite pattern (Fig. 14A). Chromatin-immunoprecipitation for the transcriptional co-activator P300, which has been shown to localize with active enhancer elements (Lee et al., 2011), showed that several of the *Otx2* DHSs were positive (>0.3% input, determined by *Irbp* positive control) for P300 binding (Fig. 15F).

To determine whether the *Otx2* DHSs function as transcriptional enhancers, we tested these elements for their ability to drive expression of a GFP reporter construct in retinal tissue. Each DHS with a P300 ChIP signal above the positive control (*Otx2* DHS #1, 2, 4, 7, 8, 10, and 15) was cloned into a minimal promoter vector and was electroporated on the day of birth along with a transfection control plasmid constitutively expressing nuclear-Cherry. We assayed

reporter expression after one day (in vitro) or after 7 days (in vivo); the TATA-box minimal promoter was used as a negative control (Fig. 14B-G; Fig 15A,B). *Otx2* DHS #1, 7, 8, and 10 showed no functional activity in driving reporter expression in the retina (data not shown). However, we found that several *Otx2* DHSs robustly drive expression of the GFP reporter in the retina. *Otx2* DHS-4, for example, drives GFP expression primarily in the progenitor zone (middle and outer retina) at P0 (Fig. 14C), while the GFP expression from the *Otx2* DHS-2 construct is concentrated in the outer retina where nascent photoreceptors reside (Fig. 14B). *Otx2* DHS-2 and *Otx2* DHS-15 GFP+ cells are nearly 100% Otx2+ at P0 (Fig. 14H; Fig. 15C), though for *Otx2* DHS-4, the percentage of Otx2+ cells was lower (Figure 14H). In the P7 retina, expression driven by *Otx2* DHS-4 is reduced (Fig. 14F), and the GFP+ cells are typically found in the inner nuclear layer, co-localized with Otx2+ bipolar cells. At this developmental time, a greater fraction of the *Otx2* DHS-4 GFP+ cells are Otx2+ (94%) (Fig. 15C). Nearly 100% of cells expressing GFP driven by *Otx2* DHSs 2 and 15 co-express OTX2 at both P0 and P7 (Fig. 14B,D,E,G,H; Fig. 15C). These results together demonstrate that DHS analysis can identify new enhancers active in distinct cell populations in developing and mature retina.

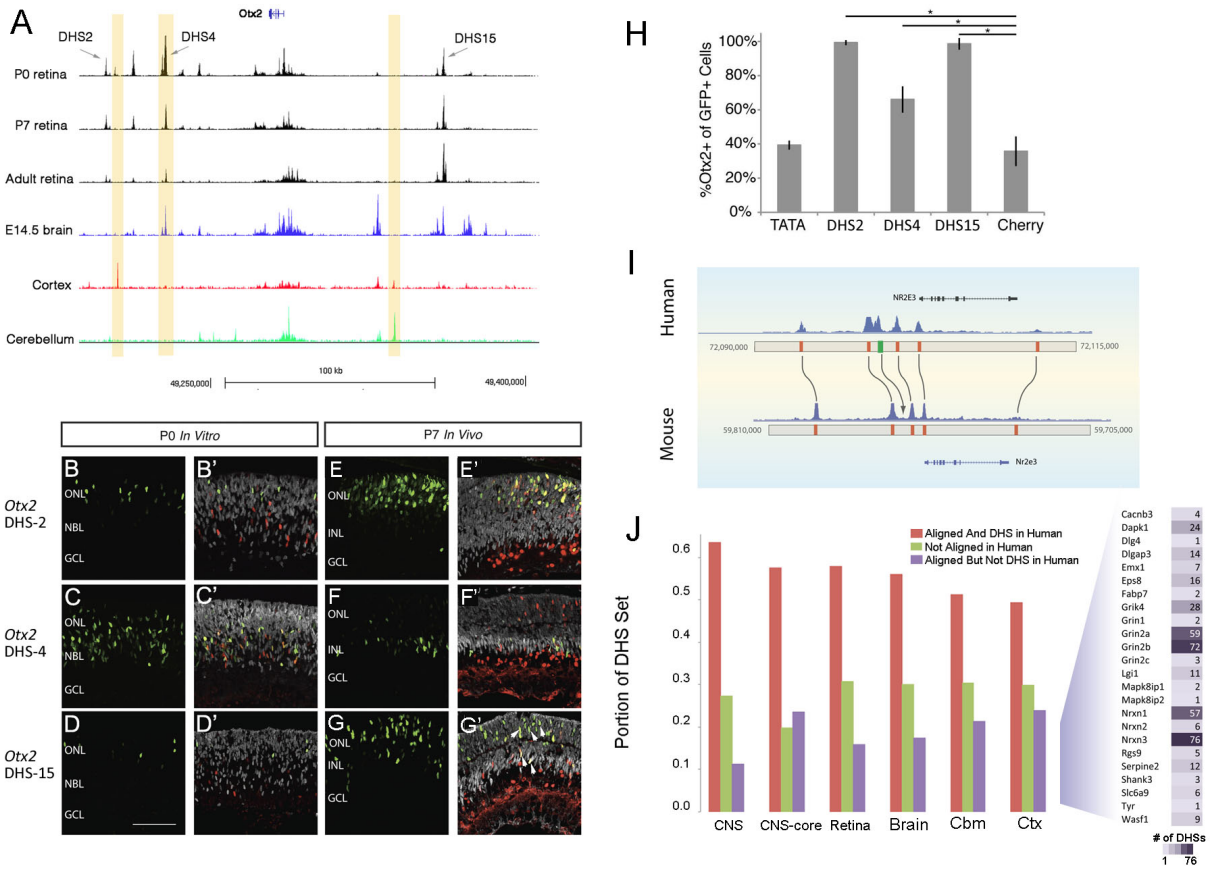


Figure 2.14: Functional testing of retinal DHSs near *Otx2*, and the conservation between mouse and human. (A) The DNase I cleavage landscape is shown surrounding the *Otx2* gene for P0, P7 and Adult (Ad) retina to highlight developmental DHS dynamics. Selected *Otx2* DHSs labeled with arrows. Tan shading highlights DHSs differentially active in retina, cerebellum and cerebral cortex. (B-G') Panels show representative images of expression from *Otx2* DHS reporter constructs (green) co-immunostained for transfection control plasmid (CHERRY, red) and endogenous OTX2 (white). Arrows (G') highlight five example OTX2+ GFP+ co-expressing cells. Left two columns show expression from indicated constructs in electroporated P0 retinal explants cultured 24 hours *in vitro* (B-D') (N=3). Right two columns show expression from indicated constructs in retinas electroporated *in vivo* at P0 and harvested at P7 (E-G') (N=2-5). ONL, outer nuclear layer; NBL, neuroblastic layer; GCL, ganglion cell layer; INL, inner nuclear layer. Scale Bar = 200µm. (H) Quantification of electroporation data showing that nearly 100% of the cells expressing *Otx2* DHS-2 or *Otx2* DHS-15 also express OTX2 in P0 retina. (I) Alignment of human and mouse genomes showing the sequence (orange rectangles) conservation of DHSs surrounding the *Nr2e3* gene, important in rod photoreceptor gene expression. (J) Comparison of functional and/or sequence conservation of human and mouse DHSs for the CNS (set of DHSs common to mature retina and brain), CNS-core (DHSs only active in the CNS), Retina, Cerebellum, adult whole Brain (Brain), and Cerebral Cortex (Cortex). Red, DHS in mouse, orthologous region is a DHS in human; Purple, DHS in mouse, orthologous region is not a DHS in human; Green, DHS in mouse, no orthologous sequence in human. Right blow-up: genes associated with mouse cortex DHSs that have orthologous sequence but no activity in the human genome; numbers of DHSs expressed as intensity of colored cells.

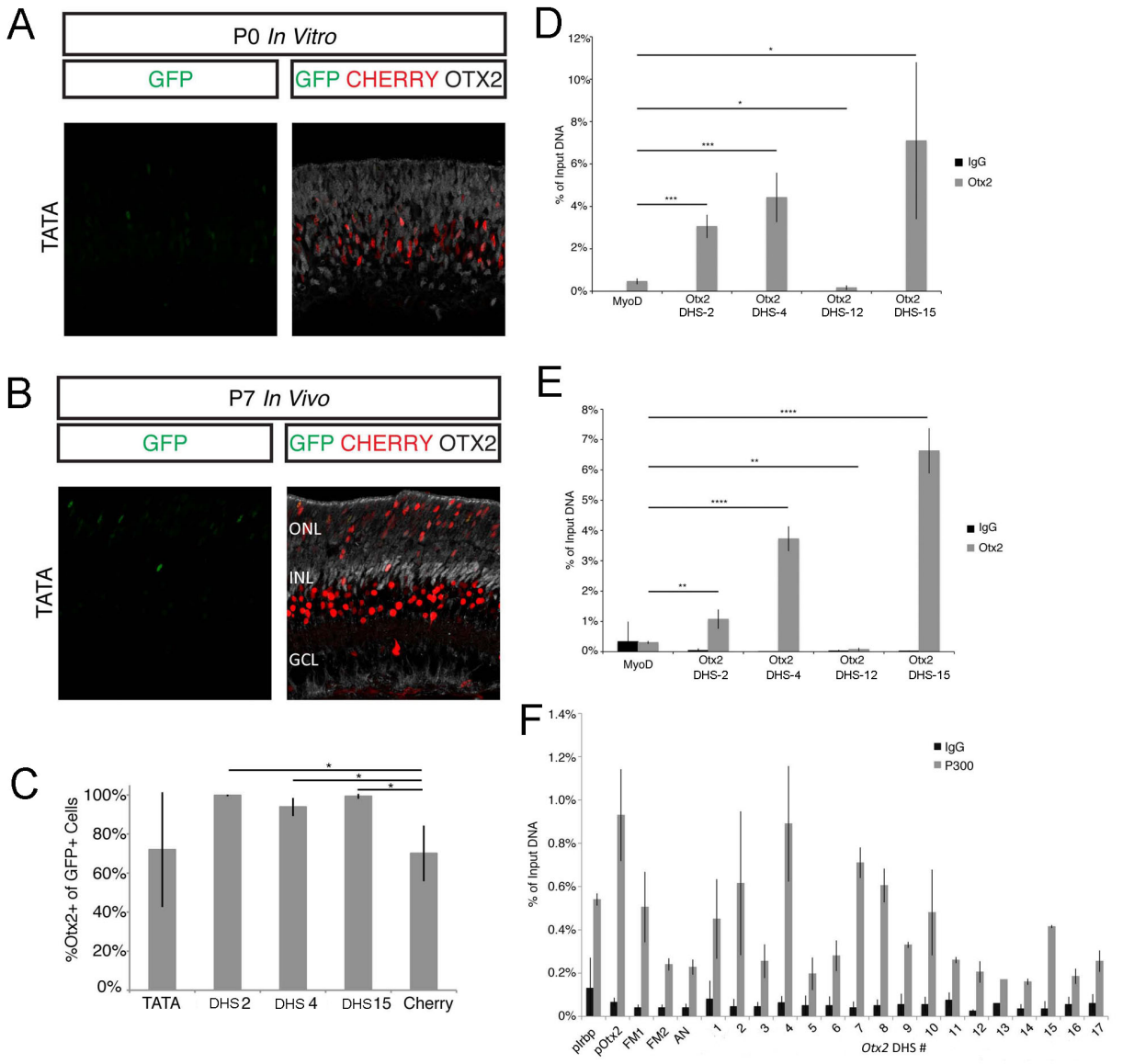


Figure 2.15: ***Otx2* DHS reporter expression and transcription factor binding**. (A,B) Panels show representative images of expression from empty minimal reporter constructs (TATA) with no *Otx2* DHS insert (green) co-immunostained for transfection control plasmid (CHERRY, red) and endogenous OTX2 (white). A. Expression from TATA in electroporated P0 retinal explants cultured 24 hours *in vitro* (N=3). B. Expression from TATA in retinas electroporated *in vivo* at P0 and harvested at P7 (N=3). ONL, outer nuclear layer; NBL, neuroblastic layer; GCL, ganglion cell layer; INL, inner nuclear layer. (C) Quantification of the percentage of GFP+ cells that co-express OTX2+ for *Otx2* DHSs, non-specific control plasmid (TATA), and transfection control (Cherry) in P7 retina *in vivo* (N=2-5). *p<0.01; error bars ± S.D. (D,E) Chromatin Immunoprecipitation for OTX2 or IgG control from P0 (D) and Adult (E) whole retina tissue

shown as a percentage of input DNA. Assayed regions are *Otx2* DHSs 2, 4, 12 and 15 with the *MyoD* promoter serving as a negative control. N=3 ± S.D. *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001. Error bars ± S.D. (F) Chromatin immunoprecipitation for P300 or IgG control shown as a percentage of input DNA for *Otx2* DHSs, the *Otx2* promoter (pOtx2), previously described enhancers (FM1, FM2, AN) and a positive control promoter (pIrbp). N=2-4 ± S.D.

Neural-tissue-specific DHSs at the Ascl1 locus drive tissue-specific reporter gene expression

Ascl1 is an important transcription factor in both developing brain and retina (Guillemot and Joyner, 1993; Jasoni and Reh, 1996; Kim et al., 2008) and is surrounded by ~290kb of gene-deficient genomic space. While BAC transgenic experiments have identified regulatory elements of *Ascl1* that drive its expression in spinal cord and brain (Battiste et al., 2007; Verma-Kurvari et al., 1998), these approaches have failed to reveal retinal enhancers for this gene. We sought to discover such enhancers by comparing our DNase I accessibility maps in the developing retina to DNase I accessibility patterns in fetal mouse brain (E14) (John et al., 2013) at the *Ascl1* locus. We identified five prominent DHSs within a 164kb region surrounding *Ascl1* that are evolutionarily conserved (*Ascl1* DHSs; Figure 16A). *Ascl1* DHSs 11 and 12 appear to show brain-specific DNase I accessibility, while DHS 6 appears to be retina-specific and DHSs 7 and 8-9 are active in both retina and brain (Figure 16A). We cloned these five sequence elements into our GFP reporter construct and examined their domains of activity in E14 forebrain slices and P0 retinal explants cultured for 24-36 hours *in vitro* (Figure 16B-G, 17A-F). As before, the TATA-box minimal promoter was used as a control and is expressed very weakly in both E14 brain and P0 retina explants (Figure 17A-B'). The retina-specific element *Ascl1* DHS6 drives GFP expression only in retina and not brain tissue (Figure 16B,C). Conversely, the brain-specific element *Ascl1* DHS12 drives reporter expression only in brain (Figure 16F,G). *Ascl1* DHS8-9, containing two DHSs active in both retina and brain, robustly drives reporter gene expression in both tissues (Figure 16D,E). Although *Ascl1* DHS11 is present as a DHS in

brain, it does not drive strong expression in either brain or retina in our reporter assay (Figure 17E,F). Similarly, *Ascl1* DHS7, a common DHS, does not drive robust reporter expression in either tissue, although faint and sporadic GFP expression is observed (Figure 17C,D). The lack of reporter gene expression from *Ascl1* DHSs 7 and 11 is still potentially consistent with a regulatory function, as they may participate in regulation, but be insufficient when isolated; other possibilities are non-enhancer regulatory elements (e.g. insulators or repressive elements not detected by enhancer assays). Additionally, DHS11 could be active in a brain region not assayed in our experiments, but included in the tissue used to generate the map of brain DHSs utilized here. These data indicate that the DNase I cleavage landscape of the *Ascl1* locus, compared between tissues, accurately predicts tissue specificity of regulatory activity, but is not absolutely predictive of reporter expression activity.

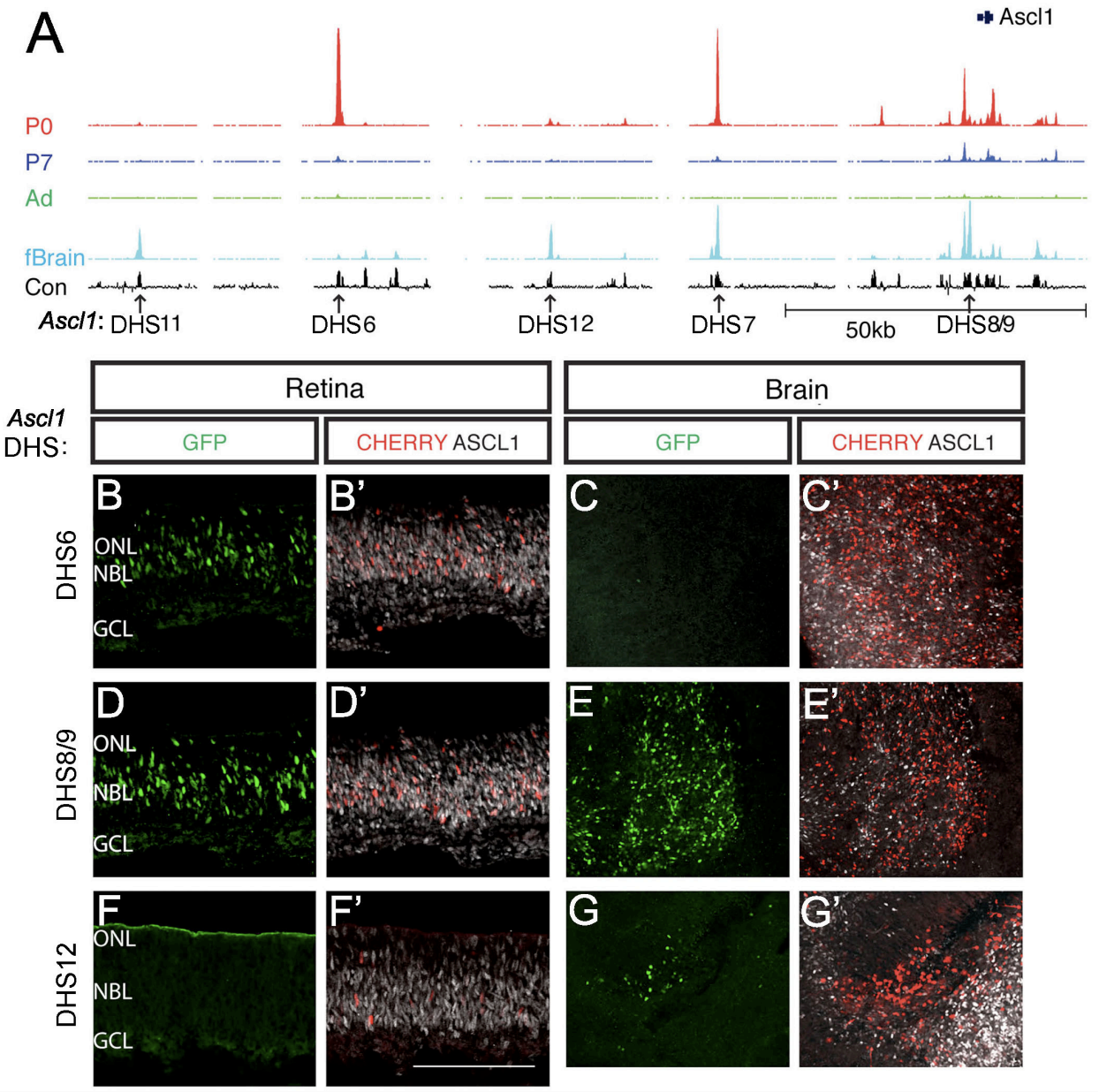


Figure 2.16: *Ascl1* DHSs display tissue-specific DNase I hypersensitivity and reporter expression patterns. (A) The DNase I cleavage landscape is shown surrounding the *Ascl1* gene for P0, P7 and Adult (Ad) retina (red, blue and green, respectively) and for fetal mouse brain (fBrain, light blue). Mammalian sequence conservation (Con) is shown in black. Labeled are *Ascl1* ECRs (ECR) that were selected for functional expression assays. The panels show representative images of expression from *Ascl1* ECR reporter constructs (green) (B-M) co-immunostained for transfection control plasmid (CHERRY, red) and endogenous ASCL1 (white) (B'-M') after electroporation and 36 hours *in vitro*. Left two columns (B-G') show electroporated mouse P0 retina. Right two columns (H-M') show electroporated mouse fetal brain. N=3. Scale Bar = 200µm.

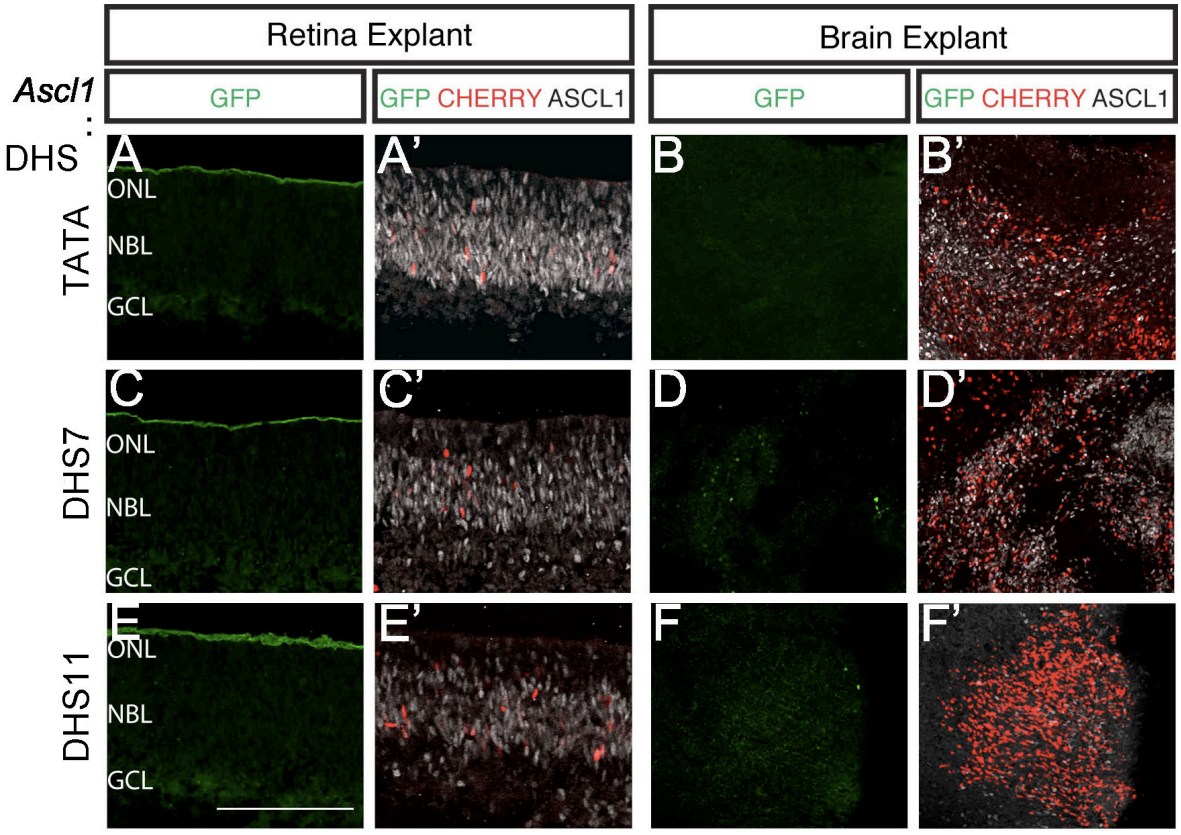


Figure 2.17: *Ascl1* DHSs displaying tissue-specific DNase I hypersensitivity, but no reporter expression pattern. Labeled are *Ascl1* DHSs that were selected for functional expression assays. The panels show representative images of expression from *Ascl1* ECR reporter constructs (green) (A-F) co-immunostained for transfection control plasmid (CHERRY, red) and endogenous ASCL1 (white) (A'-F') after electroporation and 36 hours *in vitro*. Left two columns (A-E') show electroporated mouse retina. Right two columns (B-F') show electroporated mouse fetal brain. N=3. Scale Bar = 200µm.

Shared DHS activity between mouse and human varies across brain regions

The ENCODE consortium has generated a catalog of potential functional elements in the human genome, and more recently the mouse genome (ENCODE Consortium 2012; (Vierstra et al., 2014)). Between one half and two thirds of these cis-regulatory elements are conserved between mouse and human (e.g. 61% of mouse DHSs have identifiable orthologous sequences and are also DHSs in human; Fig. 14I,J). However, despite high sequence conservation, many elements no longer have an orthologous chromatin signature, suggesting that they have diverged functionally. Interestingly, enhancers of developmental regulatory genes are among those with

the greatest amount of both functional and sequence conservation between mouse and human (Vierstra et al., 2014).

We asked whether functional conservation of CNS regulatory elements between mouse and human varies by brain region. Vierstra and colleagues (Vierstra et al., 2014) found that the median conservation of DHS activity across all tissues analyzed between mouse and human is 48%, with a range of 38.2-60.3%; referred to as "shared" DHSs. The percentage of shared DHSs is relatively high in the CNS, with over 60% of DHSs identified in this study shared between mouse and human (Fig. 14I,J). CNS-core DHSs contain a greater percentage that are unique to mouse than the overall set of CNS DHSs (Fig. 14J).

Comparing the proportions of shared DHSs within CNS regions revealed differences among their distributions (Fig. 14J). The retina and cerebellum have a somewhat lower percentage of shared DHSs than the overall CNS. However, the cerebral cortex has the lowest percentage of shared DHSs of the regions analyzed, possibly reflecting the greater extent of divergence of this structure between mice and men. We subjected mouse cerebral cortex DHSs that have conserved sequence but are not DHSs in human to GREAT analysis; among the highest scoring categories (either by gene number or p-value) are genes associated with NMDA receptors and their regulation. Figure 14J, right blow-up, shows the genes in this category with the associated number of mouse DHSs that have lost activity in human. The genes with the greatest number of unshared DHSs between mouse and human are the subunits of the NMDA receptor (*Grin2a* and *Grin2b*), critical for neuroplasticity and memory, and the Neurexins (*Nrxn1, Nrxn3*), highly differentially spliced genes involved in synapse formation (Reissner et al., 2008; Runkel et al., 2013). These results suggest that re-wiring of the cis-regulatory

elements controlling genes associated with neural plasticity and synaptogenesis may accompany evolutionary changes in brain function.

DISCUSSION

Defining the gene regulatory networks that control the vast cellular diversity and connectivity in the mammalian CNS presents a significant challenge for traditional approaches. The development of DNase I hypersensitivity mapping at the genome-wide scale has provided new approaches to characterize cis-regulatory modules (CRMs) and the transcription factors that recognize them (Mercer et al., 2013; Thurman et al., 2012). We applied this technique to the mouse CNS; by sampling different brain regions as well as developmental stages, we were able to identify CRMs associated with genes that have regional, temporal, and cell-type specificity in the CNS. A similar study using H3K27ac ChIP-seq for mouse cerebral cortex, heart, and liver demonstrated the power of comparing enhancer activity across developmental transitions and tissues (Nord et al., 2013). We found that similar conclusions can be applied to the different regions of the central nervous system and potentially to individual cell types. Moreover, the sensitivity of DNase I hypersensitivity mapping has allowed us to identify nearly ten times more putative cis-regulatory regions in the brain and retina than were identified with previous studies using ChIP.

By comparing the DHSs from the brain and retina, we were able to delineate a core set of DHSs common to the CNS, the majority of which are shared between mouse and human, representing the accessible chromatin of the brain and retina. Not surprisingly, the core set is enriched for DHSs near neural genes, like neurotransmitter receptors and ion channels, and provides a new resource for studies of their regulation. In addition there appear to be many

regulatory elements that potentially regulate expression of neural-expressed genes involved in brain disorders (eg. Parkin2, Lingo1, Dscam, Msra). In light of recent evidence that disease associated polymorphisms, identified by GWAS studies, are concentrated in DHSs (Maurano et al., 2012), the core-DHS regulome provides thousands of new candidate regions for potential disease causing mutations.

In addition to the core set of DHSs common to all regions of the CNS, when we compared different regions of the CNS, we were able to identify DHSs unique to each region. Genes expressed in many regions of the CNS, like *Nefl*, show different patterns of DNase I hypersensitivity depending on the CNS region, and these differences allow the identification of potential enhancers that regulate expression in specific CNS regions and potentially even in specific cell types. For example, of the DHSs near the *Otx2* locus that we tested experimentally, we found that there was selectivity for both developmental stage (developing vs mature) and retinal cell type (photoreceptor vs bipolar cell). It is likely that the same will hold true for other brain regions, and thus the DHSs we have identified could potentially be involved in regulating gene expression in subsets of neurons within these regions. Further testing of candidate elements in transgenic assays will be needed to validate this possibility. Although the *Otx2*-DHSs #2, 4, and 15 drive reporter expression within OTX2+ retinal cells, it is possible that these DHSs represent CREs for other neighboring genes. Thus, their statistically significant overlap with OTX2 protein could be due to their OTX2 binding sites; in fact we show that OTX2 binds to these DHSs. Therefore, more definitive evidence for direct *Otx2* regulation will required observation of a direct interaction between these DHSs and the *Otx2* promoter via chromatin conformation capture assay. Similar skepticism and need for further experimentation apply to the putative CREs of *Ascl1*.

Although these DHSs present in the genome of CNS tissue represent an enormous diversity in cell type, DNase I sensitive signals were clearly present at the promoters and enhancers of genes expressed in rare cell types highlighting the usefulness of this approach for identifying cis-regulatory elements in even highly complex tissues. In the adult mouse retina, there are approximately 7 million total cells, with 180,000 cones (~3%), 50,000 ganglion cells (~1%) and 1600 Opn4/melanosin expressing ganglion cells (Hughes et al., 2013). At birth, all ganglion cells and cones have already been generated, but only about half of the total retinal cells have been produced (Young, 1985). Therefore, the Pou4f2+ ganglion cells are 1.4% of the total retinal cells at P0, and decline to 0.36% of the retinal cells at P7 and in the adult retina. At a false discovery rate of 1%, there are DHSs at specific positions in the Pou4f2 locus at all ages. Similar conclusions can be drawn from the analysis of the cone gene DHSs. For example, there is a DHS at the Opn1sw locus at all ages examined. In mouse, the Opn1sw+ cones represent approximately one fourth of the total cones (~3% of the total cells). However, our approach is based on whole retina and brain tissue and therefore the DNase-seq landscapes represent the mixture of cells constituting the tissue with no way to distinguish which DHS is present in which cell type(s). Future experiments will determine if these DHSs can be observed only in the purified cell populations (ganglion cells and cone photoreceptors, respectively). This will require dissociation of the retinas followed by cell sorting with cell-type specific fluorescent reporter mice. If DHSs are present only in the purified cell populations and not in the retina 'negative' fraction of cells, it would suggest that DHSs can be detected from minority cell types in complex tissues even when they comprise ~1% of the total cell population. However, in the case of ganglion cell specific genes, it is possible that some non-ganglion cells that do not express these genes nevertheless contain low levels of DNase-hypersensitivity at the gene

promoters. This could be due to off-target binding of transcription factors, binding of low levels of inappropriately expressed transcription factors, or binding of non-specific promoter associated proteins. The experiment described above will be required to test the feasibility of detecting DHSs from rare cell types in a complex tissue.

Functional analyses of putative cis-regulatory regions, both *in vitro* and *in vivo*, of a key CNS gene validates this approach for the identification of enhancers for specific CNS regions and developmental stages. Several previous studies have used comparative and/or epigenetic approaches to identify CRMs in both developing and mature tissues, including the CNS (Hardison and Taylor, 2012; Hawrylycz et al., 2012; Lee et al., 2011; May et al., 2012; Shen et al., 2012; Telese et al., 2013; Vierstra et al., 2014; Visel et al., 2009; Visel et al., 2008; Visel et al., 2013; Wenger et al., 2013). With the DNase I signal alone, we were able to predict elements that drive expression in the developing brain in the Vista Browser to a similar degree as a recent H3K27ac ChIP-seq study (Nord et al., 2013). Combining the DNase-seq data with other epigenetic markers of enhancers, such as H3K27ac and P300, should continue to refine their predictive power. Moreover, since DNase-seq also identifies promoters, insulators, and virtually every class of active regulatory element, this technique provides a more comprehensive view of the epigenome, although this view is inherently non-specific to the nature and function of identified regulatory elements. Because this approach will identify insulators and repressive CREs, one future direction of this research will be to test elements (that fail in enhancer assays) in repressor and insulator assays. This will greatly expand the number and diversity of known regulatory elements in the mouse and human genome.

Combined with transcription factor motif identification, DNase I hypersensitivity mapping can also delineate transcriptional networks in the developing and mature brain. By

comparing DHSs from three different ages of retinal development, we were able to identify stage-specific transcription factor binding motifs for known developmental regulators enriched in retinal samples of each age, and generate a list of potential transcriptional regulators relevant to distinct developmental processes and mature gene regulation. However, this list is likely subject to many false positives and false negatives. First, current TF motif matching algorithms poorly distinguish between molecules within TF families, thus many of the implicated TFs are likely based on enrichment of a single motif. Indeed, many TFs on our list are not expressed in the retina. Next, the full space of TF motifs are not represented within current TF motif-matching algorithms, meaning that our list is potentially incomplete with respect to developmental regulators of the retina.

Recent studies have shown that DHS motif analysis, along with digital footprints, can be used to generate potential regulatory networks directly (Neph et al., 2012). The transcription factor networks can be generated from motif analysis within DHSs and digital genomic footprinting to validate ChIP-seq data and to generate *de novo* predictions about potential transcriptional regulators of specific genes. However, the networks that have been generated are limited in scope. Because it is not possible to definitively associated distal regulatory elements with a nearby gene, only those DHSs located within 5kb of a TSS were examined for TF footprinting and used to build the subsequent network. Therefore, to build a complete network, distal DHSs will need to be associated to specific gene(s) via chromatin conformation capture assays. Then, TF networks can be built utilizing most or all CREs that associate with a given gene. However, even these networks will suffer from imperfect footprint calling and incomplete TF motif databases. In addition, the effect (activation or repression of transcription) of binding by individual factors (or individual CREs) is not captured by motif enrichment or footprinting

assays. Therefore, experiments will be required to determine the 'sign' (positive or negative) of each putative interaction.

By comparing the core DHS set from mouse CNS with DHSs of human tissues, we found that the conservation of CNS DHSs between mouse and human is only about 60%. This is close to that observed across all tissues by Vierstra and colleagues (Vierstra et al., 2014) and reflects the rapidly evolving cis-regulatory landscape revealed by DNase-seq analysis and other approaches (Nord et al., 2013). There are region-specific differences in DHS divergence between these species, with the cerebral cortex having the lowest percentage of shared DHSs of the regions analyzed. This might be due to the greater extent of divergence of this structure between mice and men than other CNS regions, like cerebellum and retina. Although this conclusion is speculative at this time, our analysis suggests that divergence in cis-regulatory elements near genes associated with neural plasticity and synaptogenesis might be important in brain evolution. Focusing studies of evolution to the sequence content within DHSs may provide a more efficient approach to studying the evolution of gene regulation across species. The complexity of the brain is generated in part through transcriptional regulation of gene expression, and the data in this report provide an additional approach to the identification of genomic regions involved in this process.

Chapter 3:

Ascl1 partially reprograms Muller glia into retinal progenitor-like cells.

INTRODUCTION

Ever since the first rigorous observations of retinal regeneration in amphibians, zebrafish, and other species, scientists have been puzzled by the lack of endogenous retinal regeneration in mammals. The goal of the studies in this chapter is to demonstrate that Muller glia cells from the mammalian retina can be reprogrammed into retinal neurons and to highlight mechanistic aspects of this process.

The Muller glia cells of zebrafish respond to retinal injury by undergoing de-differentiation, proliferation, and re-differentiation into retinal neurons to regenerate and restore retinal tissue and function. Many injury induced signaling pathways have been shown to be necessary and sufficient for this process and result in re-expression of the transcription factor, *ascl1a*, in the Muller glia (Goldman, 2014). This factor is necessary for reprogramming the Muller cells into retinal progenitors and may serve as a point of convergence for the injury induced signaling cascades (see Introduction).

In contrast to the zebrafish, mammalian Muller glia respond to retinal injury by undergoing reactive gliosis, but have an extremely limited native capacity to proliferate and transdifferentiate into retinal neurons. This failure to regenerate substantial numbers of neurons correlates with a lack of *Ascl1* re-expression, as observed in the fish (Karl et al., 2008). In addition, *Ascl1* has been used extensively as a reprogramming factor for converting fibroblasts and astrocytes into neuron-like cells capable of synaptic connections and electrophysiological function (Vierbuchen and Wernig, 2012). Therefore, we wondered if transduction of Ascl1 into mammalian Muller glia would cause reprogramming into retinal neurons.

In this chapter, we test the hypothesis that misexpression of Ascl1 in mouse Muller glia will result in reprogramming to a neuronal fate. We show that we are able to grow purified

cultures of mouse Muller glia and, through viral transduction of *Ascl1*, these cells are partially reprogrammed into retinal neurons. The mechanism of this reprogramming includes direct binding of ASCL1, remodeling of inhibitory histone modifications, and transactivation of retinal progenitor genes in a manner similar to what occurs in P0 retinal progenitor cells. Further, misexpression of Ascl1 in young versus adult Muller glia *in vivo* demonstrates that young Muller glia have a higher intrinsic capacity to be reprogrammed and this correlates with chromatin accessibility at progenitor genes.

RESULTS

Muller glia from the post-natal mouse grow in vitro

In order to test hypotheses regarding Ascl1 action in mouse Muller glial cells, we need a stable and reproducible source of pure Muller glia. Previous work has demonstrated that Muller glia exit from the cell cycle by post-natal day 7 (P7) and up-regulate genes associated with the mature Muller glial cell state by P10 (Nelson et al., 2011). By P14 and through adulthood, Muller glia are only capable of very limited proliferation in cell culture (Ueki et al., 2012). However, Muller glia isolated from P12 retinas are capable of re-entering the cell cycle undergoing several rounds of proliferation in cell culture when grown in medium supplemented with 10% fetal bovine serum (FBS) and 10ng/mL EGF. The majority of Muller glia exit the cell cycle and cease proliferation after 1-week *in vitro* (Ueki et al., 2012).

Contamination with retinal neurons would complicate the analysis of reprogramming; therefore, we sought to determine the purity of Muller glia in these cultures. First, cells in S-phase of the cell cycle incorporate the nucleotide analog, EdU, marking the proliferating fraction of the cells in culture. Because differentiated neurons have permanently exited the cell cycle,

EdU labels the Muller glia fraction of cells. As shown in Figure 3.1A, EdU labels the majority of cells. Second, we determined the purity of Muller glial cells by immunohistochemistry for markers specific to Muller glia. Figure 3.1A demonstrates that the vast majority of cells from the P12 retinal cell culture co-label with the Muller glial specific combination of S100β and Pax6. To further validate the purity of these cultures, we quantified the percentage of all DAPI+ cells that are positive for the Muller glia markers: S100β (99.1±0.36%), Pax6 (97.5±0.63%), and Sox2 (96.8±1%) (Figure 3.1B). In addition, microarray gene expression analysis and immunohistochemistry for endothelial and astrocytic markers demonstrate that these cultures are free from contamination by these cell types (Ueki et al., 2012).

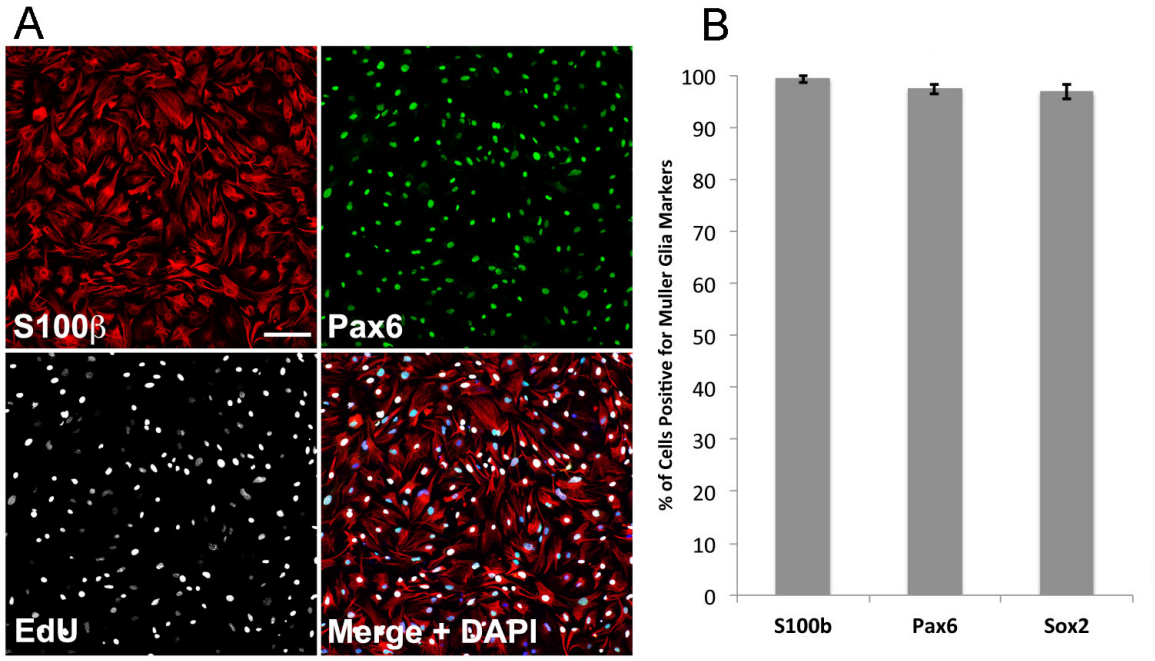


Figure 3.1: Pure populations of Muller glia are derived from the P12 mouse retina. (data published in Ueki et al., 2012) A. Confocal images of Muller glia cell culture from P12 retinas, 5 days *in vitro*. Immunohistochemistry for S100β (red), Pax6 (green), EdU (White), and DAPI (Blue); image on the bottom right is an overlay of all 4 colors. Scale bar = 100µm. B. Quantification of the percentage of cells positive for the Muller glia markers: S100β, Pax6, and Sox2. Error bars indicated standard deviation. N = 4 biological replicates.

Muller glia respond to Ascl1 expression

In order to test the hypothesis that Ascl1 reprograms Muller glia cells into retinal progenitors or retinal neurons, we sought to misexpress this transcription factor in Muller glia and examine cellular morphology and the state of gene expression. To accomplish this, we cloned the coding sequence for ASCL1 into a lentiviral construct containing a cytomegalovirus (CMV) promoter, which drives strong expression in the Muller glia, and an internal ribosomal entry site followed by green fluorescent protein (IRES-GFP), allowing us to visualize the transduced cells. We subsequently made lentiviral particles and infected the P12 Muller glia cultures, resulting in ASCL1 transduction and misexpression.

Our experimental paradigm starts with dissociating P12 mouse retinas and allowing the Muller glia to grow for 5-days in cell culture medium supplemented with 10% FBS and EGF. The cells are then passaged to remove surviving neurons. After 2 more days in culture, the remaining population of Muller glia are transduced with GFP or ASCL1-GFP lentiviral particles and the culture medium is switched to 1% FBS supplemented with 10ng/mL BDNF to support neuron survival. The cells are then analyzed between 4 and 21 days later.

We observed a strikingly change in morphology in a subset of Muller glia that were infected with the ASCL1 lentivirus, as indicated by GFP expression (Figure 3.2A). These cells transformed from a broad, flat, fibroblast like shape (compare to shape outlined by S100b staining in Figure 3.1A) into a small, round cell body with long, thin projections, characteristic of neurons. We examined the reprogrammed Muller glia for formation of multicellular rosette formation, indicative of proliferating retinal progenitors in culture, but no rosettes were

observed. However, ASCL1-GFP expressing were observed dividing through the use of time-lapse imaging (data not shown, Pollak et al., 2013).

Next, we hypothesized that ASCL1 misexpression in the Muller glia reactivates the retinal progenitor gene expression program. To determine the change in gene expression resulting from ASCL1 misexpression, we subjected GFP-control or ASCL1 transduced Muller glia to gene expression microarrays and compared the results to retinal progenitors sorted from P0 retina. These microarray experiments were conducted in collaboration with Julia Pollak. We observed that by 4-days post transduction, a subset of progenitor genes, including Notch pathway components and developmental transcription factors, are up-regulated in ASCL1 expressing Muller glia (Figure 3.2B). However, most genes were not re-expressed to the level found in retinal progenitors. A representative group of up-regulated genes is shown in Figure 3.2B.

While these changes are consistent with reprogramming to a progenitor state, we observed that many progenitor genes were not up-regulated following ASCL1 transduction (Figure 3.2C). These data indicate that the reprogramming process is only partially complete at the transcriptional level.

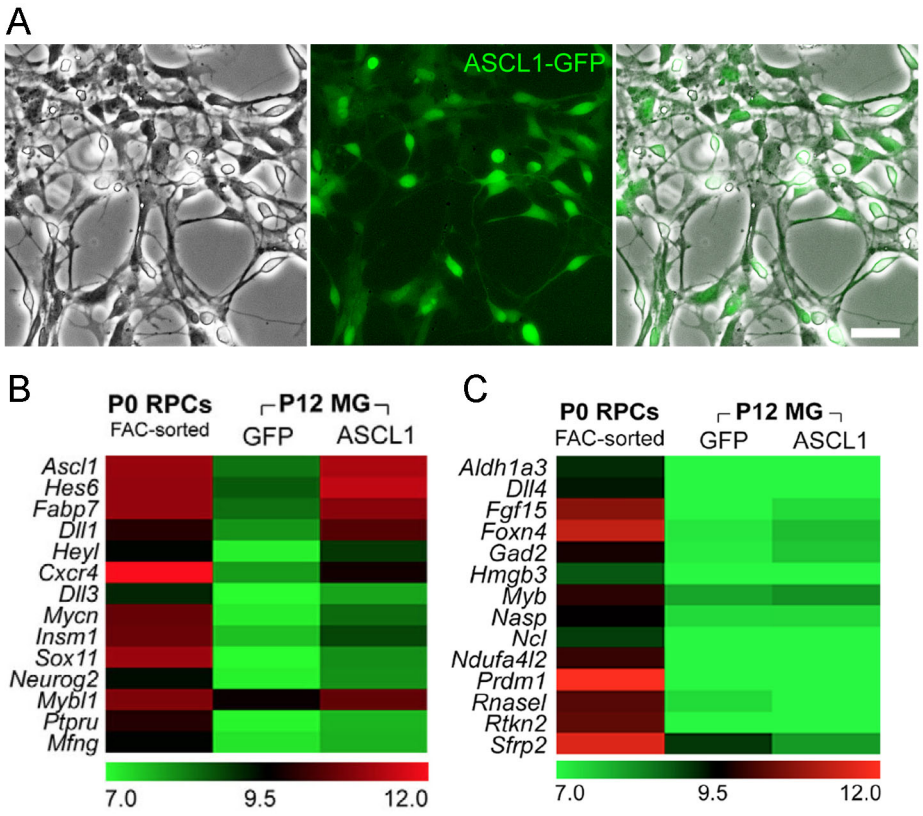


Figure 3.2: **ASCL1 causes a morphological and gene expression state change towards retinal neurons.** (data published in Pollak et al., 2013) A. Bright-field, green fluorescence, and merged images of Muller glia 18-days post transduction with ASCL1-GFP. B,C. Gene expression microarray comparing P0 retinal progenitors (FACS-sorted from Hes5-GFP mice), and P12 Muller glia 4-days after transduction with either GFP control or ASCL1 lentivirus. Panel B shows selected retinal progenitor genes that are up-regulated after ASCL1 expression. Panel C shows retinal progenitor genes that are not up-regulated after ASCL1 expression. Expression levels are log transformed and normalized.

Ascl1 directly transactivates endogenous target genes in Muller glia

To begin to characterize the mechanism of ASCL1 mediated reprogramming, I turned to chromatin immunoprecipitation (ChIP) for ASCL1. Specifically, we hypothesized that ASCL1 is binding the proximal promoter regions and thus, directly regulating progenitor genes in Muller glia.

It has been demonstrated by our lab and others that Ascl1 acts as a transcriptional activator of several components of the Notch signaling pathway in the retina (Nelson et al.,

2009) and in the brain (Castro et al., 2011). Specifically, the progenitor genes, *Dll1*, *Dll3*, *Hes5*, and *Hes6*, are all expressed at a lower level in *Ascl1* knockout retinas. Additionally, these genes are up-regulated in the ASCL1 expressing Muller glia (Figure 3.2B). Therefore, I examined these genes, and another Notch pathway component and progenitor gene, *Mfng*, for direct regulation by ASCL1 in the Muller glia by performing ChIP for ASCL1 followed by qPCR (ChIP-qPCR) on Muller glia 4-days post viral transduction. I observed that ASCL1 is significantly enriched at 5' promoter region of *Dll1, Dll3, Hes5, Hes6*, and *Mfng*, in ASCL1 expressing Muller glia, but not in GFP expressing Muller glia (Figure 3.3A). The *Myod1* promoter served as our negative control region because it is not expressed in the retina, its expression level change following ASCL1 expression, and it has not been identified as a direct target in brain tissue (Castro et al., 2011). In correspondence with this prior knowledge, we did not observe ASCL1 binding at the *Myod1* promoter in ASCL1 expressing Muller glia (Figure 3.3A).

In order to determine whether the observed ASCL1 binding Muller glia is appropriate to endogenous ASCL1 binding patterns in retinal progenitors, I performed ChIP-qPCR for ASCL1 in post-natal day 0 (P0) retina. At this age, Ascl1 is expressed in the progenitor cells, which constitute approximately 40% of total cells in the retina. In accordance with ASCL1 enrichment at progenitor genes in the Muller glia, these genes are similarly enriched for ASCL1 binding in retinal progenitor cells (Figure 3.3B). These data indicate that ASCL1 is directly regulating predicted target genes in Muller glia as it does endogenously in retinal progenitors. However, this conclusion is limited to a small subset of target genes and it is undetermined here if ASCL1 is able to directly regulate the full set of direct target genes in retinal progenitor cells.

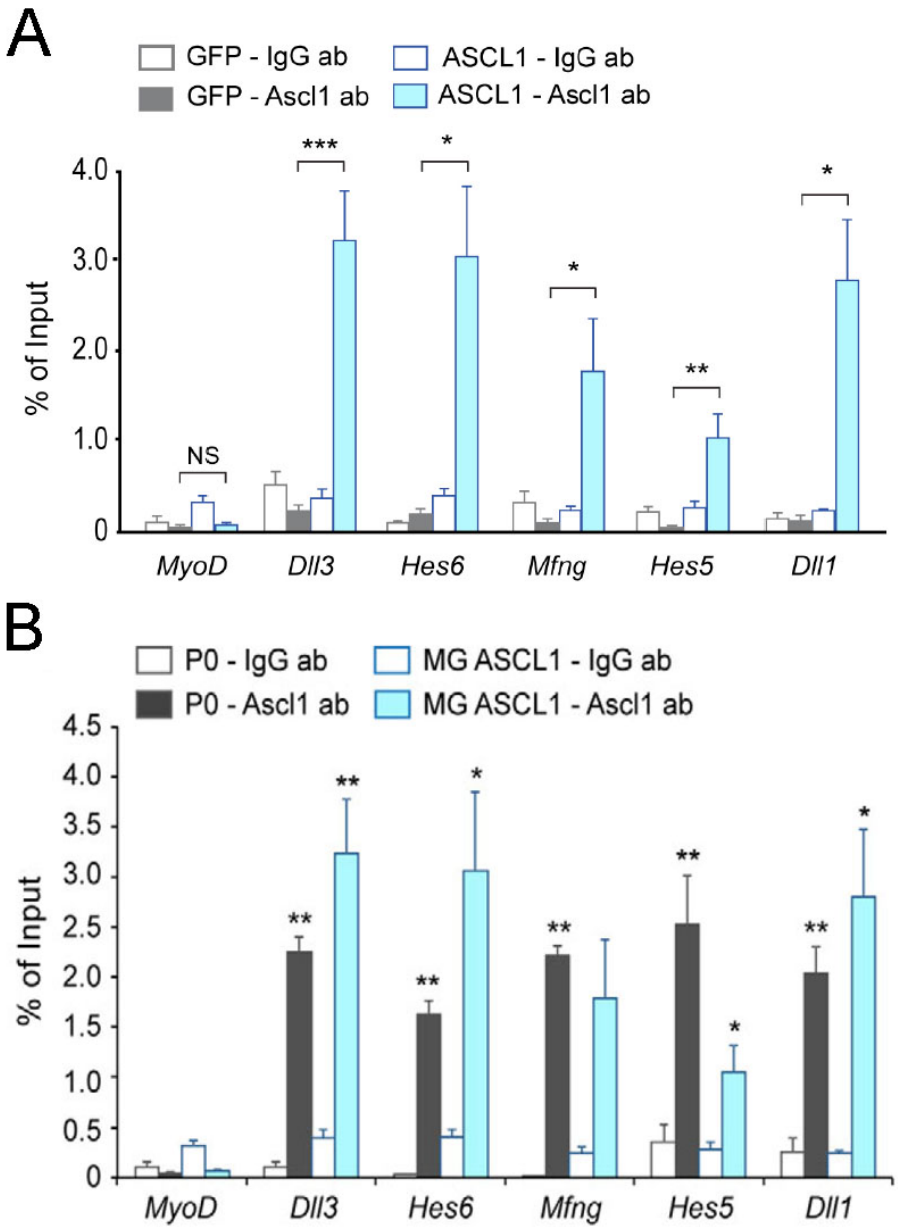


Figure 3.3: **ASCL1 binds to the promoter of target progenitor genes.** (data published in Pollak et al., 2013) A. ChIP-qPCR with anti-ASCL1 or IgG control (white bars) antibodies in Muller glia 4-days after viral transduction of ASCL1 (blue bars) or GFP control (grey bars) shown as a % of input DNA (Y-axis). Enrichment was measured at the 5' proximal promoter regions of the genes indicated on the X-axis. B. ChIP-qPCR with anti-ASCL1 or IgG control (white bars) antibodies in P0 retina (grey bars) or in Muller glia 4-days after viral transduction of ASCL1 (blue bars) shown as a % of input DNA (Y-axis). Enrichment was measured at the 5'

proximal promoter regions of the genes indicated on the X-axis. n = 3-4 biological replicates. Error bars indicate standard error of the mean. *p<0.05; **p<0.01; ***p<0.001; student's t-test.

Ascl1 reverses inhibitory chromatin environments at target genes

In addition to its role as a direct transactivator, Ascl1 has been implicated in epigenetic remodeling of target genes (Wapinski et al., 2013; Raposo et al., 2015). Therefore, I hypothesized that Ascl1 regulates the epigenetic environment of target genes by remodeling the local histone modifications. To test this hypothesis, I performed ChIP-qPCR for two histone modifications at the 5' proximal promoter region of 4 progenitor genes (*Hes5, Dll1, Hes6, and Dll3*) that ASCL1 directly binds and transactivates (Figure 3.3). The modifications we chose were histone 3 lysine 27 trimethylation (H3K27me3) and acetylation (H3K27ac). H3K27me3 is associated with genes in a transcriptionally repressed or poised state (Cao et al., 2002; Conway et al., 2015); whereas H3K27ac is associated with genes and enhancer regions in a transcriptionally active state (Karlić et al., 2010).

First, I looked to see if these chromatin modifications change and correlate with the gene expression state of progenitor genes between P0 retina and P12 Muller glia. I observed that H3K27me3 is largely absent from progenitor gene promoters in P0 retina, but significantly increases in the P12 Muller glia (Figure 3.4A). Conversely, I observed that H3K27ac levels significantly decrease at these genes between P0 retina and P12 Muller glia (Figure 3.4B). These data indicate that the inhibitory histone modification H3K27me3 is deposited at progenitor genes and accumulates during cell differentiation in the retina. Conversely, the activation-associated modification H3K27ac is enriched at progenitor genes, but is removed during differentiation of Muller glia.

Next, I investigated whether Ascl1 misexpression in Muller glia is sufficient to reverse the developmental acquisition of inhibitory modifications and depletion of activation-associated histone modifications. To test this hypothesis, I compared histone modification differences between P12 Muller glia, 4-days after misexpression of either GFP-control or ASCL1. I performed ChIP-qPCR at the same set of progenitor genes, for the same set of histone modifications as above and observed that ASCL1 misexpression results in the reversal of the changes seen during Muller glial differentiation. Specifically, levels of H3K27me3 were significantly reduced at 3 of the 4 progenitor gene promoters after ASCL1 transduction (Figure 3.4C). The exception to this trend was *Hes6*, which displayed only a small accumulation of H3K27me3 during differentiation (Figure 3.4A,C). Conversely, levels of H3K27ac significantly increased at all 4 gene promoters after ASCL1 transduction (Figure 3.4D), correlating with direct ASCL1 binding (Figure 3.3) and activation of transcription (Figure 3.2B). These data indicate that ASCL1 is able to reverse the inhibitory epigenetic environment of target gene promoters in P12 Muller glia. Although, this conclusion is currently limited to these progenitor genes, it is tempting to speculate that ASCL1 remodels the epigenetic environment of binding sites generally, as observed for the closely related bHLH factor, *Myod1* (Cao et al., 2010; see Discussion).

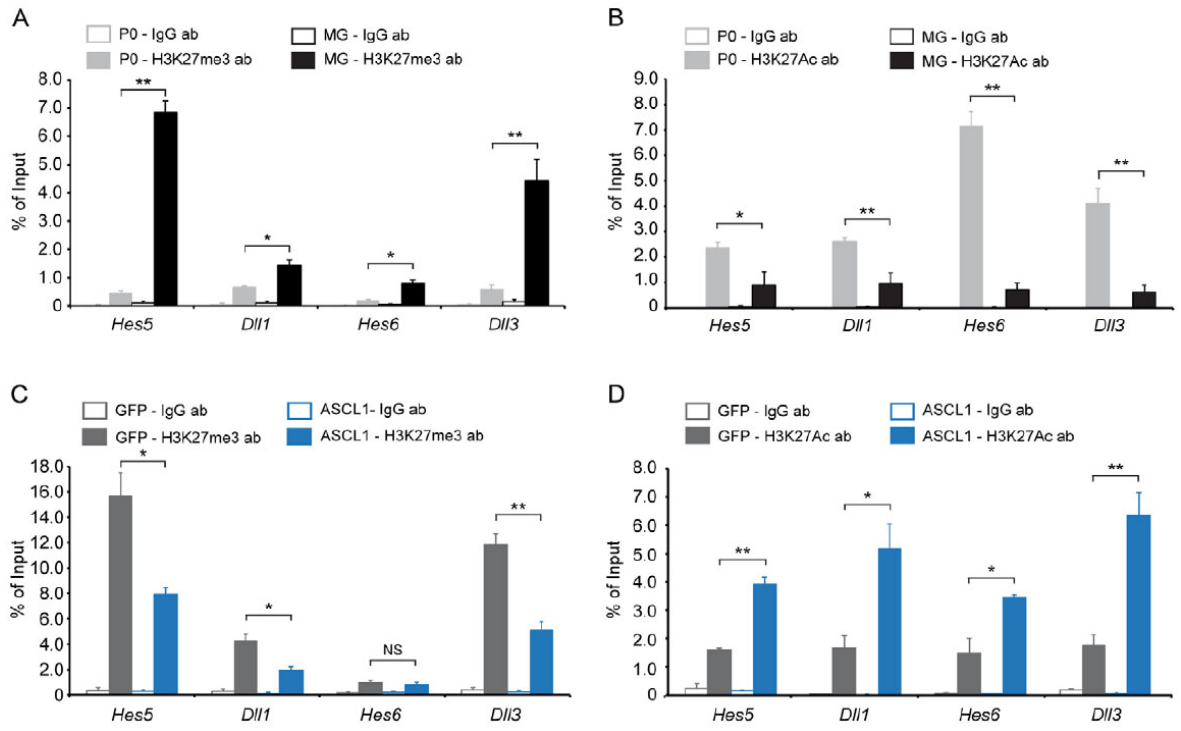


Figure 3.4: **ASCL1 reverses developmentally acquired, inhibitory chromatin modifications at progenitor genes.** (data published in Pollak et al., 2013) ChIP-qPCR for H3K27me3 (A,C), H3K27ac (B,D) or IgG control (white bars) antibodies at the 5' proximal promoter region of the indicated genes (X-axis) measured as a % of input DNA (Y-axis). Panels A and B show the change in histone modification levels between P0 retina (light grey) and P12 cultured Muller glia (black). Panels C and D show the change in histone modification levels between Muller glia 4-days after transduction with GFP control (dark grey) or ASCL1 (blue). n = 3-4 biological replicates. Error bars indicate standard error of the mean. *p<0.05; **p<0.01; student's t-test.

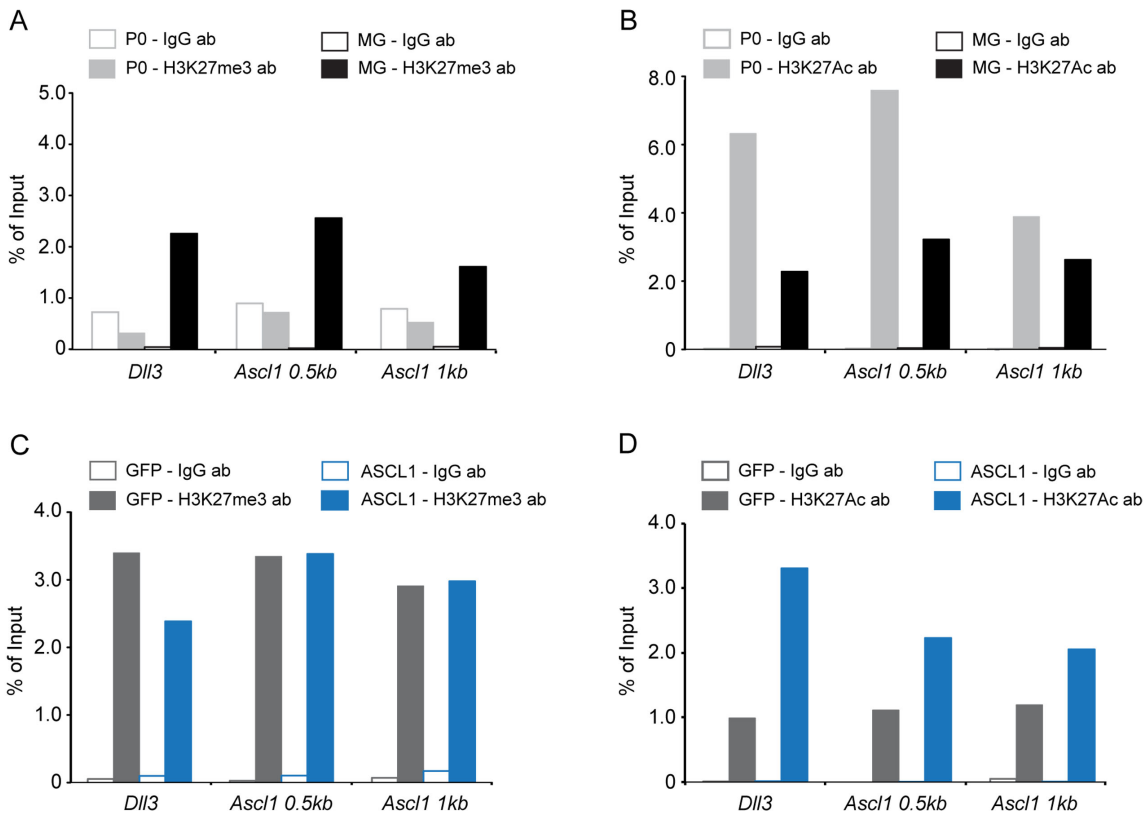


Figure 3.5: **ASCL1 partially remodels the histone modifications at the endogenous *Ascl1* locus.** (data published in Pollak et al., 2013) ChIP-qPCR for H3K27me3 (A,C), H3K27ac (B,D) or IgG control (white bars) antibodies at the 5' proximal promoter region (0.5kb and 1kb distal to the TSS) of the endogenous *Ascl1* gene. The *Dll3* promoter serves as a positive control for comparison (see Figure 3.4). Enrichment measured as a % of input DNA (Y-axis). Panels A and B show the change in histone modification levels between P0 retina (light grey) and P12 cultured Muller glia (black). Panels C and D show the change in histone modification levels between Muller glia 4-days after transduction with GFP control (dark grey) or ASCL1 (blue). n = 2 biological replicates.

I observed that endogenous *Ascl1* was not significantly up-regulated after ASCL1 misexpression (data not shown). This was not a surprise because other groups have demonstrated that *Ascl1* is not auto-regulatory in brain tissue (Castro et al., 2011). However, *Ascl1* expression is an important regulator of retinal development and its expression is a hallmark of retinal progenitor cells. Therefore, the transcriptional network of retinal progenitors that

activate and sustain *Ascl1* expression have not been re-established by ASCL1 misexpression in Muller glia.

To begin to understand this failure of re-expression, I asked whether the promoter region of *Ascl1* undergoes epigenetic remodeling during reprogramming, hypothesizing that it does not. I found that the endogenous *Ascl1* locus undergoes similar histone modification dynamics to the other progenitor genes (Figure 3.4A,C) during differentiation of the Muller glia. Specifically, H3K27me3 levels increase, and H3K27ac levels decrease between P0 retina and P12 Muller glia, measured at 0.5kb and 1kb distal to the transcription start site (Figure 3.5A,B).

Next, I observed that ASCL1 misexpression resulted in an increase in H3K27ac levels, indicating activation-associated epigenetic remodeling at the *Ascl1* locus (Figure 3.5C). However, the accumulated levels H3K27me3 were not reduced following ASCL1 misexpression (Figure 3.5D). The fact that inhibitory H3K27me3 modifications are not removed from the *Ascl1* promoter may, at least partly, explain its failure to re-express in Muller glia. However, the upstream regulator(s) of *Ascl1* in retinal cells are currently unknown and we do not know if the required transcription factors are expressed in reprogrammed Muller glia. Additional mechanisms of gene repression, such as microRNAs, may also contribute to suppression of *Ascl1* in Muller glia. Together, these data indicate that the retinal progenitor state is not completely re-established at either the transcriptional or epigenetic level.

Progenitor gene loci are relatively DNase-hypersensitive in Muller glia

One major draw back of reprogramming Muller glia in dissociated culture is that we are limited to using Muller glia from P12 retinas, a relatively young age. Ideally we would like to examine the effect of ASCL1 misexpression in Muller glia from adult retinas. To achieve this,

we created a transgenic mouse that expresses Ascl1 conditionally in Muller glia cells after treatment with tamoxifen. The transgenic mouse analysis of retinal regeneration by immunohistochemical characterization of transdifferentiation was carried out in collaboration with Yumi Ueki and lab colleagues. These mice harbored 3 critical transgenes (Figure 3.6): Glast-CreER drives Cre-recombinase from a glial specific gene which is activated in the presence of tamoxifen; Rosa-flox-Stop-tTA expresses the tTA transcriptional activator ubiquitously after Cre-mediated recombination; and tetO-Ascl1-IRES-GFP over-expresses Ascl1 and GFP in response to rTA. Thus, misexpression of Ascl1 in Muller glia could be achieved by tamoxifen injections at any age between the onset of Muller glia differentiation (~P10) and adulthood. Additionally, the Muller glia would be subject to reprogramming within the *in vivo* environment of the retina (Ueki et al., 2015).

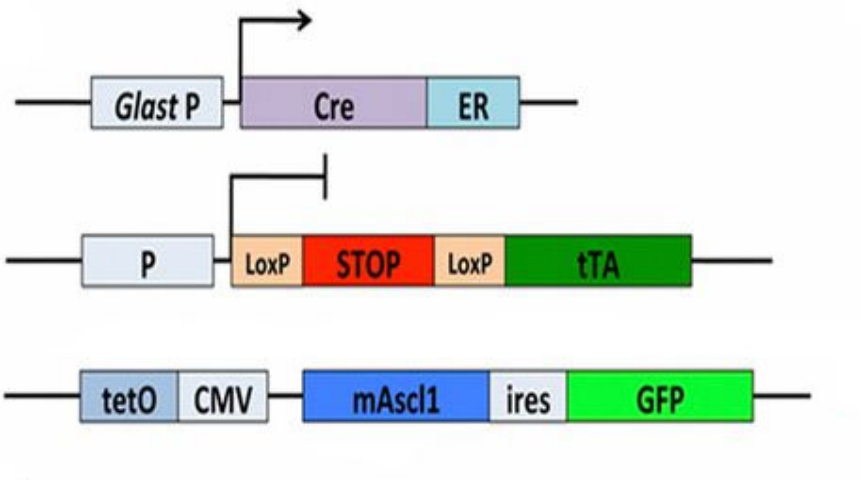


Figure 3.6: Transgenic scheme for Ascl1 misexpression in Muller glia *in vivo*. (Figure modified from Ueki et al., 2015): Schematic representation of the 3 transgenes harbored in Mice engineered to misexpress Ascl1 in Muller glia. A Muller glial specific promoter (*Glast* P) drives Cre-recombinase fused to estrogen receptor (ER). Upon addition of tamoxifen, the Cre~ER translocates to the nucleus where it recombines a stop codon, surrounded by LoxP sites, out of the genome. This allows the successful expression of the tTA protein that binds the tetO promoter and drives expression of Ascl1 and GFP, separated by an internal ribosome entry site (IRES).

When tamoxifen was injected in adult mice, the Muller glia expressed Ascl1 from the transgene. However, no morphological change was observed and the Muller glia did not co-label with *Otx2*, a direct target of Ascl1 and marker of some retinal neurons. Only when Ascl1 misexpression was combined with NMDA-induced neurotoxic damage, did the Muller glia express *Otx2* and down-regulate the glial transcription factor, *Sox9* (approximately 50% of Ascl1 expressing Muller glia). However, these adult Muller glia did not adopt a neuronal morphology or display other markers of neuronal differentiation (Ueki et al., 2015).

Similar to the adult, when Ascl1 misexpression was induced at P12 *in vivo*, no drastic morphological changes were observed. However, when Ascl1 misexpression was combined with NMDA-induced neurotoxic damage in P12-P14 mice, the Muller glia exhibited striking hallmarks of neuronal reprogramming. These changes include morphology characteristic of retinal neurons, and expression of amacrine cell genes (*HuC/D, Pax6*), and bipolar genes (*Otx2, Cabp5*). In addition, some Muller glia migrated to the outer nuclear layer, adopted a photoreceptor-like morphology, and expressed photoreceptor genes *Otx2* and *Rcvn*. Lastly, an increase in EdU was observed in these Muller glia, indicating extra proliferation above levels observed in the NMDA damage control (Ueki et al., 2015).

Given this striking contrast in the reprogrammability of Muller glia between P12 and adult ages, I hypothesized that the epigenetic environment is more conducive to Ascl1-mediated reprogramming in P12 glia versus adult. To test this hypothesis, I performed DNase-hypersensitivity sequencing (DNase-seq) on P12 cultured Muller glia and compared the chromatin hypersensitivity landscape to that of P0, P7 and adult retina. Panels A and B of Figure 3.7 show the DNase-seq landscape of Muller glia genes, *Glul* and *Rlbp1*, demonstrating chromatin accessibility at the TSS and surrounding regions of these genes in Muller glia and also

in whole retina. Panels C and D of Figure 3.7 show that the TSS of two progenitor genes, *Mfng* and *Dll1*, harbor DNase accessibility in Muller glia and developing retina (P0 and P7), but not adult retina. Interestingly, Muller glia do not express these genes, but still display accessible chromatin at their promoters.

Next I wanted to test the DNase-hypersensitivity of Ascl1 target binding sites more systematically. To accomplish this, I identified Ascl1 target regions by overlapping the DNase-hypersensitive sites (DHSs) of P0 retina with Ascl1 ChIP-seq regions from neural progenitor cells (Wapinski et al., 2013). Next, I measured the overlap between these regions and the DHSs present in the P7 retina, adult retina, and P12 Muller to measure the extent of Ascl1 target region accessibility in these tissues. I observed that the majority of Ascl1 target regions were also DHSs in P7 retina and P12 Muller glia and the majority of these DHSs were shared between these tissues. When these shared DHSs were subjected to gene ontology analysis (GREAT algorithm; McLean et al., 2010), the enriched terms were related to neurogenesis and gliogenesis (Figure 3.7F). However, far fewer of Ascl1 target regions were also DHSs in adult retina (Figure 3.7E). In addition, these overlapping regions were not enriched for gene ontology terms related to neurogenesis (data not shown, Ueki et al., 2015).

To extend this analysis we determined the DNase hypersensitivity at the TSS and proximal promoter regions of a set of 15 retinal progenitor genes. I observed that these progenitors genes displayed greater hypersensitivity in P12 Muller glia than adult retina (Figure 3.7G), indicating that the chromatin environment at progenitor genes and Ascl1 target regions is relatively accessible in the P12 Muller glia.

Together, these results suggest, although does not conclusively prove, that one reason young Muller glia are able to transdifferentiate is that the chromatin at progenitor gene loci is

relatively hypersensitive, thus allowing Ascl1 binding and transactivation. While this is a correlation and does not provide conclusive evidence that chromatin accessibility is contributing to the incomplete nature of reprogramming, it is consistent with our hypothesis and warrants further study. Future work will test this correlation and determine whether progenitor gene activation by Ascl1 is predicted by DNase-hypersensitivity in the Muller glia.

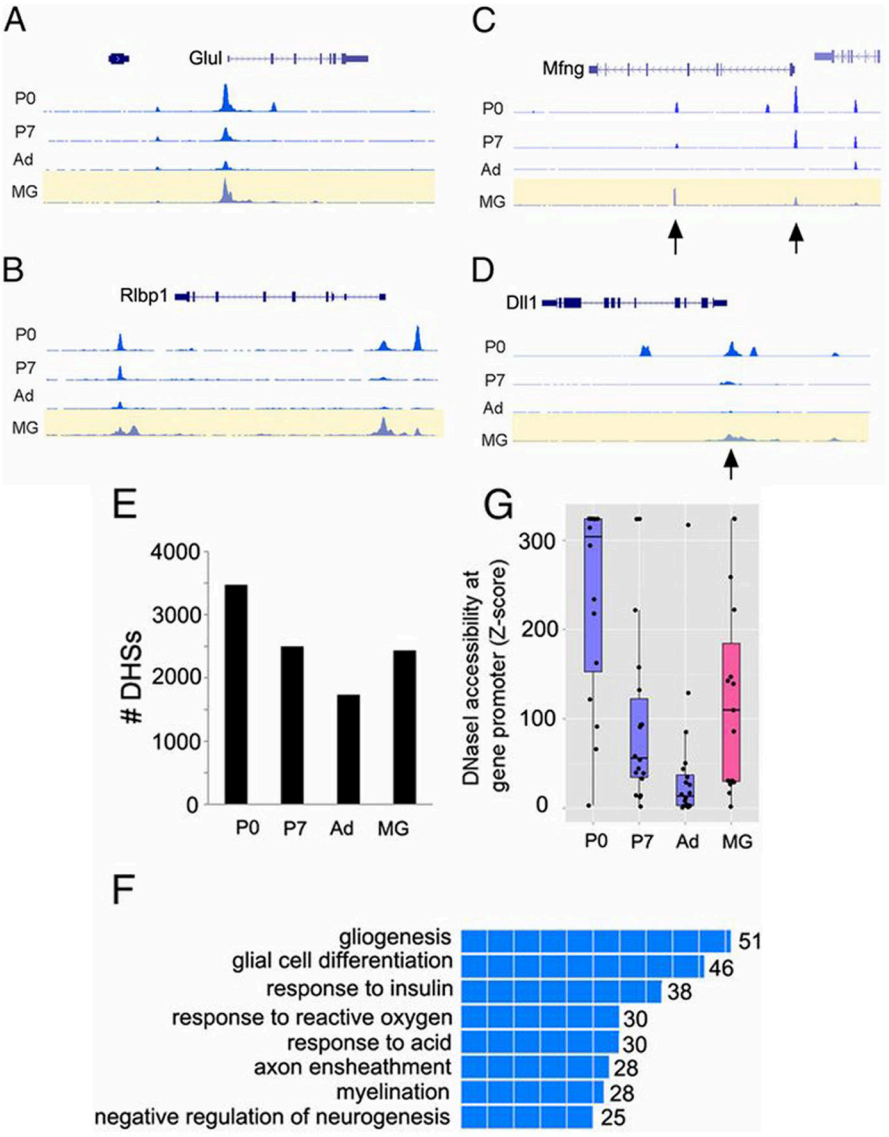


Figure 3.7: **Progenitor genes and Ascl1 target regions display DNase hypersensitivity in Muller glia.** (data published in Ueki et al., 2015): A-D. DNase-hypersensitivity signal tracks at Muller glia genes *Glul* (A) and *Rlbp1*(B) and progenitor genes *Mfng* (C) and *Dll1* (D). Indicated tissues are P0 retina, P7 retina, (Ad) adult retina, and (MG) Muller glia. E. The number of P0-DNase-hypersensitive Ascl1 binding regions that overlap a DHS in P7, Adult retina, or Muller glia. F. Gene ontology 'Biological Process' terms enriched in the set of regions in (E) that are shared between P7 retina and Muller glia. G. The Z-score of the DHS (if any) at 15 progenitor gene promoters (defined as the region between the TSS and 1kb upstream).

DISCUSSION

In summary, we found that misexpression of ASCL1 in mouse Muller glia results in partial reprogramming to a progenitor or early neuronal cell fate. Expansion and isolation of pure mouse Muller glia was demonstrated through dissociated cell culture and passaging to remove remaining retinal neurons. Next, we found that viral transduction of *Ascl1* is sufficient to activate expression of progenitor and early neuronal genes. The set of up-regulated genes partially completes the gene expression state of retinal progenitor cells, although many key genes remain transcriptionally silent. The mechanism of this reprogramming includes direct binding of ASCL1 and remodeling of inhibitory histone modifications. Further, misexpression of Ascl1 in young versus adult Muller glia *in vivo* demonstrates that young Muller glia have a higher intrinsic capacity to be reprogrammed and this correlates with chromatin accessibility at progenitor genes.

ASCL1 misexpression is able to activate the transcription of both early neuronal (e.g. Otx2) and retinal progenitor genes (e.g. *Mfng, Dll1, Dll3, Hes5, Hes6*). This result is encouraging due to the fact that these same genes, members of the Notch signaling pathway, appear to be endogenously regulated by *Ascl1* during retinal development (Nelson et al., 2011). In addition, many of the same genes that are up-regulated by microarray analysis are also targets of *Ascl1* in mouse brain tissue (Castro et al, 2011). This suggests that Ascl1 is able to activate at least some of its target genes in different neural tissues and even in glial cell types. I will investigate this question on a genome-wide scale in the following chapter. It would be an interesting experiment to misexpress Ascl1 in a variety of cell types from all 3 germ cell layers (ectoderm, mesoderm, and endoderm) and investigate the extent of convergence and divergence of ASCL1 binding in these disparate epigenetic contexts.

ASCL1 directly binds to at least some target gene promoters and this binding correlates with epigenetic remodeling. We performed ChIP for H3K27me3 or H3K27ac at the 5' proximal promoter region of 4 progenitor genes regulated by ASCL1: *Hes5*, *Dll1*, *Hes6*, and *Dll3*. H3K27me3 levels increase at these genes during glial differentiation between the P0 retina and Muller glia. This trend is reversed following ASCL1 transduction; H3K27me3 levels decrease between GFP control and ASCL1 expressing Muller glia. The opposite pattern is observed for the H3K27ac histone modification during Muller glial differentiation and reprogramming. One caveat is that the P0 retina is a heterogeneous mix of cells, approximately half of which are retinal progenitors; the other cells being early born neurons (ganglion and amacrine cells) and nascent late born neurons (photoreceptors and bipolar cells). Therefore, we cannot definitively state that the pattern of high H3K27ac and low H3K27me3, observed in P0 retina, is due to only the progenitor cells. Still, our observation of histone modification reversal during reprogramming is not complicated by cell population heterogeneity. In addition, there may be many ASCL1 target genes that fail to up-regulate due to the presence of other, unexamined epigenetic modifications that are not reversible by ASCL1

The endogenous *Ascl1* locus was subjected to the same inhibitory histone pattern during differentiation, but was not fully reversed during reprogramming. Specifically, H3K27ac levels increased, but H3K27me3 levels did not decrease following ASCL1 transduction. If the retinal progenitor cell state were fully re-established, we would expect re-expression and reversion of the epigenetic state of this critical progenitor gene. These results confirm that reprogramming is only partially complete.

It is interesting that ASCL1 does not bind directly to the endogenous Ascl1 promoter (see the following chapter) and yet levels of the activation-associated histone modification, H3K27ac,

increase after ASCL1 transduction. This suggests two possibilities: broad histone remodeling effects of ASCL1 or a downstream factor from ASCL1 is altering the *Ascl1* locus, but unable to re-activate transcription in Muller glia. In correspondence with option 1, the related bHLH factor, *Myod1*, binds around the genome broadly (up to 60,000 sites) and induces a large-scale acetylation of histone 4, at least (Cao et al., 2010). It is tempting to speculate that ASCL1 binding to neighboring regions induces a broad acetylation of histones without activating transcription. However, it is possible that factors secondary to Ascl1 feedback on the Ascl1 locus. Without knowledge of what transcription factors activate Ascl1, it is difficult to test this hypothesis.

Another possible reason for the incomplete nature of reprogramming is that in this experimental paradigm, Ascl1 misexpression is never silenced. Constitutive expression of Ascl1 may be preventing neuronal differentiation by maintaining Notch pathway and other progenitor genes such as *Hes5*. Therefore, transient expression of Ascl1 may be required where Ascl1 is misexpressed for a sufficient period of time, but then silenced to allow differentiation. This strategy would more closely resemble the developmental mechanisms in retinal progenitors where Ascl1 is only transiently expressed. Seemingly at odds with this hypothesis is that markers of mature neurons, such as *HuCD* and *Rho*, are observed from reprogrammed Muller glia *in vivo*. However, the numbers of fully transdifferentiated cells remains low (~5% HuCD+) and thus, this mechanism may be preventing differentiation in a large portion of the Muller glia

An open question is whether ascl1 is only able to bind to accessible sites or acts as a pioneer factor. We show that many developmentally appropriate Ascl1 target regions and progenitor genes are DNase-hypersensitive in the Muller glia. However, we have not determined

that this contributes to the reprogrammability of these cells. These ideas will be further tested in the following chapter.

Chapter 4:

Using the *cis*-regulatory landscape of Muller glia to inform reprogramming and retinal regeneration.

INTRODUCTION

In the previous chapters we have performed DNase-hypersensitivity sequencing on the developing retina and purified, cultured Muller glia. Additionally, we observed that Ascl1 has a substantial capacity to reprogram mouse Muller glia to a neurogenic state both in dissociated cell culture. This effect was also observed in young mice *in vivo*, if combined with retinal injury, and to a much lesser extent in adults. In this chapter we analyze the similarity and sequence features of these disparate cis-regulatory landscapes in order to inform the reprogramming process. Comparing the DNase-seq landscape of retinal progenitors and Muller glia could generate candidate transcription factors required for more complete reprogramming of the transcriptional and epigenetic state of Muller glia.

In dissociated culture, Ascl1 misexpression activated expression of many progenitor and early neuronal genes. However, this was only a partial reprogramming, as many progenitor genes, especially those of early retinal progenitors, were not re-activated in the Muller glia. Therefore, a major focus of our research has become understanding the basis of incomplete reprogramming of Muller glia.

We reasoned that the Muller glia might fail to reactivate all progenitor genes because Ascl1 is not binding to all the same regions as in retinal progenitors. Comparing the binding pattern of endogenous Ascl1 in retinal progenitors with the Ascl1 binding pattern following misexpression in Muller glia may identify critical cis-regulatory elements required for complete reprogramming. Further, examination of the underlying sequence may reveal candidate transcription factors required for conversion of the cis-regulatory landscape of Muller glia into retinal progenitors.

In this chapter I demonstrate that the partial nature of gene expression reprogramming correlates with a partially appropriate binding pattern of Ascl1 in the Muller glia. This is determined by comparative analysis of the Ascl1 ChIP-seq binding pattern in retinal progenitors and Muller glia.

Next, by comparing the sequences underlying the differential DNase-hypersensitive sites (DHSs), we identified Zic1 as a reprogramming factor that can activate extra progenitor genes beyond Ascl1-mediated reprogramming. Zic1 is a zinc-finger transcription factor involved in early neural development across vertebrate species (Aruga et al., 2004) and is expressed in embryonic retinal progenitor cells (Watabe et al., 2011). Consistent with its expression pattern, Zic1 activates the expression of some early retinal progenitor genes. However, the majority of transcription factors identified by comparative DNase-seq exhibited no enhancement of reprogramming at the assayed target genes.

Finally, we found that treating Muller glia with histone de-acetylase (HDAC) inhibitors concurrently with transcription factor mediated reprogramming can enhance the reprogramming process. When standard Ascl1 misexpression is combined with Otx2 misexpression and treatment with sodium butyrate, a small molecule inhibitor of HDACs, activation of photoreceptors genes is observed for the first time. Thus, modulating the epigenetic state of the Muller glia is a promising approach for enhancing the reprogramming of Muller glia to desired cell types.

RESULTS

The binding pattern of ASCL1 in Muller glia partially overlaps the binding pattern of endogenous ASCL1 in retinal progenitors.

In order to determine the endogenous binding pattern of ASCL1 in retinal progenitor cells on a genome-wide scale, I performed ChIP-seq for ASCL1 in post-natal day 0 (P0) retina. The subsequent sequencing reads were filtered and mapped to the mouse genome using the program 'bwa' (see Methods). This data was then applied to the peak-calling algorithm of the HOMER software suite. In total, 22,251 peaks were called with a False Discover Rate (FDR) of 0.1%. In order to confirm the validity of these ASCL1 peaks, I compared the presence of ChIP-seq peaks to previous ChIP-qPCR experiments (see previous chapter). In all cases where I observed a significant enrichment of ASCL1 binding by ChIP-qPCR, I also observed at least one ASCL1 ChIP-seq peak (Figure 4.1A; compare to Figure 3.3). As another confirmation of the fidelity of ASCL1 ChIP-seq peaks, I subjected the peak-called regions to a Gene Ontology analysis using the GREAT algorithm and observed terms related to retinal development (Figure 4.2AB). As another confirmation, I applied the DNA sequence underlying ASCL1 ChIP-seq peaks to the MEME motif enrichment algorithm. The top-scoring motif was the canonical ASCL1 E-Box (Figure 4.1B) that has been determined following ASCL1 ChIP-seq in other tissues, such as the brain (Castro et al., 2011). Interestingly, we found that 98% of all Ascl1 peaks overlap with a P0 retina DNase-Hotspot, as would be expected. These results indicate that this data set accurately represents the ASCL1 binding pattern in retinal progenitors.

In order to determine the binding pattern of ASCL1 following misexpression of Ascl1 in Muller glia on a genome-wide scale, I performed ChIP-seq for ASCL1 following misexpression via a transgenic mouse modified for doxycycline inducible expression of Ascl1 in all cells. In

this experiment, retinas from P12 mice were dissociated and the Muller glia were grown in culture as described in the previous chapter. After 7 days *in vitro* and passaging, Ascl1 expression was induced in the purified Muller glia by addition of doxycycline to the culture medium. Chromatin was then collected after 6 days post Ascl1 induction and subsequently processed for ChIP-seq. The subsequent sequencing reads were filtered and mapped to the mouse genome using the program 'bwa' (see Methods). This data was then applied to the peak-calling algorithm of the HOMER software suite. In total, 47,507 peaks were called with a False Discover Rate (FDR) of 0.1%. In order to confirm the validity of these ASCL1 peaks, I applied the DNA sequence underlying ASCL1 ChIP-seq peaks to the MEME motif enrichment algorithm. The top-scoring motif was the canonical ASCL1 E-Box (Figure 4.1B) that has been determined following ASCL1 ChIP-seq in other tissues, including the retina. Interestingly, the Gene Ontology analysis for the total set of these peaks was not strongly enriched for terms related to retinal cell development. This prompted us to perform a binding site overlap analysis with the endogenous ASCL1 peaks in retinal progenitors. As shown in Figure 4.1C, only 31% (14,578/47,507) of ASCL1 binding events in Muller glia overlap an endogenous or 'appropriate', ASCL1 binding event in retinal progenitor cells. Further, this discrepancy is not simply due to the higher number of binding events in the Muller glia because 34% (7,673/22,251) of retinal progenitor ASCL1 binding events are not bound in the Muller glia (Figure 4.1C). Therefore, there is substantial 'inappropriate' binding of ASCL1 in Muller glia that may not be aiding the process of reprogramming to retinal progenitors or neurons. In addition, many ASCL1 binding events in retinal progenitors are not occurring within Muller glia.

This discrepancy lead us to examine the gene ontology associated with the different ASCL1 binding site categories of Figure 4.1C. When we applied the ASCL1 binding sites that

are specific to retinal progenitors to the 'GREAT' gene ontology algorithm, the majority of top enriched terms related to neurogenesis (e.g. – 'neural retina development', 'layer formation in cerebral cortex') (Figure 4.2A). ASCL1 binding sites that occurred in both progenitors and Muller glia were enriched for neurogenic and gliogenic terms (e.g. – 'negative regulation of gliogenesis', 'negative regulation of oligodendrocyte differentiation') (Figure 4.2B). However, the majority of ASCL1 binding sites specific to Muller glia were inappropriate to retinal development (e.g. – 'filopodium assembly', 'regulation of mitochondrial membrane permeability') (Figure 4.2C). Although some neural-related terms were present (e.g. – commissural neuron axon guidance), these were in the minority. Therefore, while ASCL1 binds 66% of all appropriate sites in retinal progenitors, the majority of binding sites in Muller glia are inappropriate and, based on gene ontology, are not productive towards neurogenic reprogramming.

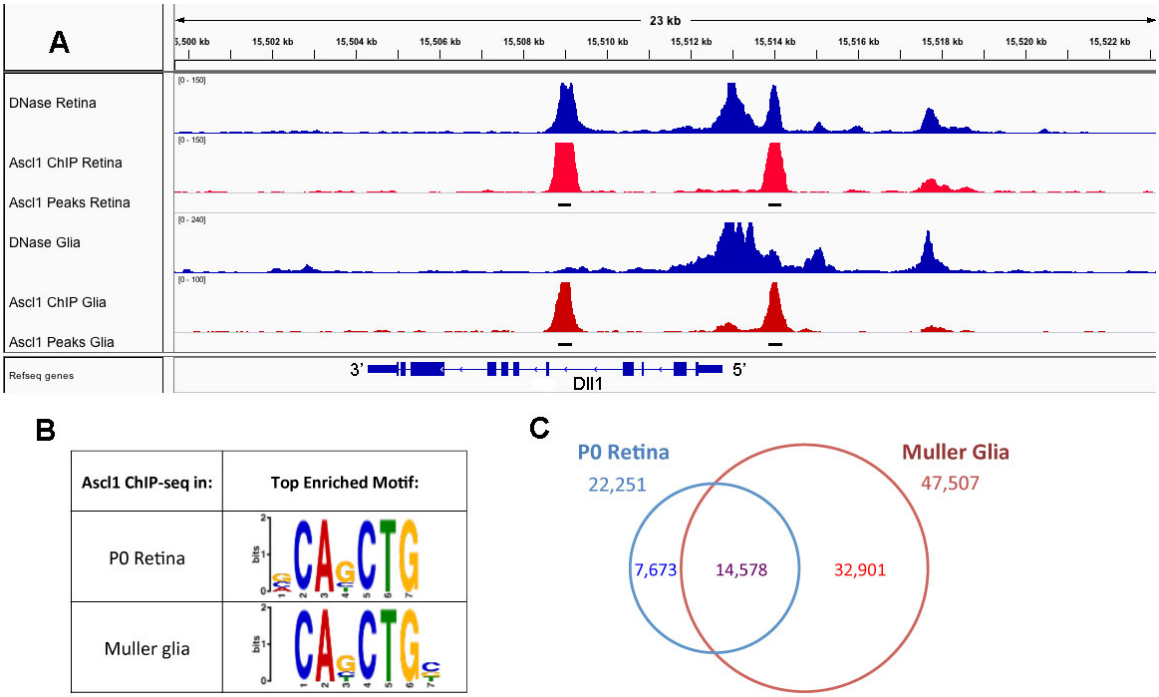


Figure 4.1: **The binding pattern of ASCL1 in Muller glia partially overlaps the binding pattern of endogenous ASCL1 in retinal progenitors.** A. Genome browser view of the *Dll1* locus with DNase-seq signal tracks (blue), ASCL1 ChIP-seq (red), and peak calls for ASCL1 ChIP-seq (black). From top to bottom: P0 retina DNase-seq; P0 retina Ascl1 ChIP-seq and peak calls; P12 dissociated Muller glia DNase-seq; Ascl1 ChIP-seq on Muller glia misexpressing Ascl1 and peak calls. B. Top enriched sequence motifs from Ascl1 ChIP-seq in P0 retina and Muller glia misexpressing Ascl1 as determined by the MEME software suite. C. Overlap between Ascl1 ChIP-seq peaks in P0 retina (endogenous Ascl1; blue) and Muller glia (misexpressing Ascl1; red) with the number of peaks in each category indicated.

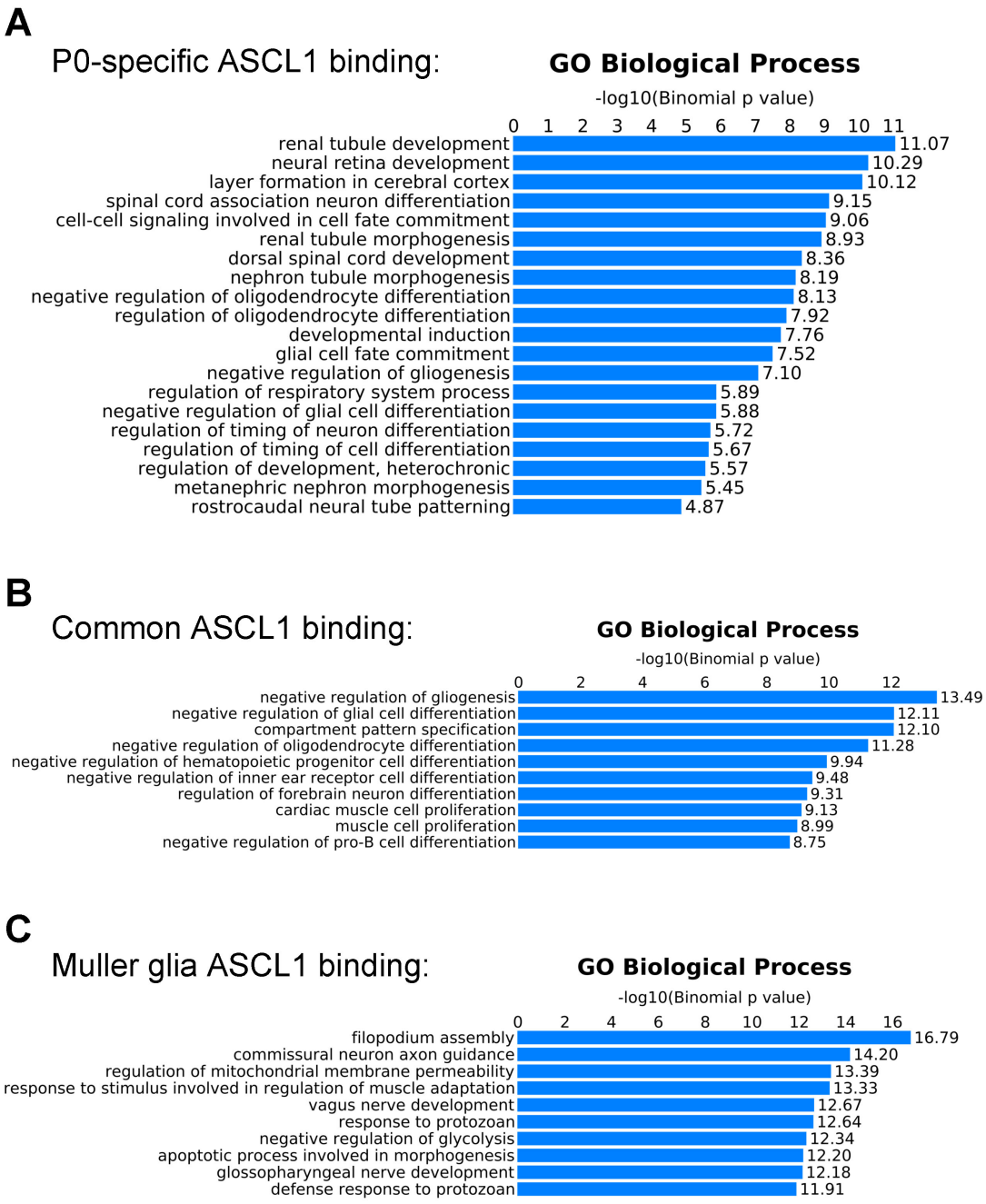


Figure 4.2: **Gene Ontology – Biological Process enrichment for differential ASCL1 binding events.** Related to Figure 4.1C. The top gene ontology – biological process enriched categories as determined by the GREAT algorithm for ASCL1 binding events that are retinal progenitor specific (A), common to both progenitors and Muller glia (B), and Muller glia specific (C).

ASCL1 binds to non-DNase-Hypersensitive Regions in Muller Glia

In the previous section, we determined that ASCL1 binds to both developmentally appropriate (i.e. – endogenous ASCl1 binding sites in P0 retina) and inappropriate regions of the genome in Muller glia. We wondered whether these inappropriate sites are due to ASCL1 binding opportunistically to DHSs that are present in the Muller glia and not in the retina. Therefore, we quantified the overlap between ASCL1 ChIP-seq peaks and DHSs that are present in Muller glia. As shown in Figure 4.3B, only 78% of ASCL1 binding sites occurred within DNase-hotspots in the Muller glia and 22% occurred within non-hypersensitive chromatin. This suggests that ASCL1 qualifies as a so-called 'pioneer factor' within the Muller glial epigenetic context. However, definitive evidence that ASCL1 rearranges chromatin at its binding sites requires DNase-seq on the Muller glia after ASCL1 misexpression. For the purposes of this dissertation, I define 'pioneer' activity as ASCL1 binding to non-DNase hypersensitive chromatin. A representative image of a possible pioneer binding site is shown in Figure 4.3A.

We hypothesized that these pioneer ASCL1 binding events in Muller glia were actually occurring at regions of DNase-hypersensitivity in the P0 retina. This scenario would be productive towards the goal of reprogramming the cis-regulatory landscape of Muller glia towards that of retinal progenitors. Therefore, we quantified the overlap between pioneer ASCL1 binding sites in Muller glia and P0 DNase-hotspots (Figure 4.3C,D). 34% (3591/10,575) of pioneer sites overlap a P0 DNase-Hotspot, whereas 66% (6984/10,575) of pioneer sites do not overlap a DNase-Hotspot in either P0 retina or Muller glia. This indicates that a third of ASCL1 pioneering events are productive for reprogramming towards a retinal progenitor state, however a majority of pioneering events appear to be unproductive and we term them 'radical' pioneering events. The determinants of the radical pioneering binding pattern remain to be discovered.

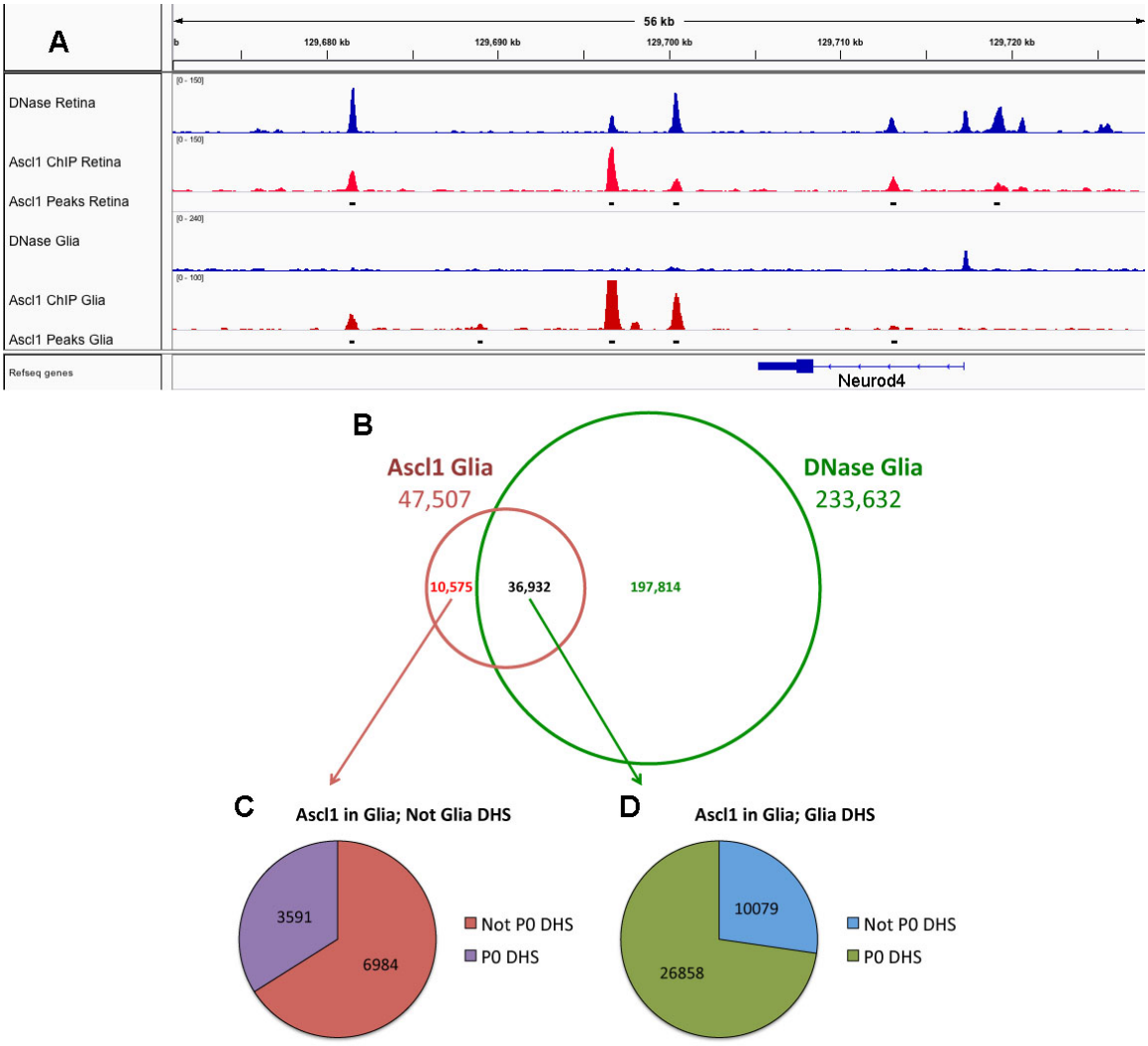


Figure 4.3: **ASCL1 binds to non-DNase-Hypersensitive Regions in Muller Glia.**
A. Genome browser view of the *Neurod4* locus with DNase-seq signal tracks (blue), ASCL1 ChIP-seq (red), and peak calls for ASCL1 ChIP-seq (black). From top to bottom: P0 retina DNase-seq; P0 retina Ascl1 ChIP-seq and peak calls; P12 dissociated Muller glia DNase-seq; Ascl1 ChIP-seq on Muller glia misexpressing Ascl1 and peak calls. B. Overlap between Ascl1 ChIP-seq peaks in Muller glia (misexpressing Ascl1; red) and DNase-Hotspots in Muller glia (not misexpressing Ascl1; green) with the number of peaks in each category indicated. C. Pie chart of Ascl1 ChIP-seq peaks in Muller glia that fall outside a DHS in Muller glia; showing the number of Ascl1 peaks that overlap (purple) or do not overlap (red) a P0 retina DHS. D. Pie chart of Ascl1 ChIP-seq peaks in Muller glia that occur within a DHS in Muller glia; showing the number of Ascl1 peaks that overlap (green) or do not overlap (blue) a P0 retina DHS.

DNase-hypersensitivity reveals the differences in cis-regulation between Muller glia and retinal progenitors.

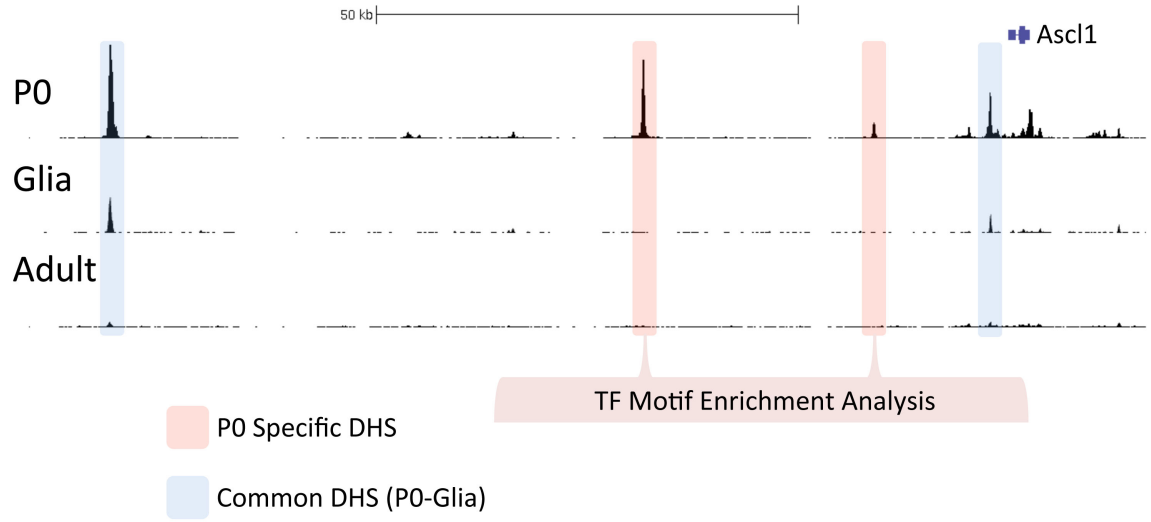


Figure 4.4: **Comparison of the DNase-hypersensitivity landscape of Muller glia and P0 retina.** DNase-hypersensitivity signal tracks surrounding the *Ascl1* locus from P0 retina, P12 cultured Muller glia, and adult mouse retina. Highlighted in blue are DHSs present in both P0 retina and Muller glia. Highlighted in red are DHSs present in just P0 retina and not Muller glia or adult retina. The sequences underlying the red, P0-specific DHSs are then applied to motif enrichment analysis (MEME software-suite).

Transcription factor motif enrichment identifies candidate reprogramming factors

Motif Found	Matching TF in Retina
	Pou3f2 / Rax
	Ascl1
	Sox2 / Sox11
	Klf7
	Meis1
	Rfx3 / Rfx7
	Zic1

Figure 4.5: **Comparative DNase-seq coupled to sequence based motif discovery identifies candidate transcription factors for reprogramming into retinal progenitors.** Chart showing discovered motif logos from the MEME software suite with the closest matched transcription factor that exhibits significant expression in retinal progenitors.

Comparison of the disparate ASCL1 binding pattern in retinal progenitors and Muller glia led us to ask about the difference in DNase-hypersensitivity between these two cell types in general. Sequence specific DNA binding proteins, primarily transcription factors, determine which cis-regulatory elements are active and DNase-hypersensitive in a given cell type. Therefore, we reasoned that applying the sequences underlying P0-specific DHSs to a transcription factor motif discovery algorithm would reveal those transcription factors

determining the cis-regulatory landscape of retinal progenitors and early neurons. These factors would then be candidates for enhancing Ascl1-mediated reprogramming of Muller glia.

Subtraction of Muller glial and adult retina DHSs from the set of P0 retina DHSs (Figure 4.4) yielded a set of sequences that was applied to the MEME-ChIP software pipeline. This generated a list of motif logos and matched them to transcription factors with similar binding motifs. This list was filtered according to a secondary criterion: the candidate factors must be expressed in the retinal progenitors (as determined by gene expression microarray on FACS-sorted P0 progenitors; see previous chapter). This removed a large majority of candidates and focused on the set of factors likely to instruct a retinal cell fate (Figure 4.5).

Confidence in this approach was bolstered when we observed that the canonical Ascl1 binding motif was identified as a candidate reprogramming factor (Figure 4.5). As expected, several transcription factors with well-known roles in retinal development were identified (e.g. – *Pou3f2*, *Sox11*); excitingly, extra factors were identified that have established roles in neurogenesis, but little or no described role in retinal development (e.g. – *Klf7*, *Rfx3*). Thus, comparative analysis of cis-regulatory elements between retinal progenitors and Muller glia can generate a plausible set of candidate transcription factors for transdifferentiation between cell types.

Many candidate transcription factors do not alter gene expression of target genes in Muller glia

Following the reprogramming paradigm that we established with Ascl1, we cloned cDNA coding for the candidate factors, identified above, into lentiviral constructs and misexpressed them in P12 Muller glia cultures by viral transduction. In addition to the candidates identified by

comparative DNase-seq analysis, we supplemented our list with several other transcription factors with known-roles in retinal neurogenesis (Figure 4.7).

In order to determine the efficacy of each candidate factor, we performed RTqPCR on the transduced Muller glia and measured the expression of canonical progenitor genes as well as candidate-specific target genes that are expressed in the P0 retina. These targets were determined by DNase-seq footprinting analysis (Stergachis et al., 2014) in the P0 retina. Specifically, a gene was assigned as a target of a candidate reprogramming factor if a DNase-footprint for the candidate factor was detected in a gene's proximal promoter DHS. These criterion gave us confidence that we would detect whether a given candidate factor would enhance the reprogramming process by activating progenitor genes.

After screening 24 factors beyond Ascl1, we were surprised that the vast majority did not show efficacy in activating the assayed progenitor genes in Muller glia (Figures 4.6, 4.7). A representative result is shown in Figure 4.6 where either *cMyb*, *Sox11*, or *Ascl1+cMyb+Sox11* was misexpressed in Muller glia and no target gene transactivation was observed. In this case and others, candidate factors were combined in a given experiment if they had common target genes so that synergistic activation could be measured, if applicable. In addition, candidate factors were tested alone or in combination with Ascl1 to examine the possibility that a given factors efficacy is dependent on Ascl1-mediated reprogramming (i.e. – a candidate factor enhances but is not sufficient for reprogramming). However, despite the tailored assays and numerous conditions, only one factor, *Zic1*, was able to enhance the reprogramming process towards retinal progenitors. One other factor, *Otx2*, activated genes characteristic of differentiating and mature photoreceptors, although this effect was dependent on exogenous small molecule inhibitors (see later section).

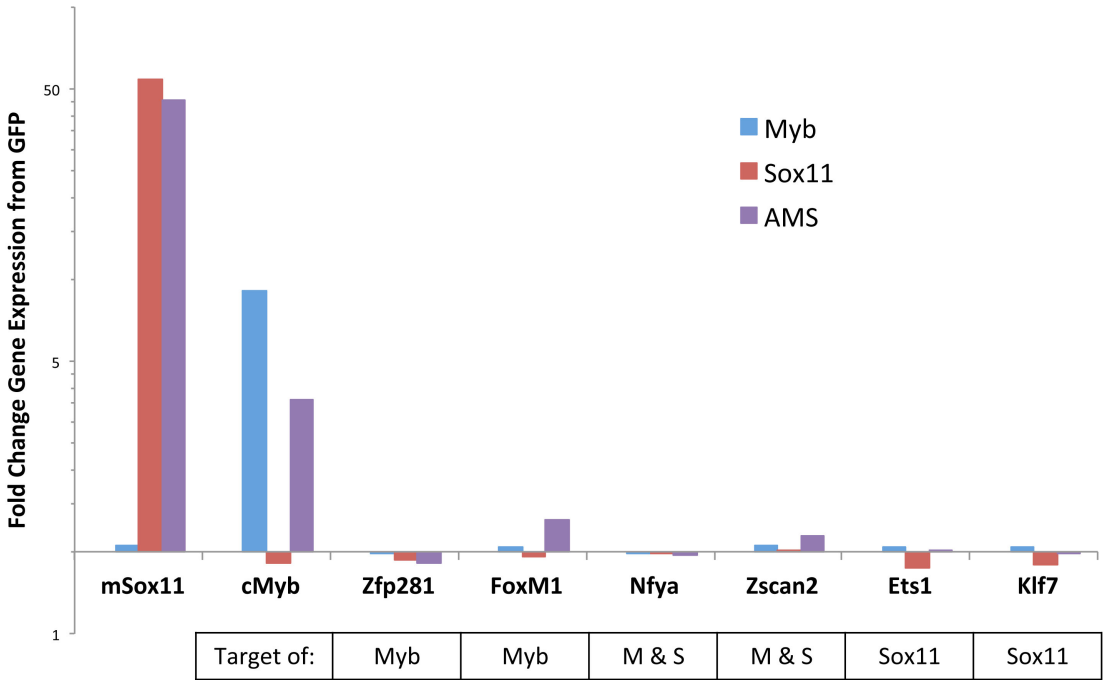


Figure 4.6: **Neither cMyb or Sox11 activate predicted target genes in Muller glia.** Fold-change of gene expression measured by RTqPCR for the genes indicated on the x-axis after 6-days post transduction with: Myb (blue); Sox11 (red); or ASCL1+Myb+Sox11 (AMS; purple). For each gene, values normalized to GFP control condition. Chart at bottom displays which transcription factor is predicted to be a direct upstream regulator of the assayed target gene. Left two conditions confirm that Sox11 and Myb are expressed in the Muller glia.

Factor	Target Gene Activation
Pou3f2	No
Rorb1	No
Pax6	No
Hmgb3	No
Klf7	No
Zfp281	No
Myb	No
Sox11	No
Zic1	Yes
Olig2	No
Neurog2	No
Lin28	No
Myt1l	No
Foxn4	No
Rfx3	No
Sox2	No
Otx2	*Yes
Otx2~VP16	*Yes
Crx	No
Nrl	No
Nr2e3	No
Prdm1	No
Neurod1	No
EBNA1	No

Figure 4.7: **Summary chart of transcription factors tested for reprogramming efficacy in P12 Muller glia.** The indicated transcription factors were identified either by comparative DNase-seq coupled to motif enrichment analysis or by comparative gene expression data in progenitor and Muller glia. These factors were virally misexpressed in cultured Muller glia alone or concurrently with Ascl1-mediated reprogramming. Changes in factor specific target genes, generic progenitor genes, and/or differentiated retinal neuron genes were assayed for increased expression.

Zic1 activates gene expression of retinal progenitor genes that are not activated by Ascl1 in Muller glia

We observed that Zic1 was capable of activating expression of several progenitor genes within the Muller glia beginning at 4-days post transduction (Figure 4.8). Interestingly, some genes were responsive to Zic1 misexpression alone, and others depended on the co-expression of Ascl1. By itself, Zic1 activated *Chd6*, *Fgf15*, and to some extent *Foxn4*, which was not affect by Ascl1-mediated reprogramming. A striking synergy between Zic1 and Ascl1 was observed in the activation of *Foxn4*, *Dll4*, and possibly *Fabp7*. In the case of *Foxn4* and *Dll4*, either Zic1 or Ascl1 was able to up-regulate expression by 10-fold or less, however when combined, expression was up-regulated by 200-300 fold (Figure 4.X). At least, two of these genes, *Foxn4* and *Fgf15*, are characteristic of early retinal progenitors. Combined with the fact that Zic1 is expressed in early retinal progenitors, this suggests that Zic1 may be directing Ascl1-mediated reprogramming towards early generated retinal cell fates; however we have no further data supporting this hypothesis at this time. These results demonstrate that a comparative analysis of the cis-regulatory landscape between retinal progenitors and Muller glia can inform and expand the approach to reprogramming.

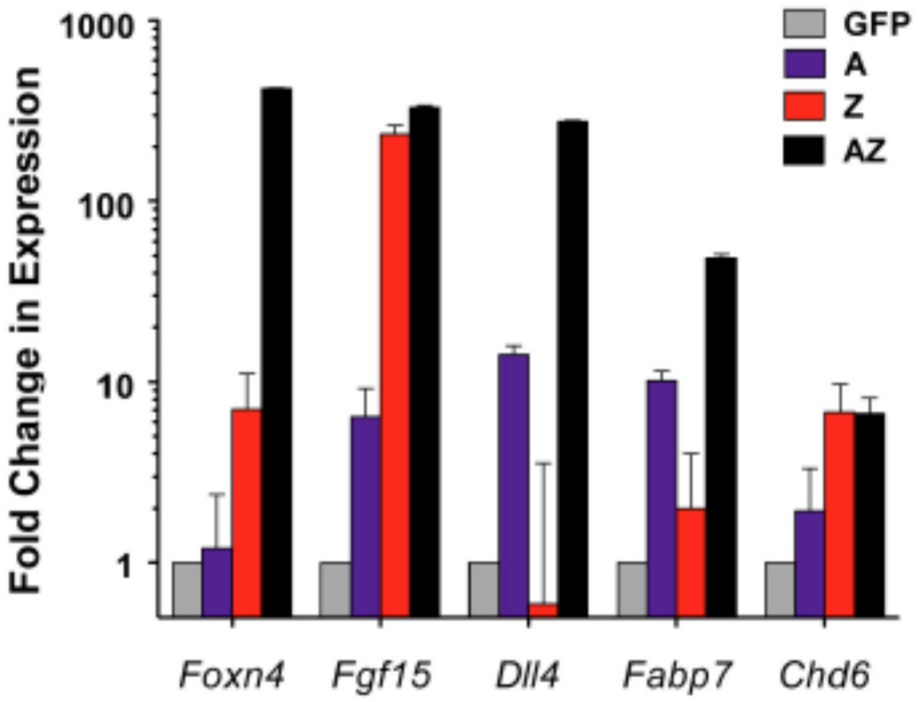


Figure 4.8: **Zic1 misexpression in Muller glia causes up-regulation of several early retinal progenitor genes.** Fold-change of gene expression measured by RTqPCR for the genes indicated on the x-axis after 7-days post transduction with: A: ASCL1 (purple); Z: Zic1 (red); AZ: ASCL1+Zic1 (black). For each gene, values normalized to GFP control condition. Error bars indicate standard error of the mean. (Data from Kristen Cox/Julia Pollack)

Targeting Epigenetics to Enhance Ascl1-Mediated Reprogramming

Because the epigenetic context is to some extent determining the activity of ASCL1 during reprogramming (i.e. – altering the binding pattern of ASCL1; see previous section), we hypothesized that modulating the epigenetic state of the Muller glia during reprogramming would change the 'reprogrammability' of the Muller glia. Several studies have shown that modulating epigenetic enzymes and protein interactions has an effect on reprogramming in numerous paradigms (Vierbuchen and Wernig, 2012). Therefore we tested small molecules for

several well-established enzymes in epigenetic regulation that represent major classes of epigenetic inhibition (Figure 4.9).

In these assays, we misexpressed Ascl1 in conjunction with a given epigenetic inhibitor and measured the expression of a set of progenitor and neuronal genes that are not affected by standard Ascl1-mediated reprogramming. In addition, we performed checks that the epigenetic modification being targeted was actually changed due to our manipulation. For example, treatment with 5-aza-cytidine was effective at removing DNA-methylation at photoreceptor genes in the Muller glia (data not shown). A172 and GSK126, the small molecule inhibitors of *Ezh2*, which catalyze the addition of methyl groups to H3K27, resulted in the reduction, although not complete ablation, of inhibitory H3K27me3 levels at the promoters of progenitor genes. However, we were not able to confirm that BIX-01294, the small molecule inhibitor of the H3K9 methyl-transferase, G9a, was efficacious at reducing this histone modification. Nevertheless, the maximum sub-toxic dose of BIX-01294 was determined, but little to no effect was observed beyond standard Ascl1-mediated change to gene expression.

We observed a small but reproducible and productive effect on reprogramming of Muller glia with the histone de-acetylase (HDAC) inhibitor, sodium butyrate. Neither butyrate alone, nor Ascl1-mediated reprogramming in combination with butyrate affected progenitor gene expression, however when further combined with misexpression of the transcription factor, *Otx2*, resulted in activation of photoreceptor genes (Figure 4.11). As discussed in previous chapters, Otx2 has an instructive role in the development of bipolar cells and photoreceptors. Recent ChIP-seq studies have demonstrated that Otx2 binds to many photoreceptor genes, including early instructive photoreceptor transcription factors (e.g. – *Nr2e3, Nrl*) as well as genes involved in mature function (e.g. – *Pde6b, Guca1a*). Figure 4.10 displays Otx2 peaks along with the

DNase-hypersensitivity landscape at 3 photoreceptor genes. Otx2 binding sites fall within DHS in the adult retina, however Muller glia exhibit essentially undetectable DNase-hypersensitivity at photoreceptor genes. This complete absence of accessible chromatin suggested further that modulating the epigenetic state of the Muller glia could be beneficial for reprogramming.

Therefore, we measured the expression of photoreceptor genes in Muller glia following ASCL1-OTX2-butyrate combination reprogramming. After 8-days post transduction/treatment, we observed small but reproducible up-regulation of several photoreceptor specific transcription factors (e.g. – *Nrl, Nr2e3*) but not *Rhodopsin* (Figure 4.11). These results were particularly encouraging due to the fact that activating expression of definitive photoreceptor genes has eluded all other reprogramming attempts in the Muller glia thus far.

Presently the histone acetylation hypothesis is supported by the fact that up to 4 other experimental HDAC inhibitors, provided by collaborators, have exhibited a nearly identical effect on PR gene expression. In addition, an alternative HDAC inhibitor, trichostatin A, exhibits a striking enhance to Ascl1-mediated reprogramming in injured retinas *in vivo* (unpublished observations). Therefore, epigenetic modulation through the use of HDAC inhibitors is a promising approach to regenerating photoreceptors from the Muller glia.

Targeted Modification	Small Molecule Inhibitor	Result
H3K27me3	A172, GSK126	Not Productive
H3K9me3	BIX-01294	Not Productive
Histone-Acetylation	Sodium Butyrate	Photoreceptor Genes
DNA-Methylation	5-aza-cytidine	Not Productive

Figure 4.9: **Summary chart of epigenetic modulating compounds tested for reprogramming enhancement.** The indicated small molecules inhibitors were applied to cultured Muller glia concurrently with Ascl1-mediated reprogramming. Changes in progenitor and differentiated retinal neuron genes were assayed for increased expression with and without the small molecule inhibitor.

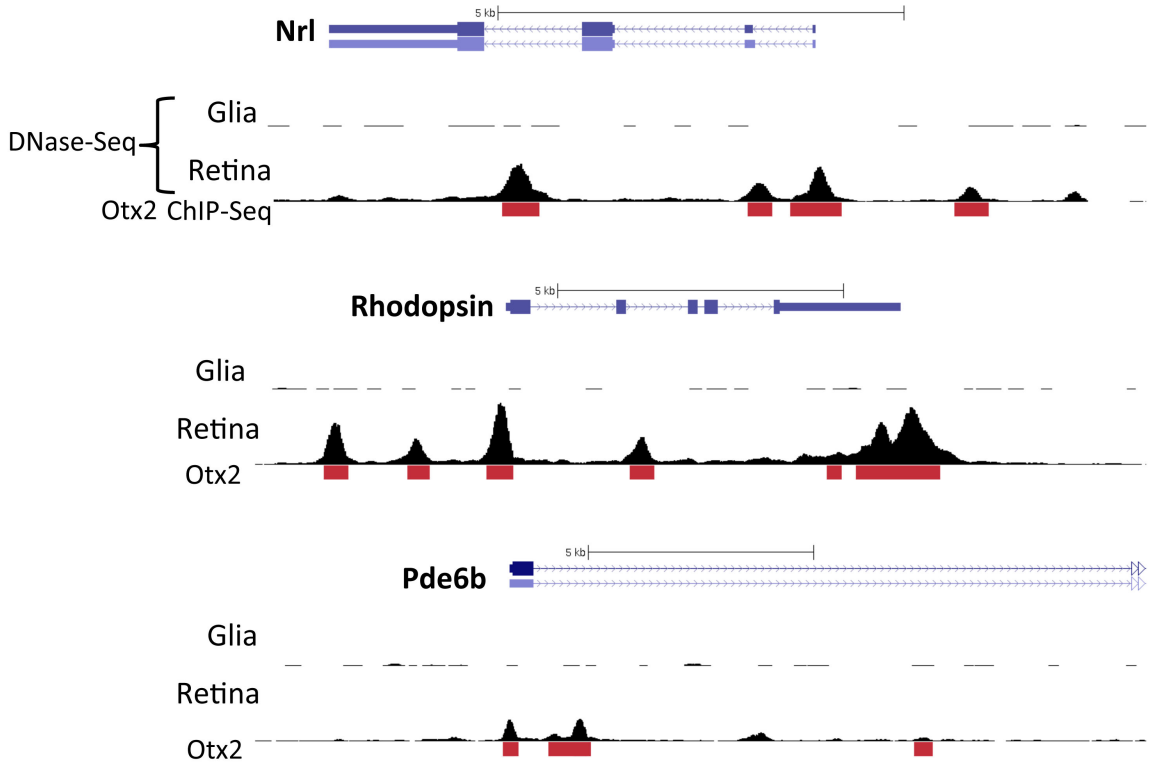
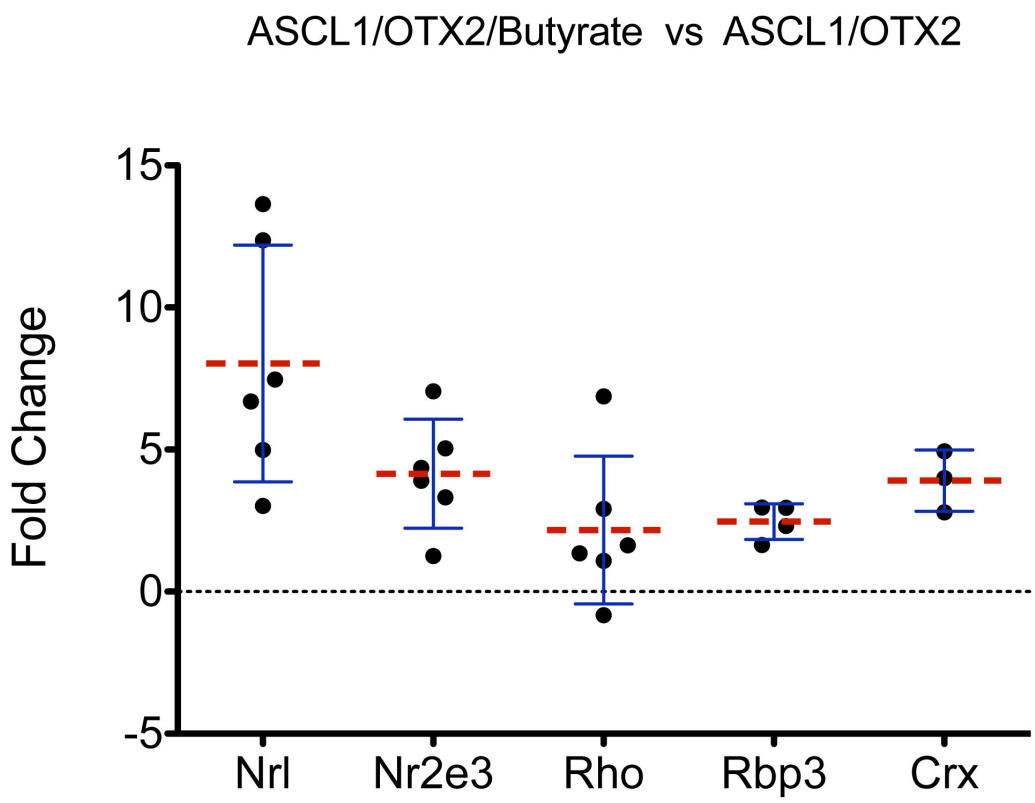


Figure 4.10: **Photoreceptor genes are not DNase-hypersensitive in Muller glia**. For each photoreceptor gene (*Nrl, Rhodopsin, Pde6b*), the DNase-hypersensitivity signal is displayed in the dissociated Muller glia (top track) and adult mouse retina (middle track); peaks from Otx2 ChIP-seq (Samuel et al., 2014) are displayed as red boxes in the bottom track.



	Nrl	Nr2e3	Rho	Rbp3	Crx
P value (two tailed)	0.0052	0.0032	0.0965	0.0043	0.0244

Figure 4.11: **Sodium Butyrate causes activation of photoreceptor genes in Muller glia during ASCL1/OTX2 mediated reprogramming.** Fold-change of gene expression in Muller glia +ASCL1+OTX2+butyrate relative to non-butyrate condition for indicated genes on the x-axis. Error bars indicated standard deviation; red dotted line indicates the mean; dots represent biological replicate measurements. Chart displays students t-test p-values for each gene versus the non-butyrate condition.

DISCUSSION

Broadly, the aim of this chapter has been to demonstrate that analysis of the epigenetic state of Muller glia can be used to understand the current limits to reprogramming and possibly be used to enhance the scope and efficiency of transdifferentiation into retinal progenitors and neurons. To this end, we have characterized the binding pattern of ASCL1 in both retinal

progenitors and in ASCL1-misexpressing Muller glia. We have compared DNase-hypersensitive landscapes to derive a set of candidate transcription factors likely to convert the cis-regulatory landscape of Muller glia into retinal progenitors and nascent neurons. Finally, we found a small molecule, known to modulate the epigenetic state of cells, which expands the range of cell-type specific genes activatable by transcription factor mediated reprogramming.

Comparison of the endogenous Ascl1 binding pattern in retinal progenitors with Ascl1 binding in Muller glia revealed substantial overlap and divergence of binding sites (Figure 4.1). Although 66% of progenitor ASCL1 binding sites were also bound in Muller glia, 34% of these developmentally appropriate sites were not bound. In addition, 69% of ASCL1 bound sites in the Muller glia did not correspond to a site bound in progenitors. Thus, ASCL1 binds both in a developmentally appropriate and inappropriate pattern in Muller glia. We speculate that this inappropriate binding is neutral to, or possibly even detrimental to the desired reprogramming process, due to the inappropriate gene ontology enrichment of Muller specific ASCL1 sites (Figure 4.2).

Possible reasons for the inappropriate ASCL1 binding sites in Muller glia are numerous. First, ASCL1 could simply be binding in an opportunistic manner to DHSs in the Muller glia not present in the retinal progenitors. While this may explain some inappropriate binding events, this possibility cannot account for the observed binding to non-hypersensitive chromatin (see next section). Second, the high level of ASCL1 in the cell resulting from transgenic over-expression may be driving binding to extra sites. Third, the necessary co-factors required to guide ASCL1 to the progenitor specific binding sites could be missing in the Muller glia. Fourth, ASCL1 may be guided to inappropriate binding sites by cooperative binding with Muller glia specific co-factors. These last two possibilities are being actively investigated. Specifically,

searching for transcription factor binding motifs that are enriched in progenitor-specific ASCL1 sites versus Muller glia-specific sites may reveal factors responsible for the differential binding patterns.

We found that ASCL1 is able to bind to regions of DNA that were not previously DNase-hypersensitive, which agrees with other reports that Ascl1 is a 'pioneer' transcription factor (Wapinksi et al., 2013; Raposo et al., 2015) (Figure 4.3). Thus, the non-progenitor Ascl1 binding sites observed in Muller glia are not simply due to opportunistic binding within DHSs present in Muller glia. However, 21% of ASCL1 sites in Muller glia fall within a Muller glia DHS, but not a P0 DHS, demonstrating that approximately one-fifth of all ASCL1 binding is opportunistic to the specific epigenetic context present in Muller glia (Figure 4.3). Interestingly, approximately one-third of pioneer ASCL1 sites in Muller glia correspond to a DHS in P0 retina, therefore in a limited set of instances, ASCL1 is able to override the lack of hypersensitive chromatin and create a cis-regulatory landscape more closely resembling a retinal progenitor. The sequence features that are enriched in these 'productive pioneering' binding sites is an area of active investigation. However, we speculate that co-factors present in both progenitors and Muller glia, which are not sufficient to induce hypersensitive sites alone, are cooperating with Ascl1 to bind to 'productive pioneering' sites. Finally, we cannot exclude the possibility that ASCL1 is binding to 'pioneer' sties, but not rearranging chromatin to render the site DNase-hypersensitive. Direct evidence of this will require performing DNase-seq on Muller glia after Ascl1 misexpression. In addition, a systematic examination of the correlation between Ascl1 'pioneering' events within a given gene's locus, and the gene's change in transcriptional state would provide evidence that Ascl1 'pioneering' binding events are functional in inducing gene expression.

One problem complicating the comparative DNase-seq landscape analysis (between P0 and Muller glia) is the possibility that many of the hypersensitive sites in dissociated Muller glia may be artificial (i.e. – induced by dissociated cell culture and soluble factors within the growth medium) relative to DHSs that exist within the cell *in vivo*. Indeed, the number of DNase-Hotspots in dissociated Muller glia is twice the number in P0 retina (233K versus 120K). These may be induced by the artificial environment and stimulation of cell culture. To determine the endogenous DNase-landscape of Muller glia, cell-isolation (by FACS) from intact retina would be required, followed by immediate DNase treatment. While the DNase-seq protocol requires approximately 10 million cells, FACS-sorting Muller glia could only reasonably generate less than 1 million per experiment (based on the census of cell numbers in the retina and FACS data not shown). In the absence of a low cell number DNase-seq protocol, ATAC-seq could be a useful approach due to the low cell numbers required as input.

Through sorting of DHSs in progenitors and Muller glia (Figure 4.4) and subsequent transcription factor binding motif discovery within progenitor specific DHSs, we identified several candidate factors to augment Ascl1-mediated reprogramming (Figure 4.5). One factor that limits the usefulness of this approach is that the current programs for identifying transcription factor binding sites are not complete and not able to distinguish between related transcription factors. Thus, the candidate reprogramming factor list is likely to be incomplete or partially inaccurate. Nevertheless, we restricted our list to those factors present in the developing retina. Despite testing of 24 candidates, only Zic1 and Otx2, which is conditional with a small molecule (see next section), were capable of activating target genes in the Muller glia (Figure 4.6 and 4.7).

Zic1 is a zinc finger transcription factor with a well-established role in neural development. During embryogenesis, it is involved in the specification of ectoderm to neuroectoderm across vertebrate species (review by Aruga et al., 2004). The role of Zic1 in the retina is much less described, however it is expressed in retinal progenitors by embryonic day 14 or earlier, gradually declines during development, and is absent from the retina early post-natal ages (Watabe et al., 2011). Additionally, overexpression of Zic1 in retinal progenitors extends the duration of (but does not increase) proliferation. Consistent with these findings, we found that Zic1 activates genes characteristic of early retinal progenitors (e.g. – *Foxn4* and *Fgf15*). In addition, it can activate genes alone (*Foxn4, Fgf15, Chd6*) or synergistically with Ascl1 (*Foxn4*, *Dll4*, *Fabp7*) (Figure 4.8). Thus, although the success rate is low, comparison of cis-regulatory elements, revealed by DNase-seq, has the potential to identify reprogramming factors.

Finally, small molecule modulators of the epigenetic state have been shown to enhance reprogramming in other systems (reviewed by Vierbuchen and Wernig, 2012). Because we observed that epigenetic context is at least a partial determinant of ASCL1's behavior in Muller glia, we tested several small molecule inhibitors for enhancing reprogramming of Muller glia (Figure 4.9). Our testing of epigenetic inhibitors was only exploratory and by no means exhaustive, therefore, a more thorough screen of small molecule inhibitors may reveal new mechanisms for de-repression of targeted genes during reprogramming.

We discovered that the HDAC inhibitor, sodium butyrate, caused activation of photoreceptor genes when combined with Ascl1 and Otx2 mediated reprogramming (Figure 4.11). The level of up-regulation was relatively small, however the effect included several 'master-regulators' of the photoreceptor cell fate (i.e. – *Nrl, Nr2e3, Crx*). This result was striking because several of these genes have resisted activation during all earlier attempts at

reprogramming the cultured Muller glia. Correspondingly, DNase-seq demonstrates that these genes are almost completely devoid of hypersensitive regions in Muller glia (Figure 4.10).

We noticed an interesting discrepancy: endogenous Otx2 activated by Ascl1 in Muller glia, fails to activate photoreceptor gene expression (even with butyrate), however, viral Otx2 overexpression did cause activation. One possible explanation is that a very high level of Otx2 is required for efficacy in Muller glia, which is achieved with Otx2 viral overexpression, but not transcription from the endogenous Otx2 locus. This hypothesis could be tested by tittering down the expression of Otx2 from the viral construct until there is no effect and compare this level to endogenous Otx2 expression levels activated by Ascl1.

In addition, we are interested in the mechanism by which butyrate enhances reprogramming. Future work will be required to confirm that the mechanism of action relevant to enhancing reprogramming is the acetylation of histones. This will be accomplished through the use of ChIP for H3K27ac and H4-acetylation. Activation of PR genes should correlate with an increase in histone acetylation levels. Further proof will require alternative inhibition of HDACs. Presently the histone acetylation hypothesis is supported by the fact that up to 4 other experimental HDAC inhibitors, provided by collaborators, have exhibited a nearly identical effect on PR gene expression. In addition, an alternative HDAC inhibitor, trichostatin A, exhibits a striking ability to enhance Ascl1-mediated reprogramming in injured retinas *in vivo* (see below).

Unpublished observations by members of our lab demonstrate that HDAC inhibition concurrently with Ascl1-mediated reprogramming generates photoreceptors *in* vivo. In these experiments, intraocular injection of TSA during Asc1 misexpression in the Muller glia of adult mice generates *Rhodopsin*+ photoreceptor like cells. Generation of photoreceptors will be an

active area of investigation for our lab, especially considering that photoreceptors regeneration is the most desired cell type from a therapeutic perspective.

Thus, we have demonstrated that investigation and modulation of the epigenetic landscape of Muller glia informs the reprogramming process towards retinal progenitors and neurons. These studies have implications for understanding reprogramming and cell fate in general and may even be useful for therapeutic applications in the future.

Chapter 5

Discussion

DISCUSSION

Summary of results

In this dissertation, I set out to begin the elucidation of the cis-regulatory landscape of mouse retinal development and regeneration. The retina provides a relatively tractable model for the rest of central nervous system development. It is part of the CNS and yet the retina is physically separated from the brain. While it contains a heterogeneous mix of neurons, the retina is composed of many fewer neuron sub-types than the brain. Thus, understanding retinal development is both fascinating in itself and deeply relevant to understanding the bewildering complexity of the brain. Further, due to the numerous pathologies and environmental assaults on the retina, understanding its physiology in molecular detail may lead to therapeutic advances in the future.

In chapter 2, I described the changes in the cis-regulatory landscape during retinal development and use this to discover new regulatory elements for key transcription factors. First, we generated an extensive, and possibly exhaustive, set of cis-regulatory elements in the developing and mature CNS that can be used to decipher cell-type specific gene regulation. Indeed, recent studies are already doing so (Shen et al., 2016; see below). We show that temporal clustering of DHSs during development can identify instructive transcription factors and implicate new factors in developmental processes. Further, we discovered potential enhancers, at distal locations, for two transcription factors with crucial roles in retinal development. Cis-regulatory elements for *Otx2* displayed temporal specificity and those for *Ascl1* exhibited tissue specificity.

In chapter 3, I described our efforts to reprogram Muller glia cells into new retinal neurons via misexpression of the transcription factor, Ascl1. Specifically, we found that viral transduction of Ascl1 in purified cultures of P12 mouse Muller glia caused a profound change in cellular physiology. The transcriptional state of the Muller glia was partially reprogrammed to that of retinal progenitors, including Notch pathway components and transcription factors indicative of neurogenesis and nascent differentiation. In addition, Ascl1 binding executed some of these changes directly and caused local histone remodeling. However, several key progenitor genes remained silent and only bipolar-like cells were generated in dissociated culture. Interestingly, when Ascl1 misexpression in Muller glia occurred *in vivo* and was combined with damage at a young age, a subset of cells exhibited characteristic morphology and gene expression markers of photoreceptors. This suggests that a young cellular age, the supportive *in vivo* environment, and especially retinal injury affect Muller glia reprogammability.

In chapter 4, I described the interaction between Ascl1 and the cis-regulatory landscape of Muller glia and attempt to use this knowledge to inform the reprogramming process. The binding pattern of Ascl1 in Muller glia overlaps about two-thirds of the retinal progenitor pattern and includes twice as many binding sites. However, there can be uncertainty in the exact number of binding sites, as these peak calls can be sensitive to IP efficiency in different cell types and sequencing depth. In addition, Ascl1 binds opportunistically to Muller glial DHSs, but also binds to closed chromatin, both at progenitor cell appropriate and inappropriate binding sites. By comparing the accessible cis-regulatory elements specific to P0 retina and Muller glia, we generated candidate reprogramming transcription factors to enhance reprogramming. Although most did not demonstrate a positive effect, we identified two factors: Zic1, which activates early progenitor cell genes, and Otx2, which activates photoreceptor genes when combined with a

histone de-acetylase (HDAC) inhibitor. Thus, epigenetic understanding can be leveraged to inform and improve the reprogramming process.

In this chapter, I will discuss some of the important considerations in the analysis of the presented data, what experiments and future approaches are warranted and likely to be informative, and also how our data relates to the literature.

Expanding the analysis of the cis-regulatory landscape of retinal development

One feature of our analysis is the temporal clustering of DHSs to discover *cis*-regulatory elements (CREs) and the transcription factors that are dynamic during retinal development. Relatively few high-throughput studies in the retina have focused on development, with most examining mature tissue. However, our approach captures genome-wide developmental processes and with further analysis, mechanisms of development. Currently our approach is limited to the late progenitor stage (P0) through mature retina (adult), however, new data in our possession will extend this analysis to an early embryonic stage (E14) that will capture a very different population of early retinal progenitors and nascent neurons. Retrieving high-throughput data from retina at this age has been problematic due to the small size of the tissue; however, we have overcome this limitation with large collection efforts. Therefore, DNase-seq analysis at this age will likely yield novel insights.

As shown in chapter 2, we showed that DNase-seq could identify DHSs near cell-type specific genes (associated by promoter proximity), even when the given cell-type represents a small minority of the total population. This situation is prevalent among CNS tissues especially, due to the vast diversity of neurons contained within. For example, at the promoters of genes: *Pou4f2* in ganglion cells and Opn1sw in cone photoreceptors, representing approximately 1% of the total retinal population (Wilken et al., 2015). This work is suggestive of cell-type specific

DHSs, however, we cannot exclude the possibility that these DHSs are present in more than one cell type in the heterogeneous retinal or brain tissue. The definitive way to ascertain the cell-type(s) of origin of any given DHS will be to sort pure populations and perform the DNase-seq protocol on both the cell type of interest, and the 'negative' fraction that is depleted of this cell type. In this way, a suspected cell-type specific DHS could be demonstrated to be present in the sorted population, but not the rest of the tissue.

One complicating factor with sorting rare cell types is the requirement for millions of cells in the standard DNase-seq protocol. Recent work provides a solution through the use of ATAC-seq, a transposon based method of discovering accessible chromatin (Buenrostro et al., 2013). This technique can be scaled down to accept single cell inputs, thus allowing CRE identification from rare cell populations isolated from a complex tissue (Buenrostro et al., 2015; Cusanovich et al., 2015). In principle, a map of CREs can be generated for every neuron sub-type in the retina.

Although we have discovered a massive number of DHSs, the bottleneck in CRE analysis is functional testing. Recently, Shen and colleagues have used our data to test retinal and brain CREs in a massively parallel reporter assay. In this study, short fragments from the retinal DHSs were used to capture full length CREs from genomic digests. Following massively parallel cloning into bar-coded reporter constructs, they performed ex vivo electroporations and adeno-associated virus transduction in the retina and cortex to assay enhancer activity quantitatively via bar-code sequencing (Shen et al., 2016). One drawback to this method is that, in heterogeneous tissues, the enhancer activity measurement will be sensitive not only to upstream regulatory activity, but also the relative proportion of cell-types a given enhancer is active within. Therefore, parallel fluorescent reporter assays are still required to determine cell-type specificity

of an enhancer. Additionally, this report only examined the top scoring 1000 retina DHSs; unsurprisingly they discovered mostly Crx/Otx2 motif enriched CREs, corresponding to the photoreceptor majority cell type in the retina. Therefore, the vast majority of retinal DHSs are still awaiting examination for activity and specificity. These types of innovative, massively parallel cloning and testing strategies will be crucial for resolving genome-wide enhancer activity in the developing retina.

Another expansion of this work was carried about by our colleagues who used deep sequencing (>250 million reads) to derive transcription factor footprints within P0 retina DHSs (Stergachis et al., 2014). The focus of this work was comparative gene regulatory networks, and therefore, the resulting transcription factor network of the retina requires examination. Still, the networks generated suggested many new connections between developmental transcription factors (e.g. – Otx2 binds the Ascl1 promoter) (Stergachis et al., 2014). Integration of these data with gene expression experiments using knockouts and overexpression of transcription factors will be required to verify the validity of DNaseI-footprint derived regulatory networks.

There are two serious limitations to the DHS mapping approach. The first is that distal (i.e. – non-promoter) DHSs cannot be reliably assigned to a given gene. Therefore, for a given gene, only those factors that bind to the promoter region will be identified as upstream regulators. This effectively removes the vast majority of CREs from incorporation into a regulatory network. In the future, integrating DNaseI-footprinting data with genome-wide chromatin conformation capture (Hi-C), which can associate a given gene to its distal CREs, may surmount this problem.

The second major limitation is that all DNase-footprints may not be reliably detected. Recent evidence suggests that transcription factors with inherently low residence times at their

binding sites, such as Sox2 and Glucocorticoid Receptor, do not reliably produce a DNaseI-footprint (Sung et al., 2014). In the future, it may be required that ChIP-seq for low-residence time transcription factors be performed and integrated with DNaseI-footprinting to derive an accurate regulatory network. Therefore, gene regulatory networks derived from DNaseI-footprinting should be integrated with other methods, including ChIP-seq and Hi-C, to build complete and accurate genome-wide networks.

Photoreceptors would be an excellent cell type for this type of integrated cis-regulatory analysis. First, there already exist several ChIP-seq studies for instructive developmental transcription factors: Crx, Otx2, and Nrl (Corbo et al., 2010; Hao et al., 2012; Samuel et al., 2014). Second, chromatin conformation capture assays have been performed in photoreceptors, albeit limited to Opsin genes (Peng and Chen, 2011; 2012). Third, the function of network connections can be tested against numerous global gene expression studies of retinas that were perturbed by transcription factor knockouts (reviewed by Swaroop et al., 2010; Brzezinski and Reh, 2015). These existing studies provide a head-start on understanding the photoreceptor regulatory network and provide important standards with which to test the validity of DNaseI-footprint derived networks.

Practical implications and applications of CREs in retinal development

A potential use for CRE mapping, beyond understanding basic biology, is their use as reporters for correct differentiation of retina neurons from stem cells. Recently, there has been intense interest in transplanting stem cells and stem cell derived retinal neurons for the treatment of human retinal degenerations (reviewed by Pearson, 2014; Wright et al., 2014). Therefore, it will be important to ascertain that retinal neurons, derived *in vitro* from stem cells, are

differentiating into the desired cell type with high fidelity. Using cell-type specific CREs, potentially in combination, is one approach to determining the accuracy and completeness of differentiation. For example, cells in a nascent photoreceptor stage, would activate a reporter gene under the regulation of an Nrl enhancer, but fail to express a second reporter from a bipolar gene specific enhancer. I envision that this technique will be used to develop and refine differentiation protocols before cells are transplanted into patients.

In a manner analogous to measuring the complete transcriptome or proteome of differentiated cells, we could examine the epigenome of *in vitro* differentiated retinal neurons, as defined by DNase-hypersensitivity. This would determine the extent to which the cis-regulatory landscape has been recapitulated by *in vitro* differentiation protocols. This would determine, for example, whether a given gene (such as Nrl) is expressed only because its promoter is actively driving transcription, or whether the complete set of Nrl CREs are present and stably maintaining expression. This may have implications for the long-term stability of cell phenotypes that have been differentiated *in vitro* from stem cells and thus be clinically relevant for transplantation. An important set of experiments to address this issue will be to measure the degree to which an *in vitro* differentiated population of retinal neurons recapitulates the hypersensitive landscape of control neurons. Then correlate this with phenotypic stability (e.g. – maintenance of correct gene expression, photoreceptor functionality) in long-term transplant experiments and after environmental perturbations.

Reprogramming Muller glia in the mouse and zebrafish

When comparing the reprogramming of Muller glia between zebrafish and mouse, two central questions emerge. First, why do zebrafish Muller glia respond to injury-induced growth

factors with regeneration, whereas mammalian Muller glia do not? Second, why does Ascl1 expression fail to completely reprogram mammalian Muller glia into retinal progenitors, as it does in zebrafish?

In regards to the first question, at least part of the answer is that these growth factors fail to up-regulate the critical reprogramming factor, Ascl1. In the fish, a variety of mitogenic growth factors, including EGF, eventually lead to activation of Ascl1 in Muller glia (Wan et al., 2012; Wan et al., 2014). However, evidence in young mice indicates that EGF treatment may even suppress Ascl1 activation (Loffler et al., 2015). In this study, explantation damaged P10 retinas activated detectable levels of Ascl1 in 10% of Muller glia, however, this dropped to 5% of Muller glia with EGF treatment. However, EGF stimulated re-entry into the cell cycle by 10-fold. This indicates that EGF does not execute the same downstream programs in zebrafish as in mice. In the fish, EGF, directly or indirectly, stimulates both Ascl1 activation and proliferation, whereas in mice EGF stimulates proliferation but not Ascl1 activation. Understanding how these signaling pathways propagate their effects to cell physiology and how these signal propagate differently in different species will be a crucial clue in translating regeneration knowledge of fish to mammals. Other prominent regeneration inducing signals (e.g. – Wnt signaling) in the zebrafish have a less consistent effect in mammals and further work is required to understand their effects on mammalian Muller glia, although to my knowledge, no study has demonstrated Ascl1 activation in Muller glia in response to Wnt agonists.

In regards to the second question above, at least part of the answer is that the trans-acting circuitry between Ascl1 and its downstream targets is different in mammals (i.e. – the transcriptional network downstream of Ascl1 has diverged during evolution). For example, one crucial event in retinal regeneration in the fish, is the up-regulation of the RNA-binding protein,

Lin-28, by Ascl1 (Ramachandran et al., 2010). Lin-28 then represses the function of the microRNA, let-7, allowing functional expression of several important progenitor genes. Other members of our lab have demonstrated that manipulation of microRNAs in Muller glia enhances reprogramming (Wohl et al., 2015). However, according to our data, Ascl1 does not activate expression of the RNA-binding protein, Lin-28, or bind to its proximal promoter region in the Muller glia. In addition, this is not simply due to an inhibitor epigenetic environment in the Muller glia because Ascl1 does not appear to bind to the Lin-28 proximal promoter in retinal progenitors either. Lin-28 has been efficacious in induced pluripotent stem cell protocols, and thus it is a tempting hypothesis that it would enhance Muller glia reprogramming. Initial experiments in our lab suggest that Lin-28 may not be necessary, however more definitive experiments are planned.

Despite transcriptional rewiring, as described in chapter 3, many interactions downstream of Ascl1 are conserved in fish and mice (e.g. – Notch pathway components). To determine the true extent of conservation and divergence of trans-acting circuitry between fish and mice, we would need to perform Ascl1 ChIP-seq in the zebrafish Muller, as we have done in mouse. A direct comparison of the transcriptional network could reveal important differences in the reprogramming response between these cell types.

Another potential difference between the fish and mice is the epigenetic context within the Muller glia. Little is known about the epigenetic state of zebrafish Muller glia due to limited cell numbers for analysis. However, Powell and colleagues demonstrated that zebrafish methylate the DNA of progenitor genes, followed by de-methylation during regeneration (Powell et al., 2013). Work in our lab has shown that progenitor gene promoters are relatively un-methylated in Muller glia; however, the photoreceptor gene promoters are highly methylated

(Russ Taylor, bisulfite sequencing). Nevertheless, DNA de-methylation by 5-azacytidine treatment followed by Ascl1 expression was not sufficient to re-activate these photoreceptor genes in my hands (data not shown). There is still much to learn about zebrafish Muller glia epigenetics, however, these limited data suggest that progenitor and neural gene regulation may be substantially different in this species.

Mechanisms and shortcomings of Ascl1-mediated reprogramming of mouse Muller glia

Abstractly, one part of the question of how to improve Ascl1-mediated reprogramming can be thought of as how to move the red-circle in Figure 5.1, to completely overlap the dark purple circle. A combination of (1) misexpression of transcription factors which bind to P0 Ascl1-bound DHSs, and (2) knockdown of transcription factors enriched in Muller glia Ascl1 bound-DHSs is an attractive strategy.

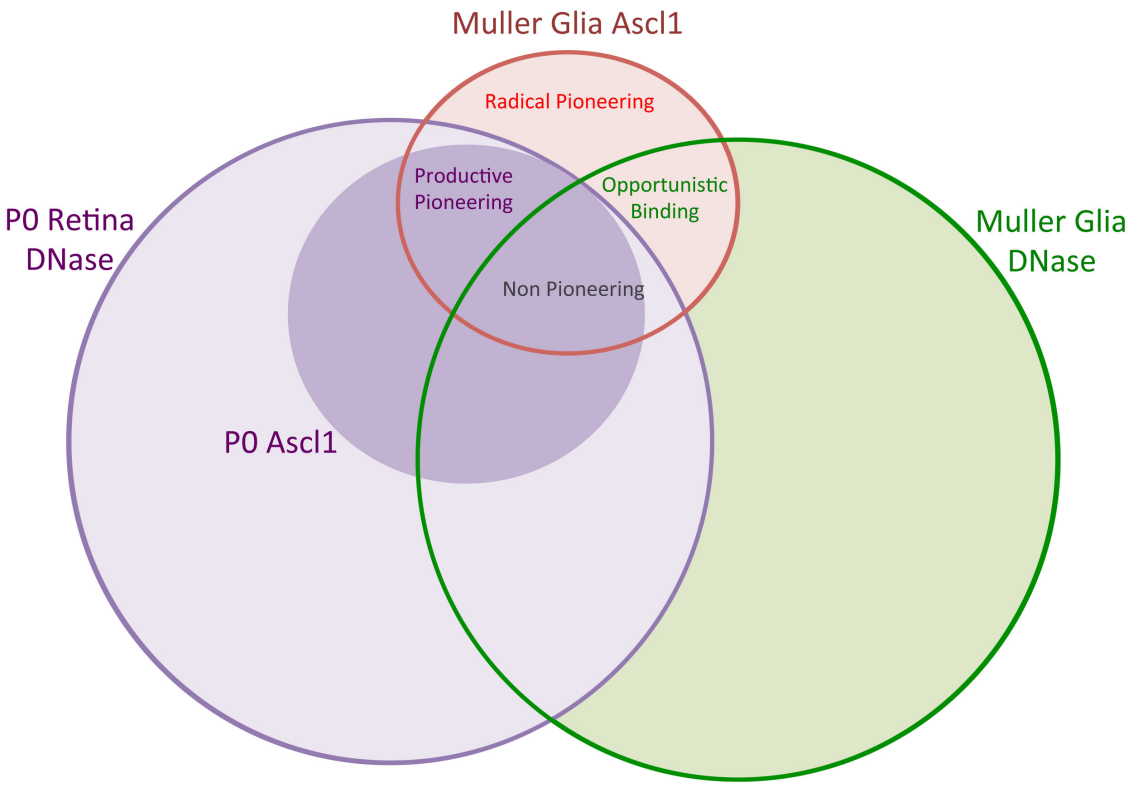


Figure 5.1: Conceptual Venn diagram of overlap between ASCL1 binding sites and DNase-hypersensitivity hotspots in P0 retina and P12 cultured Muller glia (not to scale). Green circle, Muller glia DNase-hotspots; Light purple, P0 retina DNase-hotspots; Dark purple circle, P0 retina Ascl1 binding sites; Red circle, Ascl1 binding sites in Muller glia.

As demonstrated in chapter 4, an attempt to misexpress transcription factors enriched in P0-specifc DHSs resulted in identification of Zic1 and Otx2, which were able to enhance Ascl1-mediated reprogramming to early retinal progenitor and photoreceptors, respectively. However, the majority of identified transcription factors did not enhance reprogramming. This may be due to a failure to look for the correct downstream genes. We attempted to select the best possible downstream genes to detect whether the candidate reprogramming factors were functioning in the Muller glia. This was done by DNase-footprinting in P0 retina. However, it is possible that these factors do aid reprogramming towards the retinal progenitor state, but we did not use the correct assay. A more complete, although more expensive, method would be to perform

genome-wide gene expression microarrays for each candidate transcription factor and search for changes to the transcriptional state of the cell globally.

We have observed that HDAC inhibitors, sodium butyrate and TSA, enhance reprogramming of Muller glia into more differentiated neurons (by up-regulating mature neuron genes). Experiments planned for the immediate future will address how HDAC inhibition during reprogramming changes the Ascl1 binding site profile. We speculate that the set of Ascl1 sites in HDAC inhibited Muller glia will include more P0 retina Ascl1 sites. However, this may expand the set of Ascl1 bound sites in general (i.e. – not limited to a P0 retina binding pattern). Alternatively, the effect of HDAC inhibition may only be relevant to other transcription factors in the reprogramming process (e.g. – Otx2).

Why doesn't Ascl1 bind to sites that are a DHS in Muller glia and are also Ascl1-bound DHSs in P0 retina? In Figure 1, these sites are represented by dark purple developmental Ascl1 sites overlapping green Muller glial DHSs, but not red glial Ascl1 sites. These sites are apparently accessible to Ascl1 and are bona fide binding sites. I speculate that these sites could require cooperative binding with a co-factor that is absent from the Muller glia, rendering Ascl1 unable to bind, despite accessibility. Alternatively, these sites could be strongly bound by a repressor or other factor blocking Ascl1 from its binding motif. Searching for motif enrichment in this category of sites may reveal necessary co-factors that we could misexpress to improve the reprogramming process and/or factors that must be knocked-down to allow Ascl1 to bind to appropriate retinal progenitor CREs.

There is an apparent conflict between the correlation in figure 3.6, suggesting that the capacity of young Muller glia to reprogram is due to more hypersensitive chromatin at progenitor genes, and the observation that Ascl1 is able to bind to sites without pre-existing hypersensitivity

(chapter 4). If Ascl1 is able to bind and activate genes within heterochromatin, why does the pre-existing hypersensitive landscape influence Ascl1's ability to reprogram Muller glia? One possible explanation is that Ascl1's global effect on the cell is due to a combination of the pre-existing hypersensitive landscape and it's ability to 'pioneer' heterochromatin. In addition, pre-existing hypersensitivity may be an important prerequisite for other transcription factors, besides Ascl1, to bind and activate target genes. We have also not systematically tested the hypothesis that Ascl1 binding in non-hypersensitive chromatin results in activation of nearby genes. Although we have observed activation in this scenario for a subset of genes that we have examined manually (e.g. – *Neurod4*). It may be the case that Ascl1 binding to hypersensitive versus non-hypersensitive chromatin results in a different transcriptional outcome.

Several important questions arise from Figure 1 above. First, how are the radical pioneering binding sites determined? Or, why does ASCL1 bind to these particular sites on the genome rather than any of the other, much larger, set of canonical Ascl1 E-box motifs (CAGCTG, which occurs approximately 708,000 times in the mouse genome) that occur outside hypersensitive chromatin? Several potential answers exist. First, transcription factors that are present in the Muller glia, but insufficient to bind and induce hypersensitivity alone, may cooperate with Ascl1 to bind sites where their motifs co-occur. Thus, sufficient binding energy is provided and the prerequisite for multiple proximal motifs reduces the available binding sites. Another possibility is that pioneering sites are marked or intrinsically more accessible, by an unknown mechanism, than the majority of chromatin that is not DNase-hypersensitive. This could include, but is not limited to, specific histone modifications that render DNA more accessible, although not hypersensitive, and thus more likely to be bound by Ascl1. A related possible scenario is that DNA sequences underlying the radical pioneering sites (in addition to

the E-box) are more refractory to nucleosomes than average sites. If Ascl1 were randomly sampling its canonical E-box motifs genome-wide, it would preferentially bind and reside at intrinsically more plastic regions of chromatin. Put more simply, Ascl1 may be preferentially binding specific non-hypersensitive sites because it is cooperatively guided by trans-acting factors, or guided by cis-acting factors of unknown identity.

One approach to distinguish these scenarios would be to perform a sequence motif enrichment analysis of pioneered sites relative to the set of sequences surrounding unbound Ascl1 E-boxes. Although more accurate TF motif matching knowledge and algorithms will be required to have high confidence in this approach. If the first scenario were correct, we would expect to detect one or more enriched motifs that match transcription factor(s) that are expressed in Muller glia cells. Preliminary analysis indicates that an NFI motif is enriched at pioneered sites in the Muller glia. Interestingly, Raposo and colleagues (Raposo et al., 2015) detected an NFI motif enrichment by DNaseI-footprinting within induced hypersensitive sites in P19 cells induced to differentiate by Ascl1. These data suggest that a NFI protein, several of which are expressed in the Muller glia, may be instructive to Ascl1 binding closed chromatin. In addition to specific motifs, we may observe that average sequence content (e.g. – higher A/T percentage) biases Ascl1 towards certain non-hypersensitive sites over others.

Another approach would be to search for histone modification patterns that overlap pioneered sites by performing ChIP-seq experiments in the Muller glia. In support of the histone modification hypothesis, Wapinski and colleagues (Wapinski et al., 2013) have demonstrated in fibroblasts that Ascl1 binds to relatively inaccessible DNA (as assessed by FAIRE-seq) and these sites were pre-enriched for a particular combination of histone modifications: high H3K4me1

and H3K27ac; low-intermediate H3K9me3 levels. A mechanism by which this modification pattern is established and permits Ascl1 residency has not been put forth to my knowledge.

The impact of retinal injury on Muller glia reprogrammability

As demonstrated by our observations in chapter 3 and Ueki et al., 2015, both the young age of Muller glia and especially retinal injury enhance reprogramming of Muller glia. Therefore, a characterization of epigenetic changes in Muller glia during aging and after retinal injury is warranted. Although we noticed that P12 Muller glia retain many DHSs at progenitor gene loci, we have not directly compared them to Muller glia from adult mice. In the past, the low cell numbers from FACS sorting limited us because adult glia do not expand substantially in cell culture. However, this experiment has become possible, and a future priority, with the advent of the low cell number accessibility assay, ATAC-seq.

The most striking environmental factor that enhanced reprogramming was neurotoxic damage to the retina, resulting in the generation of some photoreceptor-like cells. Therefore, it is the aim of future studies to examine the epigenetic changes within Muller glia after retinal injury. Again, the technical hurdles to obtaining sufficient material from non-expanded Muller glia can be surmounted with FACS and ATAC-seq. Numerous possibilities exist for explaining the injury-induced enhancement of reprogramming. Molecules released by dying neurons may be inducing signaling events that synergize with Ascl1 directly or complement Ascl1-mediated changes indirectly. However, by elucidating the chromatin accessibility changes during reactive gliosis, we may be able to hypothesize plausible mechanisms by examining transcription factor motif enrichment in dynamic and induced DHSs. By identifying the DHSs that allow or are required for successful Ascl1-mediated reprogramming (in young and/or reactive Muller glia),

we may identify new transcription factors or terminal signaling pathway molecules that will enhance reprogramming.

Enhancing reprogramming by epigenetic modulation from HDAC inhibition

Future work will be required to confirm that the mechanism of action relevant to enhancing reprogramming is the acetylation of histones. This will be accomplished through the use of ChIP for H3K27ac and H4-acetylation. Activation of photoreceptor genes should correlate with an increase in histone acetylation levels. Further proof will require alternative inhibition of HDACs, for example by RNA-interference knockdown of specific HDAC enzymes. Presently the histone acetylation hypothesis is supported by the fact that up to 4 other experimental HDAC inhibitors, provided by collaborators, have exhibited a nearly identical effect on photoreceptor gene expression. In addition, an alternative HDAC inhibitor, trichostatin A (TSA), exhibits a striking ability to enhance Ascl1-mediated reprogramming in injured retinas *in vivo* (unpublished observations, Nik Jorstad).

More work is needed to determine the mechanism behind HDAC-inhibition enhancement to reprogramming. Several studies have shown that HDAC inhibition can enhance reprogramming of somatic cells to pluripotent stem cells (reviewed by Feng et al., 2009; Li et al., 2013). Does HDAC inhibition "loosen" the interaction between DNA and nucleosomes in heterochromatin, thus allowing transcription factors to bind to sites they would have otherwise been excluded from? Recent studies indicate that TSA treatment substantially alters the binding pattern of Sox2 in neural stem cells (Liu et al., 2014). If these effects are general, HDAC inhibition may be allowing Otx2 to bind new regions in the Muller glia. ChIP for Otx2 in Muller glia will determine whether Otx2 can bind to photoreceptor gene promoters only with HDAC

inhibition, or whether Otx2 binds, but only activates expression after HDAC inhibition. In addition, performing DNase-seq on Muller glia with and without HDAC inhibition would reveal whether chromatin accessibility is altered, and whether the result is increased accessibility at specific sites or distributed evenly across the genome. Many pathologies of the retina, including excessive light induced injury, are the result of non-functional or dying photoreceptors. Thus, the prospect of using HDAC inhibition to regenerate this cell type from the Muller glia is particularly compelling.

Alternative paths forward for discovering extra reprogramming factors

In this dissertation, we have taken a rational approach to candidate reprogramming factor selection and testing. However, this approach is limited by the current state of knowledge and suffers from caveats inherent to any high-throughput technique. For example, the analysis of DNase-seq data is potentially limited by incomplete identification of motif enrichment and inaccurate assignment of transcription factors to discovered motifs. Beyond this, we have limited ourselves to misexpressing transcription factors and inhibiting a subset of known epigenetic inhibitory enzymes. Therefore, an unbiased, high-throughput screen should be attempted to discover effective reprogramming factors. This could be done with using the newly developed CRISPR-Cas9 systems and available, genome-wide guide RNA libraries for transcriptional activation (CRISPR/Cas9 Synergistic Activation Mediator, Cas9-SAM; Konermann et al., 2015) and knockout (Shalem et al., 2014).

A stable and representative Muller glial cell line would be invaluable for this type of large-scale experiment. However, our lab has begun stockpiling Ascl1-Cre~ER; flox-Tomato mice for this purpose. In this paradigm, Ascl1 expression acts as a reporter for re-activation of

the progenitor cell gene expression state. Thus, any single or combination of genes that are activated by Cas9-SAM or knocked-out via CRISPR-Cas9 that re-activates Ascl1 can be sorted based on red-fluorescence and the upstream guide-RNA sequenced. Libraries containing guide-RNAs for every gene in the mouse and human genome have already been created for this purpose. This may become a routine approach to problems such as these, where unknown factor(s) are either absent and required, or present and inhibiting, a desired process (e.g. – reprogramming).

Broad Considerations

There are several important reasons for choosing to reprogram Muller glia to regenerate the retina rather than transplanting retinal neurons generated from stem cells. First, the latter approach is currently being aggressively pursued by our laboratory and others, but carries similar concerns regarding the completeness of differentiation. Second, Muller glia cells are already resident to the retina and integrated into all cell layers. Therefore, no potentially disruptive transplantation is required with Muller glial reprogramming, which may be accomplished with small molecules or exogenous gene expression through viral vectors. Third, and most importantly, the Muller glia of other vertebrate species (such as the zebrafish) possess and intrinsic capacity to undergo dedifferentiation, proliferation, and reprogramming into retinal neurons. Therefore, the question of why mammalian Muller glia fail to undergo this process is important for restoring sight via retinal regeneration. In addition, if the mechanistic basis of this difference can be found and generalized, it may also have profound implications for regenerative biology and medicine in general.

Due to the apparent resistance of Muller glia to reprogramming to retinal neurons, the question has been raised of whether it would be more effective to reprogram Muller glia into a pluripotent state followed by differentiation into retinal neurons. To my knowledge, this hypothesis has not been tested to date. Although, it has been observed that embryonic stem cells can be directed to the neuronal fate by misexpression of Ascl1 (Yamamizu et al., 2013). However, the efficiency of this process is difficult to determine due to the poor characterization of the final 'neuronal' state (e.g. - only a few neuronal markers were examined). Therefore, it is unclear at this time whether a more efficient source of retinal neurons would result from reprogramming Muller glia to retinal neurons through an intermediate pluripotent state.

Implications of this work

Understanding reprogramming of Muller glia and retinal regeneration will have direct implications for regenerative approaches in the brain. Several groups are investigating regeneration of neurons in the mammalian brain from resident astrocytes, a type of glial cell (see Introduction; Heinrich et al., 2010; Niu et al., 2015). These studies also employ transcription factor overexpression, but are similarly limited in the scale and robustness of reprogramming to fully differentiated neurons. Therefore, a deeper understanding of proneural transcription factor behavior in a glial epigenetic context will have implications for reprogramming and regeneration throughout the CNS.

Chapter 6:

Materials and Methods

Animals

C57BL/6J mice (Jackson Laboratory, Bar Harbor ME) were used for all experiments., housed in the University of Washington Department of Comparative Medicine. All experiments were carried out according to approved protocols by the University of Washington IACUC.

Nuclei Isolation

I dissected retinal and brain tissue (minced into ~2 cubic mm pieces) and suspended in 3mL homogenization buffer (20mM tricine, 25mM D-sucrose, 15mM NaCl, 60mM KCl, 2mM $MgCl_2$, 0.5mM spermidine, pH7.8). Tissue was Dounce homogenized with 5-10 strokes with loose, type-A pestle (brain tissues) or with 5 and 25 strokes of loose and tight pestle, respectively (retina tissues), followed by filtration through a 100µm filter. I cryopreserved the nuclei suspension by addition of DMSO to 10%, controlled freeze to -80°C, and subsequent storage in liquid nitrogen. Then, samples were transferred to the Stamatoyannopoulos lab for subsequent processing. After thaw and before DNaseI treatment, buffer was exchanged with 15mL sucrose buffer (10mM Tris-HCl, 250mM D-sucrose, 1mM $MgCl_2$, pH 7.5), nuclei collected by centrifugation (600*g*, 10 minutes, 4°C), and resuspended in 10mL fresh sucrose buffer. Nuclei were passed through a 20µm filter and centrifuged (600*g*, 10 minutes, 4°C). The pelleted nuclei were washed with 10mL of buffer A (15mM Tris-HCl, 15mM NaCl, 60mM KCl, 1mM EDTA, 0.5mM EGTA, 0.5mM spermidine) and resuspended to 2 million nuclei per mL.

DNase I Treatment

DNaseI treatment was carried out by the Stamatoyannopoulos lab. Nuclei were incubated at 37°C for 3 minutes with limiting concentrations of DNaseI enzyme in buffer A

supplemented with Ca^{2+}. The reaction was terminated with an equal volume of stop buffer (50mM Tris-HCl, 100mM NaCl, 0.1% SDS, 100mM EDTA, 1mM spermidine, 0.5 spermine pH 8.0) and subsequently treated with proteinase K and RNase A at 55°C. Small (<750bp) DNA fragments were recovered via sucrose ultracentrifugation and subsequently end repaired and ligated with Illumina compatible adaptors. A detailed description of the mapping of DNaseI hypersensitive sites is available in reference (Hesselberth et al., 2009).

Sequence Alignment and DHS Scanning Algorithm

Sequence aligment and Hotspot peak calling were carried out by the Stamatoyannopoulos lab. Sequence reads (36 bp) were mapped to the human (GRCh37) and mouse (NCBI37) genomes using bowtie (v 0.12.7) (Langmead et al., 2009). Sequencing reads varied between samples (Supplementary Table S3). To account for this variability, we down-sampled each mouse tissue sample to 25 million reads (random sampling, no replacement) and subsequent datasets were used for DNaseI peak and hotspot calling. Reads were summed within 150bp windows in 20bp steps and normalized to the number of reads per tissue sample dataset and subsequently scaled to 1 million reads.

The Hotspot algorithm (John et al., 2013), detailed description of calculations can be found at http://www.uwencode.org/proj/hotspot/) was used to detect distinct regions of chromatin accessibility. Localized enrichments of sequence tags are identified based on a binomial distribution model computed against a local background model surrounding each tag. Regions of enrichment are termed hotspots and are further internally scanned for the local maxima, a 150bp window around local maxima are called as peaks. To generate a false discovery rate (FDR 1% for all datasets), simulated datasets are generated based on random

reads at equal sequencing depth to each sample dataset and the simulated data was subsequently scanned for hotspots to determine an estimate FDR. Dataset quality is also measured using a SPOT (signal portion of tags) score defined as the percentage of tags that fall into hotspots. (http://www.uwencode.org/proj/hotspot/)

Global Analysis of DHS landscape

The mouse Ensembl65 genomic coordinates were used as the basis for this analysis and I used BEDOPS (Neph et al., 2012) to determine overlap between DHSs and genomic features. CRX ChIP-seq peaks were determined by the intersection of peaks from CRX ChIP-seq replicates 1&2 using BEDOPS.

K-Means Clustering Analysis

A 'master list' of 197,962 non-redundant, non-overlapping retinal DHSs from all three stages was created as previously described (Thurman et al., 2012). This K-means clustering analysis was carried out by Kyle Siebenthall. The maximum read-count-normalized DNase I tag density was then determined for each master list DHS in each sample. Each sample's tag density values were divided by the sample's SPOT score, a quality metric for DNase-seq library complexity) to control for differences in sample quality. Tag density values were then transformed by log10(density+1) and row-normalized across stages at each DHS to set the maximum density value to 10. The DHSs were then subjected to k-means clustering to create 12 groups containing DHSs with similar temporal activity across the three sampled developmental stages.

Functional annotation of DHSs

DHSs within a region 1 kb upstream of Ensembl65-annotated transcription start sites were classified as promoter DHSs. CRX and NRL binding regions were obtained from ChIP-seq data in Corbo et al. 2010 and Hao et al. 2012. Master-list DHSs used for k-means clustering were considered occupied by CRX and/or NRL if the peak calls for these two factors overlapped a DHS by at least 75bp. Of 5,724 CRX binding sites, 178 failed to overlap a DHS and 224 overlapped more than one DHS; the latter were not considered for calling CRX-occupied DHSs. Of 7,303 NRL binding sites, 1,707 fail to overlap a DHS and 260 overlap more than one DHS. Retina-specific DHSs were independently determined in a study of the human and mouse regulatory landscapes (Vierstra et al., 2014); the master-list DHSs in this study were considered to be retina-specific if they overlapped a DHS called by Vierstra et al., 2014 by at least 25 bp. The list of retinal-specific DHSs was generated as described in Vierstra et al., 2014.

Motif enrichment in DHSs

Putative transcription factor binding sites were identified by scanning the entire mouse genome for consensus sequences using the FIMO tool from the MEME Suite (version 4.6) (Bailey et al., 2009) with default parameters, using motif models curated from TRANSFAC (version 11) (Matys et al., 2006), JASPAR (Bryne et al., 2008) and UniProbe (Newburger et al., 2009). Each motif model was linked to a transcription factor gene, allowing for redundancies in the motif databases; multiple TFs were allowed to be paired with the same motif, and many TFs were represented by multiple motif models. We then determined the number of DHSs containing a motif match (FIMO p-value < 10e-4) for each TF, and used a cumulative hypergeometric distribution to calculate a p-value for the enrichment of that TF's motifs within DHSs assigned to

specific k-means clusters (or retina-specific DHSs) compared to the overall prevalence of its binding sites in master-list DHSs. P-values were corrected for multiple testing using the Bonferroni method. Motif enrichment in the retina from K-means clustering of DHSs was carried out by Kyle Siebenthall. I carried out all other motif enrichment analyses (e.g. - in Muller glia and in relation to ASCL1 ChIP-seq).

Electroporations

Reporter plasmids contain the experimental ECR immediately upstream of a minimal promoter containing TATA box driving nuclear GFP; control plasmids contain the *Ef1a*-promoter driving nuclear CHERRY red fluorescent protein. I electroporated retinal explants (dissected retina tissue cultured *in vitro*) in PBS with 2μL DNA (2.33 g/μL ECR-GFP plus 1g/μL mCherry control plasmid) using an ECM830 Square Wave Electroporation System (BTX Harvard Apparatus) with settings: 35V, 5 Pulses, 50ms/pulse. Retinas were then cultured in 6-well tissue culture plates with 1mL of Neurobasal media, with 1% FBS (Clontech), 1 mM L-glutamine (Invitrogen), N2 (Invitrogen), 1% Penicilin–Streptomycin (Invitrogen) for 24-36 hours. Brains were dissected from mice and sliced with a McIlwain Tissue Chopper (Vibratome 800) set to 300micron sections. Individual brain sections were electroporated as above and cultured on 0.4micron Millicell cell culture inserts (Millipore PICM03050) in 6-well tissue culture plates containing 1mL neurobasal media (above) for 36 hours. For *in vivo* electroporations, P0 mice were anesthetized on ice and 1μL DNA (2.7 g/μL ECR-GFP and 0.3g/μL mCherry control plasmid) was injected into the vitreous of the eye (Syringe: Hamilton 7635-01; Needle: 32 Gauge Hamilton 7803-04). Electroporation was performed with head paddles connected to ECM830 Square Wave Electroporation System with settings: 90V; 5

Pulses; 50ms/pulse; 950ms intervals. Mice were revived at 37°C and retinas were harvested 7 days later.

Immunohistochemistry and Microscopy

I fixed retinas or brain explants with 2% paraformaldehyde. Retinas were cryoprotected in 30% sucrose/PBS at 4°C overnight, embedded in OCT compound (Sakura Finetek), and sectioned at 12μm using a cryostat. Immunohistochemistry (IHC) was carried out using chicken anti-GFP (1:500, Abcam, ab13970), rabbit anti-RFP (1:500, Clontech #632496), biotinylated anti-Otx2 (1:100, R&D Systems, BAF1979). All secondary antibodies were from Life Technologies or Jackson ImmunoResearch and used at 1:500. Imaging was performed using an Olympus Fluoview confocal microscope.

Otx2 Chromatin ImmunoPrecipitation

Otx2 ChIP-qPCR: I digested P0 or adult retinas with Papain into single cell suspension and fixed with 0.5% formaldehyde, 10 min, rotating at room temperature (RT). Sonication (Fisher Scientific) was performed: 12 pulses of 100J, 35 Amplitude with a 45s offset at 4°C. I performed immunoprecipitation with 11μL Protein-G coated magnetic beads (Invitrogen, #112-03D) and 2μg goat anti-hOTX2 antibody (R&D Systems BAF1979) or 2μg goat IgG (R&D Systems AB-108-C) against chromatin from $1e^6$ (P0) per IP according to LowCell # ChIP Kit (Diagenode). DNA sequences were quantified with BioRad CFX96 thermocycler using SsoFast EvaGreen Supermix (Bio-Rad) according to manufacturer's instructions. All values expressed as a percentage of input DNA averaged from at least 3 biologically independent experiments.

Histone-3 ChIP-qPCR

For histone ChIP, I fixed P0 retina or P12 Muller glia cell suspensions with 0.5% formaldehyde, 10 minutes, rotating at room temperature. Sonication (Fisher Scientific) was performed: 12 pulses of 100J, 35 Amplitude with a 45s offset at 4°C. IP was performed with 20 µl anti-rabbit IgG magnetic beads (Invitrogen) and 2 µg rabbit anti-H3K27me3 (Active Motif), rabbit anti-H3K27Ac (Abcam) or rabbit IgG (R&D Systems). Values were averaged from three to five independent experiments.

Ascl1 ChIP-qPCR

For Ascl1 ChIP, I digested P0 retinas or cultured P12 MG with papain into a single cell suspension and fixed with 1% formaldehyde for 10 minutes
at room temperature (RT). Cells were sonicated at 4°C. I performed the Ascl1 IP with 40 µl anti-mouse IgG magnetic beads (Invitrogen) and 4 µg mouse anti-Mash1 antibody (BD Pharmingen) or 4 µg mouse IgG (Millipore) against chromatin from 1°—106 (P0 retinas) or 2.5°—105 (cultured MG) cells per IP according to LowCell # ChIP Kit (Diagenode). IP and wash buffers were as described by Castro et al. (Castro et al., 2006). DNA sequences were quantified by qPCR and averaged from three to six independent experiments (as described in Pollak et al., 2013).

Ascl1 ChIP-seq

I performed this Ascl1 ChIP as described above for Ascl1 ChIP-qPCR. Cell number per experiment was approximately 5 Million cells per condition. Samples were 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). I used SAMtools (v 1.2) to merge and sort samples

from different sequencing lanes. I applied data to HOMER software suite, 'findPeaks' function (v 4.7). I performed overlap analysis of Ascl1 ChIP seq peaks and DHSs with BEDOPS (Neph et al., 2012).

Muller glia cell culture

I dissociated mouse retinas from age P11-P12 to single cells using Papain for 10 minutes at 37C (Worthinton kit) and plated and cultured for 5-days in Neurobasal Media + N2, epidermal growth factor (EGF), 10% fetal bovine serum (FBS). Cells were passaged onto a new plate to remove neurons and plated in the same medium. Lentiviral particles and/or epigenetic inhibitor treatment began 7-days *in vitro* in Neurobasal + N2, B27, 1% tetracycline (tet)-free FBS), hBDNF (R&D Systems, 10ng/ml), bFGF (R&D Systems, 100ng/ml) and rGDNF (R&D Systems, 10ng/ml). (Described in Ueki et al., 2012) Media was changed every other day from beginning to end of experiments.

Viral Transduction

ASCL1 was expressed from the viral plasmid: pLoc-hASCL1-IRES-GFP (Open Biosystems). The pLOC-IRES-GFP vector was used to express GFP as a control. Zic1, Olig2, Neurod1, Brn2, Myt1l were expressed from Tet-O-FUW-(gene) (Addgene) For other transcription factors, cDNA was cloned into pLVS-tight-Puro vector (Clontech) for rtTA-doxycycline inducible expression. pLVX-Tet-ON vector (Clontech) was used to express rtTA protein. Lentiviral particles were produced using Lenti-X HTX Packaging System (Clontech) in HEK293T cells and concentrated by ultracentrifugation at 25,000g for 1.5 hours.

Epigenetic inhibitor treatment

I treated dissociated Muller glia cultures with either the epigenetic inhibitor (below) or a control, consisting of the liquid the inhibitor is dissolved in (DMSO or water). 5-Aza-Cytidine was used at 5µM. A172 was used at 5µM. GSK-126 was used at 10µM. BIX-01339 was used at 1µM. Sodium Butyrate was used at 1mM. Treatments took place concurrently with/out Ascl1 misexpression.

Gene Expression Analysis by RTqPCR and Microarray

I lysed dissociated cells in Trizol reagent (Invitrogen) and RNA extraction/purification was performed with a mRNeasy kit (Qiagen) with DNase digestion (Qiagen). I made cDNA using iScript cDNA Synthesis Kit (Biorad). qPCR was performed using SsoFast EvaGreen Supermix (Biorad) on a 96-well plate thermocycler (Biorad). 3-technical replicates were performed per gene per sample. Values were expressed as cycle difference from beta-Actin control primer set on all samples. Biological replicates were performed as noted and statistics were performed using a student's t-test. Gene expression microarray was performed using a GeneChIP Mouse Gene 1.0 ST Array (Affymetrix) at the Institute for Systems Biology (Seattle, WA).

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Matthew S Wilken

EDUCATION

Ph.D., Molecular & Cellular Biology — Sept 2009 – June 2016
University of Washington, Seattle
Cumulative GPA: 3.96

Bachelor of Science, Biochemistry — Sept 2005 – May 2009
University of Minnesota, Minneapolis
College of Biological Sciences
Cumulative GPA: 3.98

RESEARCH EXPERIENCE

Graduate Thesis Research — Jun 2010 – Present
Laboratory of Dr. Tom Reh, University of Washington

- Using DNaseI Hypersensitivity and ChIP-Seq to study the role of transcription factors and cis-regulatory elements in mouse retinal development. Also, I study the de-differentation and reprogramming of retinal Muller Glia into neurons using the transcription factor *Ascl1*, other transcription factors, and epigenetic modulating small molecules (especially Histone De-Acetylase Inhibitors).

- **Skills Learned**: Chromatin Immunoprecipitation, DNase Hypersensitivity, High-Throughput Sequencing, Analysis of High-Throughput Sequencing Data (Mapping, File Conversion, Peak Calling Programs), Library Preparation, Molecular Cloning, Lenti-Virus Production and Transduction, RTqPCR, Primary Cell Culture (Glia, Neurons, Progenitors), Retinal Dissection and Primary Tissue Culture, Transgenic Mouse Breeding

Graduate Rotation — Mar 2010 – Jun 2010
Laboratory of Dr. Stephen Tapscott, Fred Hutchinson Cancer Research Center

- Studied DNA methylation at myogenic transcription factor loci during zebrafish embryogenesis and development.
- **Skills Learned**: 5-Aza-Cytidine Treatment, Bisulfite Sequencing, General Zebrafish Handling

Graduate Rotation — Sept 2009 – Dec 2009
Laboratory of Dr. Patrick Paddison, Fred Hutchinson Cancer Research Center

- Used RNAi screens to discover genes involved in regulation of self-renewal in neural stem cells and glioma neural stem cells.
- **Skills Learned**: Neural Stem Cell Culture, High Throughput siRNA Screening, Flourescence Microscopy, Western Blotting

Undergraduate Research Assistant — Jan 2008 – May 2009

Laboratory of Dr. Fang Li, University of Minnesota

- Studied SARS coronavirus evolution through kinetics of receptor binding.
- **Skills Learned**: DNA Transformation, Cloning and Expression, Site Directed Mutagenesis, Protein Purification (Affinity & Column Chromatography), Isothermal Titration Calorimetry, Surface Plasmon Resonance (Biacore).

Undergraduate Research Assistant May 2006 – Jan 2008
Laboratory of Dr. Kristin Hogquist, University of Minnesota

- Studied T-cell development in the thymus of wild type and transgenic mice. Specifically the kinetics of thymic emigration and negative selection in the thymic cortex.
- **Skills Learned**: Antibody Cell Staining, Tissue Section Staining and Visualization, Cell Sorting and Flow Cytometry, Intra-thymic Injection Surgery, Tissue Culture, DNA Extraction, PCR, Real-Time PCR, General Mouse Care.

High School Research Assistant Jan 2005 – Apr 2005
Laboratory of Dr. David O'Neill at 3M

- Mentorship program at engineering company, 3M, studying the properties of carbon vapor deposition into thin films for industrial applications.
- **Skills Learned**: Basic spectroscopic techniques for determining carbon film thickness and purity; exposure to science in an industrial, goal-oriented context.

CONFERENCES & WORKSHOPS

Computational Genomics Workshop; Center for Cell Circuits, Broad Institute
Sept 2015

Systems Biology: Global Regulation of Gene Expression; Cold Spring Harbor Laboratories
Jan 2015

*Poster: DNase Hypersensitivity Mapping of the Developing Mouse Central Nervous System. Wilken et al.

Gene Regulatory Networks for Development; Woods Hole MBL Oct 2012

Northwest Society for Developmental Biology Regional Meeting Mar 2012

*Poster: DNase Hypersensitivity Sequencing as Method to Identify Tissue-Specific Enhancers. Wilken et al.

INFORMAL EDUCATION (Online Courses)

Introduction to Systems Biology (Coursera) July 2013

Network Analysis in Systems Biology (Coursera) December 2013

TEACHING EXPERIENCE

Biology 355: Introduction to Molecular Biology Sept 2010 – Dec 2010

Biochemistry 442: Molecular and Cellular Biology March 2011 – Jun 2011

North West Association for Biomedical Research Mentorship Program 2010 - 2011
Mentoring high school students on the creation of a research-writing project.

HONORS & AWARDS

National Science Foundation Graduate Research Fellowship (2011 - 2014)

Best Poster: Northwest Developmental Biology Meeting 2012

University of Minnesota Presidential Scholarship (2005 - 2009)

L.M. Henderson Biochemistry Scholarship (2008 – 2009)

COMPUTER SKILLS

Basic working knowledge in the Shell and 'R' environment (bioconductor).

Microsoft Windows XP, Office, Macintosh OS, and Unix.

PUBLICATIONS

Wilken MS, Reh TA. Retinal Regeneration in Birds and Mammals. Current Opinion in Genetics & Development. ***Review.*** *Accepted with minor revisions.*

Ueki Y, **Wilken MS**, Cox KE, Chipman L, Jorstad N, Sternhagen K, Simic M, Ullom K, Nakafuku M, Reh TA. Transgenic expression of the proneural transcription factor Ascl1 in Müller glia stimulates retinal regeneration in young mice. Proc Natl Acad Sci U S A. 2015 Oct 19.

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*Co-First Author

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