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Nadean Brown, PhD
The Role of Basic-Helix-Loop-Helix Transcription Factors in Early Retinal Neurogenesis

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ABSTRACT

The retina converts visual information into neural signals that are processed and transmitted to the brain. During retinal development, seven major cell types, six neuronal and one glial, are generated from a common neuroepithelium during discrete but overlapping time periods. Here, we study the retinal expression and function of three basic helix-loop-helix (bHLH) transcription factors: *Atoh7/Math5* (atonal homologue 7), *Neurog2/Neurogenin2*, and *Ascl1/Mash1* (achaete-scute complex like 1). Proneural bHLH transcription factors are critical for neuronal differentiation and cell type specification in the retina. *Atoh7* and *Neurog2* are expressed at the initiation of retinal development, and *Atoh7* is critical for the generation of the first-born cell type, retinal ganglion cells (RGCs), which transmit visual information to the brain via the optic nerve. *Ascl1* is expressed later in retinogenesis, and is required for normal bipolar interneuron and Müller glial genesis, two later-born cell types.

First, I explored the regulation of *Atoh7* expression, using GFP-expressing transgenes under control of *Atoh7* regulatory DNA, which expressed GFP in *Atoh7*-expressing progenitor cells and nascent RGC axons as they sent projections into the optic nerve and established connections with the brain. In addition to the visual system, *Math5*-GFP transgenic expression was observed ectopically in developing auditory and proprioceptive systems in the developing brain, spinal cord, and inner ear that normally express *Atoh1/Math1*, the other atonal semi-orthologue. I found similarities in the genetic regulation of the proximal 2.1 Kb of 5’ *Atoh7* DNA and the *Atoh1* 3’ enhancers, and concluded that these highly-related bHLHs share common regulatory features that, during evolution from a common precursor, were restricted to nonoverlapping expression domains by as of yet unknown DNA repressor elements.
Second, I examined the function of Neurog2 at the initiation of retinal neurogenesis. Neurog2 and Atoh7 expression was observed sequentially in progenitor cells that give rise to the first neurons in the central retina. I determined that Neurog2, but not Atoh7, is essential for the peripheral expansion of neurogenesis and RGC genesis. In Neurog2 mutant mice, neurogenesis was delayed until the onset of retinal Ascl1/Mash1 expression, but by birth the proportions of early-born cell types are returned to normal. Ascl1 replacement of Neurog2 rescued the delay in both neural differentiation and RGC genesis, signifying that retinal development proceeds as overlapping waves of neurogenesis regulated by these bHLH factors.

Finally, I further explored the interchangeability of bHLH transcription factors. To test the hypothesis that Ascl1 and Atoh7 have distinct functions in cell cycle exit and fate specification in retinal progenitor cells, I used a previously constructed mouse model, the Atoh7\textsuperscript{Ascl1KI} allele, which misexpresses Ascl1 in Atoh7-lineage cells. Ascl1 replacement of Atoh7 did not rescue RGC development but increased bipolar interneuron and decreased Müller glia number in adult eyes. During the initiation of neurogenesis, ectopic Ascl1 prolonged proliferation of Atoh7-expressing cells that normally exit the cell cycle, dominant to endogenous Atoh7 function.

In sum, this thesis provokes new mechanisms for the divergence of bHLH regulation and function in mouse retinal development. Neurog2 and Atoh7 have separate roles in early retinal progenitor cells during the initiation of neurogenesis. While Ascl1 can compensate for neural differentiation defects in Neurog2 mutant mice, it does not promote cell cycle exit or rescue RGC specification in terminally mitotic Atoh7-lineage cells. Together, bHLH factors have overlapping and distinct functions in the mammalian retina, defined by a combination of evolutionary homology, phase of cell cycle expression, and developmental timing.
Dedicated to

Thomas Michael Essman, M.D.

&

John William Hufnagel
ACKNOWLEDGEMENTS

I have been in school for twenty-four years, with only one left to go (before residency “school”). These last four years, however, have enacted more change than the two preceding decades of classwork. It has been hard work, with strange hours and even stranger results. It would have been impossible to do this alone, so there are many people I would like to thank.

Nadean, thank you for the honor of being your first graduate student. I owe you so much, more than I’m able to express, for my development as a scientist in and out of the lab. It’s been an invaluable experience to be here through the trials of tenure, grant renewal, and changing lab directions. Five years from now, the lab will have shifted again and be off in exciting new directions. Hopefully, you’ll have another grad student in love with the “simple and accessible model” that is the retina, trying not to drown in a sea of bHLH data.

Many thanks to the rest of my committee, whose meetings shaped my scientific thinking drastically in a short time. Kenny, thanks for being a great co-mentor and for all your supportive comments. Thanks to you, I’ve started anthropomorphizing bHLH factors in conversation and poster presentations. We never drank whiskey together, but I’ll remedy that before I go. Masato, you kept me grounded in your “simple” way of thinking that was well beyond me, and never failed to point out where I needed to improve. In class, your honest answer that “I can say nothing for sure…!” helps me always keep an open mind. Noah, thanks for the great conversations at happy hours, and especially for all your career advice. We both started out in the eye, and maybe I’ll end up with you at the other end some day. Steve, you helped me become a scientific writer when I was just starting out. Your constant push to get me out of here and start my career lets me know I’m ready to start one, hopefully like yours.
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<td>4V, 4v</td>
<td>Fourth ventricle, hindbrain</td>
</tr>
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<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP2α</td>
<td>Activating enhancer-binding protein 2 alpha</td>
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<tr>
<td>Ascl1, Ash1</td>
<td>Achaete-scute complex-like 1 (Mash = Mouse Ash)</td>
</tr>
<tr>
<td>Ato, cato, nato</td>
<td>Drosophila atonal, cousin of atonal, nephew of atonal</td>
</tr>
<tr>
<td>Atoh7, Ath7</td>
<td>Atonal homologue 7 (Math = Mouse Ath, Xath = Xenopus Ath)</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>βgal, LacZ</td>
<td>Beta-galactosidase (protein product of LacZ)</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>Bhlhb</td>
<td>Basic helix-loop-helix domain containing, class B gene</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine (5-bromo-2-deoxyuridine)</td>
</tr>
<tr>
<td>CDKI</td>
<td>Cyclin-dependent kinase inhibitor (p27/Kip1, p57/Kip2)</td>
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<tr>
<td>CN, cn</td>
<td>Cochlear nucleus, hindbrain</td>
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<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
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<tr>
<td>Cre</td>
<td>Cre recombinase</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DsRed</td>
<td>Discosoma species red fluorescent protein</td>
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<td>E16</td>
<td>Embryonic day 16</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Flp</td>
<td>Flippase, excises frt sites</td>
</tr>
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<td>GCL, gel</td>
<td>Ganglion cell layer, retina</td>
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<td>GFP, gfp</td>
<td>Green fluorescent protein</td>
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<td>H3T</td>
<td>Tritiated thymidine</td>
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<tr>
<td>HC, hc</td>
<td>Hair cell, inner ear</td>
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<tr>
<td>INL, inl</td>
<td>Inner nuclear layer, retina</td>
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<tr>
<td>IPL, ipl</td>
<td>Inner plexiform layer</td>
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<td>IRES</td>
<td>Internal ribosome entry site</td>
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<td>Isl1</td>
<td>Islet lim homeobox 1</td>
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<td>L</td>
<td>Lens</td>
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<td>LG, lg, lgn</td>
<td>Lateral geniculate nucleus, thalamus</td>
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<td>LIF</td>
<td>Leukemia inhibitory factor</td>
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LL, ll  Lateral lemniscus, hindbrain
LRL, lrl  Lower rhombic lip, hindbrain
MC, mc  Merkel cells, whisker barrels
M/L-cone  Medium/Long-wavelength (green/red) cone photoreceptor
RNA, mRNA  Ribonucleic acid, messenger ribonucleic acid
Neurog2, Ngn2  Neurogenin 2 (Atonal homologue 4a)
Neurod1  Neurogenic differentiation 1
Neo  Neomycin
OC, oc  Optic chiasm
ONL, onl  Outer nuclear layer, retina
ON, on  Optic nerve
OPL, opl  Outer plexiform layer
OT, ot  Optic tract
P21  Postnatal day 21
Pax6  Paired box gene 6
PARP, cPARP  cleaved Poly-(ADP-ribose) polymerase
PCR  Polymerase chain reaction
PFA/PBS  Paraformaldehyde in Phosphate buffered saline
PN, pn  Pontine nucleus
Pou4f2/Brn3b  POU class 4 homeobox 2
Prox1  Prospero homeobox 1
RPE  Retinal pigmented epithelium
RXRγ, rrxγ  Retinoid X receptor alpha
SC, sc  Superior colliculus, midbrain
S-cone  Short-wavelength (blue) cone photoreceptor
SHH, shh  Sonic hedgehog
Sox9  SRY (Sex-determining region Y)-box 9
TB, tb  Trapezoid body, hindbrain
TGF-α, tgf-α  Transforming growth factor alpha
TUBB3, Tubb3  Tubulin, beta 3 (βIII-tubulin)
V1, v1  Primary visual cortex
Vsx2/Chx10  Visual system homeobox 2
WB, wb  Whisker barrels
Z/EG  LacZ/EGFP, stop-floxed EGFP reporter
CHAPTER 1

General Introduction:
The Adult and Developing Retina
The Adult Retina and Visual Perception

The eye is the light-detecting organ of the human body. Light passes first through the clear corneal epithelium, which forms a continuous outer layer with the sclera, the white of the eye. Inside the eye, the light travels through the pupillary opening of the iris and then the lens, where it is focused on the retina lining the posterior surface of the inner globe. The retina has two major subdivisions – the inner neural retina, which converts photic information into electrical signals, and the outer retinal pigmented epithelium (RPE) that provides metabolic support for the retina. The RPE is continuous anteriorly with the ciliary body, which alters the shape of the lens to focus on near or far objects, and secretes aqueous humor to provide nutrients to the lens and cornea and to create pressure maintaining the convex shape of the cornea. Between the retina and sclera lies the choroid that carries the vasculature supplying the eye. Outside the eye, several tissues support eye function, including the conjunctiva that covers the sclera and, with the lacrimal and other accessory glands, protects the eye surface by secreting mucous and tears (Davson, 1980).

Light processing in the retina is a complex process mediated by numerous cell types. The neural retina is composed of three layers and seven major cell types (Fig. 1C). First, light stimulation is converted into neural impulses by photoreceptor neurons in the outermost layer, the outer nuclear layer (ONL). Photoreceptors are tonically depolarized under dark conditions. Photons of light strike the outer segment of photoreceptors, causing a conformational change in the chromophore, from 11-cis to all-trans retinaldehyde, which is bound to an opsin protein, producing a hyperpolarizing response. The ONL contains rod and cone photoreceptors that transduce light in a similar manner but detect different wavelengths of light based on the type of opsin protein in the outer segment. Cone photoreceptors are capable of detecting color and have
high spatial acuity. Primates have high acuity vision due to the fovea, a localized area in the central retina populated exclusively by cones. In humans, there are three subtypes of cone photoreceptors – blue (short wavelength, S-cones), green (medium wavelength, M-cones), and red (long wavelength, L-cones), that detect overlapping ranges of wavelengths along the visible spectrum. Mice have two cone photoreceptor subtypes, blue (S-cones) and green/red (M/L-cones). Rod photoreceptors do not function in color vision and have lower acuity than cones, but rods are sensitive enough to detect a single photon of light. Hence, they function primarily in dim light conditions. Mice and humans both have many more rods than cones (35:1 mouse, 20:1 human) (Davson, 1980; Jeon et al., 1998; Masland, 2001).

Light detected by photoreceptors is transmitted to the inner nuclear layer (INL). Here, light information is synaptically transmitted to bipolar interneurons, located in the outer half of the INL. The mouse retina contains many more photoreceptors than bipolar cells (11:1) (Jeon et al., 1998). Bipolar interneurons receive input specifically from either rod or cone photoreceptors, and these synapses form the outer plexiform layer (OPL), located between the ONL and INL. In response to rod photoreceptor hyperpolarization, rod bipolars are depolarized (Davson, 1980). Many rod photoreceptors synapse on a single rod bipolar cell, increasing the light detection capability of the rod pathway. There are two classes of cone bipolar cells, each comprised of three to five distinct subtypes. ON cone bipolar cells are depolarized and activated upon photoreceptor stimulation, while OFF cone bipolar cells are hyperpolarized. In the mouse OPL, each cone photoreceptor contacts a minimum of 10 ON and OFF bipolar cells, and each bipolar neuron receives input from 5-8 different cone cells (Cheng et al., 2005; Masland, 2001; Wassle et al., 2009). Thus, convergent and divergent processing of light information occurs at the first synapse.
Horizontal and amacrine interneurons modulate visual information in the INL.

Horizontal interneurons, in the outermost layer of the INL, synapse on multiple cone photoreceptors and photoreceptor-bipolar synapses. Amacrine cells, located in the inner half of the INL, alter bipolar interneuron output. Amacrine neurons are the most diverse cell type in the retina, and over 30 different forms have been recognized. The AII amacrine subtype mediates the rod bipolar pathway, connecting rod bipolar to cone bipolar neurons to direct rod photoreceptor detection through the cone visual pathway (Lin and Masland, 2006; Masland, 2001). Müller glia, also located in the INL, provide structural and metabolic support for the neurons. Glial processes extend from the nerve fiber layer to the photoreceptor outer segments, forming the inner and outer limiting membranes. In goldfish, zebrafish, and chick, that are capable of retinal regeneration, Müller glia are a source of new neurons, but these cells do not appear to have the same properties in mammals (Hitchcock et al., 2004).

After processing in the INL, visual information is then sent to the innermost layer, the ganglion cell layer (GCL), where bipolar neurons synapse onto retinal ganglion cells (RGC) in the inner plexiform layer (IPL). More than twenty subtypes of RGCs have been identified (Masland, 2003; Rockhill et al., 2002). OFF cone bipolar cells synapse with OFF RGC dendrites in the outer portion of the IPL, and ON cone and rod bipolar cells project to ON RGC arborizations in the inner IPL (Masland, 2001). Typically, multiple bipolar neurons synapse with each RGC, and the ratio of bipolar cells to RGCs is ~12:1 in mouse (Jeon et al., 1998). RGCs axons form a nerve fiber layer across the inner surface of the retina as they course towards the optic nerve head. Displaced amacrine interneurons also populate the GCL. As in the INL, they modulate visual signals through local and lateral interactions.
The convergence of visual information from photoreceptor cells to bipolar interneurons to RGCs creates a receptive field for each ON or OFF RGC. Light stimulating the photoreceptors located in the center of an ON-center receptive field causes RGC depolarization, whereas light stimulating the periphery but not the center of that same field will inhibit transmission. The opposite is true for OFF RGCs, which are inhibited when the central receptive field is stimulated (Davson, 1980; Masland, 2001). Additional modifications of these signals by horizontal and amacrine interneurons further refine these signals and resolve contrast and directional motion, indicative of retinal preprocessing of visual signals before transmission to the brain (He et al., 2003; Olveczky et al., 2003).

RGCs are the projection neurons of the retina, their axons entering the brain via the optic nerve. The optic nerve head is located in the central retina near the fovea, and the lack of neurons there results in a blind spot. Certain RGC axons, particularly from the temporal half of the retina (lateral in humans, caudal in mice), cross the midline at the optic chiasm before entering the optic tract, terminating on several targets including the superior colliculus of the midbrain (sc) and the thalamic lateral geniculate nucleus (lgn) that projects to the primary visual cortex (V1). In non-mammalian vertebrates, particularly avian species, visual processing occurs primarily in the midbrain tectum, analogous to the sc in mammals. In mammals, the majority of visual processing occurs in V1, while the sc coordinates both eye reflexes and involuntary eye movements. Color discrimination occurs in the retina, lgn, and V1 through comparisons of the outputs of different cone subtypes in each receptive field (Rodieck, 1998).

In the tectum, sc, lgn, and V1, RGC axons and target neurons form retinotopic maps. These brain regions are subdivided into discrete regions or layers corresponding to discrete regions of the visual field. Inputs from the left visual field, viewed binocularly in the left nasal
retina (medial in humans, rostral in mice) and right temporal retina, project to the right sc (and vice-versa). Specific layers of the lgn receive projections from neighboring RGCs encoding information from overlapping receptive fields, which then project the information to a similar region in V1. As such, the spatial orientation of visual information from the external environment is conserved by the precise arrangement of RGC axons (Hubel and Wiesel, 2005; Rodieck, 1998). RGC projections do not strictly function in visual processing, however – ganglion cells that project to the pretectum control pupil size, and those to the terminal nuclei of the accessory optic system may coordinate eye with head movement. Melanopsin-expressing RGCs detect light and project to the suprachiasmatic nucleus to control circadian entrainment (Hattar et al., 2002; He et al., 2003).

Overall, the mouse retina is a uniform neuroepithelium composed of seven major cell classes, each with multiple subtypes (Fig. 1C, D). Per the total retina, rod photoreceptors are predominant (78.4%), while cone photoreceptors comprise 2.2% of cells. Bipolar interneurons (7.4%) and amacrine interneurons (7.0%) form the majority of the INL that also contains horizontal interneurons (0.6%) and Müller glia (2.9%). Besides RGCs (0.6%), the GCL also contains displaced amacrine interneurons (0.9%) (Jeon et al., 1998). Other cell types include endothelial cells of the vasculature within the retina and on the inner retinal surface, astrocytes associated with the RGC nerve fibers and the inner retinal vasculature, and microglia that function in immune responses.

**Retinal Development and Disease**

The retina is a long-standing model for the study of cell fate determination. Molecular investigations of retinal neurogenesis have produced exciting advances in understanding the
mulitpotency of progenitors and the specification of cell types. The study of retinal development has clinical relevance as well. Congenital defects such as anophthalmia and aniridia can be caused by mutations in genes critical for retinal development, such as the transcription factor Pax6 (Hever et al., 2006). Degenerative diseases including retinitis pigmentosa, macular degeneration, and glaucoma involve the loss of specific neurons, most notably photoreceptors and RGCs. Generating cells able to properly integrate themselves in the mature retina, and understanding how to maintain the health of remaining neurons are long-term therapeutic goals (reviewed in Fan et al., 2006; Zaghloul et al., 2005). Understanding the processes underlying retinal neurogenesis will elucidate genetic mechanisms of disease, as well as methods for differentiating retinal stem cells for replacement therapies.

The retina is an extension of the brain. Vertebrate eye development begins as the optic vesicle evaginates from the diencephalon and extends to the surface ectoderm (Fig. 1A). In mouse, this begins at embryonic day (E) 8.0, and in the 3rd week of gestation in humans. Encircling the invaginating lens pit, the optic vesicle folds inward, forming the bilayered optic cup (Fig. 1A). The neural retina derives from the inner layer of the optic cup, and the outer layer becomes the RPE. The optic stalk, connecting the optic cup to the diencephalon, will contain the retinal vasculature entering the eye and the optic nerve entering the brain (Chow and Lang, 2001). The optic vesicle is parsed into the structures of the posterior eye by the concerted expression of multiple transcription factors. The homeodomain transcription factors Pax2 and Pax6 are initially co-expressed in the optic vesicle, but segregate as Pax2 expression becomes restricted to the optic stalk and Pax6 expression persists in the neural retina (Baumer et al., 2003; Schwarz et al., 2000). Cross-regulation between Chx10/Vsx2 and Mitf delineate the neural retina and RPE, respectively (Horsford et al., 2005). During retinogenesis, Pax6, Chx10, and Sox2 are
ubiquitously expressed in retinal progenitor cells (Burmeister et al., 1996; Marquardt et al., 2001; Taranova et al., 2006).

The presumptive neural retina thickens as progenitor cells begin proliferating rapidly, changing from a single cell layer into a pseudostratified neuroepithelium. The nuclei of proliferating progenitor cells migrate between the outer (apical or scleral) and inner (basal or vitreal) optic cup, anchored by membrane extensions connected to both surfaces (Fig. 1B). In S-phase, nuclei are closest to the inner surface, passing through G2 phase as they travel to the apical surface, where mitosis occurs, and progress through G1 phase as they return to the basal optic cup, a process termed interkinetic nuclear migration (Rapaport, 2006). Neurogenesis begins at E11.0 in mouse, week 6 in human gestation. In order to differentiate into neurons or glia, proliferating progenitor cells undergo a sequence of events. First, retinal progenitor cells exit the cell cycle (G1 to G0 transition), partly mediated by the cyclin-dependent kinase inhibitors p27/Kip1 and p57/Kip1, and commit to a neural fate, marked by pan-neural proteins such as βIII-tubulin/Tubb3 (Brittis et al., 1995; Dyer and Cepko, 2001b; Lee et al., 1990; Levine et al., 2000). Cells express genes specific to particular fates, including Pou4f2/Brn3b in RGCs (Xiang et al., 1993), and migrate to the proper location.

Neurogenesis initiates as a wave front across the neuroepithelium. The procession of retinal neurogenesis is relatively conserved among developing invertebrate and vertebrate retinas. In Drosophila, the morphogenetic furrow crosses the eye imaginal disc, inducing differentiation from posterior to anterior (Ready et al., 1976). In zebrafish, the first cells exit the cell cycle near the presumptive optic nerve head, and differentiation sweeps around the retina (Neumann and Nuesslein-Volhard, 2000). In chicken, the first neurons appear in the dorso-central retina, and as the retina grows neurogenesis expands in dorsal-ventral and nasal-temporal
gradients (Fig. 1F) (McCabe et al., 1999; Perron et al., 2003). While the first neurons have been observed in the dorsal-central retina in mouse (Fig. 1G), this process has yet to be well characterized with modern genetic and molecular techniques.

Multiple signals coordinate the initiation and progression of neurogenesis. In zebrafish, sonic hedgehog (Shh) secreted from postmitotic RGCs also propagates neurogenesis (Neumann and Nuesslein-Volhard, 2000). In chick, fibroblast growth factors (Fgf) expression in the optic stalk and neural retina is essential for the progression of neurogenesis (Martinez-Morales et al., 2005; McCabe et al., 1999). However, neurogenesis still occurs if the peripheral retina is detached ahead of the wave of differentiation (McCabe et al., 1999), suggesting intrinsic factors are also required to propagate the wave front. Candidate genes to regulate such a process should a) be expressed in an expanding fashion, similar to neurogenesis, and b) when lost, cause an arrest in neurogenesis. For example, \textit{Chx10} mouse mutants exhibit a reduction in Shh signaling and an arrest in the neurogenic wave, but \textit{Chx10} itself is expressed throughout the neural retina, not in concert with neurogenesis (Horsford et al., 2005; Sigulinsky et al., 2008). In chick but not mouse, \textit{Meis2} is downregulated ahead of the wave front and required for the proper expansion of neurogenesis (Heine et al., 2008). An intrinsic factor that both marks and regulates the spread of neurogenesis has not been identified in the mammalian retina.

Neurogenesis proceeds from E11 until P11 in the mouse. The first RGC axons are observed in the optic stalk at E12 (Fig. 1H), and by E14 the optic nerve, chiasm, and tract are established as these RGC axonal projections migrate towards their brain targets (Hufnagel et al., 2007). Axon terminals are observed in the sc and lgn at E16. Lamination of the retina is not complete until postnatal ages. From E13-E14, the GCL and IPL are established as postmitotic RGCs and amacrine interneurons mature. The remaining outer neuroblast layer contains
proliferating progenitors and postmitotic cells of the future INL and ONL. During postnatal neurogenesis, the ONL, OPL, and INL are delineated (Marquardt, 2003).

Retinal proliferation and differentiation change remarkably across development. Across rodent retinogenesis, the length of time for a single cell cycle (S-phase to S-phase) increases from 14 hours at E14 to over 30 hours postnatally (Alexiades and Cepko, 1996). Modes of cellular division change as well. During the initial phase of retinogenesis, rapid retinal growth is achieved by symmetric proliferative divisions, giving rise to two mitotically active daughter cells. Later embryonically, when neurogenesis is established, asymmetric divisions are more common, producing one postmitotic and one mitotically active cell (Alexiades and Cepko, 1996). When retinal neurogenesis ends postnatally, symmetric divisions reoccur, generating two postmitotic cells (Alexiades and Cepko, 1996; Livesey and Cepko, 2001). The final number of cells in the adult retina is refined by apoptosis. Early RGCs (E11-E15) undergo apoptosis more regularly than late RGCs (E15-P1). Postnatally, most apoptosis occurs within the first two weeks, with a peak just after neurogenesis ends (Farah and Easter, 2005; Young, 1984).

**Retinal Histogenesis and Progenitor Cell Multipotency**

The seven retinal cell classes are generated in a conserved, overlapping birth order across developmental time. Birthdating experiments were used to correlate cell fate with the timing of cell cycle exit. Tritiated thymidine (H³T) or bromine deoxy-uridine (BrdU) are injected into pregnant mice or postnatal pups and incorporated into the DNA of cells in S-phase. If a cell exits the cell cycle shortly after the time of injection, high levels of H³T or BrdU remain in the nucleus, while subsequent divisions dilute the marker. In adult retinas, the morphology of brightly labeled cells or molecular markers reveals the cell type. Analyses of multiple timepoints
during retinogenesis of different species reveal a birth order consistent among vertebrates. Fig. 1E depicts this birth order in the mouse retina. RGCs are the first postmitotic cell type observed. Next, cone photoreceptors and amacrine and horizontal cells are generated. In different species, horizontal cells are either an earlier-born or a later-born fate. Rod photoreceptors, the most common cell type in the mammalian retina, are born across most of retinal development. Finally, bipolar interneurons and Müller glia are specified last in all species studied. The birth order of retinal neurons demonstrates that, at any given stage of development, the cell types generated are restricted at any given time (Fig. 1E). In the E11-E13 mouse retina, RGCs, cone photoreceptors, and amacrine and horizontal interneurons are the majority of retinal neurons generated. These four early fates arise almost entirely prenatally, and only rare RGC and amacrine interneurons are born after birth. Rods are generated from E13-P10, and bipolar interneurons and Müller glia are produced last, from E18-P10 (Altshuler et al., 1991; Carter-Dawson and LaVail, 1979; Rapaport et al., 2004; Sidman, 1961; Young, 1985).

Retinal progenitor cells appear initially multipotent, as all seven retinal fates are ultimately derived from the inner optic cup. However, the differentiation of each cell type is temporally restricted to a specific window of time. As early as the blastomere stage of frog development, cells can be identified that will give rise to, among other cells, the majority of a subset of a amacrine cells (Huang and Moody, 1995). Thus, certain retinal progenitor cells could be pre-determined in potential, able to give rise to only one cell type. To test whether retinal progenitor cells are multipotent or lineage-restricted, the progeny of retinal progenitor cells was evaluated by lineage tracing, permanently labeling a cell and all of its mitotic progeny. Retroviral vectors, for example, selectively infect proliferating progenitor cells, and, after
incorporation of a constitutively-expressed reporter into the DNA of the cell, labels that cell and all mitotic daughters derived from that cell (Cepko et al., 2000).

In early retinogenesis of frog and mouse, the clonal progeny of many retinal progenitor cells were found to encompass multiple cell types, including Müller glia (Cepko et al., 2000; Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wets and Fraser, 1988). During postnatal mouse development, ‘clones’ included all three of the later-born cell types, rod photoreceptors, bipolar interneurons, and Müller glia. Clones were extensively heterogeneous in size and composition, containing two to hundreds of cells and one to six cell types. However, the distribution of different cell types in a single clone correlated to their normal proportions in the retina. No clones encompassed all seven retinal fates and very few include five or six, indicating that retinal progenitor cells are not completely multipotent. Also, a minority of clones contained only one cell type, often rods only (Turner and Cepko, 1987; Turner et al., 1990). The limitations to these experiments was that clones from the earliest retinal progenitors could not be assessed due to technical difficulty of in utero injection of virus. Hence, the potential of the earliest retinal progenitors (E11-12) in rodent is still unknown.

Retinal progenitor cells appear heterogeneous in potential, and, while some cells might be lineage-restricted, the majority of cells are multipotent. Retinal fates, however, are born during discrete periods, such that only certain fates are available during early embryogenesis, before rod photoreceptors, bipolar interneurons and Müller glia (late fates) are born, and postnatally, when RGC, cone photoreceptor, and amacrine and horizontal interneuron (early fates) genesis is concluded. Thus, retinal lineages might be plastic, able to generate certain cell types depending on developmental time, or rigid, restricted to only certain fate choices at any time regardless of environment. To test this, late retinal progenitor cells were co-cultured in vitro with an excess of
early progenitor cells to simulate the early environment (Altshuler and Cepko, 1992; Austin et al., 1995; Belliveau and Cepko, 1999; Belliveau et al., 2000; Watanabe and Raff, 1992).

Consistent with a lineage-restricted model, late retinal progenitors could not be reprogrammed to generate early retinal fates. However, early embryonic retinal cells co-cultured with an excess of postnatal cells primarily adopted only early fates, indicating that early cells are not competent to precociously generate late fates. These cells would eventually adopt late fates after an appropriate period in vitro, adding further evidence that cellular properties change over time. Thus, heterochronic transplant experiments demonstrate that retinal progenitor cells are lineage-restricted during different phases of retinogenesis.

These findings have led to the competence model of retinal histogenesis (Cepko et al., 1996; Livesey and Cepko, 2001). Of the seven major cell classes, only a subset differentiates from the progenitor pool at an given time. For example, only RGCs, amarine and horizontal interneurons are generated near the initiation of neurogenesis (Gray bar, Figure 1.E). The ‘competence’ of a retinal progenitor cell refers to the fates available to that cell at that point in time. In one sense, it is a snapshot of the potential of retinal progenitor cells as a whole without regard to the lineage restrictions placed upon them already (hypothetically, rod-only clones).

The clones derived from retroviral tracings at different ages reflect that, while early progenitors can give rise to both early and late cell fates, the ability to generate early fates is lost in postnatal progenitors. However, over the entire course of retinal development, retinal progenitors are multipotent, indicating that the competence of their progeny changes over time. Recent evidence in *Xenopus* suggests that the progeny of an individual retinal progenitor cell give rise to different cell types in a restricted order that follows the birth order in the retina (Wong and Rapaport, 2009). Unlike a progressive restriction model, retina progenitor cells appear to progress through
multiple restricted states across retinal development, which constrain the temporal histogenic windows for the seven retinal cell types. Further, competence states have an intrinsic quality and cannot be immediately reprogrammed to a different state when placed in a heterochronic environment.

Missing from the competence model are the factors that regulate each competence state and the transition from one competence state to another. Potential regulators of competence appear to be predominantly intrinsic to retinal progenitor cells, as indicated by heterochronic transplant studies. One potential competence-determining intrinsic factor is the transcription factor *Ikaros*, expressed during embryonic retinogenesis only in the four early cell types, RGCs, cone photoreceptors, and amacrine and horizontal interneurons. Misexpression of *Ikaros* in postnatal cells ectopically promotes early fate specification, including horizontal cells that are not born postnatally (Elliott et al., 2008). Thus, the timing of *Ikaros* expression in retinal progenitors may define the competence windows for the early retinal fates.

Extrinsic factors, including short- and long-range signaling molecules from the extracellular matrix and neighboring cells, may also affect competence windows. The environment of the retina is highly dynamic. Postmitotic, maturing cells release signals that negatively regulate the differentiation of other progenitors. Cone photoreceptor differentiation is inhibited when early progenitor cells are co-cultured with postnatal progenitor cells, and amacrine interneurons are inhibited by postmitotic amacrine cells in vitro (Belliveau and Cepko, 1999). The secreted factor Gdf11 is released by postmitotic RGCs to negatively regulate RGC production and control the timing of RGC genesis. Loss of *Gdf11* results in increased and prolonged RGC production at the expense of amacrine interneuron and cone photoreceptor genesis (Kim et al., 2005). In the mouse retina, Shh released by differentiating RGCs negatively
regulates RGC production, thereby controlling the number of differentiating cells (Dakubo et al., 2003; Jensen and Wallace, 1997; Wang et al., 2005b). Thus, postmitotic cells change the retinal milieu and alter subsequent specification of progenitor cells.

Intrinsic qualities of retinal progenitor cells and the response to the environment signals change over time as well. Late retinal progenitors are more responsive to certain extrinsic signals including epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α), which signal through the EGF pathway. Overexpression of EGF receptors increases the proportion of Müller glia, the last cell type generated (Lillien, 1995; Lillien and Cepko, 1992; Lillien and Wancio, 1998a). Therefore, intrinsic changes in the cell regulate the competence of retinal progenitor cells, via competence-specific transcription factors or responsiveness to changing environmental signals. While extrinsic and intrinsic factors have been shown to control the temporal windows of certain cell types, a further understanding of intrinsic regulation of retinal cell fate determination will elucidate mechanisms underlying retinal neuron birth order.

**Basic Helix-Loop-Helix Transcription Factors**

The bHLH transcription factors, encoding proteins that contain a basic-helix-loop-helix motif, are excellent candidates to regulate retinal progenitor competence. In many developing tissues, these genes regulate cell-type decisions, determining whether progenitors become neurons, glia, muscle cells, endocrine cells, etc. ‘Proneural’ bHLH factors induce neural differentiation and specification of cell fate at multiple levels. Nearly every facet of neurogenesis, from cell cycle exit and neural commitment to synaptogenesis and survival, has been linked to the function of proneural bHLH factors (Bertrand et al., 2002).
Proneural bHLH factors were originally discovered in *Drosophila*, where they regulate both neural determination and subtype specification (Villares and Cabrera, 1987). In *Drosophila* nervous system development, neurons are selected equivalent groups of ectodermal cells termed ‘proneural clusters.’ Proneural bHLH expression selects cells for a neural fate, which continue to proliferate or differentiate as neurons or glia. For example, as the morphogenetic furrow crosses the eye imaginal disc, the combined actions of the proneural bHLH gene *atonal* and the Notch and Epidermal Growth Factor (EGF) signaling pathways select the first ommatidial neuron, R8, from a group of competent progenitors (reviewed in Hsiung and Moses, 2002). Later in eye development, the *achaete-scute* complex specifies the interommatidial bristle cell (Brown et al., 1996; Sun et al., 2000). Further, bHLH expression inhibits neighboring cells through cell-cell interactions, namely lateral inhibition by Notch signaling. Thus, bHLH expression allows for the selection of neural progenitors, and for the control of neural and non-neural progenitor cell number by preventing an excess of neural specification.

Vertebrates express a host of proneural bHLH factors that are conserved to those of *Drosophila*. In mouse, *atonal*-related genes include several bHLH families. *Atoh7/Math5* and *Atoh1/Math1* are semi-orthologues and as related to each other as they are to *atonal*. The *NeuroD* and *Neurogenin* family are more distantly related, and the *Neurogenin* genes are also closely homologous to *Drosophila biparous/tap*. The bHLH protein domain of Atoh1 and Atoh7 is 70% identical to that of *Drosophila* Atonal, while Neurod1 has 54% similar identity. The *Ascl/Mash* family, including Ascl1/Mash1, share 70% bHLH identity among achaete-scute complex proteins. Similar to *Drosophila*, the vertebrate proneural bHLH factors instruct cells to adopt neural fates, and are typically expressed in progenitor cells in committed neural tissues, including the neural tube, neural crest, and the olfactory, otic, and cranial ganglia placodes.
Further, while bHLH factors appear to function in both neural determination and subtype specification in *Drosophila*, these functions appear to be subdivided in vertebrates, where certain bHLH factors function in neural determination, specification, or both. As such, bHLH factors represent a complex gene network controlling multiple aspects of neurogenesis (Bertrand et al., 2002; Hassan and Bellen, 2000).

Within the basic-helix-loop-helix motif, the basic domain is critical for DNA binding, and the HLH portion for protein-protein interaction. DNA binding requires formation of heterodimeric complexes with Class I bHLH factors (homologous to *Drosophila daughterless*) that are ubiquitously expressed, and bind to an E-box DNA consensus sequence (CANNTG) (Murre et al., 1989). E-box binding sites are very common across the genome, with a probability of 1:256 that any 6 base-pair sequence matches the CANNTG sequence. To enhance specificity of bHLH factor function, DNA binding properties of bHLH proteins are modified in several ways. Id proteins, which contain only the HLH domain, bind to the HLH domain of bHLH factors to inhibit their DNA binding (Ross et al., 2003). The specificity of different bHLH factors for DNA binding sites appears to rely, in part, on the central NN positions and bases adjacent to the E-box (Roztocil et al., 1998; Singson et al., 1994). Further, neighboring binding sites promote cooperativity between bHLH proteins and other transcription factor complexes. The bHLH protein Ascl1/Mash1 interacts with POU-domain proteins Brn1 and Brn2 to activate the promoter of the Notch ligand Delta and laterally inhibit neurogenesis (Castro et al., 2006). Ptf1a, another bHLH factor, binds cooperatively with the Notch effector Rbj to promote GABAergic neuron production in the dorsal spinal cord (Hori et al., 2008). bHLH factors can also undergo posttranslational modifications. *Neurog2* is phosphorylated at particular serine residues, enabling it to complex with LIM-domain proteins Islet1 and Lhx3 and execute a motor
neuron specification program in the ventral spinal cord (Hand et al., 2005; Lai and Johnson, 2008; Ma et al., 2008). The N-terminus of the CDKI p27/Kip1 can bind and stabilize Neurog2 protein (Hand et al., 2005; Lai and Johnson, 2008; Ma et al., 2008; Nguyen et al., 2007). Certain bHLH factors are capable of autoregulating their own expression. Atoh1/Math1 negatively autoregulates its own expression in the dorsal spinal cord, but not the dorsal rhombic lip, to downregulate expression in postmitotic, migrating progenitor cells (Helms et al., 2000).

The Neurogenin family of bHLH transcription factors was discovered in mouse by two independent means, sequence homology to NeuroD and protein-protein interaction with Ascl1. There are three members of the Neurogenin family. Neurog1 and Neurog2 are expressed in regions of the developing forebrain, olfactory system, spinal cord, dorsal root ganglia, and cranial ganglia. Neurog3 is expressed in the developing spinal cord, hypothalamus, and pancreatic endocrine progenitor cells (Gradwohl et al., 1996; Sommer et al., 1996). Neurog2 and Ascl1 appear to regulate one another in several developmental contexts. In the developing forebrain, Neurog2 and Ascl1 are expressed in distinct dorsal and ventral domains, respectively. Both bHLH factors are required for the expression of domain-specific markers and common genes necessary for neurogenesis. Therefore, they appear to instruct separate forebrain identities yet similar neural determination programs. Loss of Neurog2 results in the ectopic dorsal expression of Ascl1. Furthermore, Ascl1 misexpression in dorsal progenitors is sufficient for neural determination and induction of ventral-specific markers, but Neurog2 is only able to promote neurogenesis, not dorsal neuron specification, in ventral forebrain progenitors. Thus, Neurog2-mediated repression of Ascl1 is required to maintain the identity of Neurog2-expressing progenitors, and Ascl1 can re-specify the identity of those cells (Fode et al., 2000; Parras et al., 2002). In the dorsal spinal cord, however, Ascl1 and Neurog2 act in the progenitor cell domain
during separate phases of neurogenesis. Ascl1 is expressed in proliferating progenitor cells of the dorsal interneuron (di) 3 and 5 regions, and is required for their specification. Neurog2 is expressed downstream of Ascl1, and modulates the total number of these two cell classes (Helms et al., 2005). Finally, in the ventral midbrain, Neurog2 and Ascl1 are co-expressed in precursor cells of dopaminergic neurons. These cells require Neurog2, but not Ascl1, for their specification, but Ascl1 replacement of Neurog2 can partially rescue midbrain dopaminergic neurogenesis, indicating some overlap in function between these bHLHs in midbrain development (Andersson et al., 2006; Kele et al., 2006). Overall, bHLH factors have a variety of functions throughout the developing nervous system, which rely heavily on context and cooperation with other factors.

**bHLH Transcription Factors in Early Retinal Development**

Proneural bHLH factors expressed in the mouse retina include Atoh7/Math5, Neurog2/Ngn2, Atoh3/Math3, Neurod1, and Ascl1/Mash1. They are expressed in subsets of RPCs throughout the retina, in a ‘salt-and-pepper’ pattern, during different phases of retinogenesis (Fig. 1E). The atonal-homologues Atoh7 and Neurog2 are expressed at the initiation of neurogenesis (E11), until shortly after birth (P1). Neurod1 and Atoh3 are expressed from early retinogenesis through adult ages. Ascl1/Mash1 is expressed later, from E12-P9, during the phase of rod photoreceptor, bipolar interneuron, and Müller glia development (Fig. 1E). bHLH genes also differ in their expression at different stages of progenitor cell development. Neurog2 and Ascl1 are expressed in proliferating progenitors, Atoh7 in terminally mitotic progenitors, and Neurod1 and Atoh3 in differentiating and mature cells (Ahmad et al., 1998; Brown et al., 1998; Inoue et al., 2002; Jasoni and Reh, 1996; Mao et al., 2008a; Morrow et
al., 1999; Takebayashi et al., 1997). Differences in the timing of bHLH expression in retinal development and cell fate determination suggest very different functions among these transcription factors.

bHLH factors have been linked to the specification of every major retinal cell type. *Atoh7* is critically required for RGC genesis, and in its absence mice essentially lack RGCs and optic nerves (Brown et al., 2001; Wang et al., 2001). *Ptf1a* is required for horizontal interneuron specification, *Neurod1* functions in photoreceptor and amacrine cell differentiation, and *Ascl1* is required for the proper number of rod photoreceptors, bipolar neurons, and Müller glia to form (Morrow et al., 1999; Tomita et al., 1996). The complete absence of a particular cell type sometimes requires combined loss of multiple bHLH factors. For example, *Ascl1;Atoh3* double-mutants lack bipolar neurons and *Neurod1;Atoh3* double-mutants lack amacrine neurons, and horizontal interneurons are lost in *Atoh3;Ascl1;Neurog2, Atoh3;Ascl1;NeuroD*, and *Atoh3;Neurog2;NeuroD* triple-mutant retinas (Akagi et al., 2004; Hatakeyama et al., 2001; Inoue et al., 2002). Overall, this argues strongly that bHLH factors work in concert to control multiple aspects of cell fate determination in RPCs.

Complex genetic networks regulate retinal bHLH factors. The *Pax6* gene, an orthologue of *Drosophila eyeless* and *twin of eyeless*, regulates the expression of many bHLH factors in mouse, and the cell fate specification of all retinal cell types, except a subclass of amacrine interneurons. *Pax6* binds directly to the promoters of *Atoh7, Neurog2*, and *Ascl1* genes to activate their expression (Marquardt et al., 2001; Riesenber et al., 2009). *Sox2* is also required for proper *Atoh7, Neurod1*, and *Ascl1* expression (Taranova et al., 2006). bHLH factors also act cooperatively with non-bHLH factors to specify cell fate. *Chx10* is required for normal bipolar interneuron genesis, but misexpression of *Chx10* alone produces INL cells with Müller glia
morphology. When co-expressed with Ascl1 or Atoh3, however, bipolar neurons are formed, indicating dual requirement for Chx10 and Ascl1 or Atoh3 in bipolar interneuron specification (Burmeister et al., 1996; Hatakeyama et al., 2001). bHLH factors cross-regulate one another as well, but it is unknown whether these effects are direct or indirect. In the absence of Atoh7, both Neurod1 and Neurog2 are upregulated, but Ascl1 is unaffected (Le et al., 2006). Neurog2 and Ascl1 appear to cross-repress one other in the retina, suggesting that endogenous Neurog2 inhibits Ascl1 expression and vice-versa (Akagi et al., 2004).

Correlation between the expression and function of bHLH factors in cell type specification suggests a significant role in the birth order of major retinal neuron classes. Atoh7 is expressed at the initiation of retinal neurogenesis, and is required for the formation of the first-born retinal neuron, the RGC. The absence of Atoh7 results in an increase in cone photoreceptors and displaced amacrine cells, the next two fates generated (Brown et al., 1998; Brown et al., 2001; Le et al., 2006; Wang et al., 2001). However, while Atoh7 is required primarily for early fate specification, the Atoh7-lineage contains all the major cell types in the retina, indicating multipotency of the Atoh7-lineage (Brzezinski, 2005; Yang et al., 2003). However, the total lineage is heavily skewed towards the early retinal fates. Thus, Atoh7 is an early competence factor, and does not merely instruct progenitor cells to adopt an RGC fate (Brzezinski, 2005; Yang et al., 2003).

Neurog2 is also expressed during early retinogenesis. Of the Neurogenin genes, only Neurog2 is expressed in the mouse and frog retinas, but in chick Neurog1 and Neurog3 expression have been observed as well (Ma et al., 2009; Nieber et al., 2009). Misexpression of Neurog2 in non-neuronal RPE culture generates immature RGCs and cone photoreceptors, two of the earliest fates, and induces Neurod1 expression (Yan et al., 2001). The prenatal expression
(E11-P1) and generation of early fates in vitro suggest that Neurog2 biases progenitors towards early fates, particularly RGCs and cone photoreceptors. Interestingly, lineage tracing of Atoh7 and Neurog2-expressing RPCs revealed that their progeny include all seven retinal fates (Ma and Wang, 2006). Mutation of Neurog2 in mouse has no retinal phenotype to date, but multiple cell types are affected in triple mutant combinations of Neurog2, Neurod1, Atoh3, and Ascl1 (Akagi et al., 2004). Thus, while Neurog2-lineage is also multipotent, the function of Neurog2 during retinal neurogenesis remains unknown.

Ascl1 is expressed during later embryonic and early postnatal ages. Misexpression and mutant analyses in mouse indicate a role in promoting rod photoreceptor and bipolar interneuron fates, and suppressing Müller glia (Hatakeyama et al., 2001; Tomita et al., 2000; Tomita et al., 1996). Misexpression of Ascl1 in the mouse retina alone increases rod photoreceptor specification, while combination of Ascl1 and Chx10 misexpression increases bipolar interneurons (Hatakeyama et al., 2001). In separate misexpression experiments in frog, Xenopus Ash1 reduced bipolar neurons generated in one case and increased them in another, while mouse Ascl1 misexpression increased bipolar interneurons (Brown et al., 1998; Moore et al., 2002; Wang and Harris, 2005). In all cases, Ascl1 appears to inhibit Müller gliogenesis. Aside from studies of cell type specification, little is known about the effects of Ascl1 expression on retinal progenitor cell differentiation.

**Research Objectives**

It is well-established that bHLH factors are critical for many aspects of retinal neurogenesis. However, it is unclear if and how mammalian bHLH factors function to initiate retinal neurogenesis and control the temporal order of retinal neuron specification. Ultimately, a
A firm understanding of the sequence of events involved in retinal neuron determination will provide the groundwork for preventing degenerative processes and engineering progenitor cells for replacement therapies. To this end, I explored the expression and function of Atoh7, Neurog2, and Ascl1 in early mouse retinal neurogenesis, to determine their role in early retinal progenitor cells, particularly for RGC genesis. Overall, I hypothesized that bHLH factors function coordinately at multiple levels during retinal neuron specification, promoting cell cycle exit, neural commitment, and fate specification.

The first aim explored the regulation of Atoh7 expression in light of its selective expression in the developing retina. I utilized Math5-GFP transgenic mice that express a green fluorescent reporter (GFP) under the control of specific 5’ and 3’ Atoh7 regulatory DNA. Math5-GFP expression was detected in the earliest differentiating RGCs axons as they traversed the optic stalk and migrated to their principal brain targets. These transgenes also expressed GFP ectopically in the central nervous system (CNS) in the pattern of Atoh1, the other atonal semi-orthologue. Further, Math5-GFP transgenes and Atoh1 were regulated similarly by Pax6 and Atoh1 itself in the dorsal neural tube. Thus, these highly-related bHLHs have retained similar regulatory elements, though they have divergent expression patterns and regulatory DNA.

The second aim investigated the role of Neurog2 in early retinal neurogenesis, testing the hypothesis that Neurog2 is required for the onset of neural differentiation and RGC specification. I observed the expression of Neurog2, Atoh7, and Ascl1 during the onset of retinal neurogenesis. Neurog2 and Atoh7 were expressed in subsequent phases of the cell cycle in retinal progenitors, while Ascl1 expression initiated more than a day later. In the absence of Neurog2, the progression of neurogenesis was initially halted, then resumed during the onset of Ascl1 expression. Further, I explored the functional relationship between Neurog2 and Ascl1 in retinal
development. Functionally replacing *Neurog2* with *Ascl1* using the *Neurog2*<sup>Ascl1KI</sup> allele (Fode et al., 2000) resulted in no delay in early retinal neurogenesis. This suggests that *Neurog2* and *Ascl1* have similar if not redundant functions in the retina, and that *Ascl1* may control a second wave of neurogenesis beginning at E12. In conclusion, *Neurog2* functions in commitment of the first retinal neurons and the progression of neurogenesis, thereby contributing to the spatiotemporal window of RGC specification.

The third aim further explored the interchangeability of bHLH transcription factors, testing the hypothesis that *Ascl1* and *Atoh7* have distinct, non-overlapping functions in retinal development. Here, I functionally replaced *Atoh7* with *Ascl1* expression using the *Atoh7*<sup>Ascl1KI</sup> allele. *Ascl1* cannot rescue RGC specification in the absence of *Atoh7*, but promotes bipolar neurogenesis and suppress gliogenesis. Analysis of the *Atoh7*-lineage in these mice indicates that *Ascl1* may bias progenitor cells towards later-born fates. Thus, *Ascl1*, a later-expressed bHLH factor, cannot specify RGCs when replacing *Atoh7*.

Overall, these experiments demonstrate that bHLH factors have both overlapping and exclusive expression and function in the earliest retinal progenitors (E11-12). *Neurog2* and *Atoh7* are expressed sequentially to promote neural commitment and RGC specification, respectively, and *Neurog2* expression is critical to propagate the neurogenic wave. *Ascl1*, normally not expressed at this time, can functionally substitute for *Neurog2* in neural commitment, but not for *Atoh7* in RGC specification. By focusing on the timing and pattern of bHLH expression during retinogenesis and the progenitor cell cycle, these studies elucidate the coordinate function of bHLH factors at multiple levels of RGC determination.
**Figure Legends**

**Figure 1.1.** Vertebrate retinal neurogenesis and bHLH expression. (A) The optic cup is derived from the optic vesicle, a lateral evagination from the anterior neural tube. (B) These cells proliferate to form a pseudostratified neuroepithelium, and the nuclei migrate during the cell cycle between the apical (M-phase) to the basal (S-phase) surfaces. (C) The adult retina contains seven cell types organized into three layers, photoreceptors in the ONL, interneurons and glia in the INL, and ganglion cells and displaced amacrine cells in the GCL. (D) The composition of cell types in the adult retina. Rod photoreceptors are the most prevalent cell types, and horizontal neurons the least prevalent. Early-born cell types (RGCs, cone photoreceptors, horizontal and amacrine interneurons) comprise ~10% of the retina, and late-born cell types (rod photoreceptors, bipolar interneurons, and Müller glia) comprise the remaining ~90%. (E) The temporal generation of major retinal cell types are shown with the timing of bHLH factors studied here, *Atoh7*, *Neurog2*, and *Ascl1*. At the initiation of neurogenesis (grey bar), only early fates are generated, correlating with the onset of *Atoh7* and *Neurog2* expression. *Ascl1* is expressed later, correlating with pre- and postnatal generation of later-born fates. (F) At the initiation of chick retinal neurogenesis at E3.5, RGCs (neurofilament in pink, TuJ1 in purple) first appear in the central retina and neurogenesis spreads towards the periphery. (G) Similarly, the first TuJ1+ neurons (yellow) are located in the central retina at the initiation of mouse retinal neurogenesis at E11.75. (H) *Atoh7* expression, shown by *Atoh7*^LacZ^ reporter (red) and *Math5*-GFP transgene (green), at E12 demonstrates expression in retinal cells and perdurance in RGC axons traversing the optic stalk. Scale bar 50µm in G, 200µm in F,H. A-C adapted from (Marquardt, 2003), D adapted from Joseph Brzezinski IV and Tom Glaser, E adapted from (Marquardt and Gruss, 2002), F adapted from (McCabe et al., 1999).
**Figure 1.1**

**A** and **B** show the differentiation of retinal cells during development.

**C** illustrates the layers of the retina, including:
- Rod photoreceptor
- Cone photoreceptor
- Horizontal cell
- Bipolar cell
- Müller glia
- Amacrine cell

**D** summarizes the major cell classes and their percentage of total retinal cells:

<table>
<thead>
<tr>
<th>Major Cell Class</th>
<th>% of Total Retinal Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganglion cell</td>
<td>0.6%</td>
</tr>
<tr>
<td>Horizontal cell</td>
<td>0.6%</td>
</tr>
<tr>
<td>Cone photoreceptor</td>
<td>2.2%</td>
</tr>
<tr>
<td>Amacrine cell</td>
<td>7.9%</td>
</tr>
<tr>
<td>Rod photoreceptor</td>
<td>78.4%</td>
</tr>
<tr>
<td>Bipolar cell</td>
<td>7.4%</td>
</tr>
<tr>
<td>Müller glia</td>
<td>2.9%</td>
</tr>
</tbody>
</table>

**E** shows the progression of cell types over days, with panels for Embryonic, Atoh7, Neurog2, Ascl1, and Postnatal stages.

**F** and **G** are images of retinal sections, with **H** showing a similar view.
CHAPTER 2

Conserved regulation of *Math5* and *Math1* revealed by *Math5*-GFP transgenes
Abstract

Retinal ganglion cell genesis requires the proneural bHLH transcription factor Math5 (Atoh7), but little is known about the regulatory elements that control its expression. Here, we investigate Math5 gene regulation using transgenic mice. These mice express GFP in the prenatal retina, live-labeling RGC axon migration and innervation of the brain. Unexpectedly, these Math5-GFP transgenes are also found in Math1 expression domains throughout the nervous system, intriguing since Math5 and Math1 normally exhibit nonoverlapping expression. Furthermore, Math5-GFP and Math1 are regulated similarly, by both Pax6 and Math1 itself, in the lower rhombic lip and dorsal spinal cord. We also show that Pax6 binds to particular Math5 and Math1 regulatory sequences in vitro. Together these data suggest that these atonal semi-orthologues may share conserved regulatory elements that are normally silent in the Math5 gene.
Introduction

The mammalian neural retina is composed of seven cell types, six neuronal and one glial, that differentiate from a common progenitor pool within defined temporal windows (Cepko et al., 1996; Livesey and Cepko, 2001). Proper spatial and temporal development of retinal neurons is attributed, in part, to the proper expression of proneural basic helix-loop-helix (bHLH) transcription factors in progenitors (Cepko, 1999). The bHLH factor Math5 (mouse atonal homologue 5) is expressed in retinal progenitors prenatally, beginning at E11 and continuing through P0 (Brown et al., 1998). Math5 is required for the development of RGCs, which transmit visual information to the brain via the optic nerve. In the absence of Math5 RGCs fail to form, and although the eyes appear normal externally, these mice completely lack optic nerves (Brown et al., 2001; Wang et al., 2001). Math5 expression is also critical for the timing of RGC differentiation; in its absence this temporal window is shifted such that these cells adopt late fates, predominantly Müller glia (Brzezinski, 2005; Le et al., 2006).

An understanding of the regulatory networks that control Math5 expression is crucial to elucidating its role in neurogenesis. Math5 requires the paired-domain transcription factor Pax6 for initial activation (Brown et al., 1998; Marquardt et al., 2001) and is repressed by the bHLH factor Hes1 (Lee et al., 2005). However, the cis-regulatory elements regulating Math5 expression are not yet well defined. In Xenopus, Ath5 expression (Xath5) is regulated by upstream bHLH-dependent and independent elements (Hutcheson et al., 2005). Both proximal bHLH-specific binding sites and more distal cis-regulatory Xath5 sequences each independently drive transgenic expression of a GFP reporter in the Xenopus retina. Importantly, a phylogenetically conserved 5’ distal element is required for retinal expression in the mouse.
retina and sufficient to drive retinal expression in the frog eye (Hutcheson et al., 2005; Riesenberg et al., 2007).

Here, we explore the in vivo expression patterns of Math5-GFP transgenes containing different combinations of 5’ and 3’ Math5 non-coding DNA sequences. While endogenous Math5 expression is confined to retinal progenitors, Math5-GFP persists in mature RGCs and along the length of their axons in the developing brain. We demonstrate that these transgenic mice are useful for observing RGC axon outgrowth and the establishment of the optic nerves, chiasm, and tracts within the brain. Math5-GFP is also ectopically expressed in multiple expression domains of Math1, another orthologue of Drosophila atonal (Ben-Arie et al., 1996; Jarman et al., 1993). Math5 and Math1 are critical regulators of sensory neuron circuit formation in the visual, auditory, and proprioceptive systems (Ben-Arie et al., 2000; Bermingham et al., 1999; Brown et al., 2001; Hassan and Bellen, 2000; Saul et al., 2007; Wang et al., 2001). However, these genes have mutually exclusive expression patterns; thus, the observation of Math5-GFP and Math1 coexpression was further investigated. We find that Math5-GFP is regulated similarly to Math1, by Pax6 in the lower rhombic lip and by Math1 itself in the lower rhombic lip and dorsal spinal cord. These findings provide insight into the divergence of this gene family during vertebrate evolution.

Materials and Methods

Generation of transgenic mice

Math5-GFP constructs and transgenic mice were generated as described (Hutcheson et al., 2005; Riesenberg et al., 2007). Transgenic mice are maintained in a CD-1 background. Math5-GFP1 and Math5-GFP2 were crossed with Math5LacZ+/+, Math1LacZ+/+, or Pax6Sey/+ mice to
compare \textit{Math5}-GFP expression to that of the \textit{Math5}^{\text{LacZ}} and \textit{Math1}^{\text{LacZ}} reporters and to assess changes in GFP expression in \textit{Math5}, \textit{Math1}, or \textit{Pax6} mutant embryos. Mouse embryos were harvested from timed pregnancies for GFP imaging, cryosectioning, and immunofluorescence (see below), with the observed plug date taken as E0.5. A minimum of three embryos from at least two litters was used for each experiment. Genotyping for \textit{Math5}-GFP, \textit{Math5}^{\text{LacZ}}, \textit{Math1}^{\text{LacZ}} and \textit{Pax6}^{\text{Sey}} embryos or adult mice was performed by PCR as described (Ben-Arie et al., 2000; Brown et al., 1998; Brown et al., 2001; Hutcheson et al., 2005).

\textit{GFP imaging and immunofluorescence}

Embryos were maintained in cold PBS for whole-mount imaging on a Leica MZ-FLIII dissecting microscope equipped with a GFP fluorescence lamp, digital camera, and Magnafire software. For immunofluorescence, embryos were fixed for 1-2 hours in 4\% PFA/PBS at 4\(^\circ\)C, cryoprotected in 5\% and 15\% sucrose/PBS, embedded in OCT, and cryosectioned in 10\(\mu\)m increments. Primary antibodies used include rabbit anti-GFP Alexa-Fluor 488 (1:500-1:1000, Molecular Probes), rabbit anti-\(\beta\)gal (1:5000, ICN), rabbit anti-Math1 (1:250, gift from Jane Johnson), rabbit anti-Pax6 (1:1000, Covance), rabbit anti-Pax2 (1:100, Covance), mouse anti-Islet1 (DSHB), and DAPI (1:500). Secondary antibodies used include goat anti-rabbit IgG Alexa-Fluor 594 (1:2000, Molecular Probes), biotinylated donkey anti-rabbit (1:200, Jackson), and streptavidin Texas Red (1:200, Jackson). For antibody experiments in which the direct conjugate rabbit anti-GFP antibody and another rabbit primary antibody were employed, potential cross-reactivity was eliminated by incubating the slides in 10\% rabbit serum/TST for 2-3 hours. This step was performed after the final amplification of the rabbit primary antibody and
before applying the anti-GFP antibody. Images were generated on a Zeiss Axioplan 2 fluorescent microscope with an Apotome deconvolution device and Axiovision software.

Sequence analysis

Noncoding nucleotide sequences for *Math5* (NCBI accession #AAF418923) and *Math1* (NCBI accession #AF218258) were obtained from NCBI. The 5’ 2.1 Kb sequence was extracted from a larger *Math5* sequence. The 5’ 2.1 Kb sequence was aligned with the 3’ *Math1* enhancer sequence using the VISTA program (http://genome.lbl.gov/vista). The VISTA alignment was assessed for consensus identity using a calculation window of 20 or 30 bp, and consensus identity defined as 70%. Potential *Pax6* binding site sequences were predicted using the Transfac® (http://www.biobase-international.com) MATCH™ (Matrix Search for Transcription Factor Binding Sites) program version 10.3 and matrices M00979 (VSPAX6_Q2), M00097 (VSPAX6_01), and M00808 (VSPAX6_06). The black box in the *Math5* distal conserved region represents a conserved binding site 5-J, initially tested in Riesenberg et al (2007). All other putative binding sites (shown in grey) were predicted using any of the three matrices, and selected based on their Core score and potential evolutionary conservation between *Math5* and *Math1* as shown in Figure 7. The E-box binding sites in the *Math5* and *Math1* sequences have been previously tested *in vivo* (Helms et al., 2000; Hutcheson et al., 2005).

Electrophoretic Mobility Shift Assay (EMSA)

GST and GST-Pax6 paired domain proteins (Epstein et al., 1994), were purified from BL21 bacterial lysates by incubation with glutathione agarose beads (Sigma) for 1 hour at 4°C, washed in PBS, eluted with 25 mM glutathione/0.1M Tris pH 8 and dialyzed into 50 mM Tris
pH 7.5, 250 mM NaCl, 5 mM MgCl₂, 2.5 mM EDTA, 20% glycerol. Gel-shift reactions used a 5X binding buffer (50 mM Tris pH 7.5, 250 mM NaCl, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 0.25 mg/ml poly-dI-dC, and 20% glycerol). 20 µl reactions contained 4 µl of 5X binding buffer, 0.1, 0.5 or 1 µg of recombinant protein and 75 fmol of γ³²P end-labeled, annealed, double-stranded oligonucleotides (400,000 Cerenkov counts per reaction). After DNA probe addition, reactions were incubated for 20 minutes at room temperature and run on a 4% polyacrylamide gel in 0.5X Tris borate-EDTA buffer, gels were then dried and exposed to x-ray film.

Results

Math5-GFP expression in the developing visual system

Several transgenic mouse lines expressing green fluorescent protein (GFP) under the control of non-coding 5’ (Math5-GFP1) or 5’ and 3’ (Math5-GFP2) regulatory elements of the Math5 gene have been generated (Riesenberg et al., 2007). Both Math5-GFP transgenes contain 2.1 Kb of 5’ Math5 non-coding sequence that ends 14 base pairs upstream of the ATG start codon (Fig. 1). In addition, Math5-GFP2 contains 1.6 Kb of Math5 3’ DNA inserted downstream of the GFP coding region (Fig. 1). The 5’ 2.1 Kb sequence is sufficient to drive GFP expression in the mouse retina, beginning at E11.5 (Hutcheson et al., 2005; Riesenberg et al., 2007 and Fig. 4B). However, other aspects of Math5 cis-regulation have not been investigated.

Thus, we examined Math5-GFP1 expression in the embryonic retinæ of transgenic animals. Although Math5 mRNA is only expressed by retinal progenitors that are becoming terminally mitotic (Le et al., 2006), Math5-GFP perdures longer, like Math5LacZ (Brown et al.,
As a consequence, differentiated RGCs express GFP throughout their nucleus and cytoplasm, and along the length of their axons. This allows visualization of RGC axons as they progress towards their targets in the brain. RGCs, whose cell bodies are located within the ganglion cell layer of the retina, send axons out through the optic disk into the optic nerve, which connects to the optic chiasm and optic tract (Rodieck, 1998). At E16.5, Math5-GFP1 can be observed in the retina using live GFP fluorescence (Fig. 2A). Examination of the ventral surface of the brain reveals GFP in the optic nerve, chiasm, and tract (Fig. 2B). In the lateral diencephalon, we observe the entire length of the optic tract (Fig. 2C). RGC axons in the optic tract make specific synaptic connections to the lateral geniculate nucleus (lg), superior colliculus (sc), and other processing centers to faithfully transmit visual information from the external environment to the brain (Rodieck, 1998). At E16.5, we observe GFP expression in the developing lg (Fig. 2C,E) and sc (Fig. 2D) by live fluorescence or anti-GFP immunolabeling. Figure 2F displays the relative positions of the optic tract, lg, and sc in a coronal section of the E16.5 brain (Schambra et al., 1992). Overall, we observe GFP along the entire length of RGC axons throughout the period of optic nerve formation.

The progression of RGC axon outgrowth is readily observed in Math5-GFP1 embryos, beginning with activation of Math5-GFP1 in the optic cup at E11.5 (Fig. 3A,4B). We characterized the progression of GFP-positive axons through the developing brain by immunolabeling for GFP. At E12.5, GFP-positive axons can be seen as they migrate within the optic nerve (Fig. 2G), prior to their extension to the optic chiasm. By E14.5, GFP expression within the optic chiasm and optic tracts is apparent (Fig. 2H). By E16.5, many GFP+ axons are present in the optic tracts (Fig. 2I) and some have reached the sc and lg (Fig. 2D-F). By P1, GFP is still expressed in the retina (Fig. 3C) and RGC axons, but expression is clearly diminished, and
by P5 retinal GFP expression is no longer observable (not shown). Math5-GFP2 transgenic mice also express GFP in the developing optic nerve, chiasm, and tracts of the developing brain (not shown). We conclude that the Math5-GFP transgenes allow for visualization of migrating RGC axons from cell bodies in the retina to axon terminations in the developing brain.

Next, we compared Math5-GFP1 expression to that of Math5\textsuperscript{LacZ} in the retina. The Math5\textsuperscript{LacZ} allele was created by inserting the bacterial LacZ gene into the Math5 locus by homologous recombination. Thus, βgal protein reports endogenous Math5 expression, although it perdures longer than Math5 mRNA in differentiated RGCs (Brown et al., 2001). In Math5-GFP1; Math5\textsuperscript{LacZ/+} E11.5 and E12.5 eyes, essentially all βgal+ cells coexpress GFP (arrows in 3A,B), although a few GFP only cells can be seen. At E16.5, we still observe GFP+/βgal+ colabeled cells (arrows in 3C), but also more GFP+/βgal- and GFP-/βgal+ cells. By P1, an expression difference between the reporters is very evident. At this age, most βgal+ cells reside in the ganglion cell layer, while most GFP+ cells are located either in the neuroblast layer or the forming photoreceptor layer, with only a minor subset of cells coexpressing GFP and βgal (arrow in Fig. 3D). The Math5-GFP2 transgene exhibits a similar difference in expression with Math5\textsuperscript{LacZ} at E16.5 (Fig. 3E). This expression difference suggests that both Math5-GFP transgenes may not contain all spatiotemporal information necessary to recapitulate endogenous Math5 expression. Alternatively, because two gene reporters (GFP and βgal) were compared, expression differences might also be attributed to unequal perdurance of each reporter protein.

Non-retinal expression of Math5-GFP

Math5 mRNA and Math5\textsuperscript{LacZ} are expressed in the developing retina (Brown et al., 1998; Brown et al., 2001; Wang et al., 2001), but Math5-GFP1 is also expressed in several regions of
the developing peripheral and central nervous systems. From E10.5 to E12.5, we observe GFP expression in the forebrain (not shown), spinal cord and lower rhombic lip (Fig. 4A-D). In the spinal cord, Math5-GFP1 is expressed in both the dorsal-lateral rim and in a thick ventral band of cells (Fig. 4A,B). In the rhombic lip, Math5-GFP1 expression is restricted to the lower lip (Fig. 4C,D), which gives rise to the precerebellar nuclei, including the pontine and cochlear nuclei (Engelkamp et al., 1999; Farago et al., 2006; Landsberg et al., 2005). Math5-GFP1 is not found in the upper rhombic lip, which develops into the external granular layer (EGL) of the cerebellum (Alder et al., 1996). Math5-GFP1 is also expressed in the pontine nucleus at E16.5 (4E,F), inner ear hair cells from E14.5 to E16.5 (Fig. 4G), whisker barrels from E16.5 through P1 (Fig. 4I), and the molars and the pineal gland at E16.5 (not shown). To determine if these regions normally express Math5, we compared Math5-GFP1 to Math5\textsuperscript{LacZ} in these domains. Math5\textsuperscript{LacZ} is not expressed in any of these non-retinal domains (Fig. 4, second column). This further suggests that Math5-GFP1 is deregulated, allowing expression outside the normal Math5 expression domains. Math5 lineage tracing has described in retinal, auditory system, cerebellar cortex, cerebral cortex, and hippocampal labeled adult cells (Brzezinski, 2005; Saul et al., 2007; Yang et al., 2003), but none of the ectopic Math5-GFP expression domains were found. Although Math5-GFP1 expression was seen in a few cells of the developing neocortex, we did not find GFP in the E12.5-P1 cerebellar cortex or hippocampus (not shown). However, the ages for endogenous Math5 expression in these nonretinal domains have not been determined.

To elucidate the role of putative 3’ regulatory elements in the activation or restriction of Math5 expression, we compared both the retinal and non-retinal expression of the Math5-GFP1 and Math5-GFP2 transgenes. The GFP2 construct contains an additional 1.6 Kb of 3’ Math5 non-coding DNA inserted 3’ to the GFP coding sequence (Fig. 1). Since Drosophila atonal and
mouse Math1 contain 3’ regulatory modules (Helms et al., 2000; Sun et al., 1998), we hypothesized that Math5 3’ DNA might be required to prohibit the ectopic expression domains observed in Math5-GFP1 transgenic embryos. However, the non-retinal expression domains of both Math5-GFP constructs are nearly identical. The GFP2 transgene is also expressed in the lower rhombic lip, pontine nucleus, spinal cord, and whisker barrels, at the same ages as Math5-GFP1 (data not shown). Unlike GFP1, Math5-GFP2 is not expressed in inner ear hair cells (Fig. 4H). This suggests the presence of an inner ear-specific repressor in the 3’ DNA.

In the course of these experiments, we observed a previously uncharacterized Math5\(^{\text{LacZ}}\) expression domain in the cochlear nucleus of the developing hindbrain (Fig. 4I’)(Saul et al., 2007). The cochlear nucleus receives input from the spiral (cochlear) ganglion, which integrates auditory signals received by the cochlear hair cells in the Organ of Corti. Axons from the cochlear nucleus neurons travel through the trapezoid body and lateral lemniscus to auditory processing centers including the superior olivary nucleus, the nucleus of the trapezoid body, and the inferior colliculus (Cant and Benson, 2003). Therefore, RGCs and cochlear nucleus neurons are functionally similar in that they are projection neurons for the visual and auditory sense organs, respectively. We observe Math5\(^{\text{LacZ}}\) expression in cochlear nucleus neurons (Fig. 4K’) and their axonal projections for the trapezoid body and lateral lemniscus (Fig. 4J). Interestingly, Math5-GFP1 is not coexpressed with Math5\(^{\text{LacZ}}\) in cochlear nucleus neurons or axons from E14.5-E16.5 (Fig. 4K, K’, K”, J, not shown). Math5-GFP2 is also not expressed in the cochlear nucleus (not shown), indicating that the cochlear nucleus enhancer lies outside of the 5’ and 3’ Math5 noncoding DNA examined here. Since the GFP1 and GFP2 transgenes are expressed in identical patterns, except that GFP1 is also present in inner ear hair cells, all remaining analyses were done with Math5-GFP1.
Math5-GFP1 is expressed in a subset of Math1 expression domains

Both Math5-GFP transgenes are expressed in discrete locations outside the developing visual system. The most closely related bHLH factor to Math5 is Math1, which is expressed in several progenitor populations that give rise to components of the proprioceptive system throughout the nervous system. These include inner ear hair cells, whisker barrels, the rhombic lip, and the dorsal spinal cord (Akazawa et al., 1995; Ben-Arie et al., 2000; Bermingham et al., 2001; Machold and Fishell, 2005; Wang et al., 2005a). Sensory inputs, including Merkel cells in whisker barrels and hair cells in the inner ear, transmit positional information to the brain, which is then processed in a complex circuit including the pontine nuclei, cerebellum, and cerebral cortex (Bermingham et al., 2001). Since we observed Math5-GFP expression in several regions known to express Math1, we hypothesized that Math5-GFP1 and Math1 are expressed in the same cell lineages. However, Math5-GFP1 is not expressed in every Math1 domain, since GFP expression was not found in the upper rhombic lip, which differentiates into the EGL of the cerebellum (Ben-Arie et al., 1996).

To demonstrate coexpression of Math5-GFP1 and Math1 in these regions, we performed anti-GFP, anti-Math1 double-antibody labeling experiments. Math5-GFP1 and Math1 are coexpressed in cells of the spinal cord (Fig. 5A-B”) and lower rhombic lip from E10.5-E12.5 (Fig. 5D-E”), inner ear hair cells at E14.5 (Fig. 5G-G”), and whisker barrel Merkel cells at E16.5 (Fig. 5H-H”). In hair cells and Merkel cells, Math5-GFP1 and Math1 highly overlap. However, Math5-GFP1 is expressed in only a subset of Math1+ cells in the dorsal spinal cord and lower rhombic lip (Fig. 5A”, inset 5B”,5D”, inset 5E”). At E10.5 and E12.5, Math1-expressing progenitors in the dorsal spinal cord and lower rhombic lip are located closer to the ventricular zone, while Math5-GFP1 cells lie more laterally and ventrally (Fig. 5A-B”, 5D-E”). We
hypothesized that these GFP-positive cells represent migrating cells of the Math1 lineage. In the spinal cord of E12.5 Math1LacZ embryos, migrating cells that no longer express Math1 protein do express the more stable βgal reporter (Ben-Arie et al., 2000). To determine whether these GFP+/Math1- cells in the dorsal spinal cord and lower rhombic lip had previously expressed Math1, we compared Math5-GFP1 and Math1LacZ expression in E12.5 Math5-GFP1;Math1LacZ/+ embryos. Indeed, GFP and βgal are largely coexpressed in the dorsal spinal cord and lower rhombic lip (Fig. 5C-C”, 5F-F”). Together, these findings indicate Math5-GFP expression in cells of the Math1-lineage in multiple expression domains.

Regulation of Math5-GFP1

Math5 and Math1 are semi-orthologues of Drosophila atonal that are expressed in non-overlapping domains in mice (Hassan and Bellen, 2000). Our observation that Math5-GFP is expressed in a subset of Math1 expression domains suggests that during vertebrate evolution one or more regulatory enhancers remain conserved between Math1 and Math5 regulatory DNA. Therefore, we investigated whether Math5-GFP1 might be regulated analogously to Math1 in the lower rhombic lip and dorsal spinal cord.

Math1 expression is controlled by two enhancers (A and B) located ~3 Kb 3’ to the Math1 translation stop site (Helms et al., 2000). These enhancers are required for the proper expression of Math1 in its normal pattern, and Math1 autoregulates its own expression in the dorsal neural tube by binding to a bHLH-specific E-box in Enhancer B (Helms et al., 2000). First, we tested whether Math1 might cross-regulate Math5-GFP1 in the lower rhombic lip. Extensive overlap of Math5-GFP and Math1LacZ was observed at E12.5 (Fig. 5F, 6A). In Math5-GFP1;Math1LacZ/LacZ mutants, GFP expression was obviously decreased in the lower rhombic lip
(compare 6C,C’ to 6A). While this indicates partial cross-regulation of Math1 upon Math5-GFP1, it also implies that other factors might regulate some aspects of the Math5-GFP hindbrain domain.

Math1 expression is also Pax6-dependent in the lower rhombic lip (Landsberg et al., 2005; Walther and Gruss, 1991), making Pax6 an attractive candidate to also regulate Math5-GFP. In the E12.5 wild type rhombic lip, Math5-GFP1- and Pax6-expressing cells exhibit some overlap (Fig. 6E). Therefore, we scrutinized Math5-GFP1 expression in E12.5 Math5-GFP1;Pax6Sey/Sey embryos, to determine if those Math5-GFP cells that do not require Math1 might instead require Pax6. Indeed, Math5-GFP1 expression is absent in the lower rhombic lip of Pax6-null mice (Fig. 6G). Together these data suggest that Math5-GFP transgenes have overlapping, but genetically separable, regulation by Math1 and Pax6 in the lower rhombic lip.

Then we examined Math5-GFP1 transgene regulation in the spinal cord. Again we tested for cross-regulation by Math1, since here we also found Math5-GFP coexpression with Math1^LacZ (Fig. 5D, 6B). In the dorsal spinal cord of Math5-GFP1;Math1^LacZ/LacZ mutant embryos, some Math5-GFP cells were still observed, although greatly reduced in number in the dorsal-most region (Fig. 6D). Furthermore, co-localization of GFP and βgal was nearly absent (compare Fig. 6D to 6B), indicating the loss of Math5-GFP1 expression specifically in the Math1-lineage. Although only a few spinal cord cells coexpress Pax6 and Math5-GFP1 (Fig. 6F), we compared Pax6 spinal cord regulation to compare this outcome with that for the hindbrain. In Pax6Sey/Sey embryos, Math5-GFP1 was still expressed throughout the spinal cord, although the number of GFP+ cells appeared somewhat reduced ventrally (Fig. 6H). To define the Math5-GFP1 ventral spinal cord domain better, we compared GFP expression to that of the transcription factors Pax2 and Islet1, which mark clusters of ventral interneurons and motor
neurons, respectively. In the ventral spinal cord, Math5-GFP largely colocalized with Pax2 (Fig. 6I), but exhibited no coexpression with Islet1 (6J). Pax6 is required for the generation of a population of Pax2+/En1+ ventral spinal cord interneurons (Burrill et al., 1997), meaning the loss of Pax6 may indirectly reduce the number of Math5-GFP1 cells in this domain. Overall, we conclude that Math5-GFP is regulated by both Math1 and Pax6 in the lower rhombic lip of the hindbrain, but only Math1-dependent in the dorsal spinal cord.

Finally, we asked whether Math5 and Math1 retain conserved nucleotide sequences by comparing Math5 5’ and Math1 3’ noncoding DNA. Figure 7A diagrams both Math5 5’ 2.1 Kb and Math1 3’ 4.5 Kb regulatory DNA. In the upstream region of Math5, two highly conserved regions among the Xenopus, mouse and human Ath5 genes are shown (yellow boxes in Fig. 7A)(Brown et al., 2002; Hutcheson et al., 2005; Riesenberg et al., 2007). Within these two evolutionarily conserved regions are four E-boxes, bHLH consensus binding sites (blue boxes) (Hutcheson et al., 2005; Murre et al., 1989). For Math1, Helms et al. (2000) demonstrated that two enhancers (A and B, located 3 Kb downstream) activate Math1 expression (green boxes in Fig. 7A,B). As mentioned, Math1 positively autoregulates its expression in the lower rhombic lip and spinal cord through one E-box binding site in Enhancer B (blue box). In addition, putative Pax6 paired-domain binding sites in Math5 and Math1 regulatory DNA were identified using the Transfac MATCH program (black and grey boxes in Fig. 7A,B). Overall, 20 putative Pax6 sites are predicted in 3 Kb upstream of the Math5 start codon (Riesenberg et al., 2007), but only those sites relevant for comparison with Math1 regulatory DNA are shown here (Fig. 7A-C). Math5 site 5-J, shown in black, is highly conserved among at least four vertebrate species and specifically binds Pax6 protein in vitro (Riesenberg et al., 2007). Additional predicted Pax6
binding sites depicted in grey for Math5 and Math1 DNA are listed in Figure 7C, along with their Transfac core score and the prediction matrix used.

To compare these Math5 and Math1 noncoding sequences directly, we performed a VISTA alignment of the Math5 5’ 2.1 Kb and Math1 3’ 1.5 Kb sequences (Fig. 7B). Many regions exhibited >50% identity, using 20 bp calculation windows. The relevant regulatory regions and putative binding sites from panel A are shown under the graphical depiction of this nucleotide alignment. Across this alignment four stretches of DNA contain ≥70% nucleotide identity. Interestingly, the Math5 distal conserved region, containing a retinal enhancer (Hutcheson et al., 2005; Riesenberg et al., 2007), lies within a long stretch of nonalignment between Math5 and Math1. Conversely, the most proximal Math5 E-box (E1) aligns with the Math1 autoregulatory E-box (Fig. 7B). This shared feature could account for conserved expression between Math1 and Math5-GFP1, via cross-regulation by Math1. Intriguingly, two pairs of predicted Pax6 binding sites (5-H to 1-1 and 5-T to 1-5) appear to be conserved between Math1 and Math5 regulatory DNA (Fig. 7B).

To test whether Pax6 can bind any of the predicted sites (grey boxes) in vitro, electrophoretic mobility shift assays (EMSAs), using a GST-Pax6 paired domain fusion protein, were performed on predicted Math5 sites 5-H, 5-S, 5-T and all five Math sites, using Math5 site 5-J as a positive control (Fig. 7D). Only sites 5-T and 1-4 were specifically bound by 0.5-1 µg Pax6 protein (Fig. 7D). When three of five core nucleotides were mutated within each site, Pax6 paired domain binding was completely lost (Fig. 7D). While the two pairs of putative Pax6 binding sites that aligned between Math5 and Math1 do not appear functional, both semi-orthologues are directly regulated by Pax6. In conclusion, we demonstrate correlation between
in vivo regulation of Math5-GFP1 and Math1 (by Pax6 and Math1) with bioinformatic and in vitro protein-DNA binding data.

**Discussion**

A new tool for the study of RGC axon outgrowth and visual system patterning

In this paper, we examined Math5 gene regulation in vivo using transgenic GFP reporter mice. We observed that Math5-GFP transgenes delineate migrating RGC axons from the developing retina into the brain. At E12.5, one day after RGC differentiation begins, we observed RGCs axons extended outside the eye. By 16.5 GFP-labeled RGC axons had arrived at their two major targets, the lateral geniculate nucleus and superior colliculus. Our transgenic mouse model offers multiple advantages for observing RGC axon outgrowth, including high specificity, in vivo labeling, and live fluorescence. In Math5-GFP embryos, RGC axons travel through developing brain tissue devoid of other GFP-expressing domains, allowing us to trace optic projections with great certainty. Because GFP expression is intrinsic within the axons, specificity is greater than either anterograde or retrograde RGC axon labeling techniques, and no surgical manipulations are needed. Finally, Math5-GFP expression in RGCs occurs in vivo during axonal outgrowth, guidance, and synaptogenesis, allowing for real-time visualization of these processes. In the future, Math5-GFP mice will be used to time-lapse image RGC outgrowth in retinal explants, retinal flat mount cultures and retinal-brain cocultures. We conclude that these transgenic mice are a valuable tool for understanding RGC axon pathfinding and visual system innervation of the developing brain.
What restricts Math5 expression to the developing retina and cochlear nucleus?

The upstream 2.1 Kb of Math5 noncoding DNA contains a retinal enhancer, but transgenic mice containing this DNA show no reporter expression in the cochlear nucleus, an endogenous expression domain of Math5 (Riesenberg et al., 2007; Saul et al., 2007). Therefore the cochlear nucleus enhancer is located in more distal Math5 regulatory DNA. Instead, we observed multiple ectopic Math5-GFP transgenic expression domains, at times and places where endogenous Math5 is not expressed. We did not anticipate that these upstream Math5 DNA sequences are capable of driving GFP reporter expression in the lower rhombic lip, spinal cord, inner ear, whisker barrels, pontine nucleus, molars, pineal gland, and neocortex. Importantly, none of these ectopic expression domains were found in multiple Math5 lineage studies (Brzezinski, 2005; Saul et al., 2007; Yang et al., 2003), in Math5LacZ targeted deletion mice (Brown et al., 2001; Wang et al., 2001), or by extensive in situ hybridization experiments analyzing Math5 mRNA expression from E10.5 to birth (Brown et al., 1998; Brown et al., 2001). Because both Math5-GFP transgenes display the same deregulation, the regulatory DNA responsible for suppressing endogenous Math5 must be more distal. Interestingly, the addition of the 1.6 Kb of 3’ Math5 noncoding DNA in Math5-GFP2 was sufficient to silence ectopic GFP expression in inner ear hair cells, suggesting that the elements that keep Math5 off in Math1 domains may reside 3’ to the Math5 coding exon. Future experiments will test additional Math5 5’ and 3’ DNA to identify the cochlear nucleus enhancer and those elements that normally mask Math1-like expression.
Regulatory conservation in the evolutionary divergence of bHLH factors

The bHLH protein domains of Math5 and Math1 are as closely related to each other as they are to that of Drosophila atonal (Brown et al., 1998; Hassan and Bellen, 2000). In the fly, atonal is expressed in the developing eye and specifies the first retinal neuron, R8 (Jarman et al., 1993). Both the fly R8 photoreceptor neuron and vertebrate RGC are the first retinal neurons to differentiate and project axons that innervate the brain. Atonal is also expressed in chordotonal organs, which process proprioceptive information during larval movement, and in Johnston’s organ that functions analogously to the mammalian auditory system (Jarman et al., 1993). Math1 is expressed in several components of the proprioceptive pathway in mouse, and in auditory hair cells of the inner ear (Bermingham et al., 1999; Bermingham et al., 2001). Here we report Math5\textsuperscript{LacZ} expression in the cochlear nucleus, a component of the auditory system. In this manner, the functions of atonal appear to have been divided between Math5 and Math1, with Math5 in vision, Math1 in the proprioceptive system, and both Math5 and Math1 in auditory processing.

Our Math5 transgenes are expressed in multiple Math1 domains in the auditory and proprioceptive systems. Math5-GFP1 is expressed in Math1-lineages of the spinal cord, lower rhombic lip, inner ear hair cells, and whisker barrel Merkel cells. Moreover, Math5-GFP1 is regulated in a similar manner to Math1. Math1 is regulated by Pax6 in the lower rhombic lip and autoregulated in the dorsal neural tube (Helms et al., 2000; Landsberg et al., 2005). Similarly, Math5-GFP1 expression is absent in the lower rhombic lip of Pax6-null embryos, and coexpression of Math5-GFP and Math1\textsuperscript{LacZ} is reduced in the lower rhombic lip and dorsal spinal cord of Math1-null embryos. These changes are unlikely caused by the loss of a progenitor cell population, as Math1-expressing cells likely remain undifferentiated or switch fates in the
absence of Math1 in the rhombic lip and spinal cord, or Pax6 in the rhombic lip (Ben-Arie et al., 1997; Ben-Arie et al., 2000; Bermingham et al., 2001; Landsberg et al., 2005; Machold and Fishell, 2005; Wang et al., 2005a). Alternatively, the effects of Pax6 loss on Math5-GFP lower rhombic lip expression might be due to the loss of Math1. However, unlike Math5-GFP1, Math1 is reduced but not absent in the rhombic lip of Pax6-nulls, indicating that Pax6 regulates Math5-GFP1 in part via a Math1-independent mechanism. All together, regulatory similarities found between Math5-GFP1 and Math1 further indicate that Pax6 regulation and autoregulation are well-conserved features of the atonal gene family (Sun et al., 1998; Zhang et al., 2006).

Math1 expression is largely controlled by two 3’ enhancers, identified by their ability to direct LacZ reporter gene expression in vivo (Helms et al., 2000). Subsequently, a slightly smaller 3’ DNA fragment, containing most, but not all, of the Math1 enhancers was used to create a Math1-GFP transgene (Lumpkin et al., 2003). Intriguingly, Math1-GFP expression was reported in non-Math1 domains, including the retina, suggesting the unmasking of a retinal Math1 enhancer in this particular GFP transgenic construct. That both the Math5-GFP1 and Math1-GFP2 transgenes are capable of expression in the reciprocal gene’s pattern further suggests that the Math5 5’ and Math1 3’ regulatory DNA retain conserved regulatory motifs. By contrast, Math5-GFP1 is also expressed in regions not associated with endogenous Math1 expression, including the ventral spinal cord, forebrain, pineal gland, and molars. As such, the Math5 5’ DNA may contain pan-proneural activation elements in common with other bHLH factors, such as Mash1, Ngn1, Ngn2, or NeuroD. Interestingly, all of these genes, except NeuroD, genetically require Pax6 (Blader et al., 2004; Marquardt et al., 2001). The activation of Ngn1 and Ngn2 is directly regulated by Pax6 binding to particular CNS enhancers (Blader et al., 2004; Marquardt et al., 2001; Scardigli et al., 2003).
Our bioinformatic and EMSA analyses of these regulatory sequences yielded several interesting findings. First, the distal conserved region of Math5, which contains a retinal enhancer (Riesenber et al., 2007) does not align to the Math1 enhancer sequence, suggesting that the retinal enhancer for Math5 may have been added after these genes duplicated and diverged, or it was subsequently lost from Math1. Second, the Math1 autoregulatory E-box, that maintains expression in the dorsal spinal cord, and the Ath5 E1 E-box, required for maintenance of retinal expression in frog and chick (Hutcheson et al., 2005; Skowronska-Krawczyk et al., 2004), align to one another, providing one explanation for Math1 cross-regulation of our transgenes. Finally, we demonstrate functional Pax6 binding sites for both Math5 and Math1, which could account for the similar regulation of Math5-GFP and Math1 by this factor. Although the other predicted Pax6 binding sites do not demonstrate Pax6 binding, some may yet turn out to be functional, but require tissue-specific cofactors absent from our in vitro experiments.

The duplication and divergence of Math5 and Math1 during vertebrate evolution resulted in tissue-specific, restricted expression of each gene that, when added together, recapitulates the expression domains of Drosophila atonal. As the vertebrate nervous system expanded and elaborated, these genes became segregated to mammalian visual, auditory, and proprioceptive systems. We propose one mechanism of evolutionary divergence may have occurred at the level of cis-regulation, where common enhancer elements within the Math1 and Math5 genes became silenced by an as yet unknown repressive mechanism. It is possible that these elements are also present in other vertebrate bHLH atonal gene homologues. Future elucidation of gene-specific repressor sequences, the factors that bind to them, and the overall mechanism by which semi-
orthologous genes develop complementary expression patterns will contribute important information to the evolution of the vertebrate nervous system.
Notes to Chapter 2

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Figure Legends

Figure 2.1. *Math5*-GFP transgenic reporters. Diagram of the *Math5* gene, *Math5<sup>LacZ</sup>* targeted deletion and the *Math5*-GFP1 and *Math5*-GFP2 transgenes. The 5’ DNA common to both transgenes was generated from an EcoRI to PstI 2.1 Kb fragment. The 3’ DNA contained in the *Math5*-GFP2 transgene was generated from a PstI to PstI 1.6 Kb fragment.

Figure 2.2. *Math5*-GFP1 expression in the developing visual system. *Math5*-GFP1 mice express GFP in developing RGCs and their axons. At E16.5, live embryo fluorescence and immunolabeling of GFP in the retina (A), optic nerve, chiasm and tract (B), and the major RGC targets, the superior colliculus (D) and lateral geniculate nucleus (C,E), reveals the entire length of RGC axons. The boxed area in C is shown at higher magnification in E. F is a diagram of the optic tract and its major innervation targets at E16. The progression of RGC axon migration and optic nerve formation can be followed from E12-E16 (G-I). Magnification bars in A (1 mm), B (800 µm), C (400 µm), D (50 µm), E (200 µm), G (200 µm), and I (100 µm). gcl- ganglion cell layer; lg- lateral geniculate nucleus; on- optic nerve; oc- optic chiasm; ot- optic tract; sc- superior colliculus.

Figure 2.3. Comparison of *Math5*-GFP and *Math5<sup>LacZ</sup>* expression in the retina. (A-D) *Math5*-GFP1; *Math5<sup>LacZ</sup><sup>+/+</sup> retinal cryosections co-labeled for GFP and βgal expression. At E11.5 and E12.5, βgal+ cells co-localize with *Math5*-GFP (A,B arrows), and some GFP+ only cells are evident. At E16.5, *Math5*-GFP1 and *Math5<sup>LacZ</sup>* are coexpressed in a subset of retinal cells (C, arrows), while others express GFP or βgal alone. At P1, few co-labeled cells are observed (D, arrows). Also, more GFP+ cells reside in the neuroblast and developing photoreceptor layer at
P1, while more βgal+ cells are in the ganglion cell layer. (E) Math5-GFP2 is expressed in the retina in a similar pattern to Math5-GFP1 at E16. Sclera is at the top of all panels. Magnification bars in A,B (25 μm), C,E (50 μm) and D (100 μm). gcl- ganglion cell layer.

**Figure 2.4.** Ectopic Math5-GFP expression in the developing nervous system. Math5-GFP1; Math5\textsuperscript{LacZ/+} embryos co-labeled for GFP and βgal. GFP expression (arrows) was observed in discrete domains devoid of Math5\textsuperscript{LacZ} expression, including the spinal cord (A,A’) and lower rhombic lip (C,C’) at E12.5, inner ear hair cells at E14.5 (G,G’), pontine nuclei (E,E’) and whisker barrel cells (I,I’) at E16.5. The spinal cord, lower rhombic lip, and pontine expression domains are also observable using live fluorescence (B,D,F). Math5\textsuperscript{LacZ} is expressed in the cochlear nucleus (arrowhead in K’,K”) and axons of cochlear nucleus neurons, which project to the lateral lemniscus and trapezoid body (J). Math5-GFP1 is not found in the cochlear nucleus neurons or axons (arrow in K,K”; J). The Math5-GFP2 pattern is identical to Math5-GFP1, except for the absence of GFP expression in inner ear hair cells (H). Magnification bars in A (50 μm), B (1 mm), C (100 μm), D (400 μm), E (50 μm), F (800 μm), and I (100 μm). 4V- fourth ventricle; cn- cochlear nucleus; hc- inner ear hair cell; ll- lateral lemniscus; lrl- lower rhombic lip; pn- pontine nucleus; tb- trapezoid body; wb- whisker barrels.

**Figure 2.5.** Math5-GFP1 is expressed in cell lineages of Math1. (A-B”,D-E””) Colabeling of cryosections from Math5-GFP1 embryos. GFP and Math1 proteins are coexpressed in cells of the E10.5 and E12.5 spinal cord (A-B”, arrows in A” and B” inset) and lower rhombic lip (D-E”, arrows in D” and E” inset). In E12.5 Math5-GFP1; Math1\textsuperscript{LacZ/+} embryos, the high degree of βgal-GFP co-labeling in the spinal cord (C-C””) and lower rhombic lip (F-F””) demonstrates Math5-
GFP1 expression within the Math1 cell lineage. (G-H") Math5-GFP1 and Math1^{LacZ} are also coexpressed in hair cells of the inner ear and Merkel cells of whisker barrels (arrows in G” and H”). Magnification bars in A,D,G (25 µm), B,C,E,F (50 µm), H (100 µm). 4V- fourth ventricle; hc- hair cells; lrl- lower rhombic lip; mc- Merkel cells.

**Figure 2.6.** Math5-GFP1 expression is regulated by Pax6 and Math1 in the lower rhombic lip and spinal cord. (A-D) E12.5 Math5-GFP1; Math1^{LacZ/+} and Math5-GFP1; Math1^{LacZ/LacZ} embryos were assessed for GFP expression in the lower rhombic lip and dorsal spinal cord. A and B demonstrate nearly complete co-localization of GFP and βgal proteins in Math1 heterozygotes. Math5-GFP1 expression, particularly coexpression with βgal+ cells is decreased in the lower rhombic lip (C,C’) and dorsal spinal cord (D) of Math1-null embryos. C is the same magnification as A, while C’ is a higher magnification. (E-H) E12.5 Math5-GFP1;Pax6^{+/+} and Math5-GFP1;Pax6^{sey/sey} mice were analyzed for GFP expression in the lower rhombic lip and dorsal spinal cord. GFP and Pax6 are coexpressed in particular neurons of the lower rhombic lip (E) and spinal cord (F) at E12.5 (arrows in E and F). In Pax6-null embryos, Math5-GFP1 expression is absent in the lower rhombic lip (G), but is expressed in the spinal cord (H). (I-J) In the ventral spinal cord, GFP-positive cells fall mainly within the Pax2 domain (I) and outside the Islet1 domain (J). Magnification bars in A (100 µm) and B (50 µm). 4V- fourth ventricle.

**Figure 2.7.** Comparative analyses of Math5 and Math1 regulatory DNA. (A) Two phylogenetically conserved regions (yellow boxes) in 2.1 Kb of Math5 DNA 5’ to the start codon. Blue boxes E1-E4 represent four conserved E-box binding sites reported in Hutcheson et al. (2005) and Riesenber et al. (2007). The black box 5-J denotes a functional Pax6 binding site.
Grey boxes 5-H, 5-S, and 5-T are putative Pax6 binding sites in Math5 5' regulatory DNA. The two 3' Math1 enhancers are located ~3 Kb downstream of the Math1 stop codon. The Math1 auto-regulatory E-box resides in Enhancer B (Helms et al., 2000). Grey boxes 1-1 to 1-5 are predicted Pax6 binding sites in Math1 3’ DNA. (B) VISTA analysis comparing 2.1 Kb of Math5 5’ DNA (X-axis) with the 1.6 Kb Math1 3’ enhancers (Y-axis), utilizing a 20 bp calculation window. Several regions contain ≥70% nucleotide identity (pink shading). Immediately below, the position of Math1 and Math5 enhancers and putative Pax6 or bHLH binding sites is indicated, including gaps of nonalignment. Notably, the Math5 E1 and Math1 auto-regulatory E-boxes align, while the Math5 distal conserved region (containing a retinal enhancer) lies within a stretch of nonalignment between Math5 and Math1.

(C) The nucleotide sequences of predicted Pax6 binding sites, with core nucleotides underlined, along with the Transfac Core score and the prediction matrix used. (D) EMSA of GST-Pax6 paired domain fusion protein with binding sites 5-J, 5-T, and 1-4, with 5-J serving as a positive control. For each binding site, the left lane contains free annealed ds probe, the second lane probe and 1 µg GST protein, and the next three lanes probe and 0.1, 0.5 or 1.0 µg of GST-Pax6. Pax6 binding to site 1-4 is the weakest, since this gel shift was exposed to x-ray film 5 times longer than the others. However, specific binding is lost at all three sites when 3/5 core nucleotides are mutated (red bases in J-Mut, T-Mut, 1-4Mut).
Figure 2.3

Math5-GFP1  Math5^{LacZ/+}  Merged

A E11  

B E12  

C E16  

D P1  

E E16  

Math5-GFP2  Math5^{LacZ/+}  Merged

E11  

E12  

E16  

P1  

E E16  

Scale bars: 100 μm
Figure 2.7

A

Math5 5' Regulatory DNA

Distal conserved region

E4 E3 E2 E1

E-boxes

Proximal conserved region

Math1 3' Regulatory DNA

Stop Codon

Enhancer A

Enhancer B

Start Codon

B

Math5 5' 2.1 Kb vs. Math1 3' 1.5 Kb: Criteria 70% ± 20 or 30 bp blocks

100%

50%

0%

C

Predicted Pax6 Paired Domain Binding Sites

Math5

5-H: GCAAACTTTCGAGAAGACCTT

5-S: GAGAAGTCAAAGG

5-T: CACTTCCTCAGCAT

Transfacc Core Score Transfacc Matrix

Math1

1-1: CGCGCAATGAA

1-2: TTGCTTTGAAATAT

1-3: CTAGACTGAGAGCTAGATC

1-4: AAATTCCTGAGAGAC

1-5: CTTGCTTCCCTCC

1.000 VSPAX6.Q2

1.000 VSPAX6.Q2

0.722 VSPAX6.Q1

0.861 VSPAX6.Q2

0.918 VSPAX6.Q6

D

Probes: 5-J, 5-T, 1-4

GST GST:Pax6

Protein Gel Shift Assay

Sequence Tested

Math5 J

Math5 J Mut

Math1

Math1 T Mut

Math1 1-4

Math1 1-4 Mut

AGAATGGTGAACAGGGAGAGCA

AGAATGGTGAACAGGGAGAGCA

TTCTCTGGGCTTGAGAGAGCA

TTCTCTGGGCTTGAGAGAGCA

TATTCACCTGATTTGCTGGTCTAGCTG

TATTCACCTGATTTGCTGGTCTAGCTG
CHAPTER 3

Neurog2 controls the leading edge of neurogenesis in the mammalian retina
Abstract

In the mammalian retina, neuronal differentiation begins in the dorso-central optic cup and sweeps peripherally and ventrally. While certain extrinsic factors have been implicated, little is known about the intrinsic factors that direct this process. In this study, we evaluate the expression and function of proneural bHLH transcription factors during the onset of mouse retinal neurogenesis. Dorso-central retinal progenitor cells that give rise to the first postmitotic neurons express Neurog2/Ngn2 and Atoh7/Math5. In the absence of Neurog2, the spread of neurogenesis stalls, along with Atoh7 expression and RGC differentiation. However, neurogenesis is eventually restored, and at birth Neurog2 mutant retinas are reduced in size, with only a slight increase in the retinal ganglion cell population. We find that the re-establishment of neurogenesis coincides with the onset of Ascl1 expression, and that Ascl1 can rescue the early arrest of neural development in the absence of Neurog2. Together, this study supports the hypothesis that the intrinsic factors Neurog2 and Ascl1 regulate the temporal progression of retinal neurogenesis by directing overlapping waves of neuron formation.
Introduction

Visual processing in the retina depends on proper functioning of multiple neural classes. Thus, determining how this neuronal diversity arises is critical for understanding retinal function. Seven major retinal cell classes are generated between embryonic day (E) 11 and postnatal day (P) 10 in the mouse, in a conserved temporal order (Sidman, 1961; Young, 1985). In vertebrates, retinal ganglion cells (RGCs) differentiate first, as a wave front across the neuroepithelium of the optic cup (Easter, 2000; Holt et al., 1988; Masai et al., 2000; McCabe et al., 1999). In zebrafish, this wave begins near the optic stalk and radiates outward (Hu and Easter, 1999). In avians, the first RGCs appear in the dorsal-central retina, and neurogenesis simultaneously spreads peripherally and ventrally (Prada et al., 1991). Multiple extrinsic signals, including FGFs and sonic hedgehog, are required for the spatiotemporal progression of retinal neurogenesis (Jensen and Wallace, 1997; Macdonald et al., 1995; Martinez-Morales et al., 2005; McCabe et al., 1999; Neumann and Nuesslein-Volhard, 2000; Perron et al., 2003; Picker and Brand, 2005). However, little is known about the intrinsic factors that regulate this process.

The basic-loop-helix (bHLH) transcription factors, including Atoh7/Ath5, Ascl1/Ash1, Neurog2/Ngn2, and Neurod1, regulate multiple facets of neurogenesis, including cell cycle exit, neural versus glial determination, subtype specification, and survival (Ohsawa and Kageyama, 2008). Among the first proneural bHLHs expressed in the vertebrate retina, Atoh7 (atonal homologue 7) appears at the onset of retinal neurogenesis in the dorso-central mouse retina, and loss-of-function mutations result in the reduced differentiation of early progenitor cells and nearly complete loss of RGCs (Brown et al., 1998; Brown et al., 2001; Kanekar et al., 1997; Kay et al., 2001; Matter-Sadzinski et al., 2001; Wang et al., 2001). The vertebrate bHLH factor, Neurog2 (also an atonal homologue) is expressed during early retinogenesis (Brown et al., 1998;
Ma and Wang, 2006). In the chick eye, Neurog2 can genetically activate Atoh7 and transdifferentiate cultured RPE cells into immature RGCs and photoreceptors (Matter-Sadzinski et al., 2005; Yan et al., 2001). By contrast, X-ngnr-1, a Xenopus Neurog2 homologue, promotes photoreceptor but not RGC formation (Perron et al., 1999). Recently, Neurog2/Ngn2 was demonstrated to bind to 5’ regulatory DNA and activate Atoh7/Ath5 transcription using distinct species-specific mechanisms in the mouse versus chick retina (Skowronska-Krawczyk et al., 2009). However, no individual role for Neurog2 has been uncovered, particularly in the mammalian retina (Akagi et al., 2004; Skowronska-Krawczyk et al., 2009).

In this report, we investigate intrinsic elements controlling the spatial and temporal onset of retinal neurogenesis, and define a novel role for Neurog2 during the outward expansion of retinal neurogenesis. Neurog2 and Atoh7 are simultaneously activated in cells that give rise to the first RGCs. Neurog2 is required for the spatial and temporal progression of both the expanding wave front and Atoh7 expression, but the resulting delay of neurogenesis is transient. The onset of Ascl1, a later-expressed bHLH factor, coincides with the restoration of retinal neurogenesis, and rescues neural differentiation in the absence of Neurog2. Together, these data demonstrate a critical role for bHLH factors in both propagating and maintaining the spatial and temporal progression of mammalian retinogenesis.

Materials and Methods

Animals

Neurog2\textsuperscript{GFP} mice (Seibt et al., 2003) were maintained on an ICR background, and Atoh7\textsuperscript{LacZ} (Brown et al., 2001), Ascl1\textsuperscript{KO/+} (Tomita et al., 1996) and Neurog2\textsuperscript{Ascl1KI} mice (Fode et al., 2000) on a CD-1 background. For double-mutant studies, mice were bred together for a
minimum of two generations. PCR genotyping was performed as described (Brown et al., 2001; Fode et al., 2000; Seibt et al., 2003; Tomita et al., 1996).

For embryonic studies, gestational age was determined by timed matings, with the date of the vaginal plug as E0.5. For somite counted embryos, 4-6 hour timed matings were carried out to precisely correlate somite number with gestational age. BrdU pulse labeling was performed by injecting pregnant dams with BrdU (0.1mg/g body weight of 10mg/mL BrdU in 0.9M NaCl) and harvesting embryos after 1.5 hours. P0.5 pups were collected on the morning after birth.

**Immunohistochemistry and in situ hybridization**

Immunohistochemistry was performed as described (Hufnagel et al., 2007). Antibodies used were rabbit anti-βIII-tubulin (Tubb3) (1:1000, Covance), rabbit anti-βgal (1:10000, Cappel), rat anti-βgal (1:1000, gift from Tom Glaser), rat anti-BrdU (1:100, AbD Serotec), goat anti-Pou4f2/Brn3b (1:50, Santa Cruz), rabbit anti-activated Caspase 3 (1:100, Cell Signaling), sheep anti-Chx10 (1:1000, Exalpha Biologicals), rabbit anti-GFP (1:1000, Molecular Probes), rabbit anti-Ascl1 (1:1000; Horton et al., 1999), rabbit anti-Neurog1 (1:1000, Gowan et al., 2001), rabbit anti-Neurog2 (1:1000), mouse anti-Neurog2 (1:10, Lo et al., 2002), mouse anti-Neurog3 (1:100, DSHB), mouse anti-p27 (1:200, Thermo scientific), rabbit anti-Pax6 (1:1000, Covance), rabbit anti-Pax2 (1:1000, Covance), mouse anti-AP2α (1:500, DSHB), rabbit anti-Prox1 (1:1000, Covance), rabbit anti-RXRγ (1:200, Santa Cruz), and rabbit anti-Sox2 (1:1000, Chemicon). Direct-conjugate secondary antibodies (Molecular Probes) or sequential biotinylated secondary (Jackson Immunoresearch) and streptavidin-conjugated Alexafluor tertiary antibodies (Molecular Probes) were used to visualize primary antibody labeling.
**In situ** hybridization was performed as described (Wallace and Raff, 1999). Briefly, embryos were collected and fixed in 4% PFA/PBS overnight, then cryoprotected in 30% sucrose overnight, embedded in 50:50 OCT:30% sucrose, and sectioned at a thickness of 10µm. DIG-labeled antisense *Atoh7, Ascl1*, and *Neurod1* probes were hybridized to retinal sections overnight, detected with sheep anti-DIG antibody (1:2000; Roche), and developed with NBT and BCIP.

**Measurements and cell counting**

Microscopy was performed with a Zeiss fluorescent microscope, Zeiss camera and Apotome deconvolution device. For all retinal measurements or cell counts, a minimum of 3 embryos or postnatal pups per genotype from ≥2 independent litters were analyzed, matched for somite number across genotypes. Cell counts and measurements were performed using the Zeiss Axiovision software (v5.0), using the interactive events and curve spline tools. The circumference of the Tubb3 expression was compared to the circumference of the Neurog2-GFP domain and the total outer circumference from 4 images per animal, representing both eyes, containing the optic nerve or within 50µm dorsal to the optic nerve. The percentages of BrdU+/DAPI, act Caspase+/DAPI, RXRγ+/DAPI, Pou4f2+/DAPI, AP2α+/DAPI, Prox1+/DAPI nuclei were determined in 200X fields within in the central retina. Either a paired Student’s T test with Welch posthoc test or ANOVA with Tukey-Kramer posthoc test was used to determine p values (Instat Software, v3.0). Photoshop (v7.0) was used to adjust equally the brightness and contrast of images among different genotypes.
Results

Comparison of Ngn2 and Atoh7 expression during early retinogenesis

During the initiation of retinal neurogenesis, progenitor cells exit the cell cycle, express general neuronal markers, and commit to a single cell fate. Among vertebrates, RGCs appear first (Altshuler et al., 1991), initially in the dorso-central retina of avians and mammals. In the chick retina, neurogenesis spreads in simultaneous central-peripheral and dorsal-ventral gradients, and is regulated partly by FGF signaling (McCabe et al., 1999; Prada et al., 1991). An analogous wave front in the mammalian retina has not been described, so we sought to understand the spatiotemporal kinetics of this process in the mouse eye, and test the hypothesis that bHLH factors Neurog2/Ngn2 and/or Atoh7/Math5 regulate the initial neurogenic wave.

The first Atoh7-expressing cells are found in the dorso-central retina at E11.0, preceding the appearance of RGCs that critically require this factor (Brown et al., 1998; Brown et al., 2001; Wang et al., 2001). Neurog2 expression has also been reported to appear around the time of neurogenesis initiation in the early chick and mouse retina (Ma and Wang, 2006; Matter-Sadzinski et al., 2005). First, we compared the expression pattern of Neurog2/Ngn2 to Atoh7/Math5 and the initial spread of retinal neuron differentiation. To correlate Neurog2 and Atoh7 expression directly, we assessed the onset of Atoh7\textsuperscript{LacZ} with that of Neurog2\textsuperscript{GFP} and Neurog2 protein expression (Brown et al., 2001; Seibt et al., 2003), by antibody double labeling of retina sections from double-heterozygous animals (Neurog2\textsuperscript{GFP/+},Atoh7\textsuperscript{LacZ/+} mice; Fig. 1A,B), which are identical to wild types (not shown). Brief timed matings (4-6 hours) were used to precisely correlate gestational ages of somite-counted E10.75-E12.0 embryonic litters. Prior to E11.0, Neurog2 protein and Neurog2\textsuperscript{GFP} expression were localized to the ventral thalamus and presumptive optic stalk, but excluded from the retina (Fig. 1C and data not shown). The earliest
retinal Neurog2+ and GFP+ cells were found at E11.0 in the dorso-central retina (43 somites, Fig 1E,E’). From E11.0-E11.5 (43-50 somites), 6 of 11 embryos contained both Neurog2+ and Neurog2\textsuperscript{GFP}+ cells, indicating that retinal onset of Neurog2 does not precisely correlate with somite number. All embryonic retinas at E11.75 (51-60 somites) contained Neurog2+/GFP+ cells.

Next, we asked if the onset of Neurog2 or Atoh7 expression precedes the other. We performed antibody labeling in double-heterozygous mice (Neurog2\textsuperscript{GFP}/+,Atoh7\textsuperscript{LacZ/+}) and Atoh7 mutants (Neurog2\textsuperscript{GFP}/+,Atoh7\textsuperscript{LacZ/LacZ}), since bi-allelic expression of Atoh7\textsuperscript{LacZ} enhanced the detection of βgal+ cells. We do not observe either Neurog2\textsuperscript{GFP}+ or βgal+ retinal cells prior to 43 somites (Fig. 1D), although co-labeled GFP+ and βgal+ cells were noted in the diencephalon (arrow, Fig. 1D). Neurog2\textsuperscript{GFP} and Atoh7\textsuperscript{LacZ} were extensively coexpressed in the mouse dorso-central retina at E11.0 (arrows, Fig. 1F,G). At E11.75 and E12.5, both Neurog2 and Atoh7 expression had expanded peripherally, with a bias towards the temporal/caudal retina (Fig. 1H,I). The Neurog2\textsuperscript{GFP} domain always extended more peripherally and encompassed more cells than the Atoh7\textsuperscript{LacZ} domain (GFP+/βgal– region in brackets, Fig. 1I). At all ages examined, virtually all βgal+ cells were also GFP+ (arrows, Fig. 1F-I), indicating Atoh7\textsuperscript{LacZ} was expressed in a subset of Neurog2\textsuperscript{GFP}+ cells.

To further examine the coincidence between Neurog2 and Atoh7, we compared the pattern of Neurog2 protein with Atoh7\textsuperscript{LacZ} and Neurog2\textsuperscript{GFP}. Neurog2 is largely present in S-phase progenitor cells (Fig. 1K; Ma and Wang, 2006; Yan et al., 2001). Atoh7/Ath5 is not expressed during S-phase (Fig. 1J) (Le et al., 2006; Poggi et al., 2005), and has been extensively reported to be expressed by late G2/M phase and postmitotic retinal cells (Brown et al., 1998; Le et al., 2006, Brzezinski, 2005; Yang et al, 2003). This implies that, in mitotically active retinal
progenitor cells, Neurog2 expression in S-phase precedes that of Atoh7. Consistent with this difference, very few cells co-labeled with Neurog2 and βgal proteins (fuchsia and white arrows, Fig. 1L). The extensive overlap of Neurog2\textsuperscript{GFP} and Atoh7\textsuperscript{LacZ} likely occurs because Neurog2\textsuperscript{GFP} persists longer than Neurog2 protein, thereby acting as a short-term lineage tracer (Britz et al., 2006). Indeed, while all Neurog2+ cells co-express Neurog2\textsuperscript{GFP} (fuchsia and white arrows, Fig. 1M), many GFP+/Neurog2– cells are present (yellow and white arrowheads, Fig. 1M). We conclude that Neurog2 and Atoh7 simultaneously initiate expression in dorsal-central retinal progenitor cells at E11.0, but at distinct phases of the mitotic cell cycle.

Neurog2\textsuperscript{GFP} precedes the expansion of neurogenesis and RGC specification

Prior to retinogenesis, the optic vesicle becomes compartmentalized into the neural retina, RPE, and optic stalk. Optic vesicle cells initially co-express the paired-homeobox transcription factors Pax6 and Pax2 (Baumer et al., 2003; Schwarz et al., 2000). Pax2 is subsequently downregulated in the neural retina, but not Pax6 (Baumer et al., 2003). Importantly, Pax6 directly activates Atoh7 and Neurog2 (Marquardt et al., 2001; Riesenberg et al., 2009; Willardsen et al., 2009). Before the onset of Neurog2\textsuperscript{GFP} expression from E11.0-11.5, Pax2+ cells were detected throughout the optic cup and stalk (Fig. 2A). By E11.75, after Neurog2\textsuperscript{GFP} onset in the retina, Pax2 protein was restricted to the optic stalk and central-nasal optic cup, in GFP-negative cells (Fig. 2B,C). The Neurog2\textsuperscript{GFP} domain bordered that of Pax2, and very few GFP+/Pax2+ cells were noted (arrow, Fig. 2C). Therefore, Pax2 downregulation precedes the initiation of Neurog2 expression in the presumptive neural retina. This pattern of Pax2 expression was unchanged in Neurog2 mutants (not shown), indicating that Neurog2 does not suppress Pax2 retinal expression. Pax6 protein was co-expressed with all GFP+ cells at this
age (Fig. 2D). *Neurog2*-GFP also co-localized with Sox2 and Chx10/Vsx2 proteins (not shown), two other transcription factors required for normal retinal progenitor differentiation (Burmeister et al., 1996; Taranova et al., 2006).

Next, we directly compared *Neurog2*-GFP expression with the onset and expansion of retinal neuron differentiation. Co-labeling for GFP and Tubb3 (βIII-Tubulin), a neural-specific marker (Brittis et al., 1995; Lee et al., 1990), revealed no differentiating retinal neurons prior to *Neurog2*<sup>GFP</sup> onset (Fig. 2E). Pou4f2/Brn3b, a marker of specified RGCs (Xiang et al., 1993), and p27/Kip1, a cyclin-dependent kinase inhibitor that promotes cell cycle exit of retinal progenitor cells (Dyer and Cepko, 2001a; Levine et al., 2000), were also absent prior to *Neurog2* onset (not shown). From E11.0-11.5, the first Tubb3+ and p27+ cells were detected in *Neurog2*-GFP+ retinal cells (Fig. 2F and not shown). By E11.75, the Tubb3 domain extended from the dorsal to central retina (Fig. 2I,J) but was not present ventral to the forming optic nerve (Fig. 2K). From E12.0-E13.5, the Tubb3+ region expanded peripherally and ventrally, with bias towards the temporal retina (Fig. 2G,H). The spread of the *Neurog2* domain preceded that of neural differentiation, indicated by the peripheral subdomain of GFP+/Tubb3– cells (brackets, Fig. 2G,J), which likely represents proliferating cells that subsequently differentiate into retinal neurons. Differentiating neurons highly coexpressed Tubb3 and p27 (Fig. 2L), verifying the concurrence of cell cycle exit and neural differentiation in the earliest retinal neurons. Essentially all Tubb3+ and p27+ cells co-labeled with *Neurog2*<sup>GFP</sup> (Fig. 2F-J and not shown). At E11.75, the first GFP+/Pou4f2+ RGCs were detected in the dorso-central retina, proximal to the leading edges of the *Neurog2*<sup>GFP</sup> and Tubb3 domains (Fig. 2M and not shown). No Pou4f2+ cells were detected prior to E11.75 (not shown). We conclude that the onset and peripheral
expansion of *Neurog2* expression precedes the initiation of neurogenesis and subsequent differentiation of the first RGCs.

From E11.0-E13.5, neurogenesis spreads outward across the neural retina, excluding the optic nerve head and peripheral retina that give rise to the ciliary body and iris (Rodieck, 1998). Since *Neurog2* expression correlates with the onset of neural differentiation, we predicted that *Neurog2* expression would only be present in cells undergoing retinal neurogenesis. From E13.5 to birth, *Neurog2*\textsubscript{GFP} co-localizes with Tubb3+ cells in the neuroblastic layer (NBL) and the inner forming ganglion cell layer (GCL), excluding the optic nerve head and presumptive ciliary body (Fig. 2H, 4B-C and not shown). Previous analysis of the *Neurog2*-lineage revealed that *Neurog2*-expressing cells are capable of adopting all the retinal fates (Ma and Wang, 2006). While that study found RGCs arise from the *Neurog2*-lineage starting at E14, here we found GFP+/Pou4f2+ RGCs much earlier, at E11.75 (Fig. 2M), suggesting that *Neurog2*\textsubscript{GFP} acts as a short-term lineage-tracer without the delay of Cre-mediated reporter activation by *Neurog2*\textsubscript{CreER} (Ma and Wang, 2006). We then compared GFP expression with markers of other embryonic retinal cell types: cones (RXR\textgamma), horizontals (Prox1), and amacrine (AP2\alpha) (Dyer et al., 2003; Mori et al., 2001; Yan and Wang, 2004). RXR\textgamma+/GFP+ cone photoreceptors were detected in the outer retina at E13.5 (arrows, Fig. 2N), also with a bias for the temporal retina. GFP+ amacrine (Ap2\alpha+) and horizontal (Prox1+) interneurons were also noted in the prenatal retina (Fig. 2O, P and data not shown).

*Neurog2* is required for the peripheral expansion of retinal neurogenesis

*Neurog2* expression at the leading edge of retinal neurogenesis precedes the expansion of *Atoh7*, neural commitment, and RGC differentiation. Therefore, we asked if *Neurog2* is required
for the peripheral propagation of neural development. GFP is still expressed in the absence of Neurog2 (Neurog2<sup>GFP/GFP</sup>, Fig. 3B), and marks the lineage of Neurog2-mutant cells. Thus, Neurog2<sup>GFP</sup> allows for comparison of the peripheral extent of reporter-expressing cells (GFP+ domain) and nascent neurons (Tubb3+ domain) in heterozygous (Neurog2<sup>GFP/+</sup>) and mutant (Neurog2<sup>GFP/GFP</sup>) retinas. To confirm that the size of the GFP domain is not different for single or bi-allelic GFP expression, we compared the GFP domain relative to the total retinal circumference in Neurog2<sup>GFP/+</sup> and Neurog2<sup>GFP/GFP</sup> and found no difference between genotypes (Fig. 3H; See Methods for description of domain measurements).

To determine whether loss of Neurog2 and/or Atoh7 affects the ventral-peripheral expansion of retinal neurogenesis, we examined double-heterozygote controls (Neurog2<sup>GFP/+</sup>;Atoh7<sup>LacZ/+</sup>), Neurog2 single-mutant (Neurog2<sup>GFP/GFP</sup>;Atoh7<sup>LacZ/+</sup>), Atoh7 single-mutant (Neurog2<sup>GFP/+</sup>;Atoh7<sup>LacZ/LacZ</sup>), and double-mutant (Neurog2<sup>GFP/GFP</sup>;Atoh7<sup>LacZ/LacZ</sup>) retinas. The double-heterozygotes are appropriate controls since Atoh7 heterozygotes have no phenotypes compared to wild types (Brown et al., 2001; Le et al., 2006; Wang et al., 2001), and both Neurog2<sup>GFP/+</sup> and Neurog2<sup>GFP/+</sup>;Atoh7<sup>LacZ/+</sup> retinas exhibited no significant differences from wild type eyes (not shown).

First, we evaluated the expansion of Tubb3+ cells in relation to the Neurog2<sup>GFP</sup> domain in somite-matched embryos at E11.75 (54-60 somites). In control retinas, the Tubb3 domain was slightly smaller and included within the GFP domain (Fig. 3A, brackets 3A’). In Neurog2 mutants, the Tubb3 domain was decreased (Fig. 3B), with a greater separation between the leading edge of Tubb3+ cells and the peripheral extent of the Neurog2<sup>GFP</sup> domain (brackets, Fig. 3B’). To quantify the peripheral spread of neurogenesis, we measured the outer length of the Tubb3 and GFP domains in matched central retinal sections (Fig. 3E). At E11.75, the Tubb3
domain was significantly reduced in *Neurog2* mutant retinas. In controls, the Tubb3 domain occupied 84.7±2.5% of the GFP domain (31.4±1.4% of total circumference), but in *Neurog2* mutants, the Tubb3 domain was only 34.8±7.0% of the GFP domain (11.9±2.3% of total circumference; Fig. 3F and not shown). We also noted reduced neurogenesis in the nasal half of the retina and ventral to the optic nerve (not shown). In addition to Tubb3, we also observed reduced p27/Kip1 and Pou4f2/Brn3b expression domains in the absence of *Neurog2* (Fig. 3I–J'). We conclude that *Neurog2* mutants exhibit a reduction in retinal neurogenesis concomitant with reduced RGC specification and cell cycle exit.

Intriguingly, *Atoh7* is not required to propagate the spread of neurogenesis, as the size of the Tubb3 domain was unaffected in the absence of *Atoh7* (Fig. 3C,C',F and not shown). Like *Neurog2* mutants, mice lacking both *Atoh7* and *Neurog2* had diminished expansion of Tubb3 in relation to both the GFP domain and total circumference, though not different from *Neurog2* single mutants (Fig. 3D,D',F). Therefore, *Atoh7* and *Neurog2* do not work synergistically to promote the propagation of neurogenesis. To investigate further, we assessed the percentage of differentiating neurons within the Tubb3 domain and observed fewer Tubb3+ cells in *Neurog2* mutants, *Atoh7* mutants, and double mutants compared to controls, again in a non-synergistic manner (Fig. 3G). Previous studies indicate that at E11.5 p27+ postmitotic retinal cells and Pou4f2+ RGCs are significantly reduced in *Atoh7* mutants (Le et al., 2006; Wang et al., 2001). Although *Atoh7* mutants do exhibit fewer p27+ cells, the peripheral extent of the p27 domain was not reduced (not shown). As expected, the Pou4f2/Brn3b-expressing cells were virtually absent in *Atoh7* mutants and *Neurog2;Atoh7* double mutants (not shown). Thus, the expansion of neurogenesis requires *Neurog2*, but not *Atoh7*, although each is required to produce normal numbers of differentiating neurons.
The co-localization of Neurog2 and Atoh7 reporters and the reduced propagation of neurogenesis in Neurog2 mutants from E11.0-E11.75 suggested that the peripheral spread of endogenous Atoh7 might also be affected. Indeed, Neurog2 mutants had a reduction in the width of the Atoh7 mRNA expression domain at E11.75 (Fig. 3K,K’). Further, in the diencephalon, Atoh7-expressing cells were virtually absent in Neurog2 mutants (arrows, Fig. 3K,K’). Another early bHLH factor, Neurod1, is required for normal amacrine, S-cone, and rod photoreceptor development (Inoue et al., 2002; Liu et al., 2008; Morrow et al., 1999). Although the Neurod1 and Atoh7 domains were the same width in controls, we did not observe any appreciable changes in the Neurod1 expression domain in Neurog2 mutant retinas (Fig. 3L,L’), consistent with a previous study (Akagi et al., 2004). Together, the outward spread of Neurog2 specifically affects the expansion of Atoh7 but is not required for its initial activation.

Delayed neurogenesis in Neurog2 mutants is restored

Neurog2 is required for the propagation, but not the initiation of neurogenesis, as a cluster of neural precursor cells appears in Neurog2 mutants between E11.0-E11.75. Next, we analyzed retinal development in these mice from E12.0 to E15.5. From E12.0-E12.5, the Tubb3, p27/Kip1, and Pou4f2/Brn3b domains were truncated relative to the Neurog2-GFP domain (brackets, Fig. 4A,A’ and not shown). We also noted reduced neurogenesis in the nasal half of the retina and ventral to the optic nerve (not shown), indicating that the progression of neurogenesis was affected in both central-peripheral and dorsal-ventral axes. However, by E13.5 the pattern of neurogenesis in Neurog2 mutants was very similar to that of controls. In the temporal retina of both genotypes, the neurogenic domain extended to the periphery, to the border of the Neurog2-GFP domain (Fig. 4B,B’). On the nasal side, however, the Tubb3 and
Pou4f2/Brn3b domains were still reduced relative to the GFP domain in Neurog2 mutants (brackets, Fig. 4B,B’ and not shown). By E15.5, the central to peripheral distribution of Tubb3+ or Pou4f2/Brn3b+ cells throughout the retina had caught up to that of controls (Fig. 4C,C’ and not shown). Thus, neurogenesis and RGC specification are restored in Neurog2 mutants, largely between E12.5 and E15.5.

*Neurog2 and Atoh7 coordinate normal retinal size, but control distinct aspects of fate determination*

At the initiation of retinal neurogenesis, Neurog2 is required for the propagation of the Atoh7 expression domain. Therefore, to test for cross-regulation or synergistic activities between Neurog2 and Atoh7, we compared the four earliest retinal fates (RGCs, cone photoreceptors, amacrine and horizontal interneurons) in Neurog2 and Atoh7 single and double mutants. Since loss of Neurog2 results in neonatal lethality, mutant mice were analyzed at P0.5.

First, we examined retinal thickness of single and double mutants (Fig. 5A-D,M). Adult Atoh7 mutants have reduced laminar thickness (Brown et al., 2001; Brzezinski et al., 2005), already present at P0.5 (Fig. 5C,M). Compared to controls (Fig. 5A), Neurog2 mutant mice also had significantly thinner retinas (Fig. 5B,M), similar to Atoh7 mutants (Fig. 5C,M). Furthermore, Neurog2;Atoh7 double mutant retinas were significantly reduced in thickness compared to both wild types and single mutants (Fig. 5D,M). This indicates that the loss of both Neurog2 and Atoh7 has an additive effect on retinal size, presumably representing synergistic or parallel roles in proliferation and/or survival during embryonic retinogenesis. To understand if reduced proliferation or increased cell death are responsible for the smaller retinas, we analyzed proliferating S-phase retinal progenitors by BrdU pulse-labeling cells, and apoptotic cells by
activated Caspase-3 expression at several embryonic ages. *Atoh7* single mutants had no defect in proliferation or apoptosis at E15.5 (Le et al., 2006). At both E11.5 and E15.5, there was no difference in BrdU+ cells between wild type, *Neurog2* mutant, and *Neurog2;Atoh7* double mutant retinas (Fig. 6A-D and data not shown). The percentage of Caspase-3+ cells was normal in E15.5 *Neurog2*−/− eyes (Fig. 6E-H), as well as at E12.0 during the delay in neurogenesis (not shown). However, the number of apoptotic cells was significantly increased in *Neurog2;Atoh7* double mutants (Fig. 6H), suggesting an overlapping function for these bHLH factors in regulating some aspect of cell survival. Therefore, the increased apoptosis and enhanced reduction of retinal thickness were consistent with one another in double mutants.

To understand the extent by which the four early cell types might be altered in *Neurog2* mutants, we quantified RGCs, cones, horizontal and amacrine interneurons in P0.5 retinas. Although a loss of RGCs might be expected since their progression was delayed from E11.5-E13.5, we instead found a 2% ± 0.2% increase in Pou4f2+ RGCs within P0.5 *Neurog2* mutants (Fig. 5K). As expected, *Atoh7* and *Atoh7;Neurog2* double mutants had essentially no Pou4f2/Brn3b+ RGCs at this age (not shown). We conclude that although the percentages of RGCs in P0.5 *Neurog2* mutant eyes are significantly elevated, this phenotype cannot overcome the agenesis of RGCs in the absence of *Atoh7*.

Cone photoreceptors and Neurog2+ progenitor cells are significantly increased in *Atoh7* mutants (Brown et al., 2001; Brzezinski et al., 2005; Le et al., 2006), suggesting that cone photoreceptor genesis might normally be blocked by *Atoh7* indirect suppression of *Neurog2* expression. Analysis of single and double-mutants (*Neurog2<sup>GFP/GFP</sup>; *Atoh7<sup>LacZ/LacZ</sup>*; *Atoh7<sup>LacZ/LacZ</sup>; *Neurog2<sup>GFP/GFP</sup>*; *Neurog2<sup>GFP/GFP</sup>; *Atoh7<sup>LacZ/LacZ</sup>*) showed the trend of increased RXRγ+ cone precursor cells in the outer retinas of *Atoh7<sup>LacZ/LacZ</sup>* and double mutant mice (Fig. 5G,N). However, loss of *Neurog2* alone (*Neurog2<sup>GFP/GFP</sup>; *Atoh7<sup>LacZ/LacZ</sup>*; *Neurog2<sup>GFP/GFP</sup>; *Atoh7<sup>LacZ/LacZ</sup>*) had no significant
effect on the percentage of cone photoreceptors (Fig. 5F,N), nor did it enhance or suppress the percentages of cones in Atoh7;Neurog2 double mutants (Fig. 5H,N). This suggests that although there is a simultaneously nonautonomous increase in cone photoreceptors and Neurog2+ cells in Atoh7 mutants (Le et al., 2006), these are independent events that are likely to occur in separate populations of retinal progenitor cells.

Characterization of Neurog2;Ascl1;Atoh3 and Neurog2;Neurod1;Atoh3 triple mutant mice suggested a partial requirement for Neurog2 during horizontal and amacrine interneuron differentiation (Akagi et al., 2004). To determine if loss of Neurog2 alone affects these cell types, we quantified the percentages of AP2α+ amacrine cells (arrow, Fig. 2O)(Yan and Wang, 2004) and Prox1+ cells, which give rise to a mixed population of horizontal and amacrine neurons (arrow, Fig. 2P)(Dyer et al., 2003). The AP2α protein (Fig. 5I-L,P-R) is expressed by both displaced amacrines in the GCL and amacrines that reside in the INL. We found normal distributions and percentages of AP2α+ amacrines in Neurog2 single mutants (Fig. 5J,P-R). However, Atoh7 single mutants, and the double mutants had significant increases in amacrines (Fig. 5K,L,P-R), consistent with a previous analysis of amacrines in Atoh7 mutants (Wang et al., 2001). Finally, we compared the percentages of Prox1+ horizontals and amacrines (Fig 5S-U). Here we observed only a significant increase in Prox1+ displaced amacrines in Atoh7;Neurog2 double mutants (Fig. 5S-U). The different outcomes between Prox1+ and AP2α+ amacrines in Atoh7 single and double mutants probably resulted because the Prox1+ population (0.8%) is such a small subset of AP2α+ amacrines (29%). Regardless, we conclude that Neurog2 alone is not required for the specification of prenatal cone, amacrine and horizontal interneurons.
Ascl1 can compensate for the loss of Neurog2

Removal of Neurog2 during embryonic retinal development results in a temporal delay of early retinal neurogenesis, which then returns to normal between E12.5-E15.5 (Fig. 4). Therefore, it is plausible that other factors, for example another bHLH proneural factor, compensate for loss of Neurog2 in the early retina. We tested several such candidates here. First, at E15.5 the patterns of Atoh7 and Neurod1 mRNA were indistinguishable in control and Neurog2 mutants (not shown). If one of these factors compensates for the loss of Neurog2, we should have observed overexpression of Atoh7 or Neurod1. Next, other neurogenin gene family members, Neurog1 and Neurog3, are expressed in the chick retina, but not that of frog (Ma et al., 2009; Nieber et al., 2009). So we asked whether either parologue might be ectopically upregulated in Neurog2 mutant eyes, but neither Neurog1 nor Neurog3 protein were detectable in E11.75-E15.5 control and Neurog2−/− retinas (not shown). Finally, we evaluated the onset of Ascl1 expression in wild type retinas. In the E11.5 optic cup, Ascl1 mRNA and protein are not expressed (Fig 7I and data not shown), but beginning at early E12.5, a small population of Ascl1+ cells is detectable in the dorso-central retina (arrow, Fig. 7A). By E13.5, Ascl1 expression has spread outward to the peripheral and ventral poles of the retina (not shown). Therefore, the normal onset and progression of Ascl1 expression coincides both spatially and temporally with the recovery of neurogenesis observed in Neurog2 mutants from E12.5-E13.5.

The timing of these events suggested that Ascl1 might be capable of restoring the delayed neurogenesis of Neurog2 mutants. If so, then misexpression of Ascl1 within the Neurog2-lineage should restore the peripheral expansion of retinal neurogenesis. To test this directly, we took advantage of the Neurog2^Ascl1KI allele, a homologous recombination of an IRES-Ascl1 cassette into the endogenous Neurog2 gene locus, thereby functionally replacing Neurog2 with
Ascl1 (Fode et al., 2000). By mating Neurog2<sup>Ascl1KI/+</sup> and Neurog2<sup>GFP/+</sup> heterozygotes, Neurog2<sup>GFP/Ascl1KI</sup> embryos were generated, in which Neurog2 function was removed and replaced by that of Ascl1 within the Neurog2-lineage (Fode et al., 2000). At E12.0, both Neurog2<sup>GFP/+</sup> and Neurog2<sup>GFP/GFP</sup> retinas exhibited only rare Ascl1+ cells by immunofluorescence (Fig. 7A,A’,A” and not shown), indicating that Ascl1 is not precociously expressed in the absence of Neurog2. By contrast, Neurog2<sup>GFP/Ascl1KI</sup> retinas had abundant numbers of ectopic Ascl1+ cells (Fig. 7B,B’,B”), most of which were also GFP+, indicating a substitution of Ascl1 in cells that normally express Neurog2. We then compared the width of the Tubb3 and GFP domains in Neurog2<sup>GFP/+</sup>, Neurog2<sup>GFP/GFP</sup>, and Neurog2<sup>GFP/Ascl1KI</sup> embryonic retinas at E12.0. Strikingly, upon Ascl1 replacement of Neurog2, the width of the Tubb3+ domain was now the same as in controls (Fig. 7C-E). To determine the effects of Ascl1 on RGC differentiation, we similarly evaluated the Pou4f2/Brn3b expression domain in these three genotypes. Indeed, the width of the Pou4f2/Brn3b domain in Neurog2<sup>GFP/Ascl1KI</sup> retinas was identical to controls (Fig. 7F-H). Thus, although Ascl1 normally activates in the retina after Neurog2, it is sufficient to rescue the block in the progression of early neurogenesis and RGC differentiation found in Neurog2 mutant eyes.

Because progression of the Atoh7 domain is initially delayed in Neurog2 mutants from E11.75-E12.5 (Fig. 3K,K’), we asked whether Ascl1 rescues the neurogenic wave via activation of Atoh7. The expression of Atoh7 was compared among E11.5-E12.5 Neurog2<sup>+/+</sup>, Neurog2<sup>GFP/+</sup>, Neurog2<sup>GFP/GFP</sup> and Neurog2<sup>GFP/Ascl1KI</sup> litters (Fig. 7I-P and not shown). To verify the presence of ectopic Ascl1, its expression was monitored on adjacent sections from each embryo. At E11.5, Neurog2<sup>GFP/Ascl1KI</sup> optic cups had a reduced domain of Atoh7 mRNA (compare Fig. 7K,L), but, ectopic Ascl1 was not yet present (Fig. 7I,J). A day later at E12.5,
when Ascl1 is normally expressed by a few retinal cells, we found abundant ectopic expression in Neurog2^{GFP/Ascl1KI} retinas (Fig. 7M,N), along with a normal pattern of Atoh7 mRNA (Fig. 7O,P). Although ectopic Ascl1 and Atoh7 mRNA both appeared at E12.5, we do not think that rescue occurred at the level of Atoh7 transcriptional regulation. In support of this idea, loss of Ascl1 has no effect on Atoh7 mRNA expression from E11.5 and E15.5 (Fig. 8A-D), the Ascl1 protein does not bind to Atoh7 5’ regulatory DNA (Skowronska-Krawczyk et al., 2009), and Drosophila Scute and Atonal proteins have different E Box binding site consensus sequences (Powell et al., 2004). Somewhat paradoxically, at E17.5 Ascl1 was proposed to suppress Atoh7 (Akagi et al., 2004), although Atoh7 upregulation was only found in the retinas of two bHLH triple mutant combinations that included Ascl1 mutants. Furthermore, Ascl1 and Neurog2 mutually suppress each other’s mRNA expression in the E17.5 retina (Akagi et al., 2004), which is somewhat at odds with normal expression of Ascl1 in E11.5-E15.5 Neurog2 mutants (Fig. 8E,F), and of Neurog2 GFP or protein in E11.5-E15.5 Ascl1^-/- retinas (not shown). Although particular bHLH factors can suppress one another’s expression at older stages of retinal formation, there is no evidence that these regulatory interactions are direct (Akagi et al., 2004). We hypothesize that late embryonic retinal bHLH cross suppression involves intermediate genes and/or occurs nonautonomously, particularly since these factors do not encode transcriptional repressors.

**Discussion**

Here, we investigated bHLH transcription factor expression and function during the initiation of retinogenesis in mouse, and identify Neurog2 as one intrinsic regulator of the leading edge of neurogenesis. Onset and expansion of Neurog2 and Atoh7 expression predicts
the initial wave front, concomitant with the compartmentalization of the neural retina and optic stalk by Pax6 and Pax2, respectively. The first RGCs are subsequently specified in the dorso-central retina, and differentiation spreads ventrally and peripherally, similar to that found in fish and chick (Hu and Easter, 1999; Prada et al., 1991). Neurog2 is required for the propagation of neurogenesis, and though its loss initially causes a dramatic phenotype, retinal neurogenesis becomes corrected in a few days. At P0.5, mutant retinas exhibited only a minor increase in RGCs, with no defect in cone, amacrine or horizontal neuron genesis. Interestingly, this recovery occurred during the onset and expansion of Atoh1 expression, which was sufficient to correct the initial delay in RGC genesis.

Conservation of proneural bHLH function during initiation of murine retinal neurogenesis

The initial wave of retinal neurogenesis in mouse closely resembles the same process in non-mammalian vertebrate and Drosophila eyes. In fruit flies, a morphogenetic furrow sweeps across the eye imaginal disc from posterior to anterior ahead of retinal neurogenesis (Ready et al., 1976). At the anterior edge of the morphogenetic furrow, the bHLH protein atonal specifies the first ommatidial photoreceptor (R8) and promotes the progression of the morphogenetic furrow (Brown et al., 1995; Jarman et al., 1994; Jarman et al., 1995). Like in Drosophila, the progression of neurogenesis in the vertebrate retina exhibits wave-like properties. In zebrafish, cells cease proliferation and adopt an RGC fate in a nasal-to-temporal sequence, determined by the atonal-orthologue Ath5/lakritz (Hu and Easter, 1999; Kay et al., 2001). In chick, RGC differentiation proceeds outward from the optic stalk, with a bias for the temporal half of the retina (McCabe et al., 1999; Prada et al., 1991). In chicken, neurogenin2 and Ath5 expression are present in the central retina at the onset of neurogenesis, and microarray profiling of mouse
retinal progenitor cells identified a subpopulation with Neurog2 and Atoh7 mRNA coexpression (Trimarchi et al., 2008b). More recently, chick and mouse Ngn2/Neurog2 were shown to activate directly the Ath5/Atoh7 promoter, although the number of binding sites utilized differs between these two species (Matter-Sadzinski et al., 2001; Matter-Sadzinski et al., 2005; Skowronska-Krawczyk et al., 2009).

Here, we show that these atonal family members have distinct functions in mouse where Neurog2 controls the propagation of neurogenesis, and Atoh7 regulates RGC specification. Not surprisingly, together Atoh7 and Neurog2 reconstitute the orthologous roles of atonal in the Drosophila eye. Subdivisions of atonal functions during vertebrate development were already known, since the semi-orthologues Atoh7 and Atoh1 are present in mutually exclusive regions of the nervous system, thereby parsing Drosophila atonal functions within the mouse visual, auditory, and proprioceptive systems, respectively (Helms et al., 2000; Hufnagel et al., 2007; Saul et al., 2008).

Another example of functional subdivision relates to the ability of Drosophila atonal to autoregulate its own expression, which does not occur for the Xenopus Ath5 or mouse Atoh7 genes (Hutcheson et al., 2005; Riesenberg et al., 2009). Previously, Atoh7 was reported to suppress Neurog2 expression nonautonomously in the E13-15 retina (Le et al., 2006). Here, we found that the earliest Atoh7LacZ+ cells are also in the Neurog2GFP lineage, and that Neurog2 is present in S-phase cells, slightly preceding Atoh7 expression in these cells as they become newly postmitotic. We conclude that Neurog2 is a positive regulator of Atoh7 expression, since the peripheral expansion of Atoh7 was delayed in Neurog2 mutants. Thus, in mouse these two genes cross-regulate one another, but at different stages of retinal neurogenesis. During the initial propagation of neurogenesis, Neurog2 directly activates Atoh7 expression (this paper and
Skowronska-Krawczyk et al., 2009), but several days later Atoh7 nonautonomously suppresses Neurog2 expression (Le et al., 2006). Importantly, like atonal autoregulation within committed R8 cells in the morphogenetic furrow, Neurog2 cross-regulation of Atoh7 is an integral part of wave front progression during the initiation of mammalian retinal neurogenesis.

**Directing the wavefront of mammalian retinal neurogenesis**

Neurog2 expression expands peripherally ahead of multiple markers of retinal neurogenesis. This small Neurog2\textsuperscript{GFP}+/Tubb3-negative domain likely contains Neurog2+ cells in S-phase. As these cells progress through the terminal mitosis, a subset of Neurog2\textsuperscript{GFP}+ cells express Atoh7, p27/Kip1 and Tubb3. Therefore, the spatial difference between the GFP+/Tubb3+ and peripheral GFP+/Tubb3-negative domains likely reflects the temporal difference in cell cycle status between differentiating neurons and proliferating progenitors poised to differentiate, respectively. This is also supported by Pou4f2/Brn3b onset more centrally in newly postmitotic RGCs. Hence, the outward spread of Neurog2 expression demarcates the leading edge of neurogenesis, in which progenitor cells exit the cell cycle and become specified as retinal neurons, most of which differentiate as RGCs.

We predicted that BrdU+ S phase progenitors would be increased in E11.5 Neurog2−/− eyes, since there was an obvious reduction in p27/Kip1+ postmitotic cells. The correlation of these outcomes would indicate that Neurog2 regulates retinal cell cycle progression, however this was not the case. It remains plausible that E11.5-E13.5 Neurog2−/− cells inappropriately accumulate in G2 phase. However, we currently favor a different possibility in which Neurog2 mutant cells undergo transient changes in cell cycle length. Determining percentages of individual cell cycle markers at single time points would not uncover this defect. Instead,
window labeling should be employed in the future to measure the cell cycle length of GFP+ retinal progenitors in Neurog2\textsuperscript{GFP/+}, Neurog2\textsuperscript{GFP/GFP} and Neurog2\textsuperscript{GFP/Ascl1KI} retinas. In this regard, Ascl1 may uniquely rescue the Neurog2 phenotype, since mitotically active retinal progenitors appear to only express these two bHLH factors during embryonic retinal neurogenesis. Moreover, Neurod1 only partially rescues the Atoh7 RGC phenotype, and Atoh3 not at all (Mao et al., 2008a), while Ascl1 cannot rescue the Atoh7 RGC phenotype (Hufnagel et al, in prep).

Extrinsic signal pathways, like FGF and sonic hedgehog (shh), direct key aspects of retinal patterning and neurogenesis (Martinez-Morales et al., 2005; McCabe et al., 1999; Neumann and Nuesslein-Volhard, 2000; Picker and Brand, 2005). A decade ago, shh was shown to propagate a retinal wave in the zebrafish retina (Neumann and Nuesslein-Volhard, 2000), but the mechanism for this subsequently underwent modification. Ath5 expression and RGC genesis were subsequently shown to initiate normally in sonic you (syu) mutants (Kay et al., 2005; Masai et al., 2005). However, the period for the retinal wave to progress from nasal to temporal becomes extended when postmitotic retinal neurons are unable to secrete Shh. Therefore, retinal shh maintains progression but cannot initiate retinal neurogenesis. Instead, shh in the midline appears to trigger initiation of retinogenesis and Ath5 expression. It is unknown if midline shh activates Neurog2 in the zebrafish optic cup. In the mouse retina, activation and expansion of Neurog2 and Atoh7 expression precedes the appearance of retinal derived shh at E12.5 (Jensen and Wallace, 1997). In the future, it will be important to correlate the onset of midline and retinal shh with a) the time course of Neurog2 expression, b) Neurog2 regulation of early neurogenesis, c) the period when the delay is overcome in Neurog2 mutants and d) the ability of Ascl1 to rescue the Neurog2 phenotype.
There are other signaling pathways that should be considered as well. For example, thyroid hormone signaling, which is important for photoreceptor differentiation, is deployed in multiple coordinated waves, at different phases of progenitor proliferation (Trimarchi et al., 2008a). Yet another example of extrinsic signaling is the Notch pathway, which also controls the timing of RGC differentiation and bHLH expression (Austin et al., 1995; Bao and Cepko, 1997; Nelson et al., 2006; Nelson and Reh, 2008).

Importantly in the chick eye, McCabe et al (1999) demonstrated that proximity to the wave front is not required for the progression of RGC genesis, indicating that this process depends more strongly on intrinsic components than extrinsic signals. Here, Neurog2 retinal expression was correlated with and identified as required for the spatiotemporal progression of the wave of neurogenesis in the mouse eye. Potentially, Neurog2 may act as a temporal integrator, interpreting combinations of extrinsic signals and multiple intrinsic inputs, from transcription factors such as Pax6 and Sox2 (Marquardt et al., 2001; Taranova et al., 2006), resulting in the activation and expansion of Neurog2, followed by neurogenic wave initiation.

There is evidence for other intrinsic factor regulation of spatiotemporal progression of neurogenesis. In the orJ mouse, loss of Vsx2/Chx10, which is critical for maintaining retinal progenitor proliferation, results in severe microphthalmia, lack of peripheral neurogenesis, and a delay in RGC-derived shh signaling (Bone-Larson et al., 2000; Burmeister et al., 1996; Sigulinsky et al., 2008). Although Vsx2/Chx10 is ubiquitously expressed in retinal progenitors prior to the initiation of neurogenesis, it likely acts in concert with Neurog2 and other factors to control the wave of neurogenesis in the retina. Another spatiotemporal process, cell migration is tightly coordinated for normal laminar patterning in the neocortex and retina – as cells exit the cell cycle and adopt a neural fate, they must migrate out of the ventricular zone to reach the
proper layer. Recently, Neurog2 and other proneural genes have been shown to regulate cortical migration, in part through regulation of Rnd2, a small GTP binding protein (Ge et al., 2006; Heng et al., 2008). Thus, coordinating spatiotemporal aspects of retinal development seems to require the tight coupling of multiple facets of neurogenesis by proneural bHLH and homeodomain transcription factors.

While Neurog2 is necessary for the propagation of neurogenesis and Atoh7 expression, it is not required for their initiation, clearly indicating that other factors are required. The initiation is neurogenesis is highly dependent on Pax6, critical for the expression of multiple bHLH factors (Brown et al., 1998; Marquardt et al., 2001; Riesenberg et al., 2009). The onset of proneural bHLH gene expression and retinal neurogenesis closely coincides with the downregulation of Pax2 in the nascent neural retina, a known regulator of Pax6 (Schwarz et al., 2000). It stands to reason, then, that the timing of bHLH initiation may be controlled indirectly by Pax2 regulation of Pax6 function or directly by Pax2 repression of bHLH gene expression.

A bHLH network controlling retinal neurogenesis

In different contexts of the developing nervous system, Neurog2 controls proliferation, cell cycle exit, cell fate identity, neurotransmitter specification, cell migration, axon guidance, and survival (Aaker et al., 2009; Britz et al., 2006; Cai et al., 2000; Fode et al., 1998; Fode et al., 2000; Seibt et al., 2003). However, previous to this study no phenotype was attributable solely to Neurog2 function during vertebrate retinal development. Here, we uncovered a key role for Neurog2 in regulating the initial progression of early retinal neurogenesis and RGC specification, which can be compensated for by substitution of Ascl1 for Neurog2. Throughout the CNS, Ascl1 and Neurog2 are intricately linked in a context-dependent manner. In the
forebrain, Neurog2 represses Ascl1 to maintain dorsal projection neuron identity, while in the dorsal neural tube Neurog2 appears to function temporally downstream of Ascl1 to influence the timing of cell cycle exit (Fode et al., 2000; Helms et al., 2005). Other Neurog2^Ascl1KI replacement experiments demonstrate that Ascl1 cannot rescue the Neurog2 phenotype in the dorsal forebrain or dorsal root ganglia (Fode et al., 2000; Parras et al., 2002), but can partially compensate for ventral spinal cord and midbrain dopaminergic neuron phenotypes (Kele et al., 2006; Parras et al., 2002).

In the retina, Neurog2 and Ascl1 both appear to promote cell cycle exit and neuronal determination analogously, such that Ascl1 expressed from the Neurog2 locus can rescue the temporal delay of RGC genesis. This was unexpected, since RGCs are unaffected in Ascl1 mutants, and Ascl1 is thought to function primarily in specification of later-born retinal fates, particularly rod photoreceptors and bipolar interneurons (Hatakeyama et al., 2001; Tomita et al., 1996). Here we propose that the normal onset of endogenous Ascl1 expression activates a subsequent wave of neurogenesis. In Neurog2 mutants, retinal second wave cells could either autonomously produce first wave and second wave neurons, or nonautonomously jumpstart the stalled first wave cells. The absence of increased retinal cell proliferation in Neurog2 mutants suggests the first scenario as the least likely. At present there is no hard evidence for Ascl1 regulation of a subsequent wave, although Ascl1 impressively rescues the Neurog2 phenotype. To settle this question, the Ascl1 retinal lineage and mutant phenotypes (ideally with a conditional allele) will need careful examination during prenatal retinogenesis.

Conversely, Ascl1 may compensate for the loss of Neurog2 by an unknown mechanism. Interestingly, Ascl1 performs a critical function during zebrafish retinal regeneration (Fausett et al., 2008). Both Neurog2 and Ascl1 are present in proliferating neural progenitor cells (Jasoni
and Reh, 1996; Yan et al., 2001), implying that they share a common set of downstream target genes critical for controlling cell cycle progression versus exit for neural differentiation. The expression of Neurog2 and Ascl1 at different times during retinogenesis seems integral with their context-specific functions. Intriguingly, the removal of both Neurog2 and Ascl1 did not result in the total loss of neurogenesis or Atoh7 expression (Akagi et al., 2004), suggesting that further levels of compensation exist. In postnatal Ascl1 mutant retinas, horizontal interneuron and rod photoreceptor differentiation is temporarily reduced (Tomita et al., 1996), potentially restored by yet another compensatory factor. Overall, we conclude that the spatial and temporal progression of mammalian retinal neurogenesis is regulated by the bHLH factor Neurog2, and that a remarkable compensatory potential exists in the developing retina, potentially through a secondary wave of neurogenesis directed by Ascl1.
Notes to Chapter 3


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**Figure Legends**

**Figure 3.1.** Onset of Neurog2 protein, Neurog2\(^{GFP}\) and Atoh7\(^{LacZ}\) expression in the mouse retina.  
(A-B) Whole mount micrographs of Atoh7\(^{LacZ/+}\) and Neurog2\(^{GFP/+}\) embryos at E11.5, demonstrated retinal expression for each reporter (arrows).  (C-D) Immunolabeling for GFP and βgal showed no detectable optic cup expression at E10.75, although coexpressing cells were present in the diencephalon (arrow in D).  E-H) Labeling of Neurog2 protein and GFP at onset of neurogenesis.  GFP versus βgal at E11.0 (F) and E12.5 (H) in Neurog2\(^{GFP/+}\); Atoh7\(^{LacZ/+}\) mice showed consistent overlap of reporters at these ages.  (G,I) Neurog2\(^{GFP/+}\); Atoh7\(^{LacZ/LacZ}\) embryos double-labeled for GFP and βgal at E11.0 and E11.75, respectively, demonstrated the temporal progression of each expression domain.  Arrows point to double-labeled cells, and brackets show Neurog2\(^{GFP}\) expression peripheral to the Atoh7\(^{LacZ}\) domain.  (J) There was no expression of Atoh7 mRNA (arrowheads point to cells with purple in situ reaction product) observed in BrdU pulse-labeled retinal (red) cells.  (K) However, Neurog2 protein was clearly detected in many BrdU+ S-phase cells at E11.75 and E14.5 (arrows).  (L) Most βgal+ cells were Neurog2+/βgal+ (fuchsia arrows).  Yellow arrowheads mark Neurog2+/βgal− cells peripheral to the Atoh7\(^{LacZ}\) domain.  (M) The overlap of Neurog2 and GFP (fuchsia arrows) showed GFP+/Neurog2− cells (yellow arrowheads).  Scale bars: 50 µm in C,G,H,L,J; 25 µm in F.  L = lens.

**Figure 3.2.** Initiation of retinal neurogenesis and Neurog2\(^{GFP}\) expression is coincident in the mouse eye.  (A-C) Pax2 and Neurog2\(^{GFP}\) co-labeling.  At E11.0-E11.5, Pax2+ cells are evident in the optic cup (A).  When Neurog2\(^{GFP/+}\) cells first appeared in the central retina, the Pax2 domain was restricted to the optic stalk and nasal retina (B,C).  Arrow in C marks a very rare co-labeled Neurog2\(^{GFP/+}\)/Pax2+ cell.  (D) Extensive Neurog2\(^{GFP}\) coexpression with Pax6 protein.
(E-H) Time course of the onset and expansion of neurogenesis and Neurog2<sup>GFP</sup> expression. (E) In an E11.25 retina, Neurog2<sup>GFP</sup>+/Tubb3+ cells were detected in the diencephalon, but not in the optic cup when Neurog2<sup>GFP</sup> is not present. (F) The first neurons appear from E11.0-E11.5 in Neurog2<sup>GFP</sup>+ cells. (G) Neurog2<sup>GFP</sup>+/Tubb3+ cells are present in the nasal (arrows) and temporal retina at E12.0. (H) By E13.5, the GFP and Tubb3 expression domains had reached the peripheral retina. (I-K) Dorsal, central, and ventral sections from the same eye at E11.75, demonstrating Neurog2<sup>GFP</sup>+/Tubb3+ cells in the dorsal and central (I,J), but not ventral retina (K). (L) Tubb3 and p27/Kip1 were extensively coexpressed in cells exiting the cell cycle. (M) Pou4f2/Brn3b expression onsets at E11.75 in Neurog2<sup>GFP</sup>+ cells. (N-P) GFP+ cells coexpress markers of other embryonic fates: RXRγ+ cones (N), AP2α+ amacrine (O), and Prox1+ horizontal and amacrine interneurons (P). Scale bars: 50 µm in A,C,H,N,O. Insets in F-P are 8X magnifications of boxed area in each panel. L = lens.

**Figure 3.3.** Delay of early neurogenesis in Neurog2 mutants. (A-D) Tubb3 and Neurog2<sup>GFP</sup> labeling of double-heterozygote controls (A), Neurog2<sup>GFP/GFP</sup>;Atoh7<sup>LacZ+/</sup> (Neurog2 mutants) (B), Neurog2<sup>GFP/+</sup>; Atoh7<sup>LacZ/LacZ</sup> (Atoh7 mutants) (C), and double mutant (D) embryos at E11.75. (A’-D’) Insets show higher magnification of the peripheral extent of Tubb3 expression, brackets mark GFP+/Tubb3– domain. (E) Measurement scheme for retinal circumference, Tubb3 and Neurog2<sup>GFP</sup> expression domain widths. (F) Compared to controls, the Tubb3 domain was diminished relative to the Neurog2<sup>GFP</sup> domain in Neurog2 mutants and double mutants, but not in Atoh7 mutants. (G) The percentage of Tubb3+ cells per total DAPI+ nuclei in the Tubb3 domain indicated that both Neurog2 and Atoh7 mutants had diminished neural differentiation. (H) The distal extent of GFP expression was the same in Neurog2<sup>GFP/+</sup> or Neurog2<sup>GFP/GFP</sup> eyes.
(I-J’) *Neurog*2 mutants also exhibited a reduction of the p27/Kip1 (I,I’) and Pou4f2/Brn3b (J,J’) domains.  (K-L’) *Atoh7* (K,K’) and *Neurod1* (L,L’) mRNA expression in *Neurog*2+/+ and *Neurog*2GFP/GFP retinas, indicated a smaller *Atoh7* domain, while *Neurod1* expression was unaffected. Scale bars: 50 µm in A,I,K. *p<0.05, **p<0.001; n=6 eyes (3 embryos) per genotype.

**Figure 3.4.** Arrested neurogenesis in the absence of *Neurog*2 is temporary.  (A,A’) At E12.0, the peripheral extent of the Tubb3 domain is reduced in *Neurog*2GFP/GFP retinas compared to controls (brackets, A,A’).  (B,B’) At E13.5, the peripheral extent of Tubb3 domain is reduced only on the nasal side of the optic cup, compared to the *Neurog*2GFP domain (brackets).  (C,C’) By E15.5, the peripheral extent of Tubb3 expression was indistinguishable between *Neurog*2 mutants and heterozygous controls. Scale bars: 50µm in A,B,C; n=8 eyes (4 embryos) per genotype.

**Figure 3.5.** Neonatal phenotypes of *Neurog*2 and *Neurog*2;*Atoh7* mutants.  (A-D) Retinal thickness was measured as the vitreal-scleral width of DAPI+ nuclei in the NBL and GCL at P0.5.  (E-H) Cone precursors were assessed by RXRγ labeling in the outer NBL.  (I-L) AP2α+ amacrines in the forming INL and GCL.  (M) Compared to wild type controls, *Neurog*2 mutants and *Atoh7* mutants had reduced retinal thickness, and double-mutants were significantly thinner than either single mutant.  (N) No significant change in RXRγ+ cells was found in any genotype, although there was a trend towards increased cones in *Atoh7* mutants and double-mutants.  (O) *Neurog*2 mutants had a small increase in Pou4f2+ RGCs.  (P-R) AP2α+ amacrines were unaffected in *Neurog*2 mutants, but significantly increased in *Atoh7* mutants and double mutants
in both the GCL and NBL. (S-U) Prox1+ retinal cells were significantly increased in the GCL of Neurog2;Atoh7 double-mutants, but unaffected in Neurog2 or Atoh7 single mutants. Scale bar: 50 µm in A, I. *p<0.05, **p<0.01, ***p<0.001; n=6-8 eyes (3-4 P0.5 pups) per genotype.

Figure 3.6. Cell proliferation and apoptosis in the absence of Neurog2. (A-D) The percentage of BrdU+ S-phase cells at E15.5 was normal in Neurog2 mutants (Neurog2\(^{GFP/GFP}\);Atoh7\(^{LacZ/+}\)) and double-mutants (Neurog2\(^{GFP/GFP}\);Atoh7\(^{LacZ/LacZ}\)). (E-H) The percentage of activated Caspase-3+ apoptotic cells (arrows in E-G) was significantly increased in double mutants, but not in Neurog2 single mutants. Scale bar: 50 µm in A. ***p<0.001; n=6 eyes (3 embryos) per genotype.

Figure 3.7. Ascl1 rescue of delayed neurogenesis in Neurog2 mutants. (A,A’) At E12.0, only rare Ascl1+ cells are present in Neurog2\(^{GFP/+}\) eyes. The Ascl1+ cell in A coexpresses GFP, thus is in the Neurog2 lineage (arrow in A’. and higher magnification in A’’). (B,B’) Neurog2\(^{GFP/Ascl1Kl}\) retinas have many more Ascl1+GFP+ cells (arrows in B,B’ and higher magnification in B’’). (C-E) Tubb3 and GFP co-labeling at E12.0. Delayed progression of Tubb3+ neurons in Neurog2\(^{GFP/GFP}\) mutants (D) was not found in Neurog2\(^{GFP/Ascl1Kl}\) eyes (E), which had an identical Tubb3 domain to controls (C). (F-H) Immunolabeling for Pou4f2/Brn3b and GFP showed that differentiated RGCs were also normal in Neurog2\(^{GFP/Ascl1Kl}\) mice (H), compared to Neurog2\(^{GFP/GFP}\) mutants (G). (I-L) Ectopic Ascl1 expression is delayed in Neurog2 mutants, relative to endogenous Neurog2 expression (compare to Figure 1), with a smaller domain of Atoh7 mRNA expression in Neurog2\(^{GFP/Ascl1Kl}\) retinas (K,L). (M-P) At E12.5, ectopic
Ascl1 and normal Atoh7 mRNA expression patterns are observed. Scale bars: 75 µm in A,C; 50 µm in I,M; n= 3-4 embryos per genotype; L = Lens.

**Figure 3.8.** Absence of bHLH factor cross-regulation at early stages of retinogenesis. (A-D) Atoh7 mRNA expression is normal in E12.5 (B) and E15.5 (D) Ascl1 mutant eyes. (E-F) Similarly, Ascl1 mRNA expression in E15.5 Neurog2^{GFP/GFP} retinas is indistinguishable from wild type. This is in contrast to E17.5 where the loss of Neurog2 derepresses Ascl1 mRNA expression (Akagi et al., 2004). Scale bar = 50 microns and in A,E; n = 3 embryos per genotype.
Figure 3.3

A-D: Imaging of Neurog2 and Atoh7 expression in the developing brain at E11.75-60.6S. The images show the expression of Neurog2 (GFP+; Atoh7LacZ+/GFP+) and Tubb3 in the brain tissues.

E: Schematic representation of Neurog2 and Tubb3 domains along the circumferences of the brain.

F-G: Bar graphs showing the percentage of Tubb3 domain extension relative to Neurog2 domain and the percentage of Tubb3+ cells in the Tubb3 domain.

H: Schematic representation of Neurog2 domain along the circumferences of the brain.

I-J: Additional imaging showing P21/Kip1 and Pou4f2 expression in Neurog2 and Neurog2/GFP combinations.

K-K': Immunohistochemical analysis showing Atoh7 and Neurod1 mRNA expression in the brain tissue sections.
Figure 3.6

A-E. Immunostaining of E15.5 neural tube sections for BrdU (A, B, C) and Act. caspase-3 (E, F, G) under different conditions. Scale bars: 20 μm.

D. Histogram showing the percentage of S-phase progenitor cells labeled with BrdU normalized to the total DAPI signal.

H. Histogram showing the number of apoptotic cells labeled with Casp3+/200x field.

Legend:
- Blue: Neurop2^{GFP}; Atoh7^{LacZ/+}
- Red: Neurop2^{GFP/GFP}; Atoh7^{LacZ/+}
- Green: Neurop2^{GFP/GFP}; Atoh7^{LacZ/LacZ}

Statistical significance: *** p < 0.001
Figure 3.8

**A**
Ascl1^{+/+}

**B**
Ascl1^{KO/KO}

**C**
E12.5
Atoh7 mRNA

**D**
E15.5
Atoh7 mRNA

**E**
Neurog2^{+/+}

**F**
Neurog2^{GFP/GFP}

**E15.5**
Ascl1 mRNA
CHAPTER 4

Distinct roles for Atoh7 and Ascl1 in early retinal neurogenesis
Abstract

Retinal neurons and glia differentiate in a stereotypical birth order in mice. Retinal ganglion cells (RGCs) are born first and bipolar neurons and Müller glia are generated last. The bHLH transcription factors Atoh7 and Ascl1 are required for normal RGC and bipolar development, respectively. Atoh7 expression begins at the initiation of neurogenesis, prior to onset of Ascl1 expression. To test the multipotency of the earliest retinal progenitors, we have misexpressed Ascl1 precociously in the Atoh7-lineage. Atoh7\textsuperscript{Ascl1} mice were generated by targeted replacement of Atoh7 by Ascl1 coupled to an IRES-DsRed2 reporter cassette. Atoh7\textsuperscript{Ascl1/Ascl1} mice have essentially no RGCs, like Atoh7 mutants. Thus, Ascl1 cannot compensate for Atoh7 in specifying the RGC fate. Homozygous Ascl1-misexpressing mice have increased bipolar neurons and decreased Müller glia. However, birthdating studies indicate that the onset of bipolar neurogenesis is unaffected in Ascl1-misexpressing retinas, and lineage-tracing reveals that bipolars are increased nonautonomously. At the initiation of retinal neurogenesis, Ascl1-misexpressing cells fail to differentiate and remain inappropriately mitotically active. Since Atoh7-lineage cells are normally terminally mitotic, the presence of S-phase Ascl1-misexpressing cells implies that Ascl1 promotes proliferation of early progenitors. Therefore, Ascl1 and Atoh7 encode different cues for cell fate specification and cell cycle progression.
**Introduction**

Retinal neurons in the vertebrate eye are born in a stereotypical birth order, in frog, chick, mouse, rat, monkey, and human (Altshuler et al., 1991). In all species, RGCs are born first, and bipolar interneurons and Müller glia are born last. In mouse, RGC genesis is closely followed by that of cone photoreceptors, amacrine and horizontal interneurons, which occurs largely prenatally (Sidman, 1961; Young, 1985). These four early cell types compose nearly fifteen percent of the adult mouse retina (Jeon et al., 1998). Rod photoreceptors, which make up over seventy-five percent of the adult retina, are largely born postnatally, although some appear during late embyrogenesis. Bipolar interneurons and Müller glia are almost entirely born postnatally, and compose the remaining ten percent (Jeon et al., 1998).

The birth of these seven cell types, in highly overlapping sequence from a common neuroepithelium, is an intriguing model for cell fate specification. Some retinal progenitor cells are multipotent, their clonal progeny giving rise to both early and late cell types across development (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990). The competence model of retinal development stipulates that, across developmental time, progenitors pass through a series of fate-restricted states, wherein only a subset of the total possible fates are available at any time (Cepko et al., 1996; Livesey and Cepko, 2001). In *Xenopus*, this appears to hold true within a single lineage, as the birth order of an individual clone composed of multiple cell classes reflects the overall sequence of their genesis (Wong and Rapaport, 2009). This suggests that the overlapping nature of birth dates results from heterogeneity of competence status among progenitors.

The competence model was based, in part, on data from *in vitro* heterochronic transplant studies, which indicated that embryonic progenitors are restricted to early fates when placed in a
postnatal retinal environment, and, similarly, postnatal fates are largely unavailable
embryonically (Belliveau and Cepko, 1999; Belliveau et al., 2000; Morrow et al., 1998). These
studies point to intrinsic components of competence, as environment alone could not reprogram
the temporal potential of retinal progenitor cells. Recently, the transcription factor Ikaros was
proposed to act as an early competence factor, since it induced early fate specification when
misexpressed postnatally (Elliott et al., 2008).

Here, I investigate the functions of different basic helix-loop-helix (bHLH) transcription
factors in the generation of early versus late fates in mouse retinal development. The temporal
expression of each bHLH factor was already known to correlate with distinct classes of retinal
neurons. For example, Atoh7 is expressed at the initiation of retinogenesis, and is critically
required for RGC formation and suppression of cone photoreceptors (Brown et al., 1998; Brown
et al., 2001; Wang et al., 2001). Ascl1 expression, which initiates two days later than Atoh7, is
required for normal bipolar interneurons and suppression of Müller glia differentiation (Jasoni
and Reh, 1996; Tomita et al., 1996). These data suggest that Atoh7 and Ascl1 have
fundamentally different properties in fate specification – Atoh7 during the early, prenatal phase
of retinogenesis, while Ascl1 regulates late, postnatal fates. I hypothesized that the period when
each bHLH factor is expressed influences the particular cell fates that can be produced by retinal
progenitor cells. Therefore, misexpression of Ascl1 in a population of cells that normally adopts
predominantly early fates could potentially convert them to later-born fates.

To answer this question, Ascl1 was misexpressed within Atoh7-expressing cells by
homologously recombining Ascl1 into the Atoh7 gene locus. I find that these bHLHs are not
interchangeable for early retinal fates, as Ascl1 fails to rescue RGC genesis in Atoh7^{Ascl1KI/Ascl1KI}
mice or alter the proportions of the other early fates. In both heterozygous and homozygous
Atoh7Ascl1KI mice, bipolar and Müller glia number was altered, although neither cell type was born precociously. Ascl1 misexpression in the earliest retinal progenitor cells prolonged their proliferation, indicating that Atoh7 and Ascl1 regulate retinal progenitor cell cycle progression differently. To investigate the effects of Ascl1 misexpression in Atoh7-expressing cells in directly, we permanently marked the Atoh7-lineage using a Math5-Cre transgene and a floxed GFP lineage reporter. As such, I was able to visualize the final fates adopted by the Atoh7-lineage in Atoh7Ascl1KI/Ascl1KI and Atoh7Ascl1KI/+ mice. Interestingly, in adult eyes Ascl1 misexpression resulted in the generation of both early and later-born fates in the Atoh7-lineage. Overall, I show that Atoh7 and Ascl1 have independent functions in cell fate specification and cell cycle exit, and encode potentially conflicting cues for cell fate determination.

Materials and Methods

Construction of Atoh7Ascl1 targeting vector

The Atoh7Ascl1KI mouse allele was made by MQ and NLB. The targeting strategy was similar to that of the Atoh7LacZ allele (Brown et al., 2001). Here, two regions of homologous sequence flanking the Atoh7 coding region – a 2.1 Kb EcoRI-PstI fragment for the 5’ arm and a 3.1 Kb PvuI fragment for the 3’ arm (Fig. 1A) were cloned into a targeting vector containing a PGK-neo cassette, flanked by LoxP sites. The Atoh7 coding exon was replaced with an Ascl1-IRES-dsRed2 cassette, creating a bicistronic mRNA and separately translated Ascl1 and dsRed proteins.
Generation of mutant and chimeric mice

The targeting vector was electroporated into W4 ES cells, using standard culture, transfection and G418/neomycin selection conditions (Auerbach et al., 2000). Genomic DNA from ES cells and mouse tail DNA was examined by long range PCR and Southern blotting to detect homologous recombination events. Confirmation of proper targeting to the Atoh7 locus was performed by Southern blot using a BamH1 digest and 5’ external probe as described (Brown et al., 2001), yielding a 22kb fragment for the normal allele and a 14kb fragment for the targeted replacement (Fig. 1C).

A recombinant ES clone was injected into C57BL/6J blastocysts and a resulting chimeric male bred with C57BL/6J, 129/Ola and CD-1 females to obtain germline transmission of the mutation directly into each genetic background. Mice were backcrossed five generations into their respective background, and the optic nerve phenotypes compared. No genetic background differences were observed, thus CD-1 mice were used for phenotypic analyses. Atoh7Ascl1 mice were subsequently crossed for two generations with germline EIIa-Cre mice (Lakso et al., 1996), to remove the neo cassette.

Animals

Atoh7LacZ (Brown et al., 2001) and Atoh7Ascl1KI mice were maintained on a CD-1 background. PCR genotyping of Atoh7LacZ and Ascl1− alleles was performed as described (Brown et al., 2001; Guillemot et al., 1993). PCR genotyping of Atoh7Ascl1KI mice generated a 350 bp wild type product and 232 bp targeted replacement product (Fig. 1C). For the Ascl1KI allele, we used the upper primer (AAGGTCTGTTGAATGTCGTGAAGG) and lower primer (TTGAATACGCTTGAGGAGCC), and amplification was carried out for 40 cycles of 1 min
at 94°C, 1 min at 56°C, and 1 min at 72°C. For the wild-type allele we used the upper primer (CGCCGCATGCAGGGGCTGAACACG) and lower primer (GATTGAGTTTTCTCCCCCTAGACCC), and amplification was carried out for 40 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min at 72°C, with MaterAmp in the formulation.

For embryonic studies, gestational age was determined by timed matings, with E0.5 as the date of the vaginal plug. Mitotically active cells in S-phase were labeled by injecting pregnant dams once with BrdU (0.1mg/g body weight of 10mg/mL BrdU in 0.9M NaCl) and harvesting embryos after 1.5 hours. For birthdating experiments, pregnant dams or pups were injected with BrdU at one age (0.1mg/g body weight of 10mg/mL BrdU in 0.9M NaCl), then analyzed at P21. For adult retinal proliferation studies, mice were injected once daily from P21-P27, and eyes collected on P28. Embryos and eyes were fixed for 1 hour (4%PFA/PBS), sectioned and antibody stained.

**Immunocytochemistry and in situ hybridization**

Immunohistochemistry was performed as described (Hufnagel et al., 2007). Antibodies used were rabbit anti-βgal (1:10000, Cappel), rat anti-βgal (1:1000, gift from Tom Glaser), rat anti-BrdU (1:100, AbD Serotec), sheep anti-Chx10 (1:1000, Exalpa Biologicals), rabbit anti-dsRed (1:500, Living Colors), rabbit anti-GFP (1:1000, Molecular Probes), rabbit anti-Ascl1 (1:1000; Horton et al., 1999), rabbit anti-M/L Opsin (1:500, Chemicon), rabbit anti-S-Opsin (1:500, Chemicon), rabbit anti-Pax6 (1:1000, Chemicon), mouse anti-p27 (1:200, Thermo Scientific), goat anti-Pou4f2/Brn3b (1:50, Santa Cruz), rabbit anti-RXRγ (1:200, Santa Cruz), and rabbit anti-Sox9 (1:1000, Chemicon). Direct-conjugate secondary antibodies (Molecular Probes) or sequential biotinylated secondary (Jackson Immunoresearch) and streptavidin-
conjugated Alexafluor tertiary antibodies (Molecular Probes) were used to visualize primary antibody labeling.

For immunohistochemistry experiments, a minimum of n=3 animals were used, except where noted for preliminary experiments. For the Atoh7-lineage analysis, n=2 animals were used for Math5-Cre;Z/EG, n=3 animals for Math5-Cre;Z/EG;Atoh7^{Ascl1KI/+}, and n=4 animals for Math5-Cre;Z/EG;Atoh7^{Ascl1KI/Ascl1KI} GFP and retinal marker immunolabeling.

**Cell counting**

Microscopy was performed with a Zeiss fluorescent microscope, Zeiss camera and Apotome deconvolution device. For all measures, I analyzed a minimum of 4 images per animal representing both eyes, from ≥3 embryos or postnatal pups from 2 independent litters. Preliminary counts consist of 3 images minimum from 1-2 animals per genotype. Cell counts and measurements were performed using the Zeiss Axiovision software (v5.0), using the interactive events and line tools. A paired Student’s t-test or ANOVA were performed to obtain p values (Instat v3.0). Photoshop (v7.0) was used to adjust images for brightness and contrast.

**Results**

*Precocious misexpression of Ascl1 from the Atoh7^{Ascl1KI} allele*

To understand if Ascl1 can substitute for Atoh7 during retinal development, and to test whether early retinal cells can make late fates precociously, Ascl1 was misexpressed by homologously recombining it into the Atoh7 locus (MQ and NLB, Fig. 1A). An Ascl1-IRES-dsRed2 cassette replaced the single Atoh7 coding exon. The Atoh7^{Ascl1KI} allele expressed Ascl1 a) ectopically in Atoh7-lineage cells, b) under the regulatory control of Atoh7, and c)
precociously beginning at the initiation of neurogenesis and RGC specification. Homozygous $Atoh7^{Ascl1KI/Ascl1KI}$ mice had complete replacement of $Atoh7$ expression with $Ascl1$, while heterozygous $Atoh7^{Ascl1KI/+}$ mice co-expressed $Atoh7$ and $Ascl1$ in the same cells. PCR and Southern blotting confirmed the presence of the $Atoh7^{Ascl1KI}$ allele in heterozygotes and homozygotes, and the lack of the wild type allele in homozygous animals (Fig. 1B-C, see Methods). For experimental comparisons, wild types were compared to $Atoh7^{Ascl1KI/+}$ mice, and $Atoh7$ mutants served as controls for $Atoh7^{Ascl1KI/Ascl1KI}$ mice, as both lacked $Atoh7$ expression.

To confirm that dsRed and Ascl1 proteins were each produced in the $Atoh7$-lineage, immunohistochemical analyses were performed. At E11.5, when retinal neurogenesis initiates, I found dsRed+ and Ascl1+ cells in the dorso-central retina of $Atoh7^{Ascl1KI/Ascl1KI}$ and $Atoh7^{Ascl1KI/+}$ mice (Fig. 1E-F,H), in the same pattern as βgal in $Atoh7^{LacZ/+}$ mice (Fig. 1D). To further demonstrate their concordance, I directly compared dsRed and βgal in $Atoh7^{LacZ/Ascl1KI}$ retinas, and found a high degree of overlap (not shown). By E13.5, dsRed and Ascl1 were expressed throughout the retina (Fig. 1E’-F’, H’), in the same pattern as $Atoh7^{LacZ}$ (Fig. 1D’). DsRed and Ascl1 are co-localized in many cells (arrows, Fig. 2) in $Atoh7^{Ascl1KI/Ascl1KI}$ mice at E13.5. The concordance is not 100%, likely due to the increased perdurance of the dsRed reporter compared to Ascl1 protein. In wild types, there were no Ascl1+ cells at E11.5 (Fig. 1G), and at E13.5 only rare Ascl1+ cells were observed (Fig. 1G’).

Ascl1 cannot rescue the Atoh7 RGC mutant phenotype, but does promote bipolar neurogenesis

In the retina, both loss- and gain-of-function data indicate that Ascl1 promotes bipolar and suppresses Müller glia genesis (Hatakeyama et al., 2001; Tomita et al., 1996). By contrast, $Atoh7$ is critical for RGC genesis (Brown et al., 2001; Wang et al., 2001). This implies that the
functions of Ascl1 and Atoh7 are not interchangeable. To test this hypothesis, I analyzed the eyes of P21 Atoh7Ascl1KI/+ and Atoh7Ascl1KI/Ascl1KI mice. As predicted, Ascl1 was unable to promote RGC formation in Atoh7Ascl1KI/Ascl1KI eyes, since optic nerves were absent (Fig. 3C), like Atoh7 mutants (Fig. 3D). The cranial nerves that innervate the extraocular muscles appeared grossly normal (arrowhead, Fig. 3C). Atoh7Ascl1KI/Ascl1KI eyes also had a severe reduction of RGC markers on histology, as Pou4f2/Brn3b+ cells (Xiang et al., 1993) were virtually absent in the GCL (Fig. 3G and not shown). This confirmed a massive loss of retinal ganglion cells in Atoh7Ascl1KI/Ascl1KI retinas. The eyes also had increased S-cones, like Atoh7 mutants (Fig. 3K,N). M-cones were unaffected in either Atoh7 mutant or Ascl1 replacement retinas. The increase in cones is also reflected by increased RXRγ+ nuclei in the ONL (not shown). Atoh7Ascl1KI/Ascl1KI eyes exhibited reduced INL thickness, consistent with Atoh7 mutants (Fig. 3M). Thus, Ascl1 could not compensate for the loss of Atoh7 in Atoh7Ascl1KI/Ascl1KI retinas.

Next, I asked if co-expression of Atoh7 and Ascl1 (Atoh7Ascl1KI/+) also affected these retinal fates. P21 Atoh7Ascl1KI/+ eyes had optic nerves and a normal complement of Pou4f2/Brn3b+ RGCs (Fig. 3B,F). Furthermore, these mice had both a normal percentage of S-cone photoreceptors, and INL thickness (Fig. 3J,M,N). Therefore, one allele of Atoh7 was sufficient to direct normal RGC and cone genesis, and the ectopic presence of Ascl1 was unable to alter these functions. To determine whether other early fates were affected, I evaluated the proportions of amacrine interneurons (Pax6+) and horizontal interneurons (Calbindin+, Pax6+) in the INL. Compared to wild types, both cell types were unaffected in Atoh7Ascl1KI/+ and Atoh7Ascl1KI/KI mice, similar to Atoh7 mutants (Fig. 3O). Overall, Ascl1 misexpression in Atoh7-expressing cells did not alter early fate specification.
To understand how ectopic Ascl1 affected bipolar interneurons (Chx10+) and Müller glia (CRALBP+, Sox9+), I also quantified INL cell types. In Atoh7^{Ascl1KI/Ascl1KI} mice, bipolar interneurons were increased (Fig. 4C,D,I) and Müller glia decreased (Fig. 4G,H,I) compared to Atoh7 mutant controls. On the other hand, Atoh7^{Ascl1KI/+} eyes had an overall decrease in bipolars compared to controls (Fig. 4A,B,I), with no change in Müller glia (Fig. 4E,F,I). I conclude that Ascl1 replacement was sufficient to promote bipolar and suppress Müller glia genesis, even when misexpressed during early retinogenesis.

**Bipolar neurons and Müller glia are not precociously generated**

I hypothesized that, if the effect of Ascl1 misexpression on bipolar neurons is direct, then these cells might be generated during the embryonic period in advance of normal histogenesis. Therefore, I performed a BrdU birthdating analysis in Atoh7^{Ascl1KI/Ascl1KI} mice. Pregnant dams were injected with BrdU at E12, E14, or E18, or pups injected at P2, P4, or P6, and all analyses performed at P21. Cells retaining high BrdU completed mitosis and exited the cell cycle soon after the injection, since BrdU was not diluted by subsequent mitotic divisions. To analyze the birth of bipolar interneurons, I performed double antibody labeling to mark BrdU+/Chx10+ cells. For embryonic ages E12 and E14, I noted no precocious bipolars (Fig. 5A,B). At E18, the beginning of normal bipolar and Müller glia genesis, I noted rare Chx10+/BrdU+ birthdated bipolars in homozygotes and controls (Fig. 5C). Postnatally, I observed no difference in bipolar birth dates, compared to wild types. At P2, the majority of bipolar neurons were born in the central retina (Fig. 5D), by P4 they were born more evenly throughout the retina (Fig. 5E), and by P6 the majority were born in the peripheral retina (Fig. 5F). I found no alterations in bipolar genesis in Atoh7^{Ascl1KI/+} mice, (not shown), nor was Müller glia genesis affected in heterozygotes.
or homozygotes (not shown). Therefore, the increase in bipolar interneurons observed with Ascl1 misexpression did not result from precocious generation of these cell types.

Ascl1 misexpression prevents cell cycle exit during early retinogenesis

Although Ascl1 cannot promote RGC genesis, it clearly performs a unique role postnatally during the period of bipolar and Müller glia development. One important difference between Atoh7 and Ascl1 is their expression at distinct phases of the cell cycle – Ascl1 is expressed mostly by proliferating, mitotically active retinal progenitor cells, while Atoh7 is expressed when these cells exit the cell cycle (Brzezinski, 2005; Jasoni and Reh, 1996; Le et al., 2006; Yang et al., 2003). Atoh7 mutants exhibited altered cell cycle profiles during early neurogenesis, namely a failure to exit the cell cycle properly, suggesting a critical period for Atoh7 action at the point of terminal differentiation (Le et al., 2006). To understand whether Ascl1 encodes distinct cell cycle cues during retinal progenitor differentiation, I next examined the cell cycle progression and exit of retinal cells in Atoh7^{Ascl1KI} eyes.

At the onset of retinal neurogenesis, subsets of progenitor cells cease proliferating, exit the cell cycle, and adopt a neuronal fate. First, I examined retinal cell proliferation, using a 1.5 hour BrdU pulse to label cells in S-phase. In Atoh7 mutants, proliferation was reduced at E11.5 but normal at E12.5 (Le et al., 2006). By comparison, the overall proportion of BrdU-labeled cells in the retina is not decreased at E11.5 and E12.5 in Atoh7^{Ascl1KI/+} and Atoh7^{Ascl1KI/KI} mice compared to wild types (Fig. 6A-C), and preliminary quantification suggests an increase in S-phase progenitors at these ages (Fig. 6M).

I then examined cell cycle exit, using the marker p27/Kip1, a cyclin dependent kinase inhibitor (Dyer and Cepko, 2001a; Levine et al., 2000). p27+ cells were reduced in Atoh7^
mutants at E11.5, but increased at E12.5 (Le et al., 2006). Similar to Atoh7 mutants, 
Atoh7^{Ascl1KI/Ascl1KI} retinas had fewer p27+ cells at E11.5 (Fig. 6D,E,N). However, at E12.5, this 
reduction persisted in homozygous Ascl1-misexpressing mice (Fig. 6G,H,N), indicating that 
ectopic Ascl1 perturbed the normal pattern of p27 expression. To determine if Ascl1 could block 
p27 expression, even in the presence of Atoh7, I next examined Atoh7^{Ascl1KI/+} mice. Compared to 
wild-type controls, p27+ cells were reduced in Atoh7^{Ascl1KI/+} mice, at both E11.5 and E12.5 (Fig. 
6F,I,N). Furthermore, this correlated with reduced differentiation of RGCs, since 
Pou4f2+/Brn3b+ RGCs was reduced in Atoh7^{Ascl1KI/+} eyes, compared to controls at both ages 
(Fig. 6J,K), supported by preliminary quantification (Fig. 6O). Pou4f2+ RGCs were virtually 
absent in Atoh7 mutants and Atoh7^{Ascl1KI/Ascl1KI} mice at all embryonic ages examined (Fig. 6L). 

Since RGCs and optic nerves in adult Atoh7^{Ascl1KI/+} eyes were indistinguishable from wild 
types, I investigated older embryos to determine if terminal differentiation is initially delayed but 
eventually returns to normal. At E13.5 and E14.5, p27+ cell number appears normal in 
Atoh7^{Ascl1KI/+} eyes by preliminary quantification (Fig. 6P). However, p27+ cells were reduced in 
Atoh7^{Ascl1KI/Ascl1KI} mice at both ages (Fig. 6P). While the ratio of p27+ cells returned to normal in 
Atoh7 mutants at E14.5 and at subsequent ages (Le et al., 2006), the reduction in postmitotic 
cells is prolonged to E14.5 in Atoh7^{Ascl1KI/Ascl1KI} mice (Fig. 6P). Overall, I conclude that Ascl1 
replacement of Atoh7 prevents cell cycle exit and terminal differentiation. Further, Ascl1 co-
expression with Atoh7 caused a temporary, but measurable delay in cell cycle exit. However, 
RGC differentiation was ultimately restored and adult Atoh7^{Ascl1KI/+} eyes had a normal 
complement of RGCs (Fig. 3B,F). Therefore, although the overall proportions of mitotic cells 
were unaffected in Ascl1-misexpressing heterozygotes and homozygotes, it remained possible 
that ectopic expression of Ascl1 affected the cell cycle status of only the Atoh7-lineage cells.
Ascl1 autonomously prolongs proliferation of early Atoh7-lineage cells

Alterations in cell cycle exit of early progenitor cells suggested that Ascl1-misexpressing cells failed to terminally differentiate during early retinogenesis. To determine if these cells continue through additional mitotic cycles, I co-labeled E11.5 and E12.5 retinas for dsRed and BrdU (1.5 hour pulse). There is normally no Atoh7 expression in S-phase (BrdU+) retinal progenitor cells (Le et al., 2006; Poggi et al., 2005). To our surprise, I observed many dsRed+/BrdU+ cells in Atoh7\textsuperscript{Ascl1KI/+} and Atoh7\textsuperscript{Ascl1KI/Ascl1KI} retinas, at both E11.5 and E12.5 (Fig. 7A,B), indicating that certain Atoh7-lineage cells misexpressing Ascl1 continued to proliferate, even in the heterozygotes where Ascl1 and Atoh7 were co-expressed. I conclude that Ascl1 misexpression overrode cell cycle exit cues normally present in the Atoh7-lineage cells, either allowing or promoting progression through at least one additional mitosis. Atoh7 itself, while required for normal cell cycle exit, was unable to prevent this, as heterozygous dsRed+ cells also remained proliferative. At these early ages, cell cycle progression cues encoded by Ascl1 acted dominantly over Atoh7 in these cells.

Because transcription at the Atoh7 locus ends shortly after birth (Brown et al., 1998), ectopic Ascl1 expression in the Atoh7-lineage should cease at this time. Thus, Ascl1 misexpression is unlikely to promote cell proliferation postnatally. To verify this, mice were given daily BrdU injections from P21-P28, to label any retinal cells that were inappropriately proliferating. Although a small population of cells in the ciliary body and lens remained proliferative into adulthood, proliferating cells were absent from the mouse retina (Dhomen et al., 2006; Kubota et al., 2002; Reh and Fischer, 2001; Tropepe et al., 2000; Yamamoto et al., 2008). I did not observe any BrdU+ cells in control, heterozygote, or homozygote Ascl1-
misexpressing retinas (Fig. 8A-B’). Hence, the inappropriate mitosis within the embryonic Atoh7-lineage did not persist into adulthood.

Ascl1 directs the Atoh7-lineage towards postnatal fates

To determine if Ascl1 misexpression in the Atoh7-lineage directly reprogrammed these cells to adopt a bipolar fate, it was important to mark this lineage throughout development. The dsRed reporter protein expression was transient and disappeared by P10 (not shown). Therefore, I used Math5-Cre, a BAC transgenic line carrying ~210 kb of 5’ and 3’ regulatory Atoh7 DNA that faithfully recapitulates the Atoh7 expression pattern (Brzezinski, 2005; Saul et al., 2008), and the Z/EG reporter that constitutively expresses GFP upon Cre activation. Therefore, Atoh7-expressing (or Ascl1-misexpressing) cells were permanently GFP-labeled. The Atoh7-lineage comprises 3-5% of the adult retina (Brzezinski, 2005). The proportions of cell types are skewed towards the early fates, since RGCs, cones, amacrines, and horizontals are approximately two-thirds of the Atoh7-lineage (Brzezinski, 2005; Jeon et al., 1998). Except for the loss of RGCs and increased displaced amacrine cells in Atoh7 mutants (Atoh7LacZ/LacZ) (Brown et al., 2001; Wang et al., 2001), the proportions of the other three later fates in the Atoh7-lineage are relatively similar, while increases in lineage-labeled bipolars and Müller glia are observed (Brzezinski, 2005).

Next, I analyzed all seven retinal cell classes in Math5-Cre;Z/EG, Math5-Cre;Z/EG;Atoh7Ascl1KI/+ and Math5-Cre;Z/EG;Atoh7Ascl1KI/Ascl1KI retinas by GFP and retinal marker immunolabeling. Control Cre-negative;Z/EG mice exhibited no GFP expression in the retina, and all alleles followed a Mendelian distribution (not shown). Because Atoh7Ascl1KI/Ascl1KI cells proliferated inappropriately during embryonic retinogenesis, I predicted that the Atoh7-
lineage had increased. However, in \textit{Atoh7}^{\text{Ascl1KI/}} mice, the number of GFP+ cells does not appear increased (Fig. 9A,B). In the \textit{Atoh7}^{\text{Ascl1KI/Ascl1KI}} mice analyzed, numbers of GFP+ cells appeared variable between animals (compare Fig. 9C to 8C’), which may reflect variation in the recombining efficiency of \textit{Math5-Cre} or in reporter expression. Quantification of GFP+ cells is required, and more animals need to be analyzed to properly determine the variability of GFP expression in each genotype.

In the \textit{Atoh7}^{\text{Ascl1KI}} allele, \textit{Ascl1} was misexpressed during the period of \textit{Atoh7} expression from E11.5-P1 (Brown et al., 1998), before the majority of bipolars and Müller glia are born. To determine if \textit{Ascl1} misexpression autonomously induced late fate specification of \textit{Atoh7}-lineage cells, I assessed bipolars and Müller glia in the \textit{Atoh7}^{\text{Ascl1KI}} lineage. In heterozygote or homozygote \textit{Ascl1}-misexpressing mice, I found very few GFP+/Chx10+ bipolar interneurons (Fig. 9G-I’). This did not correlate with the increase in bipolar neurons noted in \textit{Atoh7}^{\text{Ascl1KI/Ascl1KI}} mice (Fig. 3). Further, I observe few GFP+/Sox9+ Müller glia (Fig. 9J-L’) in any genotype. The rarity of GFP+ bipolar interneurons and Müller glia in all genotypes indicated that changes in the \textit{Atoh7}-lineage could not account for the retinal alterations in these cell types (Fig. 4). Therefore, I concluded that the changes in bipolar interneuron and Müller glial number in \textit{Atoh7}^{\text{Ascl1KI/}} and \textit{Atoh7}^{\text{Ascl1KI/Ascl1KI}} mice arose outside the \textit{Atoh7}-lineage. I did note, however, that the distribution of GFP+ cells was skewed towards a preponderance of GFP+ cells in the ONL of \textit{Ascl1}-misexpressing mice. Therefore, I compared cone (RXRγ+) and rod photoreceptor (Rhodopsin+) markers in GFP+ lineage-marked. The \textit{Atoh7}-lineage in \textit{Ascl1}-misexpressing eyes contained both cone (Fig. 9D-F’) and rod photoreceptors (Fig. 9G-I’). While wild type eyes had groups of spatially separated GFP+ rods, \textit{Ascl1}-misexpressing retinas had many rods inappropriately arranged in tight clusters. These rod clusters were also closely
associated with bipolars and Müller glia in the INL (Fig. 9J-O’), but not horizontal or amacrine interneurons (not shown), suggesting that these might be clonal progeny that differentiated as later-born fates. This phenotype was consistent in both $Atoh7^{Ascl1KI/+}$ and $Atoh7^{Ascl1KI/Ascl1KI}$ retinas (insets, Fig. 9 H,I,K,L,N,O).

**Discussion**

In this report, I directly compare the functions of the proneural bHLH factors $Atoh7$ and $Ascl1$, and found that they perform very distinct roles during retinogenesis. First, $Ascl1$ could not rescue the RGC or cone phenotype of $Atoh7$ mutants. In addition, although when $Ascl1$ was inappropriately co-expressed with $Atoh7$, RGC and cone differentiation appeared normal in the adult eye. Instead, ectopic $Ascl1$ recapitulated previously defined requirements for $Ascl1$ function during retinogenesis, namely promotion of bipolar interneuron specification and suppression of Müller gliogenesis. However, early misexpression of $Ascl1$ did not generate later-born fates precociously or suppress early fate specification. Paradoxically, while the presence of $Ascl1$ autonomously maintained proliferation of the $Atoh7$-lineage, the size of this population did not appear increased in heterozygous and homozygous mice. Overall, $Ascl1$ misexpression in the $Atoh7$-lineage demonstrated no effect on final numbers of early retinal fates, but, unlike $Atoh7$, $Ascl1$ opposed cell-cycle exit in early retinal progenitors.

*$Atoh7$ and $Ascl1$ are not functionally interchangeable for RGC genesis*

Because $Ascl1$ did not rescue $Atoh7$ RGC or cone phenotypes, but ectopically promoted later-born fates, I conclude these two bHLH factors perform non-overlapping functions during retinal specification. Two other bHLH genes, *NeuroD1* and *Atoh3*, have properties similar to
Atoh7, since they could partially rescue RGC specification when substituted for Atoh7 (Mao et al., 2008a). Atoh7, Atoh3, and NeuroD1 have shared homology with the Drosophila gene atonal, which specifies the first neuron, R8, during fly eye development (Bertrand et al., 2002; Jarman et al., 1994). Ascl1, on the other hand, is a mammalian orthologue of the Drosophila achaete-scute gene family. Interestingly, the achaete and scute genes do not function in neurogenesis of the Drosophila photoreceptors, though the achaete-scute complex does play a role in interommatidial bristle formation (Brown et al., 1991; Frankfort et al., 2004), implying that Ascl1 function in vertebrate retinal neurogenesis is not conserved as deeply as functions of atonal homologues during eye development. Similar to our experiments, ectopic scute expression in atonal mutants could partially rescue ommatidia, but not R8 neurogenesis (Sun et al., 2000).

The lack of interchangeability of bHLH factors in retinal development may be reflected in their distinct bHLH domain sequences and divergent functions across the evolution of eye development.

While increases in bipolar interneurons of Atoh7^Ascl1KI/Ascl1KI mice are consistent with previous phenotypic analyses of Ascl1 mutants, in our case this was not a direct effect, as tracing the Atoh7-lineage revealed no increase in GFP-labeled bipolar neurons. Further, in Atoh7^Ascl1KI/+ mice bipolar interneurons were decreased, which was also an indirect effect. These non-autonomous phenotypes were not surprising, since misexpression of Ascl1 in these mice occurs largely prenatally, well before the peaks of bipolar and Müller glial genesis. Indirect effects are not uncommon among bHLH mutants, as Atoh7 mutant mice have increased cones and decreased bipolar neurons not reflected in the mutant Atoh7-lineage (Brzezinski et al., 2005; Yang et al., 2003). How endogenous Ascl1 affects bipolar interneuron or Müller glia development is unknown, but our results suggest it can occur by a nonautonomous mechanism.
Ascl1 and Atoh7 regulate cell cycle progression of early retinal progenitors

Here, I uncovered a role for Ascl1 function in retinal progenitor cell proliferation. In Atoh7^{Ascl1KI} mice, Ascl1 was ectopically expressed within cells that are normally exiting the cell cycle. Instead, these cells undergo at least one more cell division, but do not permanently do so into adulthood. I observed this in both heterozygous and homozygous Atoh7^{Ascl1KI} eyes, suggesting that the function of Ascl1 to promote proliferation is dominant over Atoh7 regulation of cell cycle exit. This outcome differs from other misexpression analyses of Ascl1 and other proneural bHLHs in different regions of the developing nervous system, which typically result in cell cycle exit and neural commitment (Ahmad et al., 1998; Cai et al., 2000; Farah et al., 2000; Mao et al., 2008b; Tomita et al., 2000). Endogenous Ascl1 is expressed in proliferating progenitor cells. Our data suggests that Ascl1 misexpression promotes or maintains proliferation, albeit temporarily. Older embryonic ages will be examined to better define this effect.

In support of our findings, Ascl1 is required for proliferation in at least two circumstances. First, retinal injury in zebrafish results in the dedifferentiation and expansion of Müller glia to generate new photoreceptors and other retinal neurons. Here, the Müller glial expression of Ascl1a is critical for regenerating cells to properly proliferate and differentiate as neurons (Fausett et al., 2008; Yurco and Cameron, 2007). Second, many cancer models demonstrate that upregulation of Notch signaling and downregulation of bHLH expression is critical for tumor growth. However, certain neuroendocrine tumors such as small cell lung cancer had higher levels of Ascl1 than Notch components, and Ascl1 was required for proliferation in these cases (Jiang et al., 2009; Kunnimalaiyaan et al., 2006; Rapa et al., 2008; Sippel et al., 2003). In both regeneration and cancer circumstances, Ascl1 was present in
proliferating cells with a neural/neuroendocrine identity, suggesting that Ascl1 may promote proliferation in neural-restricted progenitor cells. Our findings show that Ascl1 temporarily promoted proliferation in a developmental context as well, a property that may have been overlooked in previous studies. Further investigation of endogenous and misexpressed Ascl1 will reveal whether Ascl1 directly or permissively affects cell cycle exit.

Even though Ascl1 misexpression promoted proliferation at early embryonic ages, I observed no apparent increase in the total size of the Atoh7-lineage by P21. This could occur by several mechanisms. First, it is plausible that Ascl1 autonomously or non-autonomously inhibits Atoh7 expression. Here, Ascl1 misexpression could result in negative feedback that would downregulate expression from the Math5-Cre transgene, thereby reducing the total number of lineage-labeled cells. I do not currently consider this plausible, since the dsRed reporter that temporarily marks these cells was observable in the Atoh7 expression pattern from E11-E13, and present in the retina until at least E16 (not shown). Better characterization of the Atoh7-lineage may also elucidate the effect of Ascl1 on clone proliferation and clone size.

Second, Ascl1 and Atoh7 might encode different cues for cell cycle length, which could reduce the number of differentiating cells. If Atoh7-lineage cells misexpressing Ascl1 continue to proliferate in a much longer cell cycle, the fewer cells would divide and differentiate for a given time. Though continued proliferation suggests that more cells would be generated by mitosis, if they were held in the cell cycle for a prolonged period fewer cells would divide across the period of retinogenesis. Further investigation of the coincidence of dsRed with other cell cycle markers, as well as measurements of cell cycle length are required to explore these potential mechanisms.
Third, \textit{Ascl1} misexpression could result in the death of \textit{Atoh7}-lineage cells. Misexpression of bHLH factors by retroviral infection can result in the death of neural progenitors (Cai et al., 2000), or might provide an inappropriate differentiation cue to progenitor cells. Here, if \textit{Atoh7}-expressing cells were unable to interpret ectopic \textit{Ascl1} expression, progenitor cells might enter a brief period of proliferation followed by apoptosis. I have observed dsRed+ cells throughout the retina at E16, but have not investigated later embryonic ages. Therefore, the reduction in the \textit{Atoh7}-lineage could occur by apoptosis between E16 and P21. Though increased apoptosis might occur with \textit{Ascl1} misexpression, it only comprises 5% of the retina, so I would not see a striking effect in retinal size compared to controls. I favor this possibility, and am evaluating lineage-labeled cells embryonically to determine when the reduction in the \textit{Ascl1}-misexpressing \textit{Atoh7}-lineage occurs.

\textit{Retinal mechanisms of competence and birth order are not regulated by a simple bHLH switch}

The changing competence of retinal progenitor cells across time involves a combination of intrinsic cues and extrinsic signals. The early-competence factor \textit{Ikaros} is expressed primarily in proliferating retinal progenitors and nascent RGCs, amacrines, and horizontals. Inappropriate postnatal \textit{Ikaros} expression increased the generation of these cell types, even horizontal interneurons that are strictly generated prenatally (Elliott et al., 2008). However, the molecular pathways that regulate early versus late competence states are still unknown. The proneural bHLH factors are considered prime candidates for competence factors, since their timing of expression correlates with phenotypically affected cell fates (Brown et al., 1998; Brown et al., 2001; Jasoni and Reh, 1996; Moore et al., 2002; Tomita et al., 1996).
These findings provide several insights into the mechanisms of retinal progenitor cell differentiation and multipotency. First, I do not find precocious generation of the later fates, though *Ascl1* misexpression only affects later fates, bipolar interneurons and Müller glia. This does not rule out *Ascl1* as a component of a later competence state, however, as *Ascl1* may skew the *Atoh7*-lineage autonomously from a majority of early fates towards a photoreceptor fate, which I am currently quantifying. Unknown, too, is the extent of endogenous *Ascl1* expression in *Atoh7*-lineage cells and effects at later embryonic ages. Second, misexpression of *Ascl1* in the *Atoh7*-lineage results in clusters of lineage-labeled cells in the ONL that appear to be photoreceptors (Fig. 9). While the majority of these ONL clusters express rhodopsin, it is unclear if these are fully differentiated, functional photoreceptors. Birthdating of the *Atoh7*-lineage may provide insight into the clonal nature of the ONL clusters. Whether a change in the *Atoh7*-lineage represents a shift towards a later competence state for these cells, or simply reflects the normal proportions of cell types in the adult mouse retina is not yet known.

Entry into late retinal competence states may be much more dependent on retinal environment than that of early competence. As development proceeds, cells are able to respond more fully to CNTF, LIF, and FGF, indicating that extrinsic signaling likely play a larger role in late fate specification (Ezzeddine et al., 1997; Lillien, 1995; Lillien and Cepko, 1992; Lillien and Wancio, 1998b). Bipolar neurons and Müller glia may be unable to differentiate in the early retina. These cell types are generated only postnatally in the presence of the other five cell classes. It is known that postmitotic cells influence the fates adopted by proliferating progenitor cells. This suggests that the changing milieu of the retina is critical to the genesis of later fates, and play a role in the transition into the late-only competence state of postnatal retinogenesis.
Retinal retroviral misexpression of *Ascl1* generates rod photoreceptors and bipolar interneurons via retroviral vectors, and these cell types are reduced in *Ascl1* mutants (Hatakeyama et al., 2001; Tomita et al., 1996). However, it is unknown whether the loss of these cell types occurred cell autonomously in *Ascl1* mutants. Even if *Ascl1* is a late competence factor, it may be unable to execute the bipolar specification program in the environment of the early retina. Thus, *Atoh7AsclIKI* cells might have the intrinsic competence but lack the environmental cues necessary to adopt late fates. I could test this by transplanting early retinal progenitors misexpressing *Ascl1* into the postnatal retina, and evaluate the fates they adopt. Such a heterochronic transplant experiment would provide both intrinsic and extrinsic cues specifying later-born fates, and might uncouple competence status from temporal-environmental constraints on the birth order of retinal fates.

**Progress and Future Directions**

The *Atoh7AsclIKI* mouse was made by Malgorzata Quinn, a former postdoctoral fellow in the Brown laboratory, and the *Math5-Cre* transgenic mouse was made by Joseph A Brzezinski III, a former graduate student in the Glaser laboratory. Comparison of dsRed and *Ascl1* misexpression with the *Atoh7LacZ* pattern have been performed from E11-E13 (Fig. 1-2). Colocalization of dsRed and BrdU have been performed at E11.5 (n=3 animals) and E12.5 (n=1) (Fig. 7). Counts for adult retinal cell classes, except rod photoreceptors, are complete (Fig. 3-4). Birthdating experiments from E12.5-P10 (Fig. 5) have addressed only bipolar neurons embryonically and Müller glia and bipolar neurons postnatally (n=2). Cell counting of proliferation and terminal differentiation markers during the early embryonic period (Fig. 6) are preliminary (n=1-2), except for p27/Kip1 and Brn3b staining at E12.5 (n=3-4).
The *Math5-Cre* lineage-tracing experiment (Fig. 9) has generated provocative data concerning the size and composition of the *Atoh7*-lineage with *Ascl1* misexpression. Additional animals at P21 and embryonic and early postnatal ages are being analyzed by Kate Maurer, Amy N. Riesenber, and Nadean L. Brown to determine if the *Atoh7*-lineage is shifted towards later-born fates. In light of lineage-labeled rod clusters with *Ascl1* misexpression, birthdating for rod photoreceptors may reveal if these cells differentiate in the same temporal window. In *Atoh7*<sup>Ascl1/+</sup> mice, Brn3b+ cells are reduced at E12.5 but appear normal in the adult. Evaluation of RGC genesis during late embryonic and early postnatal periods would determine if histogenesis for this cell class is altered or prolonged.

In light of a role for *Ascl1* in promoting progenitor cell proliferation, *Ascl1* mutants should be assessed embryonically for altered proliferation and terminal differentiation markers. Co-localization experiments for Ascl1 and Atoh7<sup>LacZ</sup> proteins will demonstrate overlap or exclusivity of these bHLHs. To determine the effect of endogenous *Ascl1* on the *Atoh7*<sup>Ascl1KI</sup> allele, I have generated *Atoh7*<sup>Ascl1KI/+;Ascl1</sup>+/- mice to interbreed and produce double homozygous mice that lack endogenous *Ascl1* and precociously misexpress *Ascl1*. These mice might rescue *Ascl1* function in bipolar neurogenesis, but would require explant analysis for postnatal ages as these mice are not viable. Alternatively, these mice might exhibit increased *Ascl1* misexpression if endogenous *Ascl1* normally represses the *Atoh7* allele. This is less likely as preliminary data by Tien Le indicates that *Atoh7* mRNA expression is not altered in *Ascl1* mutants at E15.5. See discussion section for other proposed experiments.
Notes to Chapter 4

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Figure Legends

Figure 4.1. The Atoh7<sup>Ascl1KI</sup> targeted replacement allele. (A) The Atoh7<sup>Ascl1KI</sup> allele precisely replaces the Atoh7 coding region with Ascl1-IRES-dsRed and neo selectivity cassette. The targeting vector contains 2.1 Kb (5’ arm) and 4 Kb (3’ arm) sequences. The neo cassette, flanked by loxP sites, was removed using EIIa-cre. Restriction sites and PCR primers are shown for the targeting allele. (B) Internal PCR primers for genotyping confirm the presence of the wild type product (350 bp) and Atoh7<sup>Ascl1KI</sup> product (232 bp). (C) Southern blot demonstrating proper targeting of Ascl1KI replacement vector in Atoh7<sup>Ascl1KI/+</sup> and Atoh7<sup>Ascl1KI/Ascl1KI</sup> mice. (D-H’) Comparison of Atoh7<sup>lacZ</sup> and Atoh7<sup>Ascl1</sup> expression patterns at E11.5 and E13.5. Atoh7<sup>lacZ</sup> is expressed in the E11.5 dorso-central retina, as shown by βgal immunostaining at E11.5 (arrow D), and throughout the retina at E13.5 (D,D’). dsRed present in a similar pattern in Atoh7<sup>Ascl1KI/+</sup> (arrow E,E’) and Atoh7<sup>Ascl1KI/Ascl1KI</sup> (arrow F,F’). Ascl1 protein is not present in E11.5 wild type eyes, and in rare cells at E13.5 (G, arrowhead G’), while observed in a similar pattern to dsRed in Atoh7<sup>Ascl1KI/Ascl1KI</sup> eyes (arrow H,H’). GCL, ganglion cell layer. Scale bar in D,D’ = 50 µm.

Figure 4.2. dsRed and Ascl1 expression patterns in the embryonic mouse retina. (A) Co-labeling for dsRed and Ascl1 proteins in Atoh7<sup>Ascl1KI/Ascl1KI</sup> embryonic retina at E13.5, arrows indicate double-labeled cells.

Figure 4.3. Ascl1 cannot rescue Atoh7 RGC or cone phenotypes. (A-H) RGC genesis is unaffected by Ascl1 misexpression from the Atoh7 locus. Compared to wild types (A,E), Atoh7<sup>Ascl1KI/+</sup> mice (B,F) have normal optic nerves and Pou4f2+ RGCs in the GCL. Atoh7<sup>Ascl1KI/Ascl1KI</sup> mice lack optic nerves and Pou4f2+ cells (C,G), like Atoh7 mutants (D,H).
Both Atoh7 mutants and Atoh7 Ascl1KI/Ascl1KI mice have increased S-cone photoreceptor outer segments (I-L, N) and thinner inner nuclear layers (M) compared to wild type and Atoh7 Ascl1KI/+ mice. Amacrine and horizontal interneurons (Pax6+) are unaffected (O). ONL, outer nuclear layer, INL, inner nuclear layer, GCL, ganglion cell layer. Scale bar in E = 25 µm, I = 50 µm.

**Figure 4.4.** Alterations in postnatal fates in Atoh7 Ascl1KI mice. Bipolars (Chx10+) and Müller glia (Sox9+) were quantified and normalized to total INL DAPI+ nuclei (I). In Atoh7 Ascl1KI/+ mice, bipolars are reduced compared to wild type controls (A,B), while Müller glia are unaffected (E,F). Atoh7 Ascl1KI/Ascl1KI mice have increased bipolars (C,D) and reduced Müller glia (G,H) compared to Atoh7 mutant controls. Scale bar in A = 50 µm.

**Figure 4.5.** Bipolar interneurons are not precociously generated in Atoh7 Ascl1KI/Ascl1KI mice. (A-C) Birthdated bipolars (BrdU+/Chx10+) are not detected embryonically in Atoh7 Ascl1KI/Ascl1KI mice at E12.5 or E14.5, but rare double-labeled cells are noted at E18.5. (D-F’) Similar to the pattern of bipolar interneuron genesis in wild types, Atoh7 Ascl1KI/Ascl1KI mice generate many bipolars in the central retina at P2 (D,D’), which is reduced by P4 (E,E’), and then nearly exclusively in the periphery at P6 (F,F’). Scale bar in A,D = 50 µm, F = 100µm.

**Figure 4.6.** Cell cycle profile of Atoh7 Ascl1KI mice during early neurogenesis. Immunolabeling for cell cycle markers at E11.5 (A-F) and E12.5 (G-L) in wild type, Atoh7 Ascl1KI/+ and Atoh7 Ascl1KI/Ascl1KI mice. Retinal progenitors in S-phase (1.5 hour BrdU pulse) in Atoh7 Ascl1KI/+ and Atoh7 Ascl1KI/KI mice at E11.5 or E12.5 (A-C,M). Progenitor cells exiting the cell cycle (p27+) are decreased (D-I, N), and the percentage of Pou4f2+ RGCs is decreased in Atoh7 Ascl1KI/+ mice.
and absent in $Atoh7^{Ascl1KI/Ascl1KI}$ at these ages (J-L, O). At E13.5 and E14.5, $Atoh7^{Ascl1KI/+}$ mice have normal p27+ cells, while $Atoh7^{Ascl1KI/Ascl1KI}$ are still reduced (P). Scale bar in A = 50 µm.

**Figure 4.7.** Continued proliferation of Ascl1-misexpressing cells. (A-B) Immunolabeling for dsRed and BrdU at E11.5 revealed co-labeled dsRed+/BrdU+ cells in $Atoh7^{Ascl1KI/+}$ (arrows, A) and $Atoh7^{Ascl1KI/Ascl1KI}$ mice (arrows, B). dsRed+/BrdU− cells were also noted (arrowheads A-B).

**Figure 4.8.** No retinal proliferation in adult $Atoh7^{Ascl1KI}$ mice. (A-B’) BrdU staining of P28 retina after daily injections from P21-P27 in central and peripheral retinas of wild type and $Atoh7^{Ascl1KI/Ascl1KI}$ mice, showing no proliferation. Arrows show proliferative cells outside the retina (A, A’, B’) or in the distal tip of the retina (B’). Scale bar in A = 50 µm, A’ = 25 µm.

**Figure 4.9.** Lineage analyses of the $Atoh7^{Ascl1}$-expressing cells. To permanently mark Ascl1-misexpressing cells, I crossed transgenic Math5-Cre and Z/EG reporter mice into the $Atoh7^{Ascl1KI}$ background. (A-C’) Analysis of GFP-marked cells at P21. (A) Wild type mice had many GFP+ cells distributed in all three layers of the retina. Inset shows lineage-marked photoreceptors spaced evenly in the ONL. (B-C’) $Atoh7^{Ascl1KI/+}$ and the majority of $Atoh7^{Ascl1KI/Ascl1KI}$ mice analyzed have a variable number of GFP+ cells per section. Insets show clusters of GFP-marked photoreceptors in the ONL. (D-I’) In the ONL, many GFP+ cone (RXRγ+) and rod (rhodopsin+) cells are detected. The ONL clusters in Ascl1-misexpressing mice are predominantly composed of rhodopsin+/RXRγ- cells (arrows in H, I, arrowheads in E, F). Bipolar interneurons (J-L’, GFP+/Chx10+) and Müller glia (M-O’, GFP+/Sox9+) are rarely labeled in all three genotypes.
(arrows), but are found in proximity to the rod clusters (arrowheads) in \textit{Atoh7}^{Ascl1KI/+} and \textit{Atoh7}^{Ascl1KI/Ascl1KI} mice. Scale bar in A = 100 \textmu m, D = 50 \textmu m.
Figure 4.4

Bipolar interneurons

Wild Type

A

B

C

D

Müller glia

E

F

G

H

I

---

Wild Type
Ascl1KI/+ 
Ascl1KI/Ascl1KI 
Lac2/LacZ

Bipolar interneurons (Chx10+/)
Müller glia (Sox9+)

---

$\text{Wild Type}$
$\text{Ascl1KI/+}$
$\text{Ascl1KI/Ascl1KI}$
$\text{Lac2/LacZ}$

---

70%
60%
50%
40%
30%
20%
10%
0%

Wild Type
Ascl1KI/+ 
Ascl1KI/Ascl1KI 
Lac2/LacZ

Bipolar interneurons (Chx10+/)
Müller glia (Sox9+)

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140
Figure 4.8

BrdU P21 - P27

Wild Type

Atoh7^{Ascl1KI/Ascl1KI}

A

P28

Central

B

Peripheral

A'

B'
CHAPTER 5

General Discussion:
A bHLH-Centric View of Retinal Neurogenesis
Purpose

This discussion considers how the previous chapters in this thesis contribute to an understanding of gene networks in the determination of retinal neuronal fate. Developmental genes that ultimately determine form and function are highly conserved among organisms with strikingly different appearance and physiology. In the central nervous system, the progression of vertebrate evolution follows an increasing complexity of tissue organization. From invertebrates to non-mammalian vertebrates to mammals to primates, more complex sensory processing, behavior, language, and consciousness have evolved. Studies of human and other genomes have revealed that these changes do not correlate with an increase in the number of genes themselves, reflected by the estimated gene number in human (20,000-25,000) and chicken (20,000-23,000) (Consortium., 2004; Lander et al., 2001). As such, many genes are homologous in nucleotide sequence and expression pattern. Therefore, it is likely that increased complexity results, in part, from changes in gene expression and small changes to protein structure, instead of gene number. Alterations in gene regulation, producing changes in the timing, level, and combination of gene expression in various developing tissues, might account for interspecies changes. In this manner, modifications of noncoding DNA enhancers and repressors regulating gene expression produce novel combinations of proteins and resultant effects on cellular differentiation and tissue formation. Thus, very different final body plans may result from the expression of homologous genes in novel combinations (Carroll, 2000; Shubin et al., 2009).

The vertebrate eye is an excellent tissue to study genetic networks in cell fate determination. The retina originates as a single layer of cells evaginating from the anterior neural tube. These cells ultimately proliferate and differentiate to form a three-layered neuroepithelium consisting of seven major cell types (Chow and Lang, 2001; Zaghloul et al.,
Even with this relatively simple organization, the retina is capable of complex visual processing, including contrast and motion, before light information is transmitted to the brain (He et al., 2003; Olveczky et al., 2003, 2007). These studies address the basic biology of RGC genesis in the mammalian retina. RGCs are critical for transmitting light information to the brain and are selectively lost in degenerative conditions such as retinal ischemia, diabetes, and glaucoma. Future therapeutics for retinal diseases may include neuroprotective and cellular replacement strategies (Fan et al., 2006; Harvey et al., 2006; Zaghloul et al., 2005).

Over the last 20 years, studies of retinogenesis have attempted to locate genes critical for the development of each retinal cell type. By genetic mutation and overexpression, the functions of a host of critical specification genes have been evaluated in the visual systems of fly, frog, fish, chick, mouse, and other organisms. The bHLH transcription factors have been of particular interest for controlling the transition of proliferating progenitors to differentiating neurons in all species and organ systems studied (Bertrand et al., 2002; Ohsawa and Kageyama, 2008; Vetter and Brown, 2001). It is also apparent that the coordinate expression of multiple bHLH factors is critical for the differentiation of each cell type (Akagi et al., 2004). However, the placement, timing, and function of bHLH factors in many of these networks have yet to be elucidated. This thesis focused on a single cell type, the RGC, and the bHLH transcription factors Atoh7, Neurog2, and Ascl1 (Fig. 1A). RGCs are the first cell type to differentiate in all vertebrates studied, like the analogous R8 cell in the Drosophila eye (Altshuler et al., 1991; Tomlinson and Ready, 1987a, b). In studying the earliest neurons, I was able to closely examine the first cells to differentiate from the pseudostratified neuroepithelium of the optic cup.

The central aim of this thesis was to evaluate the network of gene expression during the initiation of mammalian retinal neurogenesis and RGC differentiation. Much is known about the
regulation and function of the bHLH factor Atoh7, critical for RGC specification in multiple vertebrate species. However, little is known about the expression and function of other bHLH factors like Neurog2 and Ascl1. The first aim of this thesis examined Atoh7 regulatory DNA, elucidating that both positive and negative regulatory elements control Atoh7 expression. Next, these studies placed the related bHLH Neurog2 in the hierarchy of RGC differentiation, demonstrating its function in spatiotemporal regulation of neurogenesis. Finally, I studied the function of the distantly-related bHLH Ascl1 misexpressed in the earliest progenitor cells, to evaluate the effects of gene specificity and timing on RGC specification. In sum, these studies addressed the bHLH gene network in mammalian RGC genesis, and revealed complexities in the stepwise progression of RGC formation. A comprehensive understanding of RGC genesis, from proliferating progenitor cell to functional neuron, will elucidate the proper gene targets to promote neuronal survival or to engineer stem cells for cellular replacement therapies.

Conservation and Divergence of bHLH Transcription Factors

The Drosophila compound eye is dramatically different from the vertebrate complex eye. Drosophila ommatidia are essentially ~800 repetitive visual elements, each an eye itself with a lens and photoreceptor array. Each ommatidium contains the same eight neuron types, two inner color-detecting photoreceptors and six rhodopsin-expressing outer photoreceptors that project to the optic lobe lamina and medulla, respectively, in the Drosophila brain. Three different types of ommatidia are present, capable of detecting short, medium, or UV-only wavelengths of light, based on the expression of inner photoreceptor opsins (Cook and Desplan, 2001; Hsiung and Moses, 2002). The mouse eye is a continuous epithelium that receives light through a single lens. Here, photoreceptors do not directly signal to the brain. Similar to the Drosophila lamina
and medulla, light information passes from photoreceptors through bipolar interneurons in the INL to RGCs in the GCL. In this way, convergent and divergent processing occurs in the retina itself, determining contrast and object motion before visual information reaches the brain.

As organisms evolved and organs increased in complexity, so too did the molecular networks that govern organogenesis. As such, proneural bHLH genes in the mouse genome are conserved in gene families. *Drosophila atonal* and related genes *cato* (cousin of atonal), *nato3* (nephew of atonal) and *amos* are highly related to the *Atoh* genes (1,7), and more distantly related to the *Neurod (1,2,4)* and *Neurogenin (1-3)* families. The four genes in the *achaete-scute* complex, *achaete, scute, lethal of scute* and *asense*, are highly related to the *Ascl (1-3)* genes (Bertrand et al., 2002; Hassan and Bellen, 2000). In *Drosophila*, *atonal* is important for specification of R8 photoreceptors in the eye and mechanoreceptive chordotonal organ cells in the body. The *achaete-scute* complex is essential for formation of mechanoreceptive bristles in the eye and body (Brown et al., 1996; Brown et al., 1995; Jarman et al., 1993; Jarman et al., 1994; Jarman et al., 1995). These bHLH factors exhibit functional homology as well, as *Atoh1* can rescue development of photoreceptors and chordotonal organs when misexpressed in *atonal* mutant flies, and *atonal* replacement of *Atoh1* (*At1on* 1) restores *Atoh1* mouse mutant phenotypes (Ben-Arie et al., 2000; Ben-Arie et al., 1996; Wang et al., 2002a).

The homology between *Drosophila* and mammalian bHLH transcription factors is reflected in conserved gene networks that control organ development. In the *Drosophila* eye, *toy* and *eyeless* activate *atonal* similarly to mouse homologue *Pax6* activation of *atonal* homologues *Atoh7, Neurod4* (formerly *Atoh3*), and *Neurog2* and in the mouse eye (Marquardt et al., 2001; Riesenberg et al., 2009; Taranova et al., 2006; Zhang et al., 2006). *Atonal* specifies the first-born photoreceptor, R8, similar to the requirement for *Atoh7* in RGC genesis, the first-born neuron in
mouse retinal development (Brown et al., 2001; Jarman et al., 1994). However, there are notable differences in the atonal-homologues among vertebrates. The vertebrate atonal homologues exhibit interspecies differences as well. R8 determination in atonal mutant flies can be rescued by Xenopus Ath5, but not mouse Atoh7 (Sun et al., 2003). Thus, alterations in atonal-related gene function have occurred during vertebrate evolution as well.

The first aim of this thesis studied the noncoding regulatory DNA of Atoh7 using Math5-GFP transgenic mice. These transgenic mice are useful for studying early retinal Atoh7 expression in terminally mitotic cells, as well as labeling early RGCs during differentiation and axon migration. Math5-GFP expression indicates that major activating elements for Atoh7 in the developing retina are contained in the upstream 2.1 kb sequence. Further, the 2.1 kb region controls spatiotemporal expression, demonstrated by overlap with Atoh7\textsuperscript{LacZ} reporter in early retinal progenitors and RGC axons in the brain. As discussed in Chapter 2, non-overlap of Atoh7\textsuperscript{LacZ} and Math5-GFP transgenes at later ages may reflect reporter differences or absent retinal activating elements in the proximal 5’ 2.1 kb. This 2.1 kb fragment contains proximal and distal non-coding DNA segments with highly conserved nucleotide sequence from Xenopus to human (Hufnagel et al., 2007; Hutcheson et al., 2005; Riesenberg et al., 2009). In the frog 5’ 3.1 kb, two enhancers independently drive retinal expression, a distal enhancer functioning during initial retinal expression and a proximal enhancer in later expression (Hutcheson et al., 2005; Willardsen et al., 2009). In mouse, however, only the distal enhancer is sufficient for retinal expression (Hutcheson et al., 2005; Riesenberg et al., 2009). Thus, divergence of Atoh7 regulation is also present among vertebrates.

Multiple enhancer elements regulate the spatial expression of bHLH factors. Lack of cochlear nucleus expression in the GFP1 or GFP2 transgenes indicates a separate enhancer for
Atoh7 expression in the cochlear nucleus not present in the 5’ 2.1 kb or 3’ 1.6 kb sequences (Hufnagel et al., 2007). Therefore, multiple, separate Atoh7 enhancer elements regulate domain-specific expression. Studies of enhancer elements for other bHLH factors such as Neurog1 and Neurog2 have demonstrated that distinct enhancer elements direct region-specific expression, and are regulated by regionally-expressed transcription factors such as Pax6 (Blader et al., 2004; Blader et al., 2003; Marquardt et al., 2001; Scardigli et al., 2003; Scardigli et al., 2001). Analyses of the Neurog2 promoter indicates that multiple 5’ and 3’ enhancers exist to precisely restrict spatial Neurog2 expression in the spinal cord to V1-2 (Pax6+) and motor neuron (Isl1+) progenitor pools (Scardigli et al., 2003; Scardigli et al., 2001). Expression of the Neurog2 E1 enhancer, located ~10 kb upstream of the Neurog2 coding region, overlaps with Pax6+ but not Isl1+ cells and is directly regulated by Pax6 (Scardigli et al., 2003; Scardigli et al., 2001).

The level of nucleotide conservation of these activating elements is unclear. Comparisons of the 5’ Atoh7 enhancers with 5’ and 3’ Atoh1 noncoding DNA revealed no common binding site motifs. Further, expression of Math5-GFP in the pineal, molars, neocortex, and ventral spinal cord is not associated with Atoh1 expression. Recent evidence suggests enhancers, unlike coding sequences, are not evolutionarily restricted (Margulies et al., 2007). Instead of a conserved Atoh7-Atoh1 enhancer, the 5’Atoh7 DNA might contain proneural activating elements common among proneural bHLH factors. In the ventral spinal cord, Math5-GFP1 expression was localized to Pax2+, Islet1-negative cells, and is Pax6-dependent. One possibility is that the 5’ 2.1 kb of Atoh7 DNA contains regulatory elements in common with more divergent atonal-homologues such as Neurog2. Alternatively, ectopic expression of Math5-GFP may simply reflect activation of non-conserved binding sites for regionally-
expressed activators such as Pax6. Further studies will examine the regulation of the 5’ 2.1 kb sequence in non-Atoh7 and Atoh1 domains and overlapping expression with other bHLH factors.

Chapter 2 proposed that the restricted expression of Atoh7 is dependent on repressor elements as well. The 5’ 2.1 kb of Atoh7 DNA activates GFP expression in Atoh1 domains of the lower rhombic lip, dorsal spinal cord, inner ear, whisker barrels, and pontine nucleus, and non-Atoh1 domains of the ventral spinal cord, molars, pineal gland, and neocortex. Ectopic expression of Math5-GFP transgenes in domains where endogenous Atoh7 is not expressed implicates additional regulatory elements that normally suppress Atoh7 expression in these domains. Likely, the Math5-GFP1 transgene lacks repressor elements that inhibit expression of endogenous Atoh7 in a region-specific manner. Indeed, lack of inner ear hair cell expression in the Atoh7-GFP2 transgene indicates a potential inner ear repressor element for Atoh7 in the 3’ 1.6 kb DNA. However, the expression patterns of the 5’ and 5’-3’ transgenes are nearly identical, suggesting that such restrictive elements lie more distally. One repressor was recently found for Xenopus Ath5, near the distal 5’ enhancer, that prevents precocious Xath5 retinal expression (Willardsen et al., 2009). Thus, repressor elements can regulate spatial and temporal aspects of gene expression. Alternatively, the ectopic expression of Math5-GFP transgenes could result from regulation by regions surrounding the transgenic insertion site in the genome. This is unlikely, since independent insertion by both Math5-GFP transgenes had nearly identical expression patterns (Hufnagel et al., 2007).

Further analysis outside the 5’ 2.1 kb fragment is needed to identify the repressor sequences that together restrict Atoh7 expression to the retina and cochlear nucleus. A Math5-Cre BAC transgene drives Cre recombinase expression under control of 5’ 110 kb and 3’ 103 kb of Atoh7 noncoding DNA, and is faithfully expressed in the Atoh7 pattern (Brzezinski, 2005;
Saul et al., 2008). Therefore, the majority of positive and negative regulatory elements are present within that flanking ∼200 kb. One approach to locating potential Atoh7 repressor elements would be to generate Math5-GFP BAC transgenes containing different 5’ and 3’ regions of regulatory DNA in addition to the core regulatory elements in the 5’ 2.1 kb. This approach should identify candidate regions containing enhancer or repressor regulatory elements, based on the presence or absence of one or more non-Atoh7 expression domains that can then be tested for functionality and conservation. Since differences in Atoh7 function and regulation exist among vertebrates, these repressor elements may be mammalian-specific and represent further divergence of these genes across vertebrate evolution.

The divergence of proneural bHLHs Atoh7 and Atoh1 resulted in the regionally-restricted expression of each gene that together recapitulates the expression of Drosophila atonal. With the elaboration of the vertebrate nervous system, these genes diverged in a system-specific manner. I propose one mechanism for their evolutionary divergence lies in their regulation, where common activation elements drive expression in both Atoh1 and Atoh7 domains, while gene-specific repressor elements restrict their spatiotemporal expression. These regulatory elements must tightly coordinate Atoh7 expression in retinal progenitor cells to determine the first RGCs.

**Sequential Function of bHLH Factors in RGC Determination**

A second level of divergence between Drosophila atonal and the atonal-homologues in mouse is the subdivision of bHLH function. In Drosophila, bHLH factors regulate both selection of neural progenitors and subsequent specification and differentiation events (Bertrand et al., 2002; Hassan and Bellen, 2000). In the Drosophila eye, atonal is required for the
propagation of the morphogenetic furrow and specification of the first eye neurons, and *atonal* mutants lack all ommatidia (Brown et al., 1996; Brown et al., 1995; Jarman et al., 1993; Jarman et al., 1994; Jarman et al., 1995). In vertebrates, the initiation of neurogenesis also employs proneural bHLH factors. In zebrafish, a wave of *Ath5/lakritz* expression and sonic hedgehog (Shh) signaling drives RGC specification from the optic stalk to the rest of the retina (Kay et al., 2001; Masai et al., 2000). In *Xenopus*, *Ath5* expression is critical for RGC specification, and is also expressed in the ciliary marginal zone (Kanekar et al., 1997). Retinal neurogenesis in chick occurs over a longer period. The first RGCs are found in the dorsal central retina near the optic stalk, and spread along the central-peripheral and dorsal-ventral axes (Prada et al., 1991). In a similar pattern, expression of *Atoh7* and *Neurog2* initiates in the central retina and spreads to the periphery in chick (Matter-Sadzinski et al., 2005).

Chapter 3 analyzed the coincident expression and distinct functions of *Neurog2* and *Atoh7* during the initiation of retinal neurogenesis in mouse. While *Atoh7* was known to be critical for RGC genesis in all species studied, the function of *Neurog2* was previously unknown. The expression patterns of both bHLH factors has been demonstrated previously during retinal neurogenesis (Brown et al., 1998; Ma and Wang, 2006). Like *atonal*, *Neurog2* and *Atoh7* are expressed in progenitor cells that give rise to the first retinal neurons. *Neurog2* is predominantly expressed in proliferating, S-phase progenitor cells. *Atoh7* is expressed in progenitors completing their final mitotic cycle, therefore presumably its transcription is activated during G2 phase. *Neurog2* and *Atoh7* expression precede the appearance and expansion of markers for cell-cycle exit, neuronal determination, and RGC specification (Fig. 1B). However, these bHLH factors function differently during the initial wave of RGC specification. *Neurog2* is required for the propagation of the neurogenic wave, but is not required for the specification of RGCs. The
expansion of neurogenesis and RGC specification is delayed for two days in Neurog2 mutant mice, finally resuming by E13.5. Conversely, loss of Atoh7 is critical for cell cycle exit and RGC genesis, but does not affect the expansion of neurogenesis. Therefore, Neurog2 and Atoh7 have distinct functions in subsequent steps of early retinal progenitor differentiation, promoting neuronal determination and RGC specification, respectively (Fig. 1B,C).

The subdivision of function among mammalian atonal homologues appears in cochlear nucleus development as well. Atoh1 is expressed in proliferating dorsal rhombic lip progenitors that generate the cochlear nucleus, pontine and precerebellar nuclei (Ben-Arie et al., 1996; Bermingham et al., 2001; Engelkamp et al., 1999; Farago et al., 2006; Landsberg et al., 2005). Atoh1 is downregulated in these cells as they become postmitotic and migrate into the developing brainstem. Atoh7 is then expressed in the embryonic and mature cochlear nucleus (Hufnagel et al., 2007; Saul et al., 2008). Unlike the retinal phenotype, cochlear nuclei in Atoh7 mutants do not exhibit the loss of any cell type, but rather the mature neurons are morphologically and functionally impaired (Saul et al., 2008). In Drosophila, atonal is also required for development of Johnston’s organ, the auditory organ of the fly, and in axonal arborization in the adult Drosophila brain (Hassan et al., 2000). Therefore, atonal function may be subdivided in mouse cochlear nucleus development as well, with Atoh1 expression specifying the proliferating progenitor cells, and Atoh7 in maturation of postmitotic neurons.

There are several differences in the requirements for fly atonal and the mouse atonal-homologues in retinogenesis. First, published literature indicates the expression pattern of atonal is markedly different than the mouse atonal-homologues. Atonal is expressed in the morphogenetic furrow in a stripe of cells arrested in G1 phase. Initially, atonal is expressed in clusters of cells termed ‘equivalence groups,’ and subsequently its expression is refined to just
the R8 neuron which recruits the remaining ommatidial cells (Hsiung and Moses, 2002).

*Neurog2* and *Atoh7*, while expressed in overlapping populations of cells, are expressed in proliferating and terminally mitotic phases, respectively, in a ‘salt-and-pepper’ pattern in retinal progenitors. Therefore, differences in function of *atonal* and the *atonal*-homologues may be due to expression pattern, in part, and to global differences in eye development between fly and mouse. Second, unlike *Neurog2* and *Atoh7*, the loss of *atonal* is devastating to all retinal cells of the fly eye. *Atonal* mutants have a rudimentary morphogenetic furrow but completely lack photoreceptor neurons (Jarman et al., 1994). *Atoh7* mutants and *Atoh7;Neurog2* double mutants still contain six of the seven major cell types, signifying that in the mouse eye *Neurog2* and *Atoh7* together do not completely recapitulate the *atonal* phenotype. Again, this difference may relate to differences in retinal development between fly and mouse. In *Drosophila*, the other ommatidial neurons (R1-7) are recruited by signaling from R8 photoreceptors, and not directly specified by *atonal* expression. Thus, R1-7 loss is secondary to failure of R8 specification in *atonal* mutants, and not due to the loss of *atonal* function per se (Greenwood and Struhl, 1999; Hsiung and Moses, 2002; Jarman et al., 1994; Jarman et al., 1995). In vertebrates, however, RGCs are not essential for the generation of all other neurons, as demonstrated by the presence of all other cell types in *Atoh7* mutants. In the absence of *Atoh7*, these progenitors continue to proliferate and give rise to other cell types (Brown et al., 2001; Le et al., 2006; Wang et al., 2001). Therefore, different eye plans and different requirements for first-born neurons in eye development may account for the disparities between fly and mouse *atonal* homologues.

These studies demonstrate a bHLH network in RGC determination. In *Neurog2* mouse mutants, the expansion of *Atoh7* expression is delayed along with neurogenesis. In chick and mouse, *Neurog2* can bind the *Atoh7* promoter, activating the gene briefly at the initiation of
Atoh7 expression (Matter-Sadzinski et al., 2005; Skowronska-Krawczyk et al., 2009). Further, Atoh7 negatively regulates Neurog2 indirectly, as Neurog2 expression is non-autonomously increased in Atoh7 mutant mice (Le et al., 2006). Therefore, the subsequent expression and function of Neurog2 and Atoh7 in RGC specification involves cross-regulation of these genes. The findings in Chapter 3 suggested that Neurog2 activates neuronal determination programs in proliferating progenitor cells, and a subset of these express Atoh7 to become RGCs. As such, Neurog2 may spatiotemporally regulate the expansion of RGC genesis via regulation of Atoh7 in mouse (Fig. 1B,C).

Neurog2 and Atoh7 exhibit overlapping and mutually exclusive functions in retinogenesis as well. Neurog2 is required for progression of neurogenesis, which is unaffected in Atoh7 mutants. Further, this phenotype is not enhanced in Neurog2;Atoh7 double mutants at E11.75, indicating that Atoh7 does not function synergistically or redundantly for Neurog2. Thus, the propagation of neurogenesis appears to be mutually exclusive. Both Neurog2 and Atoh7 are required for normal neurogenesis, as the number of differentiating neurons is similarly decreased in both single mutants. However, double mutants do not exhibit a further reduction in differentiating neurons than single mutants, suggesting that these genes are not redundant or synergistic in neuronal determination. Therefore, it is reasonable to consider that these genes have a similar proneural effect, but cannot compensate for each other. In the neonatal mouse, Neurog2 and Atoh7 have an additive effect in determining retinal thickness, as double mutants are thinner than single mutants. Whether this represents a synergistic effect on retinal progenitor proliferation through a common mechanism or an additive effect via separate mechanisms is unknown. However, double-mutants uniquely had increased apoptosis at E15.5, unlike single mutants that did not exhibit increased apoptosis. This suggests that Neurog2 and Atoh7 are
redundant for one another for cell survival. Hence, the greater reduction in retinal size in double mutants could result from a cell survival defect during embryogenesis that occurs only with the loss of both genes. Overall, though Neurog2 and Atoh7 act sequentially in the propagation of neurogenesis and RGC determination, their redundant and nonredundant functions in neurogenesis and cell survival suggest a complex genetic relationship.

The initiation and propagation of retinal neurogenesis appear to be separate events in mouse. Neurog2 mutants and Neurog2;Atoh7 double mutant retinas contain a small, central region of neural differentiation that temporarily does not expand. This indicates that neurogenesis initiates, but the expansion of RGC genesis and Atoh7 expression is dependent on Neurog2. The mechanisms for initiating bHLH expression and neurogenesis in the dorsal central retina and the subsequent selection of cells for RGC fate are still unknown. Neurogenesis in zebrafish, chick, and mouse initiates adjacent to the optic stalk (Hu and Easter, 1999; Jensen and Wallace, 1997; Prada et al., 1991). In mouse, Neurog2 and Atoh7 expression initiate in the dorso-central retina, near the optic stalk, as Pax2 expression becomes localized to the optic stalk and Pax6 to the neural retina. Lacking still are the initiating signals from the optic stalk or extraretinal tissues. Patterning of the optic stalk and neural retinal is critical for the initiation of neurogenesis. Shh and fibroblast growth factor (FGF) are both pleiotropic signals. These signals have both been implicated as extra- and intraretinal signals regulating the neurogenic wave and bHLH factors, and are expressed from a variety of sites, including the optic stalk, RPE, lens, and retinal progenitor cells (de Iongh and McAvoy, 1993; Kay et al., 2005; Martinez-Morales et al., 2005; Masai et al., 2005; McCabe et al., 1999; Neumann and Nuesslein-Volhard, 2000; Stenkamp et al., 2000; Walshe and Mason, 2003). In zebrafish, extraretinal sonic hedgehog (shh) signaling, from the prechordal mesoderm and ventral diencephalon, is critical for division.
of the eye field, formation of the optic stalk, and initiation of neurogenesis. (Ekker et al., 1995; Masai et al., 2000; Stenkamp and Frey, 2003; Stenkamp et al., 2002). Optic stalk cells themselves are critical for zebrafish Atoh7 expression, and transplantation of optic stalk cells into the retina can ectopically induce Atoh7 expression (Masai et al., 2000; Stenkamp and Frey, 2003). In mouse, Shh released from RGCs promotes progenitor cell proliferation and laminar organization in the retina, indicating a role in limiting RGC number in the mammalian eye (Jensen and Wallace, 1997; Wang et al., 2005b; Wang et al., 2002b). Future studies will investigate the role of optic stalk, Pax2 expression, and extrinsic signaling factors on the initiation of bHLH expression and neurogenesis.

The propagation of retinal neurogenesis likely involves a combination of extrinsic and intrinsic signaling. Two hypotheses have been evaluated in the literature. First, neurogenesis may be propagated by “sequential-induction,” neurons differentiating at the front of the neurogenic wave induce differentiation in adjacent cells. This is similar to the morphogenetic furrow in the Drosophila eye. Alternatively, central and peripheral retinal cells may be preprogrammed to differentiate at a certain time. That is, early signaling events predetermine the timing of differentiation based on central-peripheral position, independent of the neurogenic wave (Kay et al., 2005; McCabe et al., 1999). These studies support the first hypothesis, as Neurog2 expression ahead of differentiating cells is required for expansion of the neurogenic wave. However, the neurogenic wave is merely delayed in this case. I hypothesize that the onset of Ascl1 represents a second wave of neurogenesis, and compensates for loss of Neurog2 during the first wave (Fig. 1B,C). The loss of both Neurog2 and Ascl1 does not result in permanent arrest of neurogenesis, either (Akagi et al., 2004). Alternatively, both the sequential-induction and pre-programming models may exist. During the propagation of neurogenesis, the expansion
of Neurog2 expression coordinates Atoh7 expression and subsequent RGC specification, but, in its absence, these cells eventually differentiate due to a second neurogenic cue, perhaps Ascl1 as predicted here. Given the evidence in favor of both theories, it is very likely that both mechanisms exist in mouse, unlike Drosophila that relies entirely on the sequential-induction of ommatidia formation via the morphogenetic furrow. I hypothesize that murine retinal neurogenesis consists of multiple, intrinsically timed waves of neurogenesis sweeping across the retina, encoding specification of the major cell types by sequential combinations of gene expression.

Still unknown are other intrinsic factors involved in the gene network regulating the initial neurogenic wave. Known regulators of Neurog2, including Pax6 and Sox2, and known regulators of spatiotemporal neurogenesis, such as Chx10, are expressed ubiquitously in retinal progenitors (Burmeister et al., 1996; Marquardt et al., 2001; Taranova et al., 2006). One group of candidates to intrinsically regulate the onset of retinal neurogenesis are those expressed in a similar spatiotemporal pattern to Neurog2 and Atoh7. The atonal-like bHLH factors Neurod1 and Neurod4 are expressed during early retinogenesis. In Chapter 3, Neurod1 expression at E11.75 was similar to Atoh7. In other studies, Neurod1 and Neurod4 retinal expression was not observed until E12.5 (Mao et al., 2008a; Takebayashi et al., 1997). Additionally, Foxn4 and Ptf1α both initiate at E11.5 in a dorso-central cluster of retinal progenitors, and regulate horizontal and amacrine cell fates (Fujitani et al., 2006; Li et al., 2004). If these transcription factors also function during the initiation of retinogenesis, then the additional loss of other initiating factors may be required to completely prevent neurogenesis.

These studies contribute to our understanding of the step-wise specification of RGCs in the mammalian retina. The coordinate expression of Neurog2 and Atoh7 controls distinct
functions in neural determination and RGC specification, respectively. While these genes work in concert to regulate the spatial and temporal aspects of retinogenesis, many other intrinsic and extrinsic factors function in RGC genesis. A careful characterization of the expression of bHLH and non-bHLH genes at the onset of neurogenesis is warranted, with close attention to timing and cellular localization of these factors with respect to Neurog2 and Atoh7.

Timing is Everything: Redundant and Distinct Functions of Retinal bHLH Factors

Chapters 3 and 4 explored the role of Ascl1 in early retinogenesis. Onset of Ascl1 expression at E12.5 proceeds in a central-to-peripheral pattern similar to Neurog2 and Atoh7 (Fig. 1A,B). The Neurog2^{Ascl1KI} and Atoh7^{Ascl1KI} mice precociously and ectopically express Ascl1 in early retinal progenitors. Homozygous mice represent a complete exchange of Ascl1 for Neurog2 or Atoh7: expression in the same cells, at the same phase in the cell cycle, and under the same regulatory control. Further, expression of Atoh7 and Neurog2 in highly overlapping cell populations allows for powerful comparisons of the two phenotypes. These Ascl1 misexpression mice have strikingly different phenotypes. The Neurog2^{Ascl1KI} homozygous mice do not have a delay in retinal neurogenesis, unlike Neurog2 mutants. Further, RGCs are specified normally, suggesting the regulation of critical RGC determinants such as Atoh7 and Pou4f2 are preserved. Therefore, one conclusion is that Ascl1 has a proneural function similar to Neurog2 as it can rescue the phenotype. This is supported by the correlation between the onset of endogenous Ascl1 expression and the delayed completion of the neurogenic wave. As such, Ascl1 may regulate a second neurogenic wave similar to the initial one, such that its functions are highly redundant with Neurog2.
*Atoh7*^Ascl1KI* mice portray very different aspects of Ascl1 function. *Atoh7*^Ascl1KI* mice lack RGCs, like *Atoh7* mutants. In regards to early-born cell types and retinal thickness, *Atoh7*^Ascl1KI/Ascl1KI* mice are not phenotypically different from *Atoh7* null animals. This is not entirely surprising as *Ascl1* has not been shown to regulate RGC genesis (Hatakeyama et al., 2001; Tomita et al., 1999). On the other hand, *Ascl1* misexpression maintained terminally mitotic cells in a proliferative state, unlike *Atoh7* mutants. *Atoh7* regulates cell cycle progression, but in its absence some progenitors are thought to stall in G2 phase, then differentiate after a delay. *Ascl1*, therefore, must promote another round of division at the expense of differentiation. Thus, in replacing *Atoh7*, *Ascl1* is incompatible with RGC specification, and promotes cell cycle progression and not neuronal differentiation.

Curiously, even though *Ascl1* misexpression can function redundantly for *Neurog2*, it cannot compensate for *Atoh7*. The functional replacements of *Neurog2* or *Atoh7* with *Ascl1* are different with respect to cell cycle exit and RGC specification. *Atoh7* is expressed in a subset of *Neurog2*-lineage cells. Therefore, in both *Neurog2*^Ascl1KI* and *Atoh7*^Ascl1KI* mice, *Ascl1* is misexpressed in the same cells, but under different gene promoters. Presumptively, *Ascl1* is also expressed in different phases of the cell cycle due to differences in the *Neurog2* and *Atoh7* expression patterns. There are several explanations for these results. *Atoh7* could be uniquely specialized for RGC genesis. This is supported by the nearly singular requirement of *Atoh7* in RGC genesis and the highly restricted expression of *Atoh7* to the retina and cochlear nucleus (Brown et al., 1998; Brown et al., 2001; Saul et al., 2008; Wang et al., 2001). However, *Atoh7* function can be partially rescued via replacement by *Neurod1* in *Atoh7*^Neurod1KI* mice (Mao et al., 2008a). Though not a complete rescue, these mice exhibit RGC and optic nerve genesis. Thus, *Atoh7* is not the only bHLH capable of RGC specification.
Another explanation is that bHLH function in the retina is highly dependent on expression during particular phases of retinal progenitor cell differentiation. Neurog2 is expressed in several contexts, in different phases of neuronal differentiation. In the dorsal forebrain, Neurog2 is expressed in proliferating progenitors, and is required for neurogenesis. Replacement of Ascl1 with Neurog2 in the ventral forebrain rescues neurogenesis but does not promote dorsal neuron specification (Fode et al., 2000). In the dorsal spinal cord, Neurog2 is expressed in postmitotic neurons in the dorsal spinal cord, and affects cell type specification but not regional identity (Helms et al., 2000). In the ventral midbrain, Neurog2 is expressed in both proliferating progenitors in the ventricular zone and in postmitotic Nurr1+ cells. Here, Neurog2 functions in both neuronal differentiation and subtype specification, as total numbers or neurons are reduced and dopaminergic neurons are lost (Andersson et al., 2006; Kele et al., 2006).

Therefore, Neurog2 appears to have context-dependent functions that parallel the phase of cell cycle expression.

Generally, bHLH factors can be classified by their activation during the cell cycle: proliferating (S-phase), terminally mitotic (G2/M phase), or postmitotic (G0 phase). In the retina, Neurog2 and Ascl1 are expressed in proliferating progenitor cells, Atoh7 in terminally mitotic cells, and Neurod1 in postmitotic cells (Ahmad et al., 1998; Brown et al., 1998; Inoue et al., 2002; Jasoni and Reh, 1996; Ma and Wang, 2006; Mao et al., 2008c; Morrow et al., 1999; Takebayashi et al., 1997). Neurog2 and Ascl1 are expressed in proliferating cells and appear to function primarily in neural commitment. Neurog2 mutants exhibit reductions in differentiating neurons, but specification programs appear intact in the remaining neurons. Similarly, Ascl1 mutants exhibit delayed differentiation of horizontal interneurons, rod photoreceptors, bipolar interneurons, and Müller glia in retinal explant culture (Tomita et al., 1996). Ultimately, bipolar
neurons are slightly reduced and Müller glia increased, but not completely lost. Retinal misexpression of *Ascl1* or *Neurog2* alone results primarily in differentiating rod photoreceptors in mouse, the most prevalent retinal cell type (Hatakeyama et al., 2001). Thus, *Neurog2* and *Ascl1* might simply function in neural commitment, and availability of bipolar determinants such as *Chx10* direct fate specification. Proneural bHLH factors expressed in terminally mitotic and postmitotic cells, including *Atoh7* and *Neurod1* are proposed to function primarily in cell fate specification. Indeed, *Atoh7* mutants lack RGCs, and *Neurod1* mutants have reduced photoreceptors (Brown et al., 1998; Brown et al., 2001; Hatakeyama et al., 2001; Inoue et al., 2002; Morrow et al., 1999; Tomita et al., 2000).

To clarify the functions of bHLH factors in different stages of differentiation, bHLH swap experiments are advantageous to misexpress proneural genes selectively in proliferating, terminally mitotic, or postmitotic cells. In this regard, the differences in ectopic bHLH expression correlated with the differences in *Neurog2*<sup>Ascl1KI/GFP</sup>, *Atoh7*<sup>Ascl1KI/Ascl1KI</sup>, and *Atoh7*<sup>Neurod1KI/Neurod4KI</sup> phenotypes. *Ascl1* rescued *Neurog2* function in the retina, and both are expressed in proliferating progenitor cells. *Ascl1* replacement of *Neurog2* did not redirect RGCs towards different fates, as *Pou4f2* expression was normal. Thus, *Ascl1* and *Neurog2* similarly affect cell cycle exit and neuronal determination, though they do not share strong homology. However, *Ascl1* cannot rescue RGC specification in the absence of *Atoh7*, as evidenced by the severe reduction in embryonic *Pou4f2*- and Islet1-immunopositive cells. On the other hand, *Atoh7* replacement by *Neurod4* and *Neurod1* (*Atoh7<sup>Neurod4KI</sup>, *Atoh7<sup>Neurod1KI</sup>*) did partially rescue RGC number (Mao et al., 2008a), even though these postmitotic bHLH factors do not promote RGC specification endogenously. In contrast, RGCs are increased in *Neurod1; Neurod4* double-
mutants, suggesting that these postmitotic bHLHs function to repress RGC specification (Inoue et al., 2002).

Alternatively, RGC rescue by bHLH replacement of *Atoh7* can be explained by homology to *atonal*. The *atonal*-homologues *Neurod4* and *Neurod1* were able to partially rescue RGC genesis, unlike the *achaete-scute* complex homologue *Ascl1*. Complementary bHLH swap experiments can also attempt to distinguish these possibilities. First, if homology to *atonal* is more predictive of bHLH function than expression phase, then replacement of *Atoh7* with a proliferation-type *atonal*-homologue such as *Neurog2* (*Atoh7^{Neurog2KI/Neurog2KI}*), should rescue RGC genesis. The alternative, phase-specific model predicts that the *Atoh7^{Neurog2KI/Neurog2KI}* mouse will lack RGCs because *Neurog2*, a proliferation-type bHLH, will not function in specification of terminally mitotic cells. Second, if bHLH function is also dependent on cell-cycle phase, then misexpression of *Atoh7* in proliferating progenitor cells will not confer RGC fate. In the theoretical *Neurog2^{Atoh7KI/Atoh7KI}*;*Atoh7^{−/−}* mouse, *Atoh7* expression would be removed from terminally mitotic cells, and inserted upstream in place of *Neurog2*. Since *Atoh7*-expressing cells comprise a subset of the *Neurog2*-lineage, this would change the timing of *Atoh7* expression in those cells that would normally adopt an RGC fate. Creation of a *Neurog2^{Atoh7KI/Atoh7KI}*;*Atoh7^{−/−}* mouse would allow us to test a) if *Atoh7* can promote general neuronal differentiation in proliferating cells, and b) if *Atoh7* can specify RGC fate when expressed in a different phase of the cell cycle. I predict that *Atoh7* will not fully prevent the delay in neurogenesis or rescue RGC development, since *Atoh7* is misexpressed in a different phase of neuronal differentiation. This result would mirror the lack of RGC specification in *Atoh7^{Ascl1KI/Ascl1KI}* mice.
Future Directions

The molecular determinants underlying the generation of retinal cells in a prescribed temporal order are poorly understood. From a global standpoint, retinal development occurs in multiple overlapping phases. With respect to cellular birth order and proneural bHLH expression, there are four stages of retinal neurogenesis in mouse. Briefly, at the initiation of neurogenesis, only RGCs are formed. This is accompanied by expression of *atonal*-homologues *Atoh7* and *Neurog2*. During the early stage, the four early-born fates, RGCs, cone photoreceptors, amacrine and horizontal interneurons are generated. Next, at E12.5-E13.5 in mouse, the middle-phase begins as *Ascl1* onset correlates with the initiation of rod photoreceptor genesis (Fig. 1A). Few bipolars or Müller glia are born embryonically. This middle stage continues until birth, when *Neurog2* and *Atoh7* are downregulated in the retina, and bipolar interneurons and Müller glial genesis increase dramatically. The late stage continues from birth through P10-P11, when the last bipolar interneurons and Müller glia are born. *Neurod1* and *Neurod4* are expressed from the early phase through adult ages (Ahmad et al., 1998; Brown et al., 1998; Inoue et al., 2002; Jasoni and Reh, 1996; Morrow et al., 1999; Sidman, 1961; Takebayashi et al., 1997; Young, 1985). Thus, retinal development can be roughly characterized by an RGC-only stage, an early stage (early fates; *Atoh7*, *Neurog2* expression), a middle stage (early fates + rod photoreceptors; *Atoh7*, *Neurog2*, and *Ascl* expression), and a late stage (rod photoreceptors + late fates; *Ascl1* expression).

The combination of a changing environment superimposed on intrinsic changes appears to set an internal clock underlying retinal neuron formation. Intrinsic factors undoubtedly play a role in determining retinal fate. In chick, neurogenesis still occurs in the peripheral retina detached ahead of the wave of differentiation and FGF signaling (McCabe et al., 1999). *Ikaros*
expression can prolong early fate genesis in the postnatal retina (Elliott et al., 2008). I hypothesize that bHLH expression does underlie the neuronal birth order of retinal cell classes. The outward expansion of atonal homologues Neurog2 and Atoh7 specifies the first RGCs. Speculatively, cones, amacrine and horizontal interneurons are subsequently specified by combinatorial expression Neurog2, Atoh7, Neurod1, and Neurod4. Then, the outward expansion of Ascl1 expression initiates rod photoreceptor development at E13. Just after birth, Neurog2 and Atoh7 expression downregulate, as Ascl1 expression continues and retinal progenitor cells become restricted to rod photoreceptor, bipolar interneuron, and Müller glia fates (Fig. 1C). As Neurod4 and Neurod1 are expressed through both early and late retinogenesis, they are predicted to act as promiscuous cofactors for the specification of both early and late fates.

Though birth order is not easily rearranged, tracking the expression of bHLH factors across time in subpopulations of proliferating and differentiating neurons can elucidate lineage relationships between bHLH factors. Interestingly, the Neurog2-lineage is composed of all seven cell types, in a normal distribution to the ONL, INL, and GCL (Ma and Wang, 2006). Atoh7-lineage cells, as noted above, comprise all seven cell types, but skewed towards early retina fates (Brzezinski, 2005; Yang et al., 2003). The Ptf1a-lineage consists of horizontal cells and a subset of amacrine interneurons (Fujitani et al., 2006; Nakhai et al., 2007). The size and distribution of the Neurog2, Atoh7 and Ptf1a lineages reflect expression in proliferating, terminally mitotic, and postmitotic cells, respectively.

Retinal lineage-tracing experiments will uncover autonomous, nonautonomous, and overlapping effects of bHLH transcription factor expression, essential for understanding their function in retinal development. First, conducting lineage analyses in wild type and mutant animals can determine whether a specific population of bHLH-expressing cells is affected
identically between genotypes and in comparison to the entire retina. Therefore, this functions as an autonomy test. In the Atoh7-lineage, RGCs are lost but cones are unaffected, indicating that the cone increase previously observed in Atoh7 mutants is an indirect effect (Brzezinski, 2005; Le et al., 2006). In Atoh7 Asc1KI mice, alterations in bipolars are nonautonomous. Since Ascl1 is thought to specify bipolar interneurons, lineage tracing would indicate whether the endogenous gene functions differently. The bHLH-lineages may also change across time. Using inducible Cre-recombinase transgenics, the lineages can be dissected across the different phases of retinal neurogenesis (Lewandoski, 2001). For instance, do the earliest Atoh7-expressing progenitors give rise solely to RGCs? Activating an inducible Cre transgene for a brief period during the initial wave of neurogenesis, from E11-E12, could demonstrate an RGC-specific phase of Atoh7-expression, in contrast to the multipotent lineage observed across all of retinal development.

bHLH genes are expressed in overlapping populations of cells, and it is difficult to characterize the single-expressing and co-expressing subpopulations. Intersectional fate mapping, using separate Cre or Flippase (Flp) transgenics with independent Cre- and Flippase-reporters to simultaneously mark two lineages, allows for the observation of overlapping and nonoverlapping populations (Farago et al., 2006; Hunter and Dymecki, 2007; Kim and Dymecki, 2009). For instance, if Neurod1 and Neurod4 are required autonomously in combination for amacrine fate specification, then amacrine interneurons in the INL would be double-labeled by the Neurod1- and Neurod4-lineage reporters.

Here, I describe the differentiation of the first retinal neurons in mouse, and the relationship between Neurog2 and Atoh7 in early retinal progenitor cells. I predict that retinal progenitor cell differentiation results from a bHLH transcription factor code. The overlapping expression of multiple bHLH transcription factors, at different phases of the cell cycle, and at
different times across retinal development, creates an immense amount of heterogeneity among retinal progenitor cells. It is likely that the combinations of bHLH factor expression, across time, are modulated by extrinsic signals and an as of yet undefined intrinsic clock mechanism. Understanding the lineage relationships between bHLH expression patterns and phenotypes is tantamount to uncovering the instructive cues guiding retinal cells from proliferating progenitors to light-capturing neurons.
Figure Legends

**Figure 5.1.** Model of retinal bHLH network in early retinal neurogenesis. (A) The birth order of the major retinal cell types in mouse, from E11-P11 (x-axis). Below, the timing of expression for *Atoh7* (E11-P1), *Neurog2* (E11-P1), and *Ascl1* (E12-P9) are depicted. The gray bar marks the time at which only *Neurog2* and *Atoh7* are expressed. (B) bHLH factors function sequentially in the specification of RGCs. *Neurog2* is expressed in proliferating progenitor cells (green), a subset of which express *Atoh7* (orange), exit the cell cycle (p27) and commit to a neural fate (Tubb3) and subsequently express the RGC marker Pou4f2. (C) Multiple staggered waves of neurogenesis are hypothesized to regulate retinal differentiation. At E11.5, *Neurog2* (green) and *Atoh7* (orange) are expressed in subsets of retinal progenitors in a central to peripheral manner. At E12.5, the onset of *Ascl1* (purple) occurs similarly, from central to peripheral, which is predicted to be a second wave of neurogenesis that persists in the absence of *Neurog2*. A adapted from (Marquardt and Gruss, 2002).


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