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Heterochronic misexpression of *Ascl1* in the *Atoh7* retinal cell lineage blocks cell cycle exit

Robert B. Hufnagel^{1,*}, Amy N. Riesenberger^{1,*}, Malgorzata Quinn², Joseph A. Brzezinski IV³, Tom Glaser^{4,#}, and Nadean L. Brown^{1,#}

¹Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation; Departments of Pediatrics and Ophthalmology, University of Cincinnati College of Medicine, Cincinnati OH

²Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Research Foundation, Cincinnati, OH

³Department of Ophthalmology, University of Colorado School of Medicine, Aurora, CO

⁴Departments of Internal Medicine and Human Genetics, University of Michigan Medical School, Ann Arbor, MI

Abstract

Retinal neurons and glia arise from a common progenitor pool in a temporal order, with retinal ganglion cells (RGCs) appearing first, and Müller glia last. The transcription factors *Atoh7/Math5* and *Ascl1/Mash1* represent divergent bHLH clades, and exhibit distinct spatial and temporal retinal expression patterns, with little overlap during early development. Here, we tested the ability of *Ascl1* to change the fate of cells in the *Atoh7* lineage when misexpressed from the *Atoh7* locus, using an *Ascl1*-IRES-DsRed2 knock-in allele. In *Atoh7^{Ascl1KI/+}* and *Atoh7^{Ascl1KI/Ascl1KI}* embryos, ectopic *Ascl1* delayed cell cycle exit and differentiation, even in cells coexpressing *Atoh7*. The heterozygous retinas recovered, and eventually produced a normal complement of RGCs, while homozygous substitution of *Ascl1* for *Atoh7* did not promote postnatal retinal fates precociously, nor rescue *Atoh7* mutant phenotypes. However, our analyses revealed two unexpected findings. First, ectopic *Ascl1* disrupted cell cycle progression within the marked *Atoh7* lineage, but also nonautonomously in other retinal cells. Second, the size of the *Atoh7* retinal lineage was unaffected, supporting the idea of a compensatory shift of the non-proliferative cohort to maintain lineage size. Overall, we conclude that *Ascl1* acts dominantly to block cell cycle exit, but is incapable of redirecting the fates of early RPCs.

Keywords

Atoh7; Ascl1; Retinal ganglion cell; cell cycle; retinal neurogenesis; retinal cell competence

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Corresponding author: Nadean L. Brown, Department of Cell Biology and Human Anatomy, University of California Davis School of Medicine, Room 4407A Tupper Hall, One Shields Avenue, Davis, CA 95616, nlbrown@ucdavis.edu, Voice: 530-752-7806.

[#]Present address: Department of Cell Biology and Human Anatomy, University of California Davis School of Medicine, Davis, CA 95616

*These authors made equal contributions

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Introduction

The murine retina is an attractive model for studying cell fate specification. From a common neuroepithelium, four cell types (retinal ganglion cells (RGCs), cone photoreceptors, amacrine and horizontal interneurons) arise largely prenatally. During late embryonic and postnatal development, the bulk of rod photoreceptors, bipolar interneurons and Müller glia are generated (Cepko et al., 1996; Jeon et al., 1998; Sidman, 1961; Young, 1985). When the mouse optic cup forms at E10.5, retinal progenitor cells (RPCs) are multipotent, but over time exhibit restricted developmental potential.

Retinal birthdating experiments demonstrated that neurons and glia exit mitosis in a regular sequence, with one or more cell types arising simultaneously, as well as from sequential cell divisions. Lineage tracing and clonal analyses have also highlighted the multipotency of individual RPCs. Moreover, when early and late RPCs were co-cultured, neither population was temporally reprogrammed, exemplifying the importance of intrinsic determinants in dictating cell fate. Although extrinsic factors influence the relative proportions of cell types produced, they cannot supersede intrinsic regulation of the order in which cell types appear (reviewed in Agathocleous and Harris, 2009; Cayouette et al., 2006; Livesey and Cepko, 2001; Rapaport, 2006).

Numerous retinal studies have focused on basic helix-loop-helix (bHLH) transcription factors, due to their prominent roles in neuronal specification. Indeed, their distinct spatiotemporal expression patterns and loss-of-function phenotypes have solidified key roles during retinal neurogenesis. For example, *Atoh7/Math5* appears at the initiation of retinogenesis, and is critically required for RGC formation, and the suppression of cone photoreceptors (Brown et al., 1998; Brown et al., 2001; Kanekar et al., 1997; Kay et al., 2001; Wang et al., 2001). *Asc1/Mash1* expression becomes apparent two days later than *Atoh7* in the mouse retina, and is required for normal bipolar interneurons and suppression of Müller glia differentiation (Brzezinski IV et al., 2011; Jasoni and Reh, 1996; Tomita et al., 1996). This suggests that *Atoh7* and *Asc1* act via inherently different mechanisms, which is further supported by the evolutionary divergence of their bHLH domains (Bertrand et al., 2002) and their segregated expression: *Asc1* within proliferative RPCs, and *Atoh7* in terminally exiting and postmitotic cells (Brzezinski IV et al., 2012; Hufnagel et al., 2010; Jasoni and Reh, 1996; Le et al., 2006; Morrow et al., 1999). Conversely, these factors may be capable of partially or totally substituting for one another, but fail to do so because they are normally segregated into largely non-overlapping lineages (Brzezinski IV et al., 2011). To understand whether these factors might be interchangeable, an in vivo functional substitution is needed, and the consequences assessed during retinal development.

Here we tested whether *Asc1* can reprogram early RPCs to acquire late-born fates, by homologously recombining an *Asc1*-IRES-DsRed2 replacement cassette into the *Atoh7* locus. The resulting mice displayed ectopic expression of *Asc1*, specifically within the *Atoh7* lineage, beginning at E11.5 when these cells would normally exit mitosis with the competence to form RGCs. We found that *Asc1* cannot substitute for *Atoh7*. In *Atoh7^{Asc1KI/Asc1KI}* embryos RGC neurons failed to develop, and the adult mice lacked optic nerves and chiasmata, like *Atoh7^{-/-}* adults (Brown et al., 2001; Wang et al., 2001). Ectopic *Asc1* failed to produce precocious or excess later-born cell types, although it did induce extra rounds of mitosis, even when coexpressed with *Atoh7*, in heterozygotes. This overproliferation was temporary, as adult heterozygous eyes contained a normal proportion of RGCs. Intriguingly, our analyses highlighted the ability of ectopic *Asc1* to block cell cycle exit and its inability to instruct RGC genesis in multiple retinal cell lineages.

Materials and Methods

Atoh7^{Ascl1} targeting

The *Atoh7*^{Ascl1KI} targeting vector was created by joining 5 fragments: 1) 2.1Kb *Atoh7* 5' EcoRI-PstI 5' arm; 2) *Ascl1* cDNA + 3'UTR; 3) IRES2-DsRed2-pA cassette (Clontech); 4) loxP-PGKneo-pA-loxP cassette in opposite orientation; and 5) a 3.1 Kb PvuI-PvuI *Atoh7* 3' genomic DNA arm. Figure 1A shows a diagram of the final targeting vector, which was confirmed by complete DNA sequencing. The linearized construct was electroporated into W4 embryonic stem (ES) cells (Auerbach et al., 2000) and colonies selected using G418. To identify homologous recombination at the *Atoh7* locus, ES cell genomic DNA was screened by long-range PCR, using one primer outside of each targeting arm and one in DsRed2 coding sequence (Fig 1A, primers C+C' and D+D'). Both the *Atoh7* coding exon and 3'UTR were recombined out of the targeted allele. Southern blotting of ES cell and mouse tail genomic DNA was performed with a 5' flanking genomic DNA probe (Figs 1A,B). Targeted ES cells were injected into C57BL/6J blastocysts, and chimeric founders mated to C57BL/6J mice. The resulting N² *Atoh7*^{Ascl1KI/+} males were mated with *Elia*-Cre female mice (Lakso et al., 1996) to delete the floxed neo cassette, and this was confirmed by PCR genotyping with Cre- or neo-specific primers. Phenotypic analyses were performed by crossing the targeted allele into C57BL/6, 129/Ola and CD-1 genetic backgrounds, then intercrossing *Atoh7*^{Ascl1KI/+} mice. No differences in adult optic nerve phenotypes were observed among homozygous mutant N⁵F² mice on C57BL/6, 129 or CD-1 genetic backgrounds.

Mouse strains and *Atoh7* lineage-marking

Atoh7^{LacZ} mice were maintained on a CD-1 background, and PCR genotyping performed as described (Brown et al., 2001). For the *Atoh7*^{Ascl1KI} targeted allele, Forward (5'-AAGGTCTGTTGAATGTCGTGAAGG-3') and Reverse (5'-TTGAATACGCTTGAGGAGAGCC-3') primers (Fig 1A,C primers B+B') were used for 40 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. *Atoh7*^{+/+} wild type allele genotyping used Forward (5'-CGCCGCATGCAGGGGCTGAACACG-3') and Reverse (5'-GATTGAGTTTTCTCCCCTAAGACCC-3') primers (Fig 1A,C primers A+A'), for 40 cycles at 95°C for 30sec, 60°C for 1 min, and 72°C for 1 min, plus 1X MasterAmp (Epicentre, Madison, WI) in the PCR assay. In some experiments, the *Atoh7* retinal lineage was marked using *Atoh7*-Cre BAC (Brzezinski IV et al., 2012) and Z/EG (Novak et al., 2000), which were crossed into the *Atoh7*^{Ascl1KI} CD-1 background. The Z/EG reporter expresses EGFP following Cre-mediated recombination.

Histology and Immunohistochemistry

Embryonic and postnatal tissues were fixed in 4% paraformaldehyde/PBS, processed through a sucrose/PBS series and cryoembedded; or processed postfixation for paraffin embedding and sectioning, with standard histology performed. Immunohistochemistry was performed on 10 micron cryosections as described (Hufnagel et al., 2007). Antibodies used were mouse anti-activated Caspase3 (1:100, Cell Signaling), mouse anti-Ascl1 (1:100, BD Pharmingen, unmasked for 10 minutes at room temperature with 0.5% TritonX/PBS), rabbit anti-βgal (1:5000, Cappel), rat anti-βgal (1:1000, (Saul et al., 2008), rat anti- BrdU (1:100, AbD Serotec, unmasked 1 hour in 1N HCl), sheep anti-Vsx2/Chx10 (1:1000, Exalpha Biologicals), rabbit anti-DsRed (1:500, Invitrogen), chicken anti-GFP (1:1000, Abcam), rabbit anti-Ki67 (1:1000, Vector Labs), rabbit anti-Tubb3 (TuJ1 1:1000, Invitrogen), rabbit anti-M Opsin (1:1000, gift from Cheryl Craft), rabbit anti-S-Opsin (1:1000, gift from Cheryl Craft), rabbit anti-Nr2e3 (1:500, gift from Anand Swaroop), rabbit anti-Pax6 (1:1000, Covance), mouse anti-Cdkn1c/p27 (1:100, BD Labs), goat anti-Pou4f2/Brn3b (1:50, Santa Cruz), rabbit anti-Rxrg/RXRγ (1:200, Santa Cruz), mouse anti-Isl1 (1:20; Developmental Systems Hybridoma Bank, Iowa) and rabbit anti-Sox9 (1:200, Chemicon) and DAPI nuclear

staining (1:1000, Sigma). Direct-conjugate secondary antibodies (Molecular Probes) or sequential biotinylated secondary (Jackson ImmunoResearch) and streptavidin-conjugated Alexa Fluor tertiary antibodies (Molecular Probes) were used to visualize primary antibody labeling.

BrdU labeling

For all timed matings, E0.5 was assumed to be the date when a vaginal plug was observed. In BrdU pulse- or window-labeling experiments, pregnant dams were injected once with 0.1mg/g body weight of 10mg/mL BrdU in 0.9M NaCl) and were harvested either 1.5 or 24 hours later. For birthdating experiments, pregnant dams or pups were injected with the same concentration of BrdU at a particular age, and then P21 eyes harvested. Images of adult eyes were captured with a Leica dissecting microscope, Optronics digital camera and software.

Cell counting

Microscopy was performed with a Zeiss fluorescent microscope, Zeiss camera and Apotome deconvolution device. For each experiment, we analyzed 2 images (200X or 400X) per animal representing both eyes, from 3 embryos or postnatal pups from 2 or more independent litters. Images were selected from central retina at equivalent depths based on anatomic landmarks. 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 with Welch's correction, or ANOVA with a Tukey post hoc test, was performed to calculate P values (Excel or InStat v3.0 software). Photoshop (v7.0) was used to adjust images for brightness and contrast.

Results

Atoh7 replacement with *Ascl1*

To test whether *Ascl1* can functionally substitute for *Atoh7* during early retinal development, and whether heterochronic misexpression of *Ascl1* alters the proliferation or fate choice of early RPCs, we recombined an *Ascl1*-IRES-DsRed2 cassette into the *Atoh7* locus (Figs. 1A-C). The solitary *Atoh7* coding exon (Prasov et al., 2010) and *Atoh7* 3'UTR were precisely replaced, preserving *cis* regulatory sequences and creating an *Atoh7* loss-of-function allele.

In the developing retinas of heterozygous *Atoh7*^{*Ascl1*KI/+} mice, *Atoh7*⁺ cells should coexpress *Atoh7* and *Ascl1*. To confirm this, we compared the expression of DsRed2, *Ascl1* and *E. coli* β -galactosidase (β gal) proteins within *Atoh7*^{*LacZ*/*Ascl1*KI} embryonic retinas (Figs 1D, 1D', 1F, 1F'), taking advantage of an *Atoh7*^{*LacZ*} allele to mark *Atoh7*⁺ cells (Brown et al., 2001). When neurogenesis begins at E11.5, we observed extensive coexpression of β gal and DsRed2 in dorsocentral optic cup (arrows in Fig 1D'). There was also very widespread coexpression at E12.5, when the initial wave of neurogenesis expanded peripherally (representative arrows in Fig 1F'). At both ages, all β gal⁺ cells coexpressed DsRed2⁺, but there were also DsRed2⁺ β gal⁻ cells, mostly at the leading edge of neurogenesis (arrowheads in Figs 1D', 1E'). Presumably, retinal cells express the DsRed2 reporter with slightly faster kinetics than β gal, or the anti-DsRed antibody is more sensitive. Importantly, there was complete coexpression of ectopic *Ascl1* and DsRed2 (Fig 1E, arrows in 1E'), plus very rare RPCs expressing endogenous *Ascl1* protein at E11.5 (arrowheads point to green-only nuclei in Fig 1E')(Hufnagel et al., 2010; Skowronska-Krawczyk et al., 2009). By E13.5, cells with endogenous *Ascl1* expression were more widespread (green only nuclei in Fig 1G). In *Atoh7*^{*Ascl1*KI/+} littermate retinas there was also a cohort of DsRed2⁺*Ascl1*⁺ colabeled cells (arrows point to colabeled cells in Fig 1H), in addition to the endogenous *Ascl1*⁺ population (arrowheads point to green-only nuclei in Fig 1H). Normally

the E13.5 *Atoh7* lineage contains few *Ascl1*⁺ cells, highlighted by *Ascl1* and β gal colabeling of *Atoh7*^{LacZ/+} retinal sections (Fig 1I, arrows). This is consistent with a recent demonstration of modest overlap between *Atoh7* and *Ascl1* lineages in *Ascl1*-GFP;*Atoh7*-Cre mice (Brzezinski IV et al., 2011). Overall, we conclude that the *Atoh7*^{Ascl1} allele drives ectopic *Ascl1* and DsRed2 reporter expression within the *Atoh7* lineage, two day earlier than endogenous *Ascl1* is observable in the retina.

Ascl1 cannot substitute for Atoh7 in the retina

During retinogenesis, *Ascl1* maintains a progenitor pool from which bipolar cells arise, and suppresses Müller glial cell formation (Brzezinski IV et al., 2011; Hatakeyama et al., 2001; Tomita et al., 1996), whereas *Atoh7* is critically required for RGC genesis (Brown et al., 2001; Wang et al., 2001). Thus, we tested whether ectopic *Ascl1* can alter the fate of *Atoh7*⁺ progenitors, or rescue the RGC deficiency in *Atoh7* mutants. The adult eyes of *Atoh7*^{Ascl1KI/+} mice were grossly similar to wild type *Atoh7*^{+/+} littermates, with normal optic nerves and retinal thickness (Fig 2B,2E). There were also no changes in the abundance of RGCs (Pou4f2/Brn3b⁺, Fig 2H, 2S, Suppl Table 1) and cone photoreceptors (S- and M-Opsin⁺, Fig 2K,2T, Suppl Table 1). Likewise, there was no shift in Pax6⁺ amacrine or horizontal cell populations in the inner nuclear layer (INL) (Fig 2U, Suppl Table 1). However, we observed a significant loss of rods (Nr2e3⁺, Fig 2V, Suppl Table 1) and bipolars (Vsx2/Chx10⁺, Fig 2N,2W, Suppl Table 1), plus an increase in Müller glia (Sox9⁺, Fig 2Q,2W, Suppl Table 1) in *Atoh7*^{Ascl1KI/+} eyes compared to wild type. To understand these shifts better, we examined their autonomy within the *Atoh7*-lineage (see below and Fig 3).

The eyes of adult *Atoh7*^{Ascl1KI/Ascl1KI} mice lack optic nerves (arrow, Fig 2C) but retain the cranial nerves that innervate extraocular muscles (arrowhead, Fig 2C), and thus resemble previously generated *Atoh7* mutants (Brown et al., 2001; Wang et al., 2001). *Atoh7*^{Ascl1KI/Ascl1KI} retinas also had reduced thickness, ectopic vasculature (arrow in Fig 2F), a near total loss of Pou4f2⁺ RGCs (Fig 2I,2S), and an excess of Rxrg⁺, S-Opsin⁺ or M-Opsin⁺ cones (Fig 2L,2T and data not shown), similar to *Atoh7* mutant retinas (Brown et al., 2001; Le et al., 2006). There was no change in INL amacrine (Fig 2U) or Nr2e3⁺ rods (Fig 2V), but Vsx2⁺ bipolars and Sox9⁺ Müller glia were each significantly reduced in *Atoh7*^{Ascl1KI/Ascl1KI} mice compared to wild type (Fig 2O,2R,2W). As a comparison, rods and bipolars are also reduced in *Atoh7*^{-/-} retinas (Brown et al., 2001; Brzezinski IV et al., 2005; Brzezinski IV et al., 2012), consistent with prenatal depletion of the RPC pool (Le et al., 2006; Mu et al., 2005). We conclude that *Ascl1* cannot substitute for *Atoh7* during retinal development.

Cell autonomous and nonautonomous retinal phenotypes from Ascl1 misexpression

To determine if the quantitative shifts observed for rods, bipolars and Müller glia in *Atoh7*^{Ascl1KI/+} eyes, or those for RGCs, cones, bipolars and Müller glia in *Atoh7*^{Ascl1KI/Ascl1KI} eyes occur cell autonomously, we scored each cell type for coexpression with an *Atoh7* lineage reporter. Because *Atoh7* is downregulated at birth, the DsRed2 reporter in *Atoh7*^{Ascl1KI/+} is uninformative in the adult retina. So we used *Atoh7*-Cre BAC (Brzezinski IV et al., 2012) and Z/EG (Hadjantonakis et al., 1998) transgenes to permanently label the *Atoh7*⁺ descendants in control, *Atoh7*^{Ascl1KI/+} and *Atoh7*^{Ascl1KI/Ascl1KI} mice, including cells derived from ectopic *Ascl1*⁺ progenitors. The size of the *Atoh7* lineage was evaluated by quantifying the percentage of DsRed2⁺ cells at E12.5 and E13.5 (Fig. 3P, Suppl Table 2), and of *Atoh7*-Cre;Z/EG (GFP⁺ cells) at E16.5 and P21 (Fig. 3Q, Suppl Table 2). There was no significant change in the *Atoh7* lineage at any age, for either *Atoh7*^{Ascl1KI} genotype. Thus the increases or decreases for particular retinal cell

types (Fig 2S-2W) cannot be explained simply by expansion or contraction of the *Atoh7* lineage, which comprises less than 10% of the adult retina (Brzezinski IV et al., 2012).

To examine the cell autonomy of each *Atoh7*^{Ascl1KI} adult phenotypes, we compared the fractional abundance of histotypic marker+ cells in the GFP+ cohort of *Atoh7*^{Ascl1/+} and *Atoh7*^{Ascl1KI/Ascl1KI} mice also carrying *Atoh7-Cre* and Z/EG transgenes. As expected, we found a cell-autonomous loss of Pou4f2+ RGCs in *Atoh7*^{Ascl1KI/Ascl1KI} adult eyes (Fig 3A-3C,3R, Suppl Table 1). Next, the pan-cone marker Rxrg/RXRγ (Roberts et al., 2005) was used to assess the autonomy of the cone phenotype in *Atoh7*^{Ascl1KI/Ascl1KI} eyes. There was no autonomous increase in Rxrg+GFP+ cones (arrows in Figs 3D-3F), although many more Rxrg+GFP- cone photoreceptors (red only) were present in the outer nuclear layer of *Atoh7*^{Ascl1KI/Ascl1KI} eyes than in controls (Fig 3F). We did note a small autonomous loss of Nr2e3+GFP+ rod photoreceptors in *Atoh7*^{Ascl1KI/+} eyes (Figs 3G-3I,3S, Suppl Table 1), which might be a consequence of earlier alterations in cell cycle exit (Fig 4). Finally, we only found rare Vsx2+GFP+; bipolar interneurons and Sox9+GFP+ Müller glia (Fig 3J-3O) in retinal sections from all three genotypes. We conclude that shifts in the total proportion of bipolars and Müller glia (Figs 2O,2R,2W) were a secondary consequence of earlier, developmental defects (see below, Fig 4).

Although *Ascl1* misexpression did not autonomously affect the number of bipolars or Müller glia within the *Atoh7* lineage, it remained possible that the birthdates of these late classes were accelerated. For this, we birthdated the offspring from *Atoh7*^{Ascl1KI/+} intercross matings at E12, E14, E18, P2, P4 and P6, and examined bipolar (Vsx2+) and Müller (Sox9+) cell birth in P21 retinal sections. Rod birthdates were uninformative in this experiment, since these cells are generated over an extended period, beginning at E13 (Cepko et al., 1996; Sidman, 1961; Young, 1985). We found no precocious bipolar neurons (BrdU+Vsx2+) in E12.5 or E14.5 birthdated *Atoh7*^{Ascl1KI/Ascl1KI} retinas (not shown). There was also no difference among the *Atoh7*^{Ascl1KI} genotypes for bipolar or Müller glial birthdates from E18.5 to P6 (not shown).

Ascl1 disrupts cell cycle progression autonomously and nonautonomously

Ascl1 is primarily expressed by dividing RPCs, while *Atoh7* is made by RPCs during (< E14) or shortly after (> E15) the terminal cell cycle (Brzezinski IV et al., 2011; Brzezinski IV et al., 2012; Jasoni and Reh, 1996; Le et al., 2006; Pennesi et al., 2003). Therefore it was possible that ectopic *Ascl1* could alter the cell cycle status of *Atoh7*+ cells. We first asked whether the total proportions of mitotic RPCs (BrdU pulse-labeled), exiting RPCs (Cdkn1b +p27+) and/or nascent RGCs (Pou4f2+) differed among wild type, *Atoh7*^{Ascl1KI/+} and *Atoh7*^{Ascl1KI/Ascl1KI} retinas, from E12.5 to E13.5. Normally at E12.5, the outer proliferative zone (BrdU pulse labeled, green nuclei) is separated from the inner neurogenic zone and nascent RGCs (Pou4f2+, red nuclei), with only sporadic BrdU+Pou4f2+ cells per retinal section (arrows in Figs 4A,4B). In *Atoh7*^{Ascl1KI/Ascl1KI} eyes, the total proportion of BrdU+ S-phase cells increased at both ages, along with a huge loss of Pou4f2+ RGCs (Figs 4C-E,H). This expansion was accompanied by a loss of both newly postmitotic Cdkn1b+ cells (Fig 4J and data not shown) and Pou4f2+ RGCs (Figs 4C, 4G, 4L). Intriguingly, the reduction in Cdkn1b/p27+ cells directly contrasts an increase observed in *Atoh7*^{-/-} retinas (Le et al., 2006). For all three markers, the changes observed were sensitive to the dosage of ectopic *Ascl1* (Figs 4H,4J,4L, Suppl Table 3).

Next, we tested the cell autonomy of the changes in proliferating, exiting and differentiating cells by quantifying BrdU, Cdkn1b and Pou4f2 coexpression with DsRed2 in E12.5 and E13.5 retinas (Figs 4D-4G, 4I,4K,4M, Suppl Table 3). A cell-autonomous increase in S-phase cells was particularly striking in heterozygous and homozygous mutant cells (arrows in Figs 4D,4E, yellow fractions in 4I), since normally BrdU+ S-phase cells do not express

Atoh7 mRNA or *Atoh7*-GFP transgenes (Hufnagel et al., 2010; Le et al., 2006; Riesenberger et al., 2009a), although a small number of BrdU+βgal+ cells have been found in *Atoh7^{LacZ/+}* eyes at this age (Brzezinski IV et al., 2012). Within both heterozygous and homozygous DsRed2-marked lineages, the excess proliferating cells were balanced by a reduction of non S-phase lineage cells (DsRed2+BrdU- red bars in right graph Fig 4I). There was no net change in the size of the *Atoh7* lineage, even with two alleles of ectopic *Ascl1* (Figs 3P, 4I, Suppl Table 2), in stark contrast to the expansion in the total S-phase population (Fig 4H).

The proportions of DsRed2+Cdkn1b+ and DsRed2+Pou4f2+ cells were also examined in adjacent sections (Figures 4J-4M, Suppl Table 3). In both cases, marker-positive cells were autonomously reduced within the *Atoh7* lineage, along with a complimentary increase in the marker-negative;DsRed2+ cohort (right graphs in Figs 4K, 4L), further demonstrating there was no fluctuation in the overall size of the *Atoh7* lineage. To explain these findings, we also examined the DsRed2-negative cells and saw nonautonomous shifts in the proportions of Cdkn1b+ and Pou4f2+ cells (left graphs in Figs 4K, 4M). This was balanced by a trend for nonautonomous increase in S-phase cells (green bars in left graph Fig 4I). We conclude that ectopic *Ascl1* blocks cell cycle exit within the *Atoh7* lineage, and that this population is both sensitive and actively responsive, to maintaining a constant proportionality within the developing retina. Simultaneously, we observed nonautonomous changes in the expression of these same markers within cells outside of the *Atoh7* lineage. Alternatively, DsRed2 might become activated earlier in the cell cycle, due to an unforeseen issue regarding knock-in allele construction, as well as *Ascl1* ectopically promoting RPC proliferation. This could account for maintaining *Atoh7* lineage size.

Lastly, we asked whether ectopic *Ascl1* caused any early RPCs to stall in the cell cycle, thereby lengthening their overall cycling time. In the developing rodent eye, RPC cell cycle length increases progressively with age (Alexiades and Cepko, 1996). If ectopic *Ascl1* induced longer cycle times, it might be interpretable as endowing the early cells with a late RPC characteristic. To test this idea, we pulsed E12.5 retinas with BrdU and scored incorporation 1.5 hours later within proliferative RPCs, which were further identified by Ki67 coexpression (Chenn and Walsh, 2002; Pei et al., 2011). Reductions in the BrdU+Ki67+/Ki67+ proliferating population suggest cell cycle lengthening, but we found no differences among the three genotypes (Suppl Fig 1A). To further examine the balance between cell cycle retention and exit, we performed a 24 hour BrdU pulse-chase and determined the cell cycle retention index (BrdU+Ki67+/BrdU+) (Chenn and Walsh, 2002; Pei et al., 2011). Here too, we found no significant difference among the three *Atoh7* genotypes (Suppl Fig 1B). An assessment of the DsRed2-marked population would be the most meaningful, but unfortunately antibody reagent incompatibility prevented us from doing so. Overall, we assume that ectopic *Ascl1* does not induce RPCs to lengthen (or shorten) their cell cycle, suggesting that the affected RPCs undergo a defined number of extra cell divisions. However, some or all of these cells might then undergo apoptosis, as a consequence of failing to differentiate correctly.

Apoptosis of *Atoh7^{Ascl1KI/Ascl1KI}* retinal cells

In *Atoh7^{Ascl1KI/+}* eyes, RGC differentiation is initially delayed (Fig. 4H) but eventually recovers, since adult eyes have a normal proportion of these neurons (Fig 2H, 2S). We therefore examined retinal sections at an intermediate time point (E16.5) from *Atoh7^{+/+}*, *Atoh7^{Ascl1KI/+}* and *Atoh7^{Ascl1KI/Ascl1KI}* embryos carrying *Atoh7*-Cre and Z/EG transgenes. In wild type and *Atoh7^{Ascl1KI/+}* eyes, the *Atoh7* lineage (GFP+) contained Pou4f2+ RGCs within the GCL (Figs 5A, 5B), indicating that differentiation was occurring in heterozygotes. But *Atoh7^{Ascl1KI/Ascl1KI}* E16.5 eyes had very few Pou4f2+ RGCs (red nuclei in Fig 5C) or Cdkn1b+ cells (not shown). This difference between the heterozygous and homozygous mutants was further confirmed by examining Isl1 localization in nascent RGCs and

displaced amacrine (Elshatory et al., 2007). The *Isl1* pattern was normal in heterozygotes, but obviously reduced in homozygotes (Figs 5G-5I). Although the recovery in heterozygotes was predicted, we remained curious about the developmental status of E16.5 homozygous mutant *Atoh7* lineage cells.

Intriguingly, E16.5 *Atoh7*-Cre; Z/EG; *Atoh7*^{Ascl1KI/Ascl1KI} retinas displayed abnormal GFP⁺ cell projections into the subretinal (ventricular) space (arrows in 5C; n=3/3 mutants compared to n=0/3 heterozygotes). Colabeling with either DAPI nuclear stain or anti-Tubb3 (β III Tubulin) demonstrated that these were GFP⁺ cellular projections, originating from *Atoh7* lineage cells displaying neuronal identity (arrows in Fig 5F). This particular phenotype is reminiscent of *Pou4f2*^{-/-} eyes, in which profound RGC axon targeting defects indirectly cause excessive RGC cell death (Bermingham et al., 1999; Erkman et al., 2000). In addition, *Atoh7*^{-/-} mutant retinas were recently shown to accumulate apoptotic cells in the GCL (Feng et al., 2010; Prasov and Glaser, 2012). Therefore, we wondered whether the aberrant axon projections in *Atoh7*^{Ascl1KI/Ascl1KI} retinas indicated that mutant cells normally fated to become RGCs, instead die. We surveyed E12.5, E14.5 and E16.5 retinal sections from wild type, *Atoh7*^{Ascl1KI/+}, and *Atoh7*^{Ascl1KI/Ascl1KI} eyes and found a rapid accumulation of activated-Caspase3⁺ cells only in *Atoh7*^{Ascl1KI/Ascl1KI} mutants from E12.5-E16.5 (Fig 5J-5M), which phenocopied *Atoh7* mutants (Feng et al., 2010; Prasov et al., 2012).

Discussion

In this paper we assessed the consequences of genetically replacing the mouse *Atoh7* coding region with that of *Ascl1* during in vivo retinal development. Our data show that *Ascl1* cannot substitute for any aspect of *Atoh7* function. Moreover, *Ascl1* misexpression within the *Atoh7*-lineage did not induce late-born retinal fates precociously or prevent RGC formation, although we did find it prolonged the proliferation of early RPCs (Figure 6). Unexpectedly, we found that ectopic *Ascl1* blocked cell cycle exit and early differentiation simultaneously within the *Atoh7* lineage and nonautonomously in other RPCs.

Atoh7^{Ascl1KI} gene replacement allele

In *Atoh7*^{Ascl1KI}; *Atoh7*^{lacZ} transheterozygous retinas, we observed more DsRed2⁺ cells than those expressing β gal, although all β gal⁺ cells coexpress DsRed2. Thus, it appears that DsRed2 overreports the *Atoh7* lineage, which could have arisen by several mechanisms: **A**) differences in knock-in allele construction, **B**) distinct rates of reporter mRNA and/or protein turnover, **C**) unequal antibody sensitivity, **D**) a miRNA in RPCs that normally suppresses *Atoh7* expression, thereby allowing DsRed2 to appear earlier, and **E**) the theoretical possibility that *Ascl1* normally suppresses *Atoh7* in the *Ascl1* lineage, thus ectopic *Ascl1* in the *Atoh7* lineage dampens the LacZ allele, possibly through an miRNA mechanism. Among these, we favor the possibility that differences in allele construction resulted in mRNAs with unique 3'UTR sequences, since the DsRed2 cassette used SV40 3'UTR, while the LacZ allele retained *Atoh7* 3'UTR. Furthermore, ectopic *Ascl1* utilized endogenous *Ascl1* 3'UTR sequences, so that the ectopic and endogenous expression closely resembles one another. In the event that there is differential miRNAs regulation of bHLH factor expression, the DsRed2 reporter expression may not completely reflect *Atoh7* regulation. There is also a rare possibility of reduced DsRed2 purdurance in reporting the consequences of misexpressing *Ascl1* in the *Atoh7* lineage. To learn the full extent to which ectopic *Ascl1* and endogenous *Atoh7* are coexpressed in normal versus gene replacement cells will require double antibody experiments that examine protein expression. Should a specific *Atoh7* antisera ever become available (Prasov et al., 2010; Suppl. Fig 2), we will

define when and where *Atoh7* protein is detectable during the final cell cycle of RPCs, and compare that to endogenous and ectopic *Ascl1* expression.

Interchangeability of *Ascl1* and *Atoh7* during retinal neurogenesis

The inability of *Ascl1* to rescue RGC development in the absence of *Atoh7* is consistent with the low frequency of *Atoh7* and *Ascl1* coexpression during the earliest phase of retinogenesis (Brown et al., 1998; Brzezinski IV et al., 2011), the absence of RGCs in the endogenous *Ascl1* retinal lineage and lack of a RGC phenotype in *Ascl1* mutant mice (Brzezinski IV et al., 2011). The population of *Ascl1*⁺ cells in the E18.5 retina that derive from the *Atoh7* lineage (~25%), presumably produce non-RGC cell types (Brzezinski IV et al., 2011). Thus, *Ascl1* has no discernible role in RGC development. Our results demonstrate that *Ascl1* also does not prevent ganglion cell development, as this cell class was present in normal abundance in *Atoh7*^{Ascl1/+} adult eyes.

One of the best examples of deep evolutionary gene family conservation is the highly conserved role of bHLH factors during retinal neurogenesis. *Atoh7* is a competence factor for RGC fate, and a semiortholog of *Drosophila atonal (ato)*, which specifies chordotonal and R8 photoreceptor sensory neurons (Bertrand et al., 2002; Brzezinski IV et al., 2012; Jarman et al., 1993; Jarman et al., 1994). *Ascl1* acts during late retinal neurogenesis in the mouse, and is a semi-ortholog of *Drosophila scute (sc)*, which specifies fly external sensory bristles, including interommatidial bristles in the pupal eye (Brown et al., 1991; Campuzano et al., 1985). Previous functional comparisons of *ato* and *sc* in *Drosophila* identified key amino acids within each bHLH domain (Chien et al., 1996; Jarman and Ahmed, 1998; Maung and Jarman, 2007), and specific nucleotides in their E-Box DNA binding sites (Powell et al., 2008; Powell et al., 2004). Together these characteristics endow Ato and Sc with unique abilities during neural specification. Heterochronic misexpression of *sc* in *ato* mutant eyes failed to produce R8 neurons, although other photoreceptor cell types were partially rescued (Sun et al., 2000). This partial rescue of non-R8 photoreceptor neurons has been attributed to a subset of downstream target genes, shared by *ato* and *sc* during sense organ development (Sun et al., 2000; Sun et al., 2003). In this study however, we found that mouse *Ascl1* could not rescue RGC genesis within the *Atoh7* lineage.

Direct comparisons of bHLH factor functions during *Xenopus* retinal development have also been made (Brown et al., 1998; Kanekar et al., 1997; Moore et al., 2002). In the frog eye, neurogenesis occurs rapidly, necessitating simultaneous or successive expression of multiple transcription factors within RPCs. The *ato*-like factors, *Xath5* and *XNeuroD* promote RGC and amacrine fates respectively, while the *sc*-like factor *Xash1* promotes bipolar neurogenesis (Kanekar et al., 1997; Moore et al., 2002). The neuronal subtype depends on the primary structure, timing of expression and unique posttranslational modifications. For example, the *XNeuroD* and *Xash1* proteins, but not *Xath5*, are modified by GSK3 β -mediated phosphorylation (Moore et al., 2002). While overexpression of either frog *Xash1* or mouse *Ascl1/Mash1* induced bipolar neurons in frogs, mouse *Ascl1* promoted RGC fates when GSK3 β activity was blocked (Moore et al., 2002). Moreover, overexpression of mouse *Atoh7/Math5* in the frog retina triggered bipolar neurogenesis, not RGC formation (Brown et al., 1998). These data strongly argue that, at least for the frog eye, temporal expression and interactions with other proteins may be more important than the bHLH domain amino acid sequence or E-Box nucleotide sequences *per se*, in determining neuronal subtype specificity. In the future, it will be important to search for posttranslational mechanisms that regulate bHLH factor activity during mammalian retinogenesis.

The interchangeability of bHLH factors has also been tested to some extent during mouse retinal neurogenesis. Two *ato*-related bHLH genes, *Neurod1* and *Atoh3*, partially rescued RGC specification when either was homologously recombined into the *Atoh7* locus (Mao et

al., 2008). The high level of amino acid similarity among *Atoh7*, *Neurod1* and *Atoh3* bHLH protein domains, the coexpression of *Neurod1* and *Atoh7^{LacZ}* within a subset of RPCs during normal development (Brzezinski IV et al., 2012; Kiyama et al., 2011), and the cell-autonomous upregulation of *Neurod1* in *Atoh7* mutants are all consistent with partial functional redundancy for these *ato*-like bHLH factors (Le et al., 2006; Mao et al., 2008). In a different study, targeted substitution of *Ascl1* into the *Neurog2* locus (Fode et al., 1998) restored the temporal progression of retinogenesis, which is *Neurog2*-dependent (Hufnagel et al., 2010). Yet, here we found that *Ascl1* cannot rescue the *Atoh7* mutant phenotypes.

We propose that mammalian bHLH factors are more likely to substitute for one another, if they are normally expressed in mitotic or nonmitotic RPCs. Whereas *Atoh7*, *Neurod1* and *Atoh3* are present in exiting RPCs, and *Ascl1* and *Neurog2* are found in proliferative RPCs. However, other mechanisms might underlie these differences. For example, *Ascl1* and *Neurog2* proteins can form complexes (Henke et al., 2009), suggesting that *Ascl1* homodimers might substitute for *Ascl1*-*Neurog2* heterodimers in activating downstream target genes. In the developing mouse retina, more work will be needed to fully understand why *Ascl1* rescues *Neurog2* retinal phenotypes, but not those of *Atoh7*, since *Atoh7* and *Neurog2* are each orthologous to *ato*.

Roles for *Ascl1* in the mouse retina

Several studies of retinal regeneration or oncogenesis have shown that high levels of *Ascl1* can drive cell cycle exit, others support a pro-proliferative function, including during regeneration. For example, zebrafish retina injury induces the expansion of Müller glia, by coordinated differentiation, division and redifferentiation into neurons. These injuries also activate *Ascl1a* within Müller glia, prior to cell cycle reentry and neuronal differentiation (Fausett et al., 2008; Yurco and Cameron, 2005). Furthermore, although tumor cell lines often upregulate Notch signaling and downregulate bHLH factors, some neuroendocrine cancer cells exhibit high levels of *Ascl1* that correlate with an increased rate of cell division (Jiang et al., 2009; Rapa et al., 2008). In both circumstances, dividing cells with neural identity express *Ascl1*, implying that it promotes growth within neural-restricted populations. Finally, *Ascl1* is normally found in proliferating neuronal progenitors throughout the developing nervous system, where it has been thought to strictly promote cell cycle exit and neural differentiation (Ahmad et al., 1998; Cai et al., 2000; Farah and Easter, 2005; Mao et al., 2009; Tomita et al., 2000). Recent genomic profiling of *Ascl1* binding sites, in either developing telencephalon or neural stem cells, indicate that *Ascl1* directly regulates a very diverse set of downstream genes, including cell cycle progression, cell cycle exit and oncogenic factors (Castro et al., 2011). *Ascl1* may coordinate progression of neuronal progenitors through multiple stages of their development, including proliferation of neural progenitors in the forming retina. Data presented in this study further support a role a pro-proliferative role for *Ascl1*.

The mechanisms by which *Ascl1* acts in the late retina may differ from those discussed here regarding early ectopic expression. When *Ascl1* was misexpressed at older ages using retroviruses, rod photoreceptors and bipolar interneurons were generated. Could this be the appropriate developmental window for *Ascl1* to promote cell cycle exit? Indeed, *Ascl1* mutant retinal explants had fewer late cell types (Hatakeyama et al., 2001; Tomita et al., 1996), and the proliferation defects in *Ascl1* mutant cells are manifested late in development (Brzezinski IV et al., 2011). Therefore, it remains unclear whether *Ascl1* actively promotes late fates, or simply influences cell cycle exit in this older context. These age-specific differences could be due to integrating low levels of Notch signaling over a long period of time. Alternatively, RPCs may require higher/longer engagement of Notch to prevent differentiation as time proceeds. The failure of *Ascl1* to reprogram the early *Atoh7* lineage to adopt late fates may reflect an inherent inability to instruct any RPC regardless of age to

adopt a late fate, may require additional late environment factors, for example CNTF, LIF or FGF (Ezzeddine et al., 1997; Lillien, 1995; Lillien and Cepko, 1992). or may require cofactors only present in cycling cells.

Perhaps the most surprising aspect of this study is the finding that ectopic *Asc11* can influence cell cycle progression, both autonomously and nonautonomously. How can the *Asc11* transcription factor effect changes in multiple lineages at once, particularly when misexpressed in just one population? Many previous studies have shown that the *Notch* pathway is influenced by the activity of proneural bHLH factors (reviewed in Pierfelice et al., 2011). In the mouse retina, reduction of *Asc11* activity is accompanied by a downregulation of *Dll1* transcription (Nelson et al., 2009; Nelson and Reh, 2008). We propose that ectopic *Asc11* elevates *Dll1* expression in the *Atoh7* lineage, thereby prolonging proliferation of an adjacent population of RPCs. The stoichiometry of *Notch* signaling between *Atoh7*⁺ cells and RPCs has not been well explored, although *Dll1* and *Dll4* are present in nascent RGCs (Rocha et al., 2009). It is plausible that *Atoh7*⁺ cells normally express a particular level of either ligand on their cell surface, but in *Atoh7*^{Asc11/+} and *Atoh7*^{Asc11KI/Asc11KI} eyes these levels become elevated and drive proliferation in RPCs. An equally intriguing question is whether *Notch* signaling among *Atoh7*-lineage cells could explain how this population maintains the correct overall proportionality. Indeed, loss of the *Notch* integrator *Rbpj* revealed active homeostasis during photoreceptor cell development (Riesenberg et al., 2009b). Future studies are needed identify and examine molecular mechanisms of *Asc11* and *Atoh7* regulation of *Dll* ligand expression within each endogenous retinal lineage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ahmad I, Acharya HR, Rogers JA, Shibata A, Smithgall TE, Dooley CM. The role of NeuroD as a differentiation factor in the mammalian retina. *J Mol Neurosci.* 1998; 11:165–178. [PubMed: 10096043]
- Alexiades MR, Cepko C. Quantitative analysis of proliferation and cell cycle length during development of the rat retina. *Dev Dyn.* 1996; 205:293–307. [PubMed: 8850565]
- Auerbach W, Dunmore JH, Fairchild-Huntress V, Fang Q, Auerbach AB, Huszar D, Joyner AL. Establishment and chimera analysis of 129/SvEv- and C57BL/6-derived mouse embryonic stem cell lines. *Biotechniques.* 2000; 29:1024–1028. 1030–1032. [PubMed: 11084865]
- Bermingham NA, Hassan BA, Price SD, Vollrath MA, Ben-Arie N, Eatock RA, Bellen HJ, Lysakowski A, Zoghbi HY. *Math1*: an essential gene for the generation of inner ear hair cells. *Science.* 1999; 284:1837–1841. [PubMed: 10364557]
- Bertrand N, Castro DS, Guillemot F. Proneural genes and the specification of neural cell types. *Nat Rev Neurosci.* 2002; 3:517–530. [PubMed: 12094208]
- Brown NL, Kanekar S, Vetter ML, Tucker PK, Gemza DL, Glaser T. *Math5* encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. *Development.* 1998; 125:4821–4833. [PubMed: 9806930]

- Brown NL, Patel S, Brzezinski J, Glaser T. Math5 is required for retinal ganglion cell and optic nerve formation. *Development*. 2001; 128:2497–2508. [PubMed: 11493566]
- Brown NL, Sattler CA, Markey DR, Carroll SB. hairy gene function in the Drosophila eye: normal expression is dispensable but ectopic expression alters cell fates. *Development*. 1991; 113:1245–1256. [PubMed: 1811940]
- Brzezinski JA IV, Brown NL, Tanikawa A, Bush RA, Sieving PA, Vitaterna MH, Takahashi JS, Glaser T. Loss of circadian photoentrainment and abnormal retinal electrophysiology in Math5 mutant mice. *Invest Ophthalmol Vis Sci*. 2005; 46:2540–2551. [PubMed: 15980246]
- Brzezinski JA IV, Kim EJ, Johnson JE, Reh TA. Ascl1 expression defines a subpopulation of lineage-restricted progenitors in the mammalian retina. *Development*. 2011; 138:3519–3531. [PubMed: 21771810]
- Brzezinski JA IV, Prasov L, Glaser T. Math5 defines the ganglion cell competence state in a subpopulation of retinal progenitor cells exiting the cell cycle. *Developmental biology*. 2012; 365:395–413. [PubMed: 22445509]
- Cai L, Morrow EM, Cepko CL. Misexpression of basic helix-loop-helix genes in the murine cerebral cortex affects cell fate choices and neuronal survival. *Development*. 2000; 127:3021–3030. [PubMed: 10862740]
- Campuzano S, Carramolino L, Cabrera CV, Ruiz-Gomez M, Villares R, Boronat A, Modolell J. Molecular genetics of the achaete-scute gene complex of *D. melanogaster*. *Cell*. 1985; 40:327–338. [PubMed: 3917860]
- Castro DS, Martynoga B, Parras C, Ramesh V, Pacary E, Johnston C, Drechsel D, Lebel-Potter M, Garcia LG, Hunt C, Dolle D, Bithell A, Ettwiller L, Buckley N, Guillemot F. A novel function of the proneural factor Ascl1 in progenitor proliferation identified by genome-wide characterization of its targets. *Genes Dev*. 2011; 25:930–945. [PubMed: 21536733]
- Cepko CL, Austin CP, Yang X, Alexiades M, Ezzeddine D. Cell fate determination in the vertebrate retina. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93:589–595. [PubMed: 8570600]
- Chenn A, Walsh CA. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science*. 2002; 297:365–369. [PubMed: 12130776]
- Chien CT, Hsiao CD, Jan LY, Jan YN. Neuronal type information encoded in the basic-helix-loop-helix domain of proneural genes. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93:13239–13244. [PubMed: 8917575]
- Elshatory Y, Deng M, Xie X, Gan L. Expression of the LIM-homeodomain protein Isl1 in the developing and mature mouse retina. *J Comp Neurol*. 2007; 503:182–197. [PubMed: 17480014]
- Erkman L, Yates PA, McLaughlin T, McEvelly RJ, Whisenhunt T, O'Connell SM, Krones AI, Kirby MA, Rapaport DH, Birmingham JR, O'Leary DD, Rosenfeld MG. A POU domain transcription factor-dependent program regulates axon pathfinding in the vertebrate visual system. *Neuron*. 2000; 28:779–792. [PubMed: 11163266]
- Ezzeddine ZD, Yang X, DeChiara T, Yancopoulos G, Cepko CL. Postmitotic cells fated to become rod photoreceptors can be respecified by CNTF treatment of the retina. *Development*. 1997; 124:1055–1067. [PubMed: 9056780]
- Farah MH, Easter SS Jr. Cell birth and death in the mouse retinal ganglion cell layer. *J Comp Neurol*. 2005; 489:120–134. [PubMed: 15977166]
- Fausett BV, Gumerson JD, Goldman D. The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. *J Neurosci*. 2008; 28:1109–1117. [PubMed: 18234889]
- Feng L, Xie ZH, Ding Q, Xie X, Libby RT, Gan L. MATH5 controls the acquisition of multiple retinal cell fates. *Mol Brain*. 2010; 3:36. [PubMed: 21087508]
- Fode C, Gradwohl G, Morin X, Dierich A, LeMeur M, Goridis C, Guillemot F. The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron*. 1998; 20:483–494. [PubMed: 9539123]
- Hadjantonakis AK, Gertsenstein M, Ikawa M, Okabe M, Nagy A. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech Dev*. 1998; 76:79–90. [PubMed: 9867352]

- Hatakeyama J, Tomita K, Inoue T, Kageyama R. Roles of homeobox and bHLH genes in specification of a retinal cell type. *Development*. 2001; 128:1313–1322. [PubMed: 11262232]
- Henke RM, Meredith DM, Borromeo MD, Savage TK, Johnson JE. Ascl1 and Neurog2 form novel complexes and regulate Delta-like3 (Dll3) expression in the neural tube. *Developmental biology*. 2009; 328:529–540. [PubMed: 19389376]
- Hufnagel RB, Le TT, Riesenberger AL, Brown NL. Neurog2 controls the leading edge of neurogenesis in the mammalian retina. *Developmental biology*. 2010; 340:490–503. [PubMed: 20144606]
- Hufnagel RB, Riesenberger AN, Saul SM, Brown NL. Conserved regulation of Math5 and Math1 revealed by Math5-GFP transgenes. *Molecular and cellular neurosciences*. 2007; 36:435–448. [PubMed: 17900924]
- Jarman AP, Ahmed I. The specificity of proneural genes in determining *Drosophila* sense organ identity. *Mech Dev*. 1998; 76:117–125. [PubMed: 9767145]
- Jarman AP, Grau Y, Jan LY, Jan YN. atonal is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. *Cell*. 1993; 73:1307–1321. [PubMed: 8324823]
- Jarman AP, Grell EH, Ackerman L, Jan LY, Jan YN. Atonal is the proneural gene for *Drosophila* photoreceptors. *Nature*. 1994; 369:398–400. [PubMed: 8196767]
- Jasoni CL, Reh TA. Temporal and spatial pattern of MASH-1 expression in the developing rat retina demonstrates progenitor cell heterogeneity. *J Comp Neurol*. 1996; 369:319–327. [PubMed: 8727003]
- Jeon CJ, Strettoi E, Masland RH. The major cell populations of the mouse retina. *J Neurosci*. 1998; 18:8936–8946. [PubMed: 9786999]
- Jiang T, Collins BJ, Jin N, Watkins DN, Brock MV, Matsui W, Nelkin BD, Ball DW. Achaete-scute complex homologue 1 regulates tumor-initiating capacity in human small cell lung cancer. *Cancer research*. 2009; 69:845–854. [PubMed: 19176379]
- Kanekar S, Perron M, Dorsky R, Harris WA, Jan LY, Jan YN, Vetter ML. Xath5 participates in a network of bHLH genes in the developing *Xenopus* retina. *Neuron*. 1997; 19:981–994. [PubMed: 9390513]
- Kay JN, Finger-Baier KC, Roeser T, Staub W, Baier H. Retinal ganglion cell genesis requires lakritz, a Zebrafish atonal Homolog. *Neuron*. 2001; 30:725–736. [PubMed: 11430806]
- Kiyama R, Fukudome K, Hiyoshi T, Umemoto A, Yoshimoto Y, Maeda T. The loss of dexterity in the bilateral lower extremities in patients with stroke. *J Appl Biomech*. 2011; 27:122–129. [PubMed: 21576720]
- Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, Alt FW, Westphal H. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93:5860–5865. [PubMed: 8650183]
- Le TT, Wroblewski E, Patel S, Riesenberger AN, Brown NL. Math5 is required for both early retinal neuron differentiation and cell cycle progression. *Developmental biology*. 2006
- Lillien L. Changes in retinal cell fate induced by overexpression of EGF receptor. *Nature*. 1995; 377:158–162. [PubMed: 7675083]
- Lillien L, Cepko C. Control of proliferation in the retina: temporal changes in responsiveness to FGF and TGF alpha. *Development*. 1992; 115:253–266. [PubMed: 1638984]
- Mao CA, Wang SW, Pan P, Klein WH. Rewiring the retinal ganglion cell gene regulatory network: Neurod1 promotes retinal ganglion cell fate in the absence of Math5. *Development*. 2008; 135:3379–3388. [PubMed: 18787067]
- Mao W, Yan RT, Wang SZ. Proneural gene ash1 promotes amacrine cell production in the chick retina. *Dev Neurobiol*. 2009; 69:88–104. [PubMed: 19067322]
- Maung SM, Jarman AP. Functional distinctness of closely related transcription factors: a comparison of the Atonal and Amos proneural factors. *Mech Dev*. 2007; 124:647–656. [PubMed: 17709231]
- Moore KB, Schneider ML, Vetter ML. Posttranslational mechanisms control the timing of bHLH function and regulate retinal cell fate. *Neuron*. 2002; 34:183–195. [PubMed: 11970861]
- Morrow EM, Furukawa T, Lee JE, Cepko CL. NeuroD regulates multiple functions in the developing neural retina in rodent. *Development*. 1999; 126:23–36. [PubMed: 9834183]

- Mu X, Fu X, Sun H, Beremand PD, Thomas TL, Klein WH. A gene network downstream of transcription factor Math5 regulates retinal progenitor cell competence and ganglion cell fate. *Developmental biology*. 2005; 280:467–481. [PubMed: 15882586]
- Nelson BR, Hartman BH, Ray CA, Hayashi T, Bermingham-McDonogh O, Reh TA. Acheate-scute like 1 (Ascl1) is required for normal delta-like (Dll) gene expression and notch signaling during retinal development. *Dev Dyn*. 2009; 238:2163–2178. [PubMed: 19191219]
- Nelson BR, Reh TA. Relationship between Delta-like and proneural bHLH genes during chick retinal development. *Dev Dyn*. 2008; 237:1565–1580. [PubMed: 18435466]
- Novak A, Guo C, Yang W, Nagy A, Lobe CG. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis*. 2000; 28:147–155. [PubMed: 11105057]
- Pei Z, Wang B, Chen G, Nagao M, Nakafuku M, Campbell K. Homeobox genes *Gsx1* and *Gsx2* differentially regulate telencephalic progenitor maturation. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:1675–1680. [PubMed: 21205889]
- Pennesi ME, Cho JH, Yang Z, Wu SH, Zhang J, Wu SM, Tsai MJ. BETA2/NeuroD1 null mice: a new model for transcription factor-dependent photoreceptor degeneration. *J Neurosci*. 2003; 23:453–461. [PubMed: 12533605]
- Pierfelice T, Alberi L, Gaiano N. Notch in the vertebrate nervous system: an old dog with new tricks. *Neuron*. 2011; 69:840–855. [PubMed: 21382546]
- Powell LM, Deaton AM, Wear MA, Jarman AP. Specificity of Atonal and Scute bHLH factors: analysis of cognate E box binding sites and the influence of Senseless. *Genes Cells*. 2008; 13:915–929. [PubMed: 18681894]
- Powell LM, Zur Lage PI, Prentice DR, Senthinathan B, Jarman AP. The proneural proteins Atonal and Scute regulate neural target genes through different E-box binding sites. *Mol Cell Biol*. 2004; 24:9517–9526. [PubMed: 15485919]
- Prasov L, Brown NL, Glaser T. A critical analysis of Atoh7 (Math5) mRNA splicing in the developing mouse retina. *PloS one*. 2010; 5:e12315. [PubMed: 20808762]
- Prasov L, Glaser T. Pushing the envelope of retinal ganglion cell genesis: context dependent function of Math5 (Atoh7). *Developmental biology*. 2012 In Press.
- Prasov L, Nagy M, Rudolph DD, Glaser T. Math5 (Atoh7) gene dosage limits retinal ganglion cell genesis. *Neuroreport*. 2012; 23:631–634. [PubMed: 22660169]
- Rapa I, Ceppi P, Bollito E, Rosas R, Cappia S, Bacillo E, Porpiglia F, Berruti A, Papotti M, Volante M. Human ASH1 expression in prostate cancer with neuroendocrine differentiation. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*. 2008; 21:700–707.
- Riesenberg AN, Le TT, Willardsen MI, Blackburn DC, Vetter ML, Brown NL. Pax6 regulation of Math5 during mouse retinal neurogenesis. *Genesis*. 2009a; 47:175–187. [PubMed: 19208436]
- Riesenberg AN, Liu Z, Kopan R, Brown NL. Rbpj cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. *J Neurosci*. 2009b; 29:12865–12877. [PubMed: 19828801]
- Roberts MR, Hendrickson A, McGuire CR, Reh TA. Retinoid X receptor (gamma) is necessary to establish the S-opsin gradient in cone photoreceptors of the developing mouse retina. *Invest Ophthalmol Vis Sci*. 2005; 46:2897–2904. [PubMed: 16043864]
- Rocha SF, Lopes SS, Gossler A, Henrique D. Dll1 and Dll4 function sequentially in the retina and pV2 domain of the spinal cord to regulate neurogenesis and create cell diversity. *Developmental biology*. 2009; 328:54–65. [PubMed: 19389377]
- Saul SM, Brzezinski JAt, Altschuler RA, Shore SE, Rudolph DD, Kabara LL, Halsey KE, Hufnagel RB, Zhou J, Dolan DF, Glaser T. Math5 expression and function in the central auditory system. *Molecular and cellular neurosciences*. 2008; 37:153–169. [PubMed: 17977745]
- Sidman, R. Histogenesis of mouse retina studied with thymidine-H3. In: Smelser, G., editor. *Structure of the Eye*. New York: Academic Press; 1961. p. 487-506.
- Skowronska-Krawczyk D, Chiodini F, Ebeling M, Alliod C, Kundzewicz A, Castro D, Ballivet M, Guillemot F, Matter-Sadzinski L, Matter JM. Conserved regulatory sequences in Atoh7 mediate

- non-conserved regulatory responses in retina ontogenesis. *Development*. 2009; 136:3767–3777. [PubMed: 19855019]
- Sun Y, Jan LY, Jan YN. Ectopic scute induces *Drosophila* ommatidia development without R8 founder photoreceptors. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:6815–6819. [PubMed: 10823908]
- Sun Y, Kanekar SL, Vetter ML, Gorski S, Jan YN, Glaser T, Brown NL. Conserved and divergent functions of *Drosophila* atonal, amphibian, and mammalian *Ath5* genes. *Evol Dev*. 2003; 5:532–541. [PubMed: 12950631]
- Tomita K, Moriyoshi K, Nakanishi S, Guillemot F, Kageyama R. Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. *Embo J*. 2000; 19:5460–5472. [PubMed: 11032813]
- Tomita K, Nakanishi S, Guillemot F, Kageyama R. Mash1 promotes neuronal differentiation in the retina. *Genes Cells*. 1996; 1:765–774. [PubMed: 9077445]
- Wang SW, Kim BS, Ding K, Wang H, Sun D, Johnson RL, Klein WH, Gan L. Requirement for *math5* in the development of retinal ganglion cells. *Genes Dev*. 2001; 15:24–29. [PubMed: 11156601]
- Young RW. Cell differentiation in the retina of the mouse. *Anat Rec*. 1985; 212:199–205. [PubMed: 3842042]
- Yurco P, Cameron DA. Responses of Muller glia to retinal injury in adult zebrafish. *Vision research*. 2005; 45:991–1002. [PubMed: 15695184]

Figure 1. Homologous recombination of *Ascl1* into the *Atoh7* gene locus

A) Targeting strategy for *Atoh7* coding exon + 3' UTR replacement with an *Ascl1* coding + 3'UTR-IRES-DsRed2 expression cassette. The positions of single-copy genomic DNA probe, routine genotyping PCR primers (A+A', B+B'), and long range PCR primers (C+C', D+D') are indicated. A reverse orientation PGK-neo-pA cassette was removed by Cre-LoxP mediated recombination, by crossing to the EIIa-Cre driver mouse. B) Southern analysis of Bam HI digested mouse tail genomic DNA yielded predicted 22Kb wild type and 14Kb targeted bands. C) Routine PCR genotyping of mouse tail DNA from F² crosses. Internal primers amplified allele-specific PCR products (A+A' = 350 bp wild type band, B+B' = 232bp mutant band). DD') Direct comparison of DsRed2 and β gal expression in the E11.5

optic cup, showing all β gal+ cells coexpress DsRed2 (arrows), but also some DsRed2+; β gal-negative (red-only) cells (arrowhead) at the leading edges of neurogenesis. E-E') At E11.5, all DsRed2 cells also coexpress ectopic Ascl1 (arrows). A rare cell presumably expressing endogenous Ascl1 (green-only) can be seen in E' (arrowhead). F-F') E12.5 retinal sections also exhibit complete overlap of β gal and DsRed2 (arrows), as well as some cells only expressing DsRed2 (red-only, arrowheads). G) Endogenous Ascl1 protein (green-only) in a population of E13.5 retinal cells. H) Extensive coexpression of ectopic DsRed2 and Ascl1 in *Atoh7^{Ascl1KI/+}* retinal lineage (arrows). A smaller population of endogenous Ascl1+ (green-only) cells is also apparent (arrowheads). I) In E13.5 *Atoh7^{LacZ}* cells (green-only), there are very few β gal+Ascl1+ coexpressing cells (arrows). H=Hind III; E=Eco RI, P=Pst I, X=Xba I, Pv=Pvu I, B=Bam HI; Scleral is up in D-I; Mag bars = 20 microns in all panels.

Figure 2. Adult retinal phenotypes of *Atoh7*^{Ascl1KI/+} and *Atoh7*^{Ascl1KI/Ascl1KI} eyes
A-C) *Atoh7*^{Ascl1KI/+} eyes were externally indistinguishable from those of wild type littermates, while *Atoh7*^{Ascl1KI/Ascl1KI} eyes lacked optic nerves (arrows point to position of optic nerve, arrowhead points to a cranial nerve innervating extraocular muscles). D-F) Histologic sections indicate normal retinal lamination in all genotypes, but, *Atoh7*^{Ascl1KI/Ascl1KI} retinas were obviously thinner. Arrow in F points to an ectopic blood vessel, also found in *Atoh7* mutant retinas (Brown et al., 2001). G-I) Substitution of *Ascl1* for *Atoh7* does not rescue loss of Pou4f2+ RGCs (arrows in G,H) in *Atoh7*^{Ascl1KI/Ascl1KI} retinas. J-L) *Ascl1* does not prevent overproduction of cone photoreceptors in *Atoh7*^{Ascl1KI/Ascl1KI} eyes, like *Atoh7* mutants (Brown et al., 2001). Arrow in J points to

cone outer segments quantified in T. M-R) *Atoh7*^{Ascl1KI/+} retinas contain significantly rods, bipolars and excess Müller glia. Paradoxically, complete replacement of *Atoh7* with *Ascl1* resulted in a wild type proportion of these cell types. S-W) Quantification of each retinal cell class among wild type, *Atoh7*^{Ascl1KI/+}, and *Atoh7*^{Ascl1KI/Ascl1KI} eyes. Scleral is up in panels D-R; n = 6 eyes/genotype; onl= outer nuclear layer; inl = inner nuclear layer; gcl = ganglion cell layer; **p < 0.01; ***p < 0.001; Mag bar in A = 500 microns, in D,G = 20 microns.

Figure 3. Cell autonomous versus nonautonomous retinal phenotypes

To follow the fates of cells misexpressing *Ascl1*, *Atoh7-Cre* BAC and Z/EG transgenes were crossed into the *Atoh7^{Ascl1KI/+}* and *Atoh7^{Ascl1KI/Ascl1KI}* genetic backgrounds. P21 retinal cryosections were colabeled with anti-GFP and cell type specific markers. A-C) The *Atoh7-Cre* lineage (GFP+) spans across all three retinal cell layers. Pou4f2+GFP+ RGCs are easily identified (arrows in A,B) except in *Atoh7^{Ascl1KI/Ascl1KI}* mutants (C). D-F) Rxrg+GFP+ cones photoreceptors are present (arrows) in all three genotypes but, excess cones in *Atoh7^{Ascl1KI/Ascl1KI}* mutants (red nuclei) arise nonautonomously. G-I) Arrows point to Nr2e3+GFP+ rods for all three genotypes. Clusters of these colabeled cells were more frequently observed in *Atoh7^{Ascl1KI/Ascl1KI}* eyes. J-O) Vsx2+GFP+ bipolars or Sox9+GFP+

Müller glia were very rare in the *Atoh7* lineages for all genotypes. P,Q) At E12.5 and E13.5, the heterozygous and homozygous lineages (DsRed2+) are the same size (data represent mean $\pm$ SEM, n = 18/age and genotype). At older ages, there was fluctuation of *Atoh7*-Cre lineage among genotypes, although the differences were not significant. R,S) P21 *Atoh7^{Ascl1KI/+}* heterozygotes had an autonomous loss of rod photoreceptors. For *Atoh7^{Ascl1/Ascl1}* retinas, RGCs were completely missing from the *Atoh7* lineage, which had a normal proportion of rods (S). Proportions of rods, bipolars and Muller glia in the wild type *Atoh7*-Cre lineage, and those in another recent study using *Atoh7*-Cre BAC Tg mice (Brzezinski IV et al., 2012), differ from one another. This can be attributed to the lineage reporters (Z/EG versus R26/*loxGFP*), different imaging and cell counting methodologies, and/or the specificity of antibodies used. Data in Q-S represent the mean $\pm$ SEM (n=6/genotype); **p 0.01 and ***p 0.001; onl= outer nuclear layer; inl = inner nuclear layer; gcl = ganglion cell layer; scleral is up in all panels; Mag bars in A-C, D-O = 20 microns.

Figure 4. *Ascl1* acts dominantly to block cell cycle exit and RGC differentiation in the *Atoh7* lineage at E12.5 and E13.5

A,B) In 1.5-hour, BrdU-labeled retinas, S phase cells (green) occupy the central neurogenic region, while differentiating Pou4f2+ RGCs (red) migrate to the inner GCL. Arrows point to rare BrdU+Pou4f2+ cells. B,C,H) At both ages, increasing ectopic *Ascl1* and decreasing *Atoh7* gene dosage correlates with an increased numbers of S phase (BrdU+) cells (H). D-E,I) Both *Atoh7*^{Ascl1KI/+} and *Atoh7*^{Ascl1/Ascl1} retinal lineages exhibit a significant, cell autonomous increase in S phase cells (arrows in D,E; yellow fraction of bars), compared to the *Atoh7*^{+/+} lineage (Brzezinski IV et al., 2012; Hufnagel et al., 2010). By contrast, no S-phase *Atoh7* mRNA+ or *Atoh7*-GFP+ retinal cells have been observed (Brzezinski IV et al., 2012; Hufnagel et al., 2010; Le et al., 2006). J-K) Overall, progressive loss of postmitotic Cdkn1b+ cells in heterozygotes to homozygotes. Interestingly, there were both autonomous (yellow bars) and nonautonomous (green bars left graph in K) reductions in Cdkn1b+ cells. F,G,L,M) Extensive loss of Pou4f2+RGCs in heterozygotes and homozygotes (L). Although the biggest loss was in the DsRed2+Pou4f2+ cohort (arrows in F,G; yellow bars in M), there was also a nonautonomous loss of RGCs (green bars, left graph in M). Yet, the total size of the DsRed2+ population was unaffected (height of right graphs in I, K, M). Data in H-M are the mean $\pm$ SEM (n= 6/genotype); *p 0.05; **p 0.01; ***p 0.001. GCL = ganglion cell layer; Mag bars in A and D = 20 microns.

Figure 5. By E16.5 RGC genesis resumes in *Atoh7*^{Ascl1KI/+} eyes, but apoptosis occurs in *Atoh7*^{Ascl1KI/Ascl1KI} eyes

A-C) At E16.5 *Atoh7*-Cre-Z/EG marked lineage cells in green are largely in the GCL, many colabeled with Pou4f2 (red) in control and *Atoh7*^{Ascl1KI/+} eyes. Arrows in C point to inappropriate accumulation of GFP-labeled axons in the subretinal space of homozygous mutants. D-F) The majority of *Atoh7*-Cre-Z/EG;*Atoh7*^{Ascl1KI/+} cells are GFP+Tubb3+ in the GCL (compare E to D). However, arrows in F points to aberrant GFP+ axons in subretinal space of *Atoh7*^{Ascl1KI/Ascl1KI} eyes. G-I) Isl1+ nascent RGCs and displaced amacrine appear nearly normal in *Atoh7*^{Ascl1KI/+} inner retina, but are drastically reduced in *Atoh7*^{Ascl1KI/Ascl1KI} eyes. J-L) At E14.5 there is an obvious increase in inner retinal

apoptotic cells in *Atoh7^{Ascl1KI/Ascl1KI}* eyes (arrows point to act. Caspase 3 expression in red). M) There is an increase in act. Caspase3+ cells between E12.5 and E16.5 in *Atoh7^{Ascl1KI/Ascl1KI}* homozygous retinas. **p < 0.01; ***p < 0.001; n= 3 animals/genotype; Mag bars in A,J = 20 microns.

Figure 6. Model for consequences of ectopic *Ascl1* in the *Atoh7* retinal cell lineage

In the prenatal retina, RPCs express *Atoh7* during their final mitotic cell cycle and as newly postmitotic cells. *Atoh7*-expressing cells that give rise to RGCs, rapidly activate both *Cdkn1b* and *Pou4f2* expression. Ectopic *Ascl1* induces a pulse of inappropriate cell division. In *Atoh7*^{Ascl1KI/+} cells, the normal complement of RGCs is ultimately produced. By contrast, *Atoh7*^{Ascl1KI/Ascl1KI} cells neither proliferate uncontrollably, nor differentiate, particularly as RGCs, in the absence of *Atoh7* expression. These mutant cells extend aberrant neuronal processes, but ultimately undergo apoptosis.