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Acheate-scute like 1 (Ascl1) is required for normal Delta-like (Dll) gene expression and Notch signaling during retinal development

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Summary

Delta gene expression in *Drosophila* is regulated by proneural bHLH transcription factors, such as *acheate-scute*. In vertebrates, multiple Delta-like and proneural bHLH genes are expressed during neurogenesis, especially in the retina. We recently uncovered a relationship between *Acheate-scute like 1* (*Ascl1*), Delta-like genes, and Notch in chick retinal progenitors. Here, we report that mammalian retinal progenitors are also the primary source of Delta-like genes, likely signaling through Notch among themselves, while differentiating neurons expressed *Jagged2*. Ascl1 is co-expressed in Delta-like and Notch active progenitors, and required for normal Delta-like gene expression and Notch signaling. We also reveal a role for *Ascl1* in the regulation of *Hes6*, a pro-neurogenic factor that inhibits Notch signaling to promote neural rather than glial differentiation. Thus, these results suggest a molecular mechanism whereby attenuated Notch levels coupled with reduced proneurogenic activity in progenitors leads to increased gliogenesis and decreased neurogenesis in the *Ascl1* deficient retina.

Keywords

Notch activity; Delta-like; *Dll1*; *Dll4*; *Dll3*; *Ascl1*; *Neurog2*; *Hes1*; *Hes5*; *Hes6*; retinal development; mutual and lateral inhibition; molecular circuit

Introduction

Studies of neurogenesis in *Drosophila* have revealed that neuroblasts in the proneural cluster express *Delta*, a Notch ligand induced by a proneural bHLH transcription factor such as *acheate-scute*, which sends a lateral inhibitory signal to neighboring cells through the Notch receptor to prevent them from acquiring this fate (reviewed by Skeath and Carroll, 1994; Bertrand et al., 2002). The activation of Notch in neighboring cells induces a signal transduction cascade that upregulates expression of *hairy and enhancer of split* genes, which ultimately lead to the repression of proneural function. Since many components of the Notch signal transduction pathway are expressed in the vertebrate nervous system, and manipulations that activate or inhibit Notch signaling maintain progenitors in an undifferentiated state or promote their differentiation into neurons, respectively, the paradigm of Notch-Delta mediated lateral inhibition in *Drosophila* has been extended to vertebrate neurogenesis (reviewed by Lewis, 1996; Lowell, 2000; Louvi and Artavanis-Tsakonas, 2006).

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The vertebrate retina has served as an excellent model system for studies of neurogenesis, and Notch signaling in particular. Notch signaling maintains the progenitor pool during the course of retinal development, and regulates the evolutionary conserved sequence of progenitor cell differentiation into the six types of neurons and one type of glia (Dorsky et al., 1995; Austin et al., 1995; Tomita et al., 1996a; Henrique et al., 1997; Dorsky et al., 1997; Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001; Silva et al., 2003; Takatsuka et al., 2004; Nelson et al., 2006; Jadhev et al., 2006; Yaron et al., 2006; Nelson et al., 2007a). While most of these studies have demonstrated key functions of Notch and the downstream signaling components in progenitor cells, relatively little attention has been given to upstream components, such as the source of Delta ligands and their respective regulation. Over ten years ago, it was discovered that *Delta-like 1* (*Dll1*) plays a key role in regulating Notch activity and maintaining a pool of progenitors during the period of retinogenesis (Dorsky et al., 1997; Henrique et al., 1997). Thus, a relatively simple lateral inhibitory model was proposed for vertebrates based on *Drosophila*, whereby differentiating neurons in the retina express *Dll1* to laterally inhibit and maintain neighboring progenitors through activation of Notch signaling (Henrique et al., 1997).

Multiple Notch ligands are present during vertebrate retinal development, complicating this simple lateral inhibitory model (Lindsell et al., 1996; Bao and Cepko, 1997; Valsecchi et al., 1997; Henrique et al., 1997; Wang et al., 1998; Benedito and Duarte, 2005; Nimmagadda et al., 2007; Nelson et al., 2007a; Nelson and Reh, 2008). Vertebrates have two orthologous families of canonical Notch ligands, Delta-like and Jagged, but have different numbers of paralogs within each family depending on the species. For example, avians (chicken) have two Delta-like genes, *Dll1* and *Dll4*, while mammals have three, *Dll1*, *Dll3*, and *Dll4*. Both chickens and mammals have two Jagged genes, *Jagged1* and *Jagged2* (*Serrate1* and *Serrate2* in chicken).

To understand how the functions of multiple Delta-like genes are coordinated during retinal development, we recently investigated how their expression patterns related to the pattern of neural differentiation in the chick. According to a previous model, Delta-like genes should be expressed in the differentiating neurons (Henrique et al., 1997). Surprisingly, we found that *Dll1* was expressed in progenitor cells, and while *Dll4* was also expressed in some progenitors, the cohort of differentiating newborn neurons primarily expressed *Dll4* (Nelson and Reh, 2008). These results suggested that progenitor cells are themselves a primary source of Notch ligands and may mutually inhibit their own differentiation through activation of Notch receptors expressed in neighboring progenitors. Thus, together with lateral inhibition from newborn neurons, the mutual inhibition between progenitors may also be critical to maintain the progenitor pool and coordinate retinal histogenesis (Nelson and Reh, 2008).

From the analysis of developing chick retina, we also described a relationship between *Dll1*, *Dll4*, *Acheate-scute like 1* (*Ascl1*) and *Neurogenin2* (*Neurog2*), two vertebrate homologs of *Drosophila* proneural bHLH genes *acheate-scute* and *atonal*, respectively. *Dll1*, *Dll4*, *Ascl1*, and *Neurog2* are all expressed in progenitors and exhibit similar expression kinetics during neuronal differentiation (Nelson and Reh, 2008). Moreover, we reported that over-expression of Ascl1 led to an increase in both *Dll1* and *Dll4* gene expression and Notch signaling activity (Nelson and Reh, 2008). These data suggested that *Ascl1* and/or *Neurog2* might normally regulate expression of *Dll1* and *Dll4* in the retina.

Here, we test the hypothesis that *Ascl1* and/or *Neurog2* are required for Delta-like gene expression and Notch signaling activity during mouse retinal development. We confirm and extend into mouse, our previous observations in the chick, that Delta-like genes are expressed in retinal progenitors, while newborn neurons express *Jagged2*. We report that

Delta-like gene expression is significantly reduced in *Ascl1* deficient mouse retinas, and that Ascl1 and Delta-like are co-expressed in progenitors. We also uncovered an additional role for *Ascl1* in the regulation of *Hes6*, a pro-neurogenic factor that functions to promote neural differentiation by inhibiting Notch signaling and glial differentiation. These results establish that a conserved Ascl1/Delta-like/Notch/Hes molecular circuitry operates within the progenitor pool itself to coordinate retinal histogenesis. They also provide a molecular mechanism whereby attenuated Notch levels coupled with reduced proneurogenic activity may lead to increased gliogenesis and decreased neurogenesis in the *Ascl1* knockout retina (Tomita et al., 1996b).

Methods

Animals

All mice were housed in the Department of Comparative Medicine and procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Washington, Seattle, WA. Wildtype C57BL and/or Swiss Webster mice were used for *in situ* hybridization, immunolabeling, and transfection experiments. *Ascl1* knockout animals (B6.129-*Ascl1*^{tm1And}/J, available from Jackson Labs; Guillemot et al., 1993) and *Neurog2* knockout animals on a Swiss Webster background (gift from D. Anderson; Fode et al., 1998) were used for QPCR analysis (and *Ascl1* was also used for *in situ* hybridization). Delta-like 3 *pudgy* (*Dll3*^{pu}) mutant mice were obtained from Jackson Labs (stock no. 00306; Kusumi et al., 1998). Animals were genotyped according to their respective protocols (Guillemot et al., 1995; Fode et al., 1998; Kusumi et al., 1998; Hartman et al., 2007). Embryonic age was staged according the morning of vaginal plug date (E0.5) and Theiler Staging criteria. Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU, Sigma, 10mg/ml, .15ml/animal) was injected intraperitoneally 2h prior to sacrifice into some animals.

In situ hybridization and immunolabeling

In situ hybridization and post-immunolabeling was performed as previously described (Nelson et al., 2007b). IMAGE clones (Open Biosystems) corresponding to Delta-like 1 (*Dll1* (BC057400, IMAGE:6402691), Delta-like 3 (*Dll3*, BC052002, IMAGE:6404029), and *Jagged2* (*Jagged2*, BC009082, IMAGE:3598850) were used to generate DIG-labeled riboprobes as previously described (Hartman et al., 2007). We also obtained the following IMAGE clones corresponding to Delta-like 4 (*Dll4*, BC042497, IMAGE:4017786), Hairy and enhancer of split 5 (*Hes5*, BC103539, IMAGE:40039948), Hairy and enhancer of split 6 (*Hes6*, BC012897, IMAGE:4011223), and *Notch1* (BC010325, IMAGE: 2651506), all of which were confirmed by DNA sequencing. Antibodies used for immunolabeling after *in situ* hybridization were rat anti-BrdU (1:200 dilution, Accurate Chemical, including 100 Kunitz units/ml DNAse1, Sigma), neuronal specific mouse anti-acetylated beta-III tubulin (Tuj1, 1:750 dilution, Covance), mouse anti-proliferating cellular nuclear antigen (PCNA, 1:100 dilution, DAKO), rabbit anti-phosphorylated Histone H3 (PH3, 1:750, Chemicon), and secondary species specific ALEXA 488 or 568 antibodies (Invitrogen). Colorimetric immuno-detection of Hes1 antigen with rabbit anti-Hes*1* (H140; 1:250 dilution, Santa Cruz, Inc.) was performed as described (Hartman et al., 2007). Antibodies used for immunolabeling of cryosections and whole-mount retinas include goat anti-Jagged1 (1:300, Santa Cruz Biotechnology, Jag1 C-20 Cat. No. SC-6011); goat anti-Sox2 (1:300, Santa Cruz, Inc.), rabbit anti-Sox9 (1:300, Chemicon), rabbit anti-GFP (1:1000, U of Alberta, CA), chick anti-GFP (1:300, Abcam), rat anti-beta-galactosidase (LacZ gene product, 1:500 dilution, Saul et al., 2008), mouse anti-Ascl1 (1:100, Chemicon), and were detected with species-specific ALEXA 488/568/594 conjugated secondary antibodies (1:500, Invitrogen). Images were acquired with a Zeiss Axioplan2 epifluorescent microscope equipped with

Normarski/DIC optics and a Spot camera, a Zeiss LSM 5 Pascal laser scanning confocal microscope, or Olympus FV1000 multiphoton microscope (MPM). Images were assembled in Adobe Photoshop and Illustrator.

Reporter constructs

We used a mouse *Notch1* 14.3Kb promoter construct driving GFP to identify Notch1 expressing progenitor cells (Notch1GFP; Lewis et al., 1998), a mouse *Hes5* 0.76Kb promoter driving destabilized GFP to identify Hes5 and active Notch signaling in progenitor cells (Hes5d2GFP, Takebayashi et al., 1995; Ohtsuka et al., 2006; Nelson et al., 2006), and a mouse *Dll1* 4.3Kb promoter construct driving LacZ to identify Dll1 expressing progenitor cells (Dll1LacZ; Beckers et al., 2000; Castro et al., 2006). We also created a *Dll3* reporter construct, since *Ascl1* loss of function especially affected *Dll3* expression. To make this construct, we analyzed multiple alignments of vertebrate genomes to identify evolutionarily conserved regions (ECR, ECR Browser, http://ecrbrowser.dcode.org, Ovcharenko et al., 2004), and found one ~400bp proximal ECR with 68.6% identity between mouse and human. Within this ECR lies the *Ascl1*-specific E-box/octamer motif identified by Castro and colleagues, similar to the *Ascl1*-specific enhancer in the *Dll1* locus (Castro et al., 2006). We used PCR (LA Taq, TaKaRa) to amplify ~1.0Kb of sequence upstream of the mouse *Dll3* locus just proximal to the *Dll3* start codon containing this ECR. BglII and EcoR1 restriction sites were included in the forward and reverse primer, respectively: BglII forward primer CGCGCGAGATCTTGGGATTACAGGTCTGCCAT, EcoR1 reverse primer CCCGGGGAATTCCAGGATGGGGAAATAGTCTCA. PCR product was restriction enzyme digested, and cloned into the BglII/EcoR1 sites in a destabilized CFP expression plasmid (pd2CFP, Invitrogen) to create Dll3d2CFP, which was verified by DNA sequencing.

Retinal transfection and explant culture

Transfection and explant culture of retinal explants was performed as previously described (Nelson et al., 2006; Nelson et al., 2007b). Briefly, mouse retinas from different embryonic to postnatal ages (E13.5, E17.5-P0) were collected, extra-ocular tissue and retinal pigmented epithelium were removed and transfered to a Milli-cell culture insert (Millipore) in a custom-built electroporation chamber. Explants were electroporated (5 pulses, 35V, 50ms pulse length, BTX ECM830 electroporator) with DNA solutions (~1μl of 2-5μg/μl per explant), and cultured overnight at 37°C 5%CO_2 with nutation as described (Nelson et al., 2007b).

QPCR

Quantitave polymerase chain reaction (QPCR) was performed as previously described (Nelson et al., 2006; Nelson et al., 2007a; Nelson et al., 2007b). All primer sequences for QPCR were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank, Wang and Seed, 2003), and obtained from Invitrogen. Eye pairs from *Ascl1* and *Neurog2* P0 animals were individually collected in HBSS+. Extra-ocular tissue, retinal pigmented epithelium, and lens were dissected, and *Neurog2* sister retinas were pooled, and lysed in Trizol (Invitrogen). For the *Ascl1* eye pairs, one eye was fixed in modified Carnoy's solution and prepared for *in situ* hybridization as described above, while the sister retina was dissected and lysed in Trizol. Total RNA was extracted, genomic DNA contamination was removed by digesting with RQ1 Rnase-free DNase (Promega), isolated (RNeasy RNA isolation, Qiagen), and converted to cDNA with SuperScriptII reverse transcriptase (RT, Invitrogen), except for RT minus controls. All sample concentrations were normalized to wildtype sibling *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) gene expression levels by QPCR with SYBR green PCR master mix (Applied Biosystems) and a DNA Engine Opticon System (Bio-Rad). For statistical analysis, gene expression levels from *Ascl1*+/+ (n=1) and

Ascl1+/− (n=2) littermates were combined (*Ascl1*+/+,−/−, n=3) and compared to *Ascl1*−/− (n=5) expression levels. Gene expression levels from *Neurog2*+/+ (n=4) were compared to *Neurog2*−/− (n=5) littermates; no statistically significant differences were observed between *Neurog2*+/+ and *Neurog2*+/− (data not shown). Student's T-test was used to determine significant differences between samples means, error bars represent standard deviation of the mean, and differences with $p \leq 0.05$ were considered significant.

Results

All Delta-like and Jagged genes are expressed in the developing mouse eye

As a first step towards determining the nature of cells expressing Notch ligands in the developing mouse eye, we examined *Dll1*, *Dll3*, *Dll4*, Jagged1, and *Jagged2* expression at early-, mid-, and late-stages of embryonic eye development. As a control for probe specificity, we first examined expression patterns for *Dll1*, *Dll3*, and *Dll4* in the cortex, diencephalon, and rostral spinal cord (Supp Fig 1), which reveal probe specific signals. Within the eye, *Dll1*, *Dll3*, and *Dll4* expression also exhibited unique expression patterns. At E12.5, both *Dll1* and *Dll4* were strongly expressed in single cells in the neurogenic region of the presumptive neural retina, compared to weaker *Dll3* expression (Fig 1 A, G, D, respectively). *Dll4* expression could also be detected in the vasculature (Fig 1 G, arrowheads). At E14.5 (Fig 1 B, E, H) and E17.5 (Fig 1 C, F, I), all Delta-like genes were strongly expressed in the neurogenic zone. Interestingly, at E17.5 *Dll1* was also expressed in the peripheral non-neurogenic ciliary epithelium (Fig 1 C arrow). Jagged genes also exhibited unique expression patterns. At both E13.5 (Fig 1 J) and E17.5 (Fig 1 K), *Jagged2* expression was strongest in the developing ganglion cell layer (gcl), but occasional cells were observed in the neural progenitor layer (Fig 1 J, K, arrows). *Jagged2* was also expressed in the vasculature (Fig 1 J arrowheads), similar to *Dll4*. Jagged1 immunolabeling revealed expression in the embryonic lens at E12.5, E13.5, and postnatal day 3 (P3, Fig 1 L-N, respectively, arrows). These results confirm and extend earlier reports by showing that not only are all of the canonical Notch ligands expressed in the developing mouse eye, they have both overlapping and unique patterns that change over time.

Differentiating neurons express *Jagged2*

To determine the identity of *Dll1*, *Dll3*, *Dll4*, and *Jagged2* cells in the neurogenic zone, we combined *in situ* hybridization with immunolabeling for neuronal-specific acetylated beta-III tubulin (Tuj1). When we analyzed the expression of Notch ligands with respect to differentiating neurons at E14.5, we found that strongest *Jagged2* expression was localized to Tuj1+ neurons in the ganglion cell layer (gcl), and could even be detected in migrating Tuj1+ neurons (Fig 2 A, arrow and arrowheads, respectively). However, *Dll1*, *Dll3*, or *Dll4* expression was not observed in Tuj1+ neurons in the ganglion cell layer, or in newborn Tuj1+ neurons in the progenitor zone (Fig 2 B-D, arrowheads and arrow, respectively). These data indicate that differentiating neurons express *Jagged2* but not Delta-like genes.

Progenitor cells express Delta-like genes

At the early stages of retinal development, most cells in the neurogenic region are either Tuj1+ neurons or Tuj1− progenitors, and the above analysis suggests the Delta-like expressing cells are actually progenitors. To confirm Delta-like genes are expressed in retinal progenitor cells, we first used *in situ* hybridization to detect *Dll1*, *Dll3*, or *Dll4* expression coupled with immunolabeling for BrdU incorporation to detect S-phase progenitors. E14.5 and E17.5 embryos received a 2h pulse of BrdU *in utero* prior to sacrifice, and were prepared for *in situ* hybridization (Nelson et al., 2007b). At E14.5, analysis of *Dll1*, *Dll3*, and *Dll4* expressing cells revealed that many had incorporated BrdU (Fig 2 E-G, arrowheads). Similarly, at E17.5, many *Dll1*, *Dll3*, and *Dll4* expressing cells

were found that had also incorporated BrdU (Supp Fig 2). We also noticed that *Dll1*, *Dll3*, and *Dll4* cells were located at the apical surface, suggesting that they may be in M-phase; however, at E17.5, phospho-histone 3 (PH3) immunolabeling revealed few *Dll1* and *Dll4* cells that were PH3+ (Fig 3 A, C, asterisks, respectively). Thus, the majority of progenitors undergoing mitosis do not express Delta-like genes (Fig 3 A-C, arrowheads). Nevertheless, immunolabeling for proliferating cellular nuclear antigen (PCNA), a marker present throughout the progenitor cell cycle, revealed strong correlation between Delta-like gene expression and PCNA labeling at E18.5 (Fig 3 D-F, arrowheads), although we did observe occasional *Dll3*+ cells that were not PCNA+ (Fig 3 E, arrow). Hes1 immunolabeling at E17.5 confirmed that Notch signaling activity is normally lowest in mitotic progenitors at the apical surface, and highest in progenitors located in the neuroblast layer (Fig 3 G, arrowheads; Nelson et al., 2007a). These results indicate that progenitor cells in the retina are themselves the primary source of Delta-like genes, and likely regulate Notch signaling within their own pool; however, they may also receive lateral inhibitory signals via *Jagged2* from differentiating neurons.

Ascl1 regulates Delta-like gene expression and Notch signaling activity

When we investigated the relationship between Delta-like and proneural bHLH genes during chick retinal development, we found that of all of the proneural bHLH genes, both *Ascl1* and *Neurog2* were expressed in progenitors that may also express Delta-like genes; however, only *Ascl1* was found to upregulate Delta-like gene expression and Notch signaling activity in a gain-of-function assay (Nelson and Reh, 2008). To determine whether *Ascl1* or *Neurog2* are required for Delta-like gene expression, we used QPCR to measure the level of expression of Notch pathway components in the retinas from *Ascl1* and *Neurog2* mutant mice. Both *Ascl1* and *Neurog2* null (*Ascl1*−/− or *Neurog2*−/−) animals die at birth (Guillemot et al., 1995; Fode et al., 1998), so we collected animals at P0 and prepared one or both retinas from individual animals for QPCR (*Ascl1* and *Neurog2*, respectively). We chose this age for our molecular analyses because the reported phenotype of the loss of Ascl1 in the retina arises after birth (Tomita et al., 1996b), and we did not want to introduce molecular changes that might arise due long-term explant cultures. QPCR analysis of *Ascl1* and *Neurog2* gene expression levels in retinas from newborn *Ascl1* and *Neurog2* +/+, +/−, and −/− animals, respectively, confirmed the changes predicted from genotyping (data not shown).

To test whether *Ascl1* or *Neurog2* regulated Notch ligands and Notch signaling, we first assayed for changes in expression of the Notch ligands *Dll1*, *Dll3*, *Dll4*, and *Jagged2*. QPCR analysis revealed that *Dll1*, *Dll4*, and particularly *Dll3* gene expression levels were significantly decreased with loss of *Ascl1*, but not with loss of *Neurog2* (Fig 4 A). By contrast, a small, but significant decrease in *Jagged2* expression was detected in *Neurog2* deficient, but not in *Ascl1* deficient retinas (Fig 4 A). Decreased Notch ligand expression would predict a concomitant decrease in Notch signaling activity. To test whether Notch signaling activity was decreased as well, we measured expression levels of Notch target genes *Hes1*, *Hes5*, and *Id3* (Nelson et al., 2007a). *Hes1*, *Id3*, and particularly *Hes5* gene expression levels were significantly decreased with loss of *Ascl1*, but were unchanged in *Neurog2* deficient reitnas (Fig 4 B).

To determine whether *Neurog2* contributed any role to this molecular circuitry, we analyzed *Hes6* gene expression levels, a known target of *Neurog2* in the spinal cord involved in a negative feedback inhibitory loop with *Hes5* to regulate Notch signaling (Fior and Henrique, 2005). Surprisingly, *Hes6* gene expression was strongly decreased with loss of *Ascl1*, rather than *Neurog2* function (Fig 4 B). To determine whether loss of either *Ascl1* or *Neurog2* affected progenitor neural differentiation, we measured levels of *Tis21*, a gene that marks progenitors biased towards neurogenic divisions (Iacopetti et al., 1999; Attardo et al., 2008).

Tis21 gene expression was decreased with loss of either *Ascl1* or *Neurog2* function (Fig 4 C). To determine whether the decreased bias towards neural differentiation resulted in an increased bias towards progenitor/glia differentiation, we measured *Glast* expression levels, a marker of progenitors at this age, and a Muller Glia marker later (Gotz and Huttner, 2005). *Glast* was significantly upregulated in the *Ascl1* deficient mouse retina, but was unchanged in retinas lacking *Neurog2* (Fig 4 C). These data indicate that *Ascl1*, rather than *Neurog2*, plays the primary role in regulating Notch ligands and Notch signaling in retinal progenitors, and biases them towards neural differentiation by regulating the proneurogenic factor *Hes6*.

To confirm the changes in expression of Notch pathway components due to loss of *Ascl1* function, we used *in situ* hybridization to visualize expression levels of *Dll1*, *Dll3*, *Hes5*, and *Hes6* genes in the sister *Ascl1*+/+ and *Ascl1* −/− retinas. Neighboring sections from *Ascl1*+/+ (Fig 5 A-D) and *Ascl1*−/− retinas (Fig 5 A′-D′) were hybridized with *Dll1*, *Dll3*, *Hes5*, and *Hes6* riboprobes, respectively, incubated in equal amounts of substrate, and developed for equivalent periods of time. Comparison of *Dll1* (Fig 5 A, A′), *Dll3* (Fig 5 B, B′), *Hes5* (Fig 5 C, C′), and *Hes6* (Fig 5 D, D′) gene expression levels demonstrates that these genes are downregulated with loss of *Ascl1* function, confirming the gene expression changes quantified in the sister retinas by QPCR analysis. Thus, *Ascl1* is required for normal levels of Delta-like gene expression and Notch signaling activity in the developing mouse retina.

Delta-like genes and Notch signaling components are expressed in Ascl1 retinal progenitors

Since reliable reagents for immunolabeling most Notch pathway components are not available, we used reporter plasmids containing cis-regulatory elements from *Notch1*, *Hes5*, *Dll1*, and *Dll3* to drive expression of fluorescent proteins (GFP/CFP) or beta-galactosidase (LacZ gene product) to further investigate the relationship between Delta-like genes, Notch signaling components, and Ascl1 expression in retinal progenitor cells Previous studies have demonstrated the specificity of these cis-regulatory elements, and we provide further documentation in Supp Fig 3 (Notch1GFP, Lewis et al., 1998), (Hes5d2GFP, Takebayashi et al., 1995; Ohtsuka et al., 2006; Nelson et al., 2006), (Dll1LacZ, Beckers et al., 2000; Castro et al., 2006), (Dll3d2CFP, this report, see methods). We used electroporation to transfect Notch1GFP, Hes5d2GFP, Dll1LacZ and Dll3d2CFP into embryonic retinal explants and cultured them overnight (≤24h) to allow reporter expression (Nelson et al., 2007b). High-resolution laser scanning confocal microscopy (LSCM) revealed that cells expressing Notch1GFP, Hes5d2GFP, Dll1LacZ, or Dll3d2CFP reporters in intact retinas typically have both an apical and basal process, indicative of progenitor cells (Supp Fig 3). We also noticed a surprising degree of morphological complexity in regards to the presence of multiple, short extensions and varicosities often observed along both apical and basal processes (Supp Fig 3). To confirm that Notch1GFP, Hes5d2GFP, Dll1LacZ, and Dll3d2CFP reporters are active in progenitor cells, we used antibodies to Sox2 and Sox9 to immunolabel retinal progenitors (Le Rouëdec et al., 2002; Taranova et al., 2006; Sakami et al., 2008; Moshiri et al., 2008; this report), along with BrdU incorporation. Immunolabeling of E17.5 retinal sections revealed that Sox2 and Sox9 identify the vast majority of the progenitor pool (Fig 6 A-C). Transfection of Notch1GFP, Hes5d2GFP, Dll1LacZ, and Dll3d2CFP reporters into E17.5 retinas revealed that transfected cells were also immunolabeled with the Sox9 antibody (Fig 6 D-G arrowheads) and could incorporate BrdU in a 2h pulse (data not shown). We next confirmed that Ascl1 is expressed in progenitor cells (Jasoni et al., 1994; Jasoni and Reh, 1996; Nelson and Reh, 2008) by labeling retinas with antibodies against Ascl1 and Sox9 (Fig 6 H-J), and then tested whether Dll1 and Dll3 are expressed in the Ascl1 progenitors by transfecting E17.5 retinas with the reporter constructs and immunolabeling with Ascl1 antibodies. We found that cells expressing either Dll1LacZ, and Dll3d2CFP were also

labeled with Ascl1 (Fig 6 M,N); moreover, both Notch1GFP and Hes5d2GFP reporters were also expressed in Ascl1 labeled cells (Fig 6 K,L). These data altogether show that Delta-like genes and Notch signaling components are expressed in Ascl1 expressing retinal progenitors.

Ascl1 and *Neurog2* are upstream proneural bHLH transcription factors

Synchronizing progenitor cell differentiation by timing Notch signaling inactivation revealed that a transient and sequential cascade of proneural bHLH transcription factors underlies the progenitor neural differentiation program (Nelson et al., 2007a; Nelson and Reh, 2008). For example, an immediate transient wave of increased *Ascl1* and *Neurog2* expression is observed in progenitors due to a rapid loss of Notch activity that precedes the later transient increases observed in downstream proneural bHLH genes such as *NeuroD4*, *NeuroD1*, and *Atoh7*, which are normally expressed in differentiating, postmitotic neurons (Nelson et al., 2007a; Nelson and Reh, 2008).

To determine whether the loss of *Ascl1* or *Neurog2* affects other downstream proneural bHLH transcription factors, we quantified differences in expression levels of *Ascl1*, *Neurog2*, *Olig2*, *NeuroD1*, *Atoh7*, *NeuroD4*, *Ptf1a*, and *Bhlhb5* between *Ascl1* or *Neurog2* deficient retinas using QPCR as described above. As expected *Ascl1* and *Neurog2* expression were not detected in *Ascl1* or *Neuorg2* deficient retinas, respectively (Fig 7). *Ascl1* expression levels were unchanged in *Neurog2* deficient retinas (Fig 7). By contrast, *Neurog2* expression was decreased in *Ascl1* deficient retinas (Fig 7). *Olig2* expression levels were decreased in both *Ascl1* and *Neuorg2* deficient retinas (Fig 7). *NeuroD1* expression levels were unchanged (Fig 7). *Atoh7*, *NeuroD4*, and *Ptf1a* expression was downregulated in *Neurog2* deficient retinas, but only *Ptf1a* expression was decreased in *Ascl1* deficient retinas (Fig 7). *Bhlhb5* expression was increased with loss of *Ascl1* function, but was unchanged in *Neurog2* deficient retinas (Fig 7). Upregulation of other *Ascl1* and *Neuorg2* bHLH family paralogs normally not expressed in the retina was not observed (data not shown). Thus, loss of the upstream *Ascl1* or *Neurog2* bHLH transcription factors can affect expression of other downstream bHLH transcription factors (Akagi et al., 2004;Cho et al., 2007), and is consistent with their placement, particularly *Ascl1*, at the top of a molecular hierarchy regulating neural differentiation in the retina.

Discussion

We report here that progenitor cells in the mammalian retina are the primary source of Delta-like expression, and likely regulate Notch signaling among themselves. The progenitors may also receive lateral inhibitory signals via *Jagged2* from differentiating neurons. We also found that loss of *Ascl1* function down regulates Delta-like gene expression and Notch signaling in progenitors. These data together with our previous studies (Nelson et al., 2006; Nelson et al., 2007a; Nelson and Reh, 2008), demonstrate that a conserved Ascl1/Delta-like/Notch/Hes molecular circuitry operates within the progenitor pool itself to coordinate retinal histogenesis. This conserved *Ascl1* function, combined with its proneural role, may be one molecular mechanism that underlies the phenotype observed in the *Ascl1* mutant retina: a loss of late arising neurons with a concomitant increase in gliogenesis (Tomita et al., 1996b).

Mouse retinal progenitor cells express Delta-like genes

Within the neurogenic domain of the developing mouse retina, we found that Delta-like genes were expressed in progenitors throughout the embryonic period of histogenesis. Cells expressing *Dll1*, *Dll3*, or *Dll4* genes were not labeled with Tuj1, which marks differentiating neurons, but rather incorporated BrdU (Fig 2, Supp Fig 2) and were immunoreactive for

PCNA (Fig 3), identifying them as progenitors. Using a different approach, based on plasmids expressing reporter molecules under control of cis-regulatory elements from *Dll1* and *Dll3* (as well as *Notch1* and *Hes5*), we found that Dll1+ and Dll3+ cells were progenitors based on morphology (Supp Fig 3) and immunoreactivity for Sox9 and Ascl1 (Fig 6) and BrdU incorporation (data not shown). By contrast, *Jagged2* expression was restricted to the inner region of the Tuj1+ ganglion cell layer, and in scattered Tuj1+ cells in the outer neuroblast region (Fig 2), which are likely newly generated ganglion or amacrine cells migrating to their final position. Thus, mouse retinal progenitor cells are the source of Delta-like genes and likely mutually inhibit themselves, while newborn neurons express *Jagged2* that may feedback to laterally inhibit neighboring progenitors, and function together to maintain the progenitor pool and regulate retinal histogenesis.

The notion that Delta-like genes are expressed in retinal progenitor cells in both chick and mouse (Nelson and Reh, 2008; this report) shows that some, and perhaps most, of the Notch signaling in the developing retina works through a “mutual inhibition” rather than a “lateral inhibition” mechanism, as is observed in the developing proneural clusters of *Drosophila*. Although this concept is novel to the vertebrate retina, Muskavitch and colleagues proposed a similar model for the *Drosophila* eye imaginal disc over a decade ago. In this system, the majority of *undifferentiated* cells in the morphogenetic furrow initially express *Delta*, suggesting that rather than a lateral inhibitory signal from the initial differentiated photoreceptor (R8), Delta-Notch functions in a mutual inhibitory manner to prevent them from responding to the initial neural inductive cue and maintain their uncommitted state (Parks et al., 1995). The reliance on mutual inhibition of differentiation may thus represent a conserved mechanism for patterning larger neurogenic epithelia in which not all of the progenitor cells can maintain contact with the differentiating neuroblast, as they do in the proneural clusters.

Although our studies in both chick and mouse show that vertebrate retinal progenitor cells express Notch ligands, there are differences between mouse and chick. First, the chick retina does not express Jagged (Serrate) genes (Myat et al., 1996; Hayashi et al., 1996). Second, the mammalian-specific ligand *Dll3* is expressed in the mouse retina (this report), while the chicken genome does not have a *Dll3* homolog (Pintar et al., 2007; Nelson and Reh, 2008). Third, newborn neurons in the chick express *Dll4*, whereas newborn neurons in the mouse express *Jagged2* (Nelson and Reh, 2008; this report), which may be a consequence of the more limited repertoire of available Notch-ligands in the chick retina compared to the mouse retina. Nevertheless, one emerging theme from these studies is that within the neurogenic zone of the developing retina, different cell types express different Notch-ligands specifically during the transition from progenitor to differentiating neuron.

This theme may extend into the progenitor pool, particularly in mammals, since all three Delta-like genes are expressed in progenitors. These results raise an interesting possibility that different Delta-like genes may sort out different subpopulations, or mark different steps in the transition from multipotency to neurogenic dividing progenitors in the retina. In this regard, it is interesting that occasional *Dll3* expressing cells were not labeled with progenitor markers (PCNA); *Dll3* is not able to activate Notch signaling, and may even attenuate Notch signaling levels (Ladi et al., 2005; Geffers et al., 2007). These data suggest that *Dll3* may be involved in the terminal neurogenic step of progenitor differentiation. However, we did not observe any major phenotype in retinas deficient for *Dll3* (data not shown), similar to our observations in the cochlea (Hartman et al., 2007). *Dll3* may mark progenitors that will undergo a neurogenic division like the neurogenic progenitor marker *Tis21* (Iacopetti et al., 1999; Attardo et al., 2008), although live-cell imaging would be necessary to test this hypothesis. Nevertheless, our demonstration that retinal progenitors from both chick and mouse are a source of Notch-ligands (this report; Nelson and Reh, 2008), together with

recent studies showing that mouse cortical progenitors themselves are also a source of Notch-ligands (Shimojo et al., 2008; Yoon et al., 2008; Kawaguchi et al., 2008a; Kawaguchi et al., 2008b), change the view of Notch signaling in the vertebrate nervous system.

Conserved Ascl1/Delta-like/Notch/Hes molecular circuitry in retinal progenitors

Another aspect of Notch signaling in the retina that has received little attention is the factor(s) that regulate Delta-like gene expression. Studies of neurogenesis in *Drosophila* demonstrate that *Delta* is regulated by proneural bHLH transcription factors such as *acheate-scute* or *atonal* (reviewed by Skeath and Carroll, 1994; Bertrand et al., 2002). Many bHLH homologs are expressed in the vertebrate retina, but only *Ascl1* and *Neurog2* are expressed in mitotically active progenitors (Jasoni et al., 1994; Jasoni and Reh, 1996; Perron et al., 1998; Yan et al., 2001; Marquardt, et al., 2001; Ma and Wang, 2006; Le et al., 2006; Nelson and Reh, 2008). While the functions of proneural bHLH genes have been extensively investigated with respect to their role in retinal cell fate specification (reviewed by Cepko, 1999; Vetter and Brown, 2001; Hatakeyama and Kageyama, 2004; Yan et al., 2005; Ohsawa and Kageyama, 2007; Harada et al., 2007), their function in the regulation Notch ligands have not been described in the developing retina. Recently, we found in the developing chick retina that the expression pattern and expression kinetics of *Dll1* during synchronized progenitor differentiation most closely matched that of *Ascl1*, similar to observations in the frog retina (Perron et al., 1998). Although these patterns suggested a specific role for *Ascl1* in *Dll1* regulation, over-expression of Ascl1, but not Neurog2, upregulated both *Dll1* and *Dll4*, as well as *Hes5* gene expression levels, indicating that Ascl1 may play a more general role in regulating Delta-like genes and Notch signaling activity in the retina (Nelson and Reh, 2008).

In this report, we analyzed retinas from *Ascl1* or *Neurog2* mutant mice for changes in Delta-like gene expression and Notch signaling. *Dll1*, *Dll4*, and particularly *Dll3* gene expression levels were significantly downregulated in *Ascl1*$-/-$ mice, but not in *Neurog2*$-/-$ mice. These results demonstrate that Ascl1 does have a conserved regulatory input into Delta-like gene expression and Notch signaling activity, although there must be additional positive regulators of at least *Dll1* and *Dll4* expression in the mouse retina. Nevertheless, decreased expression of Delta-like genes predicts decreased levels of Notch signaling activity. In support of this notion, the classic Notch target genes *Hes1*, *Id3*, and particularly *Hes5* were all downregulated in *Ascl1*$-/-$ mice, indicative of decreased Notch signaling activity. These results are complementary to our over-expression experiment in the chick retina (Nelson and Reh, 2008). These results are also consistent with the well-documented role and pattern of *Ascl1* regulation of Delta-like gene expression and Notch signaling in other regions of the mammalian nervous system (Ma et al., 1997; Casarosa et al., 1999; Beckers et al., 2000; Cau et al., 2002; Yun et al., 2002; Mizuguchi et al., 2006; Wildner et al., 2006; Castro et al., 2006). *Neurog2* also has well-documented roles in regulating Delta-like gene expression (Ma et al., 1996; Fode et al., 1998; Beckers et al., 2000; Bertrand et al,. 2002; Castro et al., 2006); however, it is not required for Delta-like gene expression in the retina. Interestingly, regions of high Jagged expression are correlated with Neurogenin expression in the CNS (Ma et al., 1997), and we did observe decreased *Jagged2* expression in the *Neurog2*, but not *Ascl1*, mutant retina.

Levels of Notch signaling regulate maintenance of the progenitor pool

Our data show that the loss of *Ascl1* leads to a decrease in Notch signaling. Previous analyses of loss of Notch signaling during retinal development have consistently shown increased neural differentiation and inhibition of progenitors/gliogenesis (Dorsky et al., 1995; Austin et al., 1995; Tomita et al., 1996a; Henrique et al., 1997; Dorsky et al., 1997; Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001; Silva et al., 2003; Takatsuka et

al., 2004; Nelson et al., 2006; Jadhev et al., 2006; Yaron et al., 2006; Nelson et al., 2007a). Notch inactivation in early embryonic progenitors increases early retinal neurons (Nelson et al., 2007a), whereas Notch inactivation in later postnatal progenitors increases differentiation of late retinal cell types and decreases glial differentiation (Fig 8 A normal, Fig 8 B Notch inactivation in LP; Nelson et al., 2007a). By contrast, loss of *Ascl1* results in *decreased* neural differentiation of retinal cell types, such as rods and bipolar cells in particular, with a concomitant *increase* in gliogenesis (Fig 8 C; Tomita et al., 1996b; Tomita et al., 2000 Hatakeyama et al., 2001). How might these opposing Notch and Ascl1 phenotypes be resolved? One answer may lie in the fact that *Hes6* gene expression is strongly downregulated with loss of *Ascl1* function. *Hes6* is regulated by upstream proneural bHLH genes, and is sufficient to promote neural and prevent glia differentiation (Bae et al et al., 2000; Koyano-Nakagawa et al., 2000; Gratton et al., 2003; Fior and Henrique, 2005; Jhas et al., 2006). Thus, decreased expression of pro-neurogenic *Hes6* coupled with attenuated Notch signaling levels in the progenitor pool provides one molecular mechanism that may explain the observed increase in gliogenesis in the *Ascl1* deficient retina (Fig 8 C). In support of this view, although the cellular phenotype of decreased neurogenesis and increased gliogenesis arises somewhat later in the postnatal *Ascl1* mutant retina, we can already detect molecular changes indicative of this switch at birth, with reduced *Tis21* and increased *Glast* expression levels in *Ascl1* mutant retina.

Taken together with previous studies, our data show that an Ascl1/Delta-like/Notch/Hes molecular circuitry regulates neurogenesis. In vertebrates, this pathway regulates retinal histogenesis by coordinating progenitor differentiation, and in higher order vertebrates, this circuitry is used to maintain the progenitor pool during their protracted period of retinogenesis. The fact that this molecular circuit operates within progenitor cells themselves shines new light into how vertebrate visual systems normally develop, and provides a simple framework for understanding neurogenesis in the retina. Additionally, recent findings indicate that this normally quiescent developmental circuit is reactivated following damage in adult retinas, regulating the capacity for retinal regeneration in fish and chick (Fischer and Reh, 2001; Hayes et al., 2007; Fausett et al., 2008). Since retinal regeneration is more limited in mammals (see Lamba et al., 2008 for review), re-establishment of this Ascl1/Delta-like/Notch/Hes molecular circuit may serve as a focal point for developing strategies to stimulate endogenous mechanisms or guide cell-based approaches toward mammalian retinal repair therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Akagi T, Inoue T, Miyoshi G, Bessho Y, Takahashi M, Lee JE, Guillemot F, Kageyama R. Requirement of multiple basic helix-loop-helix genes for retinal neuronal subtype specification. J Biol Chem. 2004; 279(27):28492–8. [PubMed: 15105417]

Attardo A, Calegari F, Haubensak W, Wilsch-Bräuninger M, Huttner WB. Live imaging at the onset of cortical neurogenesis reveals differential appearance of the neuronal phenotype in apical versus basal progenitor progeny. PLoS ONE. 2008; 3(6):e2388. [PubMed: 18545663]

Austin CP, Feldman DE, Ida JA Jr, Cepko CL. Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. Development. 1995; 121(11):3637–50. [PubMed: 8582277]

Bae S, Bessho Y, Hojo M, Kageyama R. The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. Development. 2000; 127(13):2933–43. [PubMed: 10851137]

Bao ZZ, Cepko CL. The expression and function of Notch pathway genes in the developing rat eye. J Neurosci. 1997; 17(4):1425–34. [PubMed: 9006984]

Beckers J, Caron A, Hrabé de Angelis M, Hans S, Campos-Ortega JA, Gossler A. Distinct regulatory elements direct delta1 expression in the nervous system and paraxial mesoderm of transgenic mice. Mech Dev. 2000; 95(1-2):23–34. [PubMed: 10906447]

Benedito R, Duarte A. Expression of Dll4 during mouse embryogenesis suggests multiple developmental roles. Gene Expr Patterns. 2005; 5(6):750–5. [PubMed: 15923152]

Bertrand N, Castro DS, Guillemot F. Proneural genes and the specification of neural cell types. Nat Rev Neurosci. 2002; 3(7):517–30. [PubMed: 12094208]

Casarosa S, Fode C, Guillemot F. Mash1 regulates neurogenesis in the ventral telencephalon. Development. 1999; 126(3):525–34. [PubMed: 9876181]

Castro DS, Skowronska-Krawczyk D, Armant O, Donaldson IJ, Parras C, Hunt C, Critchley JA, Nguyen L, Gossler A, Gottgens B, Matter JM, Guillemot F. Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev Cell. 2006; 11(6):831–44. [PubMed: 17141158]

Cau E, Casarosa S, Guillemot F. Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development. 2002; 129(8):1871–80. [PubMed: 11934853]

Cepko CL. The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. Curr Opin Neurobiol. 1999; 9(1):37–46. [PubMed: 10072376]

Cho JH, Klein WH, Tsai MJ. Compensational regulation of bHLH transcription factors in the postnatal development of BETA2/NeuroD1-null retina. Mech Dev. 2007; 124(7-8):543–50. [PubMed: 17629466]

Dorsky RI, Rapaport DH, Harris WA. Xotch inhibits cell differentiation in the Xenopus retina. Neuron. 1995; 14(3):487–96. [PubMed: 7695895]

Dorsky RI, Chang WS, Rapaport DH, Harris WA. Regulation of neuronal diversity in the Xenopus retina by Delta signalling. Nature. 1997; 385(6611):67–70. [PubMed: 8985247]

Fausett BV, Gumerson JD, Goldman D. The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. J Neurosci. 2008; 28(5):1109–17. [PubMed: 18234889]

Fior R, Henrique D. A novel hes5/hes6 circuitry of negative regulation controls Notch activity during neurogenesis. Dev Biol. 2005; 281(2):318–33. [PubMed: 15893982]

Fischer AJ, Reh TA. Müller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat Neurosci. 2001; 4(3):247–52. [PubMed: 11224540]

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(3):483–94. [PubMed: 9539123]

Furukawa T, Mukherjee S, Bao ZZ, Morrow EM, Cepko CL. rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. Neuron. 2000; 26(2):383–94. [PubMed: 10839357]

Geffers I, Serth K, Chapman G, Jaekel R, Schuster-Gossler K, Cordes R, Sparrow DB, Kremmer E, Dunwoodie SL, Klein T, Gossler A. Divergent functions and distinct localization of the Notch ligands DLL1 and DLL3 in vivo. J Cell Biol. 2007; 178(3):465–76. [PubMed: 17664336]

Götz M, Huttner WB. The cell biology of neurogenesis. Nat Rev Mol Cell Biol. 2005; 6(10):777–88. [PubMed: 16314867]

Gratton MO, Torban E, Jasmin SB, Theriault FM, German MS, Stifani S. Hes6 promotes cortical neurogenesis and inhibits Hes1 transcription repression activity by multiple mechanisms. Mol Cell Biol. 2003; 23(19):6922–35. [PubMed: 12972610]

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell. 1993; 75(3):463–76. [PubMed: 8221886]

Harada T, Harada C, Parada LF. Molecular regulation of visual system development: more than meets the eye. Genes Dev. 2007; 21(4):367–78. [PubMed: 17322396]

Hartman BH, Hayashi T, Nelson BR, Bermingham-McDonogh O, Reh TA. Dll3 is expressed in developing hair cells in the mammalian cochlea. Dev Dyn. 2007; 236(10):2875–83. [PubMed: 17823936]

Hatakeyama J, Kageyama R. Retinal cell fate determination and bHLH factors. Semin Cell Dev Biol. 2004; 15(1):83–9. [PubMed: 15036211]

Hatakeyama J, Bessho Y, Katoh K, Ookawara S, Fujioka M, Guillemot F, Kageyama R. Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development. 2004; 131(22):5539–50. [PubMed: 15496443]

Hayashi H, Mochii M, Kodama R, Hamada Y, Mizuno N, Eguchi G, Tachi C. Isolation of a novel chick homolog of Serrate and its coexpression with C-Notch-1 in chick development. Int J Dev Biol. 1996; 40(6):1089–96. [PubMed: 9032014]

Hayes S, Nelson BR, Buckingham B, Reh TA. Notch signaling regulates regeneration in the avian retina. Dev Biol. 2007; 312(1):300–11. [PubMed: 18028900]

Henrique D, Hirsinger E, Adam J, Le Roux I, Pourquie O, Ish-Horowicz D, Lewis J. Maintenance of neuroepithelial progenitor cells by Delta-Notch signaling in the embryonic chick retina. Curr Biol. 1997; 7(9):661–70. [PubMed: 9285721]

Hojo M, Ohtsuka T, Hashimoto N, Gradwohl G, Guillemot F, Kageyama R. Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina. Development. 2000; 127(12): 2515–22. [PubMed: 10821751]

Iacopetti P, Michelini M, Stuckmann I, Oback B, Aaku-Saraste E, Huttner WB. Expression of the antiproliferative gene TIS21 at the onset of neurogenesis identifies single neuroepithelial cells that switch from proliferative to neuron-generating division. PNAS. 1999; 96(8):4639–44. [PubMed: 10200315]

Jadhav AP, Mason HA, Cepko CL. Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development. 2006; 133(5):913–23. [PubMed: 16452096]

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(2):319–27. [PubMed: 8727003]

Jasoni CL, Walker MB, Morris MD, Reh TA. A chicken achaete-scute homolog (CASH-1) is expressed in a temporally and spatially discrete manner in the developing nervous system. Development. 1994; 120(4):769–83. [PubMed: 7600956]

Jhas S, Ciura S, Belanger-Jasmin S, Dong Z, Llamosas E, Theriault FM, Joachim K, Tang Y, Liu L, Liu J, Stifani S. Hes6 inhibits astrocyte differentiation and promotes neurogenesis through different mechanisms. J Neurosci. 2006; 26(43):11061–71. [PubMed: 17065448]

Kawaguchi A, Ikawa T, Kasukawa T, Ueda HR, Kurimoto K, Saitou M, Matsuzaki F. Single-cell gene profiling defines differential progenitor subclasses in mammalian neurogenesis. Development. 2008a; 135(18):3113–24. [PubMed: 18725516]

Kawaguchi D, Yoshimatsu T, Hozumi K, Gotoh Y. Selection of differentiating cells by different levels of delta-like 1 among neural precursor cells in the developing mouse telencephalon. Development. 2008b; 135(23):3849–58. [PubMed: 18997111]

Koyano-Nakagawa N, Kim J, Anderson D, Kintner C. Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. Development. 2000; 127(19):4203–16. [PubMed: 10976052]

Kusumi K, Sun ES, Kerrebrock AW, Bronson RT, Chi DC, Bulotsky MS, Spencer JB, Birren BW, Frankel WN, Lander ES. The mouse pudgy mutation disrupts Delta homologue Dll3 and initiation of early somite boundaries. Nat Genet. 1998; 19(3):274–8. [PubMed: 9662403]

Ladi E, Nichols JT, Ge W, Miyamoto A, Yao C, Yang LT, Boulter J, Sun YE, Kintner C, Weinmaster G. The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously

attenuates signaling induced by other DSL ligands. J Cell Biol. 2005; 170(6):983–92. [PubMed: 16144902]

Lamba D, Karl M, Reh T. Neural regeneration and cell replacement: a view from the eye. Cell Stem Cell. 2008; 2(6):538–49. [PubMed: 18522847]

Le TT, Wroblewski E, Patel S, Riesenberg AN, Brown NL. Math5 is required for both early retinal neuron differentiation and cell cycle progression. Dev Biol. 2006; 295(2):764–78. [PubMed: 16690048]

Le Rouëdec D, Rayner K, Rex M, Wigmore PM, Scotting PJ. The transcription factor cSox2 and Neuropeptide Y define a novel subgroup of amacrine cells in the retina. J Anat. 2002; 200(Pt 1): 51–6. [PubMed: 11833654]

Lewis AK, Frantz GD, Carpenter DA, de Sauvage FJ, Gao WQ. Distinct expression patterns of notch family receptors and ligands during development of the mammalian inner ear. Mech Dev. 1998; 78(1-2):159–63. [PubMed: 9858718]

Lewis J. Neurogenic genes and vertebrate neurogenesis. Curr Opin Neurobiol. 1996; 6(1):3–10. [PubMed: 8794055]

Lindsell CE, Boulter J, diSibio G, Gossler A, Weinmaster G. Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. Mol Cell Neurosci. 1996; 8(1):14–27. [PubMed: 8923452]

Louvi A, Artavanis-Tsakonas S. Notch signaling in vertebrate neural development. Nat Rev Neurosci. 2006; 7(2):93–102. [PubMed: 16429119]

Lowell S. Stem cells: You make me feel so glial. Curr Biol. 2000; 10(16):R595–7. [PubMed: 10985376]

Ma W, Wang SZ. The final fates of neurogenin2-expressing cells include all major neuron types in the mouse retina. Mol Cell Neurosci. 2006; 31(3):463–9. [PubMed: 16364654]

Ma Q, Kintner C, Anderson DJ. Identification of neurogenin, a vertebrate neuronal determination gene. Cell. 1996; 87(1):43–52. [PubMed: 8858147]

Ma Q, Sommer L, Cserjesi P, Anderson DJ. Mash1 and neurogenin1 expression patterns define complementary domains of neuroepithelium in the developing CNS and are correlated with regions expressing notch ligands. J Neurosci. 1997; 17(10):3644–52. [PubMed: 9133387]

Marquardt T, Ashery-Padan R, Andrejewski N, Scardigli R, Guillemot F, Gruss P. Pax6 is required for the multipotent state of retinal progenitor cells. Cell. 2001; 105(1):43–55. [PubMed: 11301001]

Mizuguchi R, Kriks S, Cordes R, Gossler A, Ma Q, Goulding M. Ascl1 and Gsh1/2 control inhibitory and excitatory cell fate in spinal sensory interneurons. Nat Neurosci. 2006; 9(6):770–8. [PubMed: 16715081]

Moshiri A, Gonzalez E, Tagawa K, Maeda H, Wang M, Frishman LJ, Wang SW. Near complete loss of retinal ganglion cells in the math5/brn3b double knockout elicits severe reductions of other cell types during retinal development. Dev Biol. Apr 15; 2008 316(2):214–27. 2008. [PubMed: 18321480]

Myat A, Henrique D, Ish-Horowicz D, Lewis J. A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. Dev Biol. 1996; 174(2):233–47. [PubMed: 8631496]

Nelson BR, Reh TA. Relationship between Delta-like and proneural bHLH genes during chick retinal development. Dev Dyn. 2008; 237(6):1565–80. [PubMed: 18435466]

Nelson BR, Gumuscu B, Hartman BH, Reh TA. Notch Activity Is Downregulated Just prior to Retinal Ganglion Cell Differentiation. Dev Neurosci. 2006; 28(1-2):128–41. [PubMed: 16508310]

Nelson BR, Hartman BH, Georgi SA, Lan MS, Reh TA. Transient inactivation of Notch signaling synchronizes differentiation of neural progenitor cells. Dev Biol. 2007a; 304(2):479–98. [PubMed: 17280659]

Nelson BR, Hayes S, Hartman BH, Reh TA. Fliesler SJ, Kisselev O. Signal transduction in retinal progenitor cells. Signal transduction in the retina. 2007b CRC Press.

Nimmagadda S, Geetha-Loganathan P, Prols F, Scaal M, Christ B, Huang R. Expression pattern of Dll4 during chick embryogenesis. Histochem Cell Biol. 2007; 128(2):147–52. [PubMed: 17605027]

Ohsawa R, Kageyama R. Regulation of retinal cell fate specification by multiple transcription factors. Brain Res. 2007; 1192:90–8. [PubMed: 17488643]

Ohtsuka T, Imayoshi I, Shimojo H, Nishi E, Kageyama R, McConnell SK. Visualization of embryonic neural stem cells using Hes promoters in transgenic mice. Mol Cell Neurosci. 2006; 31(1):109–22. [PubMed: 16214363]

Ovcharenko I, Nobrega MA, Loots GG, Stubbs L. ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. Nucleic Acids Res. 2004; 1:W280–6. 32 (Web Server issue). [PubMed: 15215395]

Parks AL, Turner R, Muskavitch MAT. Relationships between complex Delta expression and the specification of retinal cell fates during Drosophila eye development. Mech Dev. 1995; 50:201–216. [PubMed: 7619731]

Perron M, Kanekar S, Vetter ML, Harris WA. The genetic sequence of retinal development in the ciliary margin of the Xenopus eye. Dev Biol. 1998; 199(2):185–200. [PubMed: 9698439]

Pintar A, De Biasio A, Popovic M, Ivanova N, Pongor S. The intracellular region of Notch ligands: does the tail make the difference? Biol Direct. 2007; 2:19. [PubMed: 17623096]

Reh TA, Fischer AJ. Retinal stem cells. Methods Enzymol. 2006; 419:52–73. [PubMed: 17141051]

Sakami S, Etter P, Reh TA. Activin signaling limits the competence for retinal regeneration from the pigmented epithelium. Mech Dev. 2008; 125(1-2):106–16. [PubMed: 18042353]

Satow T, Bae SK, Inoue T, Inoue C, Miyoshi G, Tomita K, Bessho Y, Hashimoto N, Kageyama R. The basic helix-loop-helix gene hesr2 promotes gliogenesis in mouse retina. J Neurosci. 2001; 21(4):1265–73. [PubMed: 11160397]

Saul SM, Brzezinski JA 4th, 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. Mol Cell Neurosci. 2008; 37(1):153–69. [PubMed: 17977745]

Shimojo H, Ohtsuka T, Kageyama R. Oscillations in notch signaling regulate maintenance of neural progenitors. Neuron. 2008; 58(1):52–64. [PubMed: 18400163]

Silva AO, Ercole CE, McLoon SC. Regulation of ganglion cell production by Notch signaling during retinal development. J Neurobiol. 2003; 54(3):511–24. [PubMed: 12532401]

Skeath JB, Carroll SB. The achaete-scute complex: generation of cellular pattern and fate within the Drosophila nervous system. FASEB J. 1994; 8(10):714–21. [PubMed: 8050670]

Takatsuka K, Hatakeyama J, Bessho Y, Kageyama R. Roles of the bHLH gene Hes1 in retinal morphogenesis. Brain Res. 2004; 1004(1-2):148–55. [PubMed: 15033430]

Takebayashi K, Akazawa C, Nakanishi S, Kageyama R. Structure and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-5. Identification of the neural precursor cell-specific promoter element. J Biol Chem. 1995; 270(3):1342–9. [PubMed: 7836401]

Taranova OV, Magness ST, Fagan BM, Wu Y, Surzenko N, Hutton SR, Pevny LH. SOX2 is a dose-dependent regulator of retinal neural progenitor competence. Genes Dev. 2006; 20(9):1187–202. [PubMed: 16651659]

Tomita K, Ishibashi M, Nakahara K, Ang SL, Nakanishi S, Guillemot F, Kageyama R. Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron. 1996a; 16(4):723–34. [PubMed: 8607991]

Tomita K, Nakanishi S, Guillemot F, Kageyama R. Mash1 promotes neuronal differentiation in the retina. Genes Cells. 1996b; 1(8):765–74. [PubMed: 9077445]

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(20):5460–72. [PubMed: 11032813]

Valsecchi C, Ghezzi C, Ballabio A, Rugarli EI. JAGGED2: a putative Notch ligand expressed in the apical ectodermal ridge and in sites of epithelial-mesenchymal interactions. Mech Dev. 1997; 69(1-2):203–7. [PubMed: 9486542]

Vetter ML, Brown NL. The role of basic helix-loop-helix genes in vertebrate retinogenesis. Semin Cell Dev Biol. 2001; 12(6):491–8. [PubMed: 11735385]

Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Research. 2003; 31(24):1–8. [PubMed: 12519937]

Wang S, Sdrulla AD, diSibio G, Bush G, Nofziger D, Hicks C, Weinmaster G, Barres BA. Notch receptor activation inhibits oligodendrocyte differentiation. Neuron. 1998; 21(1):63–75. [PubMed: 9697852]

Wildner H, Müller T, Cho SH, Bröhl D, Cepko CL, Guillemot F, Birchmeier C. dILA neurons in the dorsal spinal cord are the product of terminal and non-terminal asymmetric progenitor cell divisions, and require Mash1 for their development. Development. 2006; 133(11):2105–13. [PubMed: 16690754]

Yan RT, Ma WX, Wang SZ. neurogenin2 elicits the genesis of retinal neurons from cultures of nonneural cells. PNAS. 2001; 98(26):15014–9. [PubMed: 11752450]

Yan RT, Ma W, Liang L, Wang SZ. bHLH genes and retinal cell fate specification. Mol Neurobiol. 2005; 32(2):157–71. [PubMed: 16215280]

Yaron O, Farhy C, Marquardt T, Applebury M, Ashery-Padan R. Notch1 functions to Suppress cone-photoreceptor fate specification in the developing mouse retina. Development. 2006; 133(7):1367–78. [PubMed: 16510501]

Yoon KJ, Koo BK, Im SK, Jeong HW, Ghim J, Kwon MC, Moon JS, Miyata T, Kong YY. Mind bomb 1-expressing intermediate progenitors generate notch signaling to maintain radial glial cells. Neuron. 2008; 58(4):519–31. [PubMed: 18498734]

Yun K, Fischman S, Johnson J, Hrabe de Angelis M, Weinmaster G, Rubenstein JL. Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. Development. 2002; 129(21):5029–40. [PubMed: 12397111]

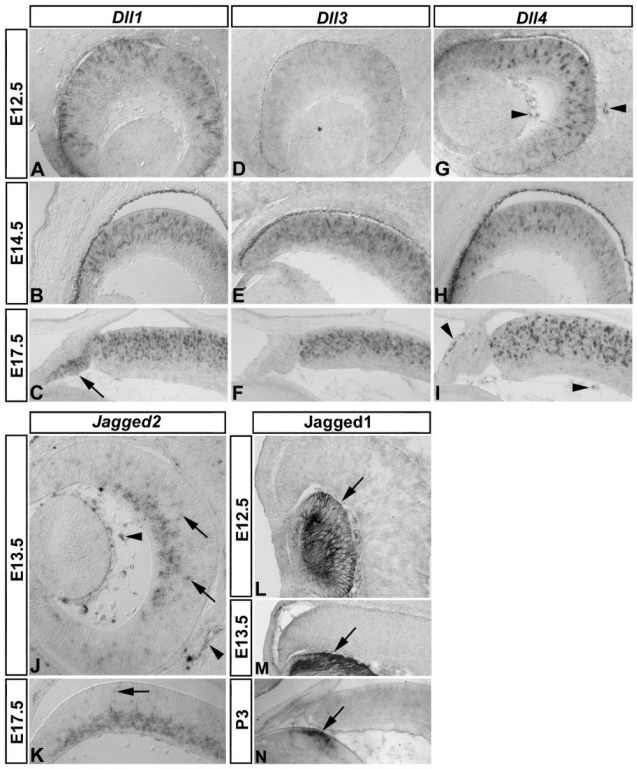

Figure 1. Notch ligand expression defines discrete regions of the developing mouse eye

In situ hybridization was used to detect *Dll1* (**A-C**), *Dll3* (**D-F**), *Dll4* (**G-I**), *Jagged2* (**J, K**), and immunolabeling was used to detect Jagged1 (**L-N**) expression at the indicated early stages of mouse eye development. *Dll1* (**A-C**) and *Dll4* (**G-I**) are strongly expressed in cells restricted to the central neurogenic region of the presumptive retina at embryonic day 12.5 (E12.5), E14.5, and E17.5: note that *Dll1* expression is upregaluted in the peripheral non-neurogenic ciliary epithelium at E17.5 (**C**, arrow), and that *Dll4* is expressed in the vasculature (**G, I**, arrowheads). Weak *Dll3* expression at E12.5 (**D**) becomes upregulated by E14.5 (E), and within the developing retina, all Delta-like genes are restricted to the neuroblast layer (*Dll1* **B, C**; *Dll3* **E, F**; *Dll4* **H, I**). *Jagged2* (**J, K**) expression is restricted to the developing ganglion cell layer of the neurogenic zone, and in isolated cells in the neuroblast layer (arrows), as well as extra-ocular vasculature and cells in the vitreous (arrowheads), similar to *Dll4*. Jagged1 (**L-N**) immunolabeling was restricted to the lens at these ages (arrows).

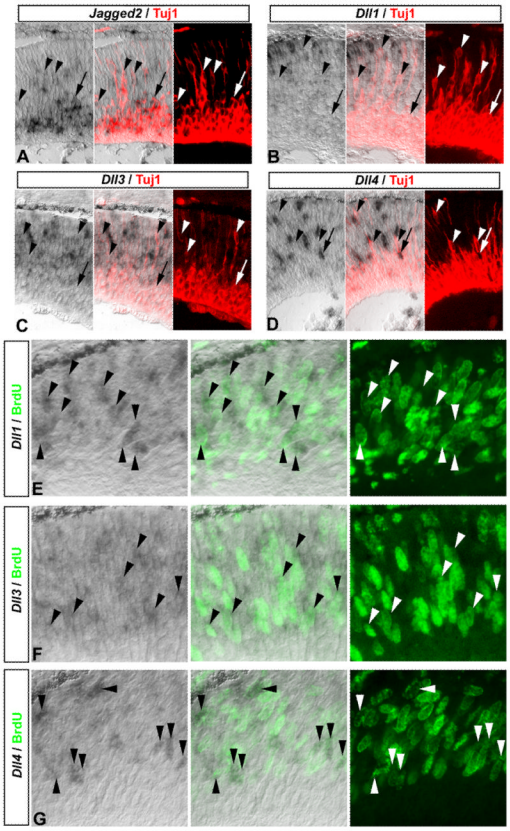

Figure 2. Newborn neurons and proliferating progenitors express different Notch ligands

(**A-D**) *Jagged2* is expressed in postmitotic differentiating neurons. In situ hybridization for *Jagged2* (**A**), *Dll1* (**B**), *Dll3* (**C**), and *Dll4* (**D**) combined with acetylated beta-III tubulin immunolabeling (Tuj1, red), reveals that *Jagged2*, but not Delta-like genes, is expressed in Tuj1+ postmitotic differentiating neurons migrating to (arrowheads), and within the ganglion cell layer (arrow) at E14.5: note that the *Dll4*+ cell near the ganglion cell layer in **D** is not Tuj1+ (arrow). (**E-G**) Delta-like genes are expressed in retinal progenitor cells. E14.5 embryos received a 2h pulse of BrdU *in utero* prior to sacrifice, and in situ hybridization was used to detect *Dll1* (**E**), *Dll3* (**F**), and *Dll4* (**G**) expression, followed by immunolabeling to detect BrdU incorporation (green). Individual and merged channels are shown, which reveal that *Dll1* (**E**), *Dll3* (**F**), and *Dll4* (**G**) expressing cells at E14.5 can incorporate BrdU (arrowheads).

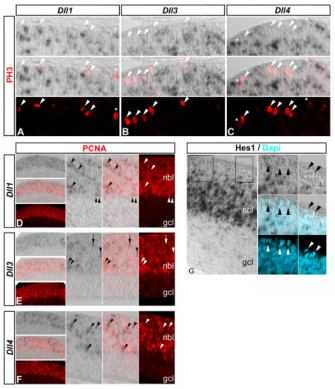

Figure 3. Delta-like gene expression and Notch signaling change during progenitor cell cycles

In situ hybridization was used to detect *Dll1* (**A, D**), *Dll3* (**B, E**), and *Dll4* (**C, F**) gene expression followed by phospho-histone H3 (PH3, E17.5, **A-C**, red) or proliferating cellular nuclear antigen (PCNA, E18.5, **D-F**, red) immunolabeling. (**A-C**) The majority of PH3+ mitotic progenitors at the apical surface do not express Delta-like genes (**A-C**, arrowheads): asterisks denote a few double-positive cells with weak *Dll1*, *Dll4*, and/or PH3 labeling. (**D-F**) Delta-like gene expression is restricted to the neuroblast layer (nbl) defined by PCNA immunolableing in contrast to the ganglion cell layer (gcl), and higher magnification views reveal that Delta-like genes are expressed in PCNA+ progenitors (arrowheads): note that one *Dll3* cell is not labeled with PCNA (E, arrow). (G) Hes1 immunolabeling is also restricted to the region of active Notch signaling in progenitors in the nbl and not in the gcl: note that Hes1 protein is not detected in mitotic progenitors at the apical surface (arrowheads, Dapi counterstain, blue), similar to the pattern observed for Delta-like genes (A-C) and active Notch signaling (Nelson et al., 2006; Nelson et al., 2007a).

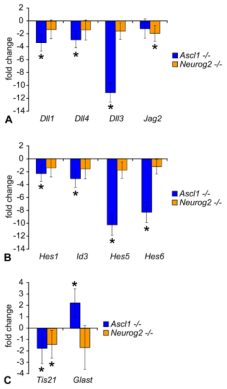

Figure 4. Delta-like gene expression and Notch signaling are downregulated in *Ascl1*, but not *Neurog2*, deficient retina

(**A-C**) QPCR analysis of *Ascl1* and *Neurog2* mutant retina. Individual retinas from postnatal day 0 (P0) *Ascl1* and *Neurog2* mice were prepared for QPCR analysis (see methods), and sample concentrations were normalized to *Gapdh* gene expression levels in sibling controls. Graphs depict fold change in expression levels of the indicated genes between $Ascl1^{-/-}$ (n=5) and littermate control $Ascl1^{+/+,+/-}$ (n=3) retinas (blue), and $Neurog2^{-/-}$ (n=5) and littermate control $Neurog2^{+/+}$ (n=4) retinas (orange, error bars represent standard deviation of the mean, student's T-test was used to determine significant differences between sample means, and changes with $p \leq 0.05$ were considered significant (asterisks). Changes in gene expression levels of the Notch ligands *Dll1*, *Dll4*, *Dll3*, and *Jagged2* (**A**); the Notch targets *Hes1*, *Id3*, *Hes5*, and the proneural target *Hes6* (**B**); and neurogenic versus gliogenic progenitor markers *Tis21* and *Glast*, respectively (**C**) due to loss of *Ascl1* or *Neurog2* function are depicted.

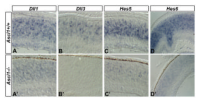

Figure 5. Visualization of key downregulated genes in *Ascl1* deficient retina

(**A-D**) *In situ* hybridization was used to visualize changes in *Dll1*, *Dll3*, *Hes5*, and *Hes6* gene expression levels between *Ascl1*+/+ (**A-D**) and *Ascl1*−/− (**A′-D′**) in the sister P0 eyes from the previous QPCR analysis. *Dll1*, *Dll3*, *Hes5*, and *Hes6* gene expression levels are decreased in the *Ascl1*−/− retina compared to normal expression levels observed in the *Ascl1*+/+ retina.

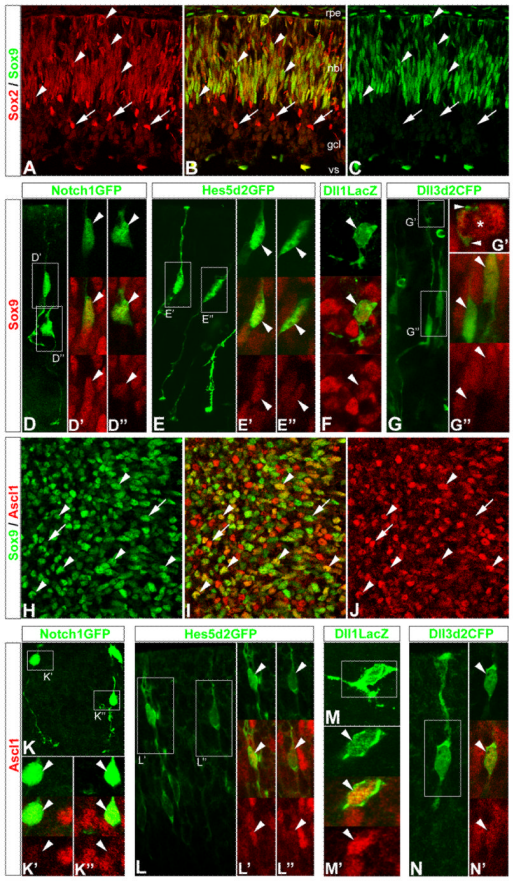

Figure 6. Delta-like and Notch pathway reporters are active in Ascl1 expressing retinal progenitor cells

(**A-C**) Laser scanning confocal microscopy (LSCM) was used to image E17.5 retinal section co-immunolabeled with antibodies to Sox2 (**A, B** red) and Sox9 (**B, C** green). Sox2 and Sox9 co-expression identify the vast majority of the progenitor pool in the neuroblast layer (nbl, arrowheads), although some postmitotic amacrine cells retain high expression of Sox2 (arrows): note that Sox9 is also expressed in the retinal pigmented epithelium (rpe, top), both Sox2 and Sox9 are expressed in cells along the inner/vitreal surface (vs) outside of the ganglion cell layer (gcl), and that Sox9 expression levels in retinal progenitors are observed at low or high levels. (**D-G**) LSCM was used to image E17.5 wholemount mouse retinas transfected with Delta-like and Notch pathway reporters, and co-immunolabeled with antibodies to GFP or LacZ, and Sox9. LSCM was used to visualize transfected cells in their intact environment, either in transverse orientation with apical and basal processes located at the top and bottom, respectively (**D, E, G**), or enface (top-down, **F**). (**D**) Maximum intensity projection (MIP) of neighboring Notch1GFP transfected cells (green) that are Sox9+ (red): **D′**, high Sox9, **D″** lower Sox9, arrowheads; single optical slices showing individual and merged channels, respectively. (**E**) MIP of neighboring Hes5d2GFP transfected cells (green) that are also Sox9+ (red): **E′** high Sox9, **E″**, lower Sox9, arrowheads; single optical slices showing individual and merged channels, respectively. (**F**) Enface view of a Dll1LacZ transfected cell (green) that is Sox9+ (red, arrowhead): single optical slices showing individual and merged channels, respectively. (**G**) Single optical slice of neighboring Dll3d2CFP transfected cells (green) that are Sox9+ (red): **G″** high Sox9, top arrowhead; low Sox9, bottom arrowhead; single optical slices showing individual and merged channels, respectively: note that some basal progenitors can make extensive contact with apical

progenitors at the ventricular surface (**G′** arrowheads and asterisk, respectively). (**H-J**) LSCM was used to image E17.5 whole-mount mouse retinas co-immunolabeled with antibodies to Sox9 (green) and Ascl1 (red). Enface view of single optical slice through the neuroblast layer identifies many progenitors that co-express Sox9 and Ascl1 (arrowheads), and subsets of progenitors that express higher-to-lower levels of either Sox9 or Ascl1, respectively (arrows). (**K-N**) LSCM was used to image E17.5 whole-mount mouse retinas transfected with Delta-like and Notch pathway reporters, and co-immunolabeled with antibodies to GFP or LacZ, and Ascl1. LSCM was used to visualize transfected cells in their intact environment, either in transverse orientation with apical and basal processes located at the top and bottom, respectively (**K, L, N**), or enface (**M**). (**K**) MIP of neighboring Notch1GFP transfected cells (green) that are Ascl1+ (red): **K′**, low Ascl1, **K″** higher Ascl1, arrowheads; single optical slices showing individual and merged channels, respectively. (**L**) MIP of neighboring Hes5d2GFP transfected cells (green) that are also Ascl1+ (red): **L′** higher Ascl1, **L″**, lower Ascl1, arrowheads; single optical slices showing individual and merged channels, respectively. (**M**) Enface MIP view of a Dll1LacZ transfected cell (green) that is Ascl1+ (red, arrowhead): **M′** single optical slices showing individual and merged channels, respectively. (**N**) MIP of a Dll3d2CFP transfected cell (green) that is Ascl1+ (red, arrowhead): **N″** single optical slices showing merged and individual (red) channels, respectively; note that **N′** depicts a Dll3d2CFP+ progenitor contacting a Sox9+ mitotic progenitor at the ventricular surface.

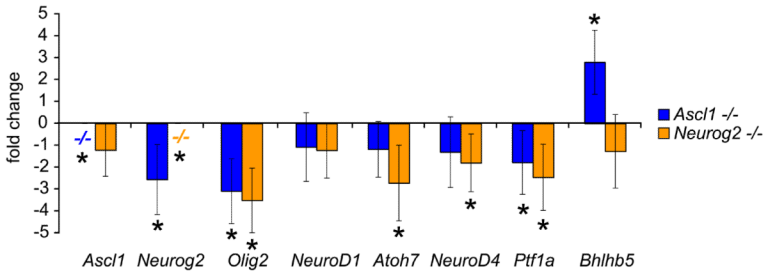

Figure 7. *Ascl1* and *Neuorg2* are upstream bHLH transcription factors

QPCR was used to measure changes in other bHLH transcription factor family members expressed during retinal development due to loss of *Ascl1* or *Neurog2* (P0, see Figure 4, and Methods). *Ascl1* was not expressed in the *Ascl1*−/− retina, and was not changed with loss of *Neurog2* function. Likewise, *Neurog2* was not expressed in the *Neurog2*−/− retina, however *Neurog2* expression was decreased in the *Ascl1*−/− retina. *Olig2* expression was decreased in both *Ascl1* and *Neurog2* knockout retina. *NeuroD1* expression was not changed in either *Ascl1* or *Neurog2* knockout retina. *Atoh7* and *NeuroD4* expression were decreased in the *Neurog2*−/− retina, but not changed in the *Ascl1*−/− retina. *Ptf1a* expression was decreased in both *Ascl1*−/− and *Neurog2*−/− retina. *Bhlhb5* expression was increased in the *Ascl1*−/− retina, but was not changed in the *Neurog2*−/− retina. These changes are consistent with the roles of *Ascl1* in particular, as well as *Neurog2*, as upstream bHLH transcription factors expressed in progenitor cells that regulate a downstream proneural bHLH transcription factor cascade underlying the transition to differentiating neurons (Nelson et al., 2007a).

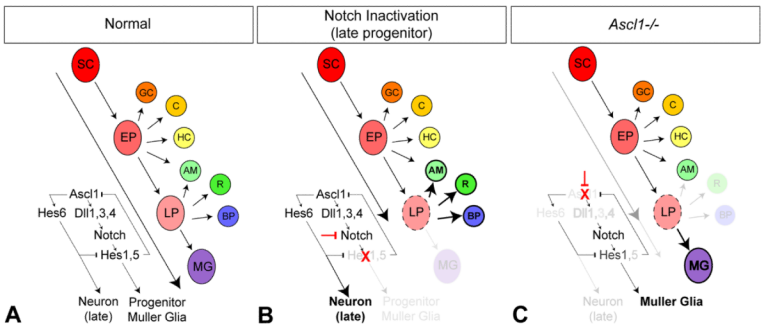

Figure 8. Summary of Ascl1 function in the conserved Delta-like/Notch/Hes molecular circuitry, and comparison of retinal phenotypes due to loss of Notch versus Ascl1 activity

(**A**) Model of vertebrate retinal development under normal conditions (adapted from Reh and Fischer, 2006). Ascl1 contributes to the regulation of Delta-like genes to activate Notch signaling. Ascl1 also drives proneurogenic Hes6, which functions to inhibit Notch signaling, creating a balance between neural differentiation and maintenance of progenitors. (**B**) Inactivating Notch signal transduction in late progenitors (LP, broken circle) blocks Hes1/5 gene function, and increases Ascl1/Hes6 activity, shifting the balance to force differentiation of later born neurons at the expense of Muller glia (Nelson et al., 2007a). (**C**) By contrast, loss of Ascl1 decreases Delta-like gene expression and attenuates Notch activity in progenitors. However, the balance of neurogenic versus gliogenic differentiation signals shift in the LP (broken circle) towards gliogenesis due to downregulation of proneurogenic Hes6 and other upstream proneural differentiation functions of Ascl1, while residual lower-levels of Notch activity (Hes1) suffice to generate Muller glia. Hence Muller glia are increased at the expense of later born neurons with loss of Ascl1 (Tomita et al., 1996). Abbreviations: SC, stem cell; EP, early progenitor; LP, late progenitor; GC, ganglion cell; C, cone; HC, horizontal cell; AM, amacrine; R, rod; BP, bipolar cell; MG, Muller glia.