RESEARCH ARTICLE

Notch signaling differentially regulates Atoh7 and Neurog2 in the distal mouse retina

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ABSTRACT

Notch signaling regulates basic helix-loop-helix (bHLH) factors as an evolutionarily conserved module, but the tissue-specific mechanisms are incompletely elucidated. In the mouse retina, bHLH genes Atoh7 and Neurog2 have distinct functions, with Atoh7 regulating retinal competence and Neurog2 required for progression of neurogenesis. These transcription factors are extensively co-expressed, suggesting similar regulation. We directly compared Atoh7 and Neurog2 regulation at the earliest stages of retinal neurogenesis in a broad spectrum of Notch pathway mutants. Notch1 and Rbpj normally block Atoh7 and Neurog2 expression. However, the combined activities of Notch1, Notch3 and Rbpj regulate Neurog2 patterning in the distal retina. Downstream of the Notch complex, we found the Hes1 repressor mediates Atoh7 suppression, but Hes1, Hes3 and Hes5 do not regulate Neurog2 expression. We also tested Notch-mediated regulation of Jag1 and Pax6 in the distal retina, to establish the appropriate context for Neurog2 patterning. We found that Notch1:Notch3 and Rbpj block co-expression of Jag1 and Neurog2, while specifically stimulating Pax6 within an adjacent domain. Our data suggest that Notch signaling controls the overall tempo of retinogenesis, by integrating cell fate specification, the wave of neurogenesis and the developmental status of cells ahead of this wave.

KEY WORDS: Retinal neurogenesis, Notch signaling, Atoh7, Neurog2, Jagged1, Mouse

INTRODUCTION

The mammalian retina comprises six neuronal and one glial cell type that originate from a common pool of retinal progenitor cells (RPCs). In mice, neurogenesis initiates in the dorsocentral optic cup on embryonic day 11.0 (E11.0), and spreads to the periphery by E13.5. Birthdating and retroviral lineage studies demonstrated that cell types arise in a stereotypical, but partially overlapping sequence (Carter-Dawson and Lavaud, 1979; Young, 1985; Turner and Cepko, 1987; Turner et al., 1990; Rapaport et al., 2004). In rodents, retinal ganglion cells (RGCs) appear first, closely followed by cone photoreceptor, amacrine and horizontal cells. Rod photoreceptor genesis begins prenatally and peaks around birth, whereas bipolar interneurons and Müller glia largely appear postnatally. RPCs are initially pluripotent for all neuronal and glial cell fates, but over time they become biased to produce only the later cell fates (reviewed by Cepko et al., 1996). Elucidation of molecular mechanisms controlling progenitor cell cycle exit, differentiation and maturation of cell types is a fundamental problem for retinogenesis.

Basic helix-loop-helix (bHLH) transcription factors specify cell fates throughout the vertebrate nervous system, in part by regulating cell cycle exit and/or neuronal differentiation (reviewed by Ohssawa and Kageyama, 2008). As retinal histogenesis initiates, the two bHLH factors Atoh7 (atohal homologue 7; also known as Math5, Ath5) and Neurog2 (neurogenin 2; also known as Ngn2, Ath4) are activated within the same cells, and simultaneously expand their expression domains along the leading edge of neurogenesis (Brown et al., 1998; Hufnagel et al., 2010). Lineage tracing studies demonstrated that RPCs expressing either Atoh7 and/or Neurog2 give rise to all seven major cell classes (Ma and Wang, 2006; Feng et al., 2010; Brzezinski et al., 2012). Importantly, Neurog2 directly activates Atoh7 transcription by binding to an evolutionarily conserved E box in the primary Atoh7 retinal enhancer, and in Neurog2 mutants, Atoh7 expression is delayed along with advancement of neurogenesis (Skowronska-Krawczyk et al., 2009; Hufnagel et al., 2010). The individual requirements of Atoh7 and Neurog2 account for those of the Drosophila orthologue, atonal (ato), in the fly eye (Jarman et al., 1993, 1994, 1995; Brown et al., 2001; Wang et al., 2001; Hufnagel et al., 2010).

Owing to coincident onset and co-expression in the mammalian eye, it is conceivable that Atoh7 and Neurog2 are similarly regulated. Evolutionary conserved co-regulation is evident, as Pax6 is a direct transcriptional activator of Atoh7 and Neurog2 (Marquardt et al., 2001; Riesenberg et al., 2009a; Willardsen et al., 2009), while the Pax6 orthologue eyeless directly regulates ato (Zhang et al., 2006). In the fly eye, Notch signaling regulates ato in multiple ways, by genetically enhancing ato expression ahead of the morphogenetic furrow, but suppressing ato within and behind the furrow (Baker et al., 1996; Ligoxygakis et al., 1998; Li and Baker, 2001).

There is strong conservation of Notch signaling, wherein cells signal to one another through the binding of transmembrane ligands and receptors (reviewed by Fortini, 2009; Kwon and Ilegan, 2009; Guruharsha et al., 2012). Upon ligand binding, a Notch receptor intracellular domain (NICD) is released, forming a complex with the DNA-binding protein Rbpj/Su(H) and co-factor MAML/mad/mandelmind. Within the nucleus, this complex binds the DNA of target genes, e.g. the Hes transcriptional repressors (reviewed by Iso et al., 2003; Kageyama et al., 2008). In the prenatal mouse retina, Notch signaling components include: the ligands Jagged1 (Jag1), Delta-like1 (Dll1) and Delta-like4 (Dll4); the receptors Notch1 and Notch3; the DNA-binding protein Rbpj; and the downstream effectors Hes1, Hes3 and Hes5 (Lindsell et al., 1996; Baio and Cepko, 1997; Hojo et al., 2000; Rocha et al., 2009). Loss-of-function studies for Dll1, Dll4, Notch1, Rbpj and Hes1 highlighted the central role for this pathway in promoting RPC proliferation and foetal cell types. As bHLH factors largely

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promote neuronal fates, the Notch pathway is likely to regulate their expression or action. However, these mechanisms remain incompletely defined.

Other unresolved issues in the mouse retina include fully defining the genetic hierarchy that triggers the outset of neurogenesis, understanding how the neurogenic wave is propagated (McCabe et al., 1999; Masai et al., 2000, 2005; Martinez-Morales et al., 2005; Hufnagel et al., 2010) and how the boundary between the neural retina and ciliary body is established and maintained. Undoubtedly both extrinsic and intrinsic factors control these processes, but only a few genes are known, and their activities are insufficient to explain the underlying mechanisms. One intrinsic factor required for the progression of neurogenesis is Neurog2 (Hufnagel et al., 2010). Another is Pax6, which is expressed by all optic cup cells, exhibits multiple functions and yet is differentially required by distal optic cup cells. Oron-Karni et al. (2008) specifically removed Pax6 in the distal retina and uncovered a complex relationship with another factor, Crx, that is crucial for cone and rod photoreceptor genesis. At E15.5, Pax6 suppresses Crx expression in the distal-most optic cup cells, bordering the presumptive ciliary body, but activates Crx in an adjacent, more proximal domain.

Here, we have examined the genetic requirements for Notch signaling in controlling Atoh7 and Neurog2 expression during early neurogenesis. We employed nine different germline or conditional mouse mutants, the α-Cre transgene, and the Z/EG lineage reporter to correlate phenotypic changes in Atoh7 mRNA or Atoh7lacZ and Neurog2 protein expression. Loss of Notch1;Notch3 or Rbpj caused cell-autonomous derepression of both bHLH factors and unique mispattering of Neurog2+ cells. These changes in number and location of Neurog2+ cells are independent of Hes1, Hes3 and Hes5 activity, suggesting a distinct Neurog2 regulation downstream of Rbpj. We also found that Notch1;Notch3 and Rbpj are required for Pax6 expression in a specific group of RPCs, where Neurog2 expression is lost, and to suppress Jag1 more distally. Our findings significantly extend known roles of Notch signaling in the vertebrate retina, by connecting this pathway to the spatiotemporal progression of neurogenesis.

RESULTS
Distinct Hes1 colocalization patterns with Atoh7 and Neurog2
During Drosophila retinogenesis, Notch signaling sequentially activates or suppresses expression of the bHLH factor ato, which is crucial for R8 photoreceptor specification and neurogenesis progression (Li and Baker, 2001). However, in the mammalian eye, little is known about the regulatory relationships between Notch signaling and the ato orthologues, Atoh7 and Neurog2. We therefore compared the early retinal expression patterns of Atoh7 and Neurog2 with the Notch pathway transcriptional mediator Rbpj and known downstream effector Hes1. We took advantage of an Atoh7lacZ allele, using β-gal as a proxy for Atoh7 expression (Brown et al., 2001). Neurog2 expression was monitored with a specific monoclonal antibody (Lo et al., 2002). As Rbpj is ubiquitously expressed in the developing retina (Zheng et al., 2009), there was complete coexpression of Rbpj with both β-gal (Fig. 1A–A″, arrows) and Neurog2 (Fig. 1B–B″, arrows). Consistent with previous demonstrations that Hes1 suppresses Atoh7 mRNA expression (Takatsuka et al., 2004; Lee et al., 2005), we found no overlap between Hes1 and β-gal proteins (Fig. 1C–C″, arrowheads). At E11.5 we observed abundant coexpression of Hes1 and Neurog2

Fig. 1. Comparison of Atoh7lacZ, Neurog2, Rbpj and Hes1 retinal expression. (A–A″) In Atoh7lacZ+ eyes, every β-gal+ cell co-expresses Rbpj (arrows). (B–B″) Similarly, all Neurog2+ cells co-express Rbpj protein (arrows). (C–C″) No β-gal+Hes1+ cells were observed (arrowheads indicate β-gal+ cells). (D–E″) At E11.5, Neurog2 and Hes1 proteins are extensively co-expressed (arrows), but by E13.5, their patterns are mutually exclusive (E–E″; arrowheads). Scale bar: 100 µm for all panels; insets on the right are higher magnifications of the panels on the left.
proteins (Fig. 1D′-D″, arrows). This was surprising, as Hes1 and Neurog2 exhibit opposite expression profiles during telencephalon development; such that, when Hes1 levels are high, Neurog2 levels are low, and vice versa (Shimojo et al., 2008; Sansom et al., 2009).

However, by E13.5 Neurog2 and Hes1 proteins were no longer co-expressed (Fig. 1E′-E″, arrowheads). We conclude that only RPCs initiating neurogenesis are Hes1+Neurog2+. These cells then transit into Neurog2+/Atoh7+ RPCs that terminally exit the cell cycle (Le et al., 2006; Hufnagel et al., 2010; Brzezinski et al., 2012). Therefore, it is plausible that Notch signaling uses distinct mechanisms to regulate Atoh7 and Neurog2 in the early mouse retina.

**Differential Rbpj regulation of Atoh7 and Neurog2**

To understand how Atoh7 and Neurog2 retinal expression globally depends on Notch signaling, we conditionally deleted Rbpj, as it complexes with the intracellular domain of all Notch receptors, and is required for all Notch-mediated transcriptional regulation. For this we used the α-Cre driver, which initiates Cre recombinase and IRES-GFP expression in the E10.5 distal optic cup (Marquardt et al., 2001), and Rbpj<sup>fl/fl</sup> conditional mutants (Han et al., 2002). We also used Z/EG transgenic mice (Novak et al., 2000) to comprehensively mark cells with Cre activity. We initially surveyed E13.5 and E16.5 eyes for phenotypes, but subsequently focused on the earlier age, when retinal defects are first detected. Moreover, the profound mispattering and apoptosis in older mutant retinas, made quantifications of bHLH factors and cell type markers difficult (Jadhav et al., 2006b; Yaron et al., 2006; Riesenberg et al., 2009b).

We previously demonstrated that Hes1+ RPCs were dramatically reduced, and that Pou4f+ RGCs and Crx+ bipotential photoreceptor precursors were each increased, in E16.5 cells lacking Rbpj (Riesenberg et al., 2009b). To interpret Atoh7 or Neurog2 expression within this context, E13.5 Rbpj control and mutant retinal sections were scrutinized with the same markers, ensuring continuity among multiple studies (Fig. 2A-F). Interestingly, in α-Cre-lineage cells, triple-labeled for GFP, β-gal (Atoh7<sup>lacZ</sup>) and Neurog2, there were unique changes in the expression of each bHLH factor (Fig. 2G,H). We observed a cell-autonomous and uniform expansion of β-gal+GFP+ cells in Rbpj mutant retinas [Fig. 2H′ (arrows), H″ (bracket), I], compared with controls. Although the percentage of Neurog2+ cells increased autonomously in Rbpj mutants (Fig. 2I), Neurog2+ cells were also obviously mispatterned. In the most distal neural retina (zone 1, see Oron-Karni et al., 2008 and Fig. 8A,B), we found a dramatic increase in Neurog2-expressing cells. However, in the adjacent, more-proximal domain (zone 2) there was a dramatic loss of Neurog2+ cells (arrowhead and arrow in Fig. 2H). This cannot be explained by a distal displacement of Neurog2+ cells from zone 2 to 1, because the overall ratio of Neurog2+ cells was elevated (Fig. 2I). This suggests that RPCs in different areas of the retina are not equivalent, consistent with previous demonstration that distal RPCs respond differently to Pax6 (Oron-Karni et al., 2008). We conclude that Rbpj regulates Atoh7 and Neurog2 differently in distal optic cup.

**Neurog2 expression does not undergo Hes1/3/5-mediated repression in the retina**

Hes1 germline mutants exhibit premature and ectopic expression of Atoh7 in E9.5 optic vesicles, which persists to later stages of retina development (Takatsuka et al., 2004; Lee et al., 2005). Hes1 also suppresses Pou4f+ RGCs (Fig. 3A,D), the expression of which is also suppressed by microarray analysis (Wang et al., 2001). In Notch1 and Rbpj mutant retinas, there is a virtually complete, cell-autonomous, loss of Hes1 (Fig. 2D, Fig. 4D and Fig. 5D) (Riesenberg et al., 2009b). This is consistent with Hes1 acting as a canonical target of activated Notch complexes to suppress Atoh7. However, the prevalence of RPCs co-expressing Hes1 and Neurog2 at E11.5 (Fig. 1D′-D″) made it difficult to determine whether Neurog2 expression was regulated by Rbpj, as Neurog2 expression was already elevated in Rbpj mutant retinas (Fig. 3A,D). Therefore, we next determined whether Neurog2 expression is regulated independently of Hes1 by using a conditional Neurog2 line (Atoh7<sup>lacZ</sup>+/gfp<sup>−/−</sup> (Atoh7lacZ<sup>−/−</sup>-gal)). In Atoh7lacZ<sup>−/−</sup>-gal/+ neurons, there was a virtual complete loss of Neurog2 expression (Fig. 3E,F), consistent with Neurog2 expression being regulated independently of Hes1 and Rbpj.

**Quantifying Rbpj regulation of Atoh7 and Neurog2**

We previously observed a cell-autonomous and uniform expansion of β-gal+GFP+ cells in Rbpj mutant retinas [Fig. 2H′ (arrows), H″ (bracket), I], compared with controls. Although the percentage of Neurog2+ cells increased autonomously in Rbpj mutants (Fig. 2I), Neurog2+ cells were also obviously mispatterned. In the most distal neural retina (zone 1, see Oron-Karni et al., 2008 and Fig. 8A,B), we found a dramatic increase in Neurog2-expressing cells. However, in the adjacent, more-proximal domain (zone 2) there was a dramatic loss of Neurog2+ cells (arrowhead and arrow in Fig. 2H). This cannot be explained by a distal displacement of Neurog2+ cells from zone 2 to 1, because the overall ratio of Neurog2+ cells was elevated (Fig. 2I). This suggests that RPCs in different areas of the retina are not equivalent, consistent with previous demonstration that distal RPCs respond differently to Pax6 (Oron-Karni et al., 2008). We conclude that Rbpj regulates Atoh7 and Neurog2 differently in distal optic cup.

**Fig. 2. Differential Rbpj-mediated regulation of Atoh7<sup>lacZ</sup> and Neurog2.** (A-F) E13.5 Hes1+ RPCs, Pou4f+ RGCs and Crx+ photoreceptor precursors RPCs were autonomously reduced (D), whereas RGCs (E) and photoreceptor precursors (F) were autonomously increased. Inset arrows indicate α-Cre lineage cells (GFP+) co-labeled with anti-Hes1 (A,D), anti-Pou4f (B,E) or anti-Crx (C,F). (G-G″) Overlapping Atoh7<sup>lacZ</sup> (anti-β-gal) and Neurog2 expression in the α-Cre (GFP+/) lineage. Arrow and arrowhead in G″ (G″) in the absence of Rbpj, Atoh7<sup>lacZ</sup>−/+ cells are increased (bracket in H″). Although Neurog2+ and the β-gal+Neurog2+ double-positive cohorts increased (J), Neurog2 expression was also mispatterned (H+′H″). In H″, the arrow indicates an abnormal accumulation of Neurog2+ cells, yet the arrowhead denotes an area with no Neurog2+ cells. Insets show co-expression of GFP, β-gal and/or Neurog2 (arrows). (I,J) Quantification of co-labeling between the white lines in G and H. Insets in G-G″ and H-H″ are boxed areas shown at higher magnification. Scale bar: 100 µm; n≥3 embryos per genotype; ***P<0.001; *P<0.05; error bars represent s.e.m.
unlikely that Rbpj would regulate Neurog2 analogously (Fig. 2H). To verify this, we quantified the percentage of Neurog2+ cells in E13.5 control and Hes1−/− retinas, and found no significant difference between genotypes (Fig. 3C,F,G). We conclude that Hes1 does not regulate Neurog2 in the retina.

There is the possibility that another Hes family member acts downstream of Notch complex activation to suppress Neurog2. Hes5 is expressed in the prenatal mouse retina, with known functions to maintain RPCs and specify Müller glia (Hojo et al., 2000). Furthermore, Hes3 is present in the embryonic retina (A.N.R., unpublished), but with no discernible role. We compared Neurog2 expression between E13.5 control and Hes3−/−;Hes5−/− retinas, and found no difference between genotypes (supplementary material Fig. S1D,H). Additionally, we observed no change in Hes1+ RPCs, Pou4f+ RGCs or Crx+ bipotential precursors in Hes3−/−;Hes5−/− retinas, compared with double heterozygous controls (supplementary material Fig. S1D,H).

Fig. 3. Hes1 does not regulate Neurog2 in the prenatal retina. Pou4f+ RGCs (A,D) are expanded and Crx+ photoreceptor precursors are reduced (B,E) in E13.5 Hes1−/− retinas, consistent with previous reports (Takatsuka et al., 2004; Riesenberg et al., 2009b). (C,F) The Neurog2 expression pattern is not altered by loss of Hes1. (G) There was no significant change in the percentage of Neurog2+ cells. Scale bar: 100 μm; n≥3 embryos per genotype; NS, not significant; error bars represent s.e.m.

Fig. 4. Notch1 regulates Atoh7lacZ and Neurog2 identically. (A–F) The loss of Notch1 autonomously reduced Hes1+ RPCs (D) and expanded Crx+ photoreceptor precursors (F), with no effect on Pou4f+ RGCs (E) (Jadhav et al., 2006b; Yaron et al., 2006). (G–J) Highly overlapping domains for Atoh7lacZ(β-gal) and Neurog2, in α-Cre; Notch1−/−;Z/EG;Atoh7lacZ+/+ retinas. (H–J) In α-Cre;Notch1−/−;Z/EG;Atoh7lacZ+/+ retinas, however (H–J), the Atoh7lacZ- and Neurog2-expressing cells are autonomously increased (H−”, I−”, brackets, quantified in J), (K–T) Notch1 ICD overexpression induced an autonomous increase of Hes1+ RPCs, and nearly abolished Pou4f+ RGCs (P) and Crx+ photoreceptor precursors (R). Both β-gal+ (S) and Neurog2+ cell populations (T,T′) were dramatically reduced (arrowhead). Scale bar: 100 μm; insets show boxed areas at higher magnification. Arrows indicate n≥3 embryos per genotype; errors bars show s.e.m.; ***P≤0.001.
material Fig. S1A-C,E-G). Therefore, Hes3 and Hes5 also do not regulate Neurog2 expression in this context.

**Loss of either Jag1 orDll1 ligands has no effect on Atoh7 or Neurog2 retinal expression**

During mammalian neurogenesis, it remains unclear which Notch pathway ligand-receptor combinations orchestrate specific cellular events. The Notch ligands, Jagged1 (Jagged1) or Delta-like1 (Dll1) and Delta-like4 (Dll4) are each present in the early optic cup (Lindsell et al., 1996; Rocha et al., 2009; Le et al., 2012). Intriguingly, Jag1 is specifically localized in the distal optic cup from E9.0 to E12.5 (Le et al., 2009), making it a good candidate to control the spatial patterning of Neurog2. However, α-Cre-mediated deletion of Jag1 had no effect on the ratios of RPCs, early retinal cell fates or Neurog2+ cells (supplementary material Fig. S2; data not shown). We then asked to what extent Dll1 regulates bHLH factor expression, as it is required for RPC maintenance and for RGC suppression (supplementary material Fig. S3A,B,D,E) (Rocha et al., 2009) (A.N.R. and N.L.B., unpublished). At E13.5, we found that Atoh7 in Dll1+ expression was unaffected in α-Cre; Dll1CKO/+,Z/EG; Atoh7lacZ/+ control and α-Cre; Dll1CKO/+,Z/EG; Atoh7lacZ/+ mutant eyes (supplementary material Fig. S3G-I). Additionally, the proportion and patterning of Neurog2+GFP+ and Neurog2+/β-gal+GFP+ cells were unchanged in the absence of Dll1 (supplementary material Fig. S3J). We conclude that neither Jag1 nor Dll1 individually regulate Atoh7 or Neurog2 in the early retina, suggesting another ligand, or the redundant activity of multiple ligands, is required.

**Notch1 suppresses both Atoh7 and Neurog2**

Notch1 and Notch3 are expressed at the initiation of mouse retinal neurogenesis (Bao and Cepko, 1997). While Notch1 alone may be sufficient to maintain RPCs and block cone photoreceptor genesis, both receptors control RGC formation (Jadhav et al., 2006b; Yaron et al., 2006; Riesen et al., 2009). To tease apart the distinct versus redundant activities of Notch1 and Notch3 in the developing retina, we first explored Atoh7 and Neurog2 expression in Notch1 and Notch3 single mutants. Previously, Yaron et al. (2006) demonstrated that Atoh7 and Neurog2 mRNA are derepressed in α-Cre; Notch1CKO/+;Z/EG; Atoh7lacZ/+ and α-Cre; Notch1CKO/+;Z/EG; Atoh7lacZ/+; RosaGFP mice, and quantified β-gal+ and/or Neurog2+ cells within the GFP-marked α-Cre lineage (Fig. 4). First, we confirmed the cell-autonomous loss of Hes1+ RPCs and Pou4f+ RGCs and an autonomous increase in the Crx+ population (Fig. 4A-F) (Yaron et al., 2006). We also saw a cell-autonomous increase in Atoh7lacZ/+ (β-gal+) and Neurog2+ cells in Notch1 conditional mutants (Fig. 4G-J). Notably, there was no distal mispatterning of Neurog2+ cells (Fig. 4H”). We conclude that Notch1 and Rbpj conditional mutants phenocopy one another regarding the loss of RPCs, expansion of bipotential photoreceptors, and increase in Atoh7lacZ/+ and Neurog2+ cells. Yet there are clear differences in Notch1 and Rbpj conditional mutants regarding RGC neurogenesis and Neurog2 patterning.

In the vertebrate retina, misexpression of the Notch1 intracellular domain (Notch1ICD) promotes a persistent progenitor-like state, abolishes neurogenesis and increases the proportion of Müller glia (Jadhav et al., 2006a). We were curious to learn the extent to which α-Cre-induced activation of a flox-stop RosaNotch1IRES-GFP transgene (Murtaugh et al., 2003) suppresses Atoh7 and Neurog2 expression. To calibrate our findings with those of Jadhav et al. (2006a), who analyzed Chx10-Cre; RosaNotch1+/+; RosaIRES-GFP retinas, we scrutinized Hes1+ RPCs, Pou4f+ RGCs and Crx+ photoreceptor precursors in α-Cre; RosaNotch1IRES-GFP; Atoh7lacZ/+; α-Cre; Atoh7lacZ/+ control retinas. We saw a profound expansion of Hes1+ RPCs (Fig. 4K,P) and the cell-autonomous suppression of both Pou4f+ RGCs (Fig. 4L,Q) and Crx+ photoreceptors (Fig. 4M,R). Moreover, Atoh7lacZ/+ (β-gal+ cells in Fig. 4N,S) and Neurog2 (Fig. 4O,T) expression were autonomously missing from cells overexpressing the Notch1ICD. Our examination of Notch1
loss- and gain-of-function retinal mutants extends previous studies, by defining particular contexts in which Notch1 mutants do not phenocopy Rbpj mutants. This strongly implicates the activities of multiple receptors to regulate particular developmental events during retinogenesis.

**Notch3 is not required for Atoh7 or Neurog2 retinal expression**

We also explored Notch3-mediated regulation of RGC genesis and Neurog2 distal patterning, using a germline viable Notch3lacZ mutant mouse, in which a β-geo reporter accumulates in secretory vesicles and axonal processes (Leighton et al., 2001; Mitchell et al., 2001). Previously, we reported that at E16.5, the loss of Notch3 resulted in fewer Hes1+ RPCs and excess Pou4f+ RGCs, with no effect on Crx+ bipotential photoreceptor precursors (Riesenber et al., 2009b). We found the same outcomes (Fig. 5A-F), and also quantified the Atoh7+ and Neurog2+ populations at E13.5 (Fig. 5G-L). For Atoh7 expression, we placed a single copy of an Atoh7GFP transgene in the Notch3 mutant background and monitored GFP expression by anti-GFP labeling. Within the Notch3 lineage (cells with β-gal+ vesicles), there was no difference in either the Atoh7+ (GFP+) or Neurog2+ populations between E13.5 Notch3lacZ/+, Atoh7GFP Tg control and Notch3lacZ/lacZ, Atoh7GFP Tg mutants (Fig. 5G-K). There is no cell-autonomous requirement for Notch3 regarding the expression of either bHLH factor, including regulation of Neurog2 distal patterning.

**Simultaneous removal of Notch1 and Notch3 during early retinogenesis**

Next, we generated a Notch1;Notch3 allelic series, consisting of α-Cre; Notch1CKO/+; Z/EG; Notch3lacZ/+ (Fig. 6A-E), α-Cre; Notch1CKO/−; Z/EG; Notch3lacZ/+ (Fig. 6F-J), α-Cre; Notch1CKO/−; Z/EG; Notch3lacZ/lacZ (Fig. 6K-O) and α-Cre; Notch1CKO/−; Z/EG; Notch3lacZ/lacZ (Fig. 6P-T) littermates. At E13.5, we searched for quantitative or qualitative changes in Hes1+ RPCs, Pou4f+ RGCs, Crx+ photoreceptor precursors, Neurog2+ cells and Atoh7 mRNA expression. We found that one wild-type allele of Notch1 maintains the progenitor pool (Fig. 6A,F,K,P, yellow bar in U), whereas complete loss of Notch1 induces a dramatic reduction in RPCs (Fig. 6U, green and red bars). Furthermore, Notch3 is not required for RPC maintenance, nor does it regulate Crx+ photoreceptors (Fig. 6C,H,M,R,W). Interestingly, the combined activity of both receptors is crucial for proper RGC
neurogenesis. We saw that, although one wild-type allele of either receptor could maintain the correct ratio of RGCs, only the double mutants exhibited an increase in Pou4f+ RGCs (Fig. 6V), which phenocopies the loss of Rbpj (Fig. 2B,E) (Riesenberg et al., 2009b).

We also scrutinized Atoh7 and Neurog2 retinal expression as the gene doses of Notch1 and Notch3 varied. We observed that derepression of Atoh7 mRNA correlates with the loss of Notch1 (Fig. 6O,T), further supporting the idea that a Notch1-Rbpj-Hes1 signal normally suppresses Atoh7 expression. However, Neurog2 regulation was clearly different (Fig. 6N,S,X). Whereas the Neurog2+ population expanded in the absence of Notch1 alone (Fig. 6X, green, red bars), only removal of both receptors disrupted Neurog2 distal patterning in zones 1 and 2 (Fig. 6S), as in Rbpj mutants.

**Notch signaling regulation in the distal retina**

Because the Notch1/Notch3 and Rbpj spatial regulation of Neurog2 was unexpected, we wished to integrate these findings with other retinal genes required in the distal retina. Most relevant was to compare Jag1 and Neurog2 expression patterns. This is because among the Notch genes expressed at the onset of neurogenesis, only Jag1 is present in a discrete distal domain, from E9.0 to E12.5 (Bao and Cepko, 1997; Le et al., 2009) (Fig. 8A). Jag1 abruptly shuts off in the wild-type E13.5 retina, when Neurog2+ cells first appear in zone 1 (Bao and Cepko, 1997; Hufnagel et al., 2010) (Fig. 8B). We verified that distal retinal cells do not normally co-express Jag1 and Neurog2 (Fig. 7E–E′). This is consistent with a Jag1-mediated signal influencing non-neurogenic cells ahead of the progression of neurogenesis. We also wondered whether the downregulation of Jag1 at E13.5 was...
mediated by loss of Notch signaling. Therefore, we examined Rbpj conditional mutants, and found that when zone 1 cells downregulate Hes1 expression, Jag1 is derepressed (Fig. 7A,A′, white brackets in 7C′). We also found that E13.5 Hes1−/− distal retinal cells ectopically express Jag1 in both zones (Fig. 7B,D, white bracket). Finally, we compared Jag1 and Neurog2 expression in E13.5 Rbpj control and conditional mutant retinas, and observed that many cells inappropriately co-express these proteins in zone 1 (Fig. 7E–F′, bracket).

Next, we addressed the loss of Neurog2 expression in proximal zone 2 cells. To understand how Neurog2 is regulated in this region, we focused on the Pax6 transcription factor, which is differentially required by zone 1 and zone 2 retinal cells (Oron-Karni et al., 2008). Because Pax6 directly activates Neurog2 (Marquardt et al., 2001), it is plausible that Notch signaling affects Pax6 expression, which in turn regulates Neurog2 expression. First, we asked whether loss of Rbpj affected Pax6 expression, and found that when E13.5 distal retinal cells lack Rbpj activity, Pax6 is downregulated exclusively in zone 2, accompanied by ectopic Crx expression (Fig. 7G–J′, quantified in Fig. 7N and Fig. 8B: supplementary material Fig. S5). This is in contrast to Pax6 conditional mutants, where only zone 1 cells exhibited ectopic Crx expression (Oron-Karni et al., 2008). We also examined Pax6 expression in the distal retinas from the Notch1:Notch3 allelic series. In the absence of Notch1, there were fewer Pax6+ cells in zone 2, again accompanied by ectopic Crx upregulation (Fig. 7M–M′, quantified in Fig. 7O; Fig. 8C). Interestingly, in receptor double mutants, zone 2 cells exhibited the greatest loss of Pax6+ expression (Fig. 7M′,O). In Rbpj conditional mutants, the zone 2 cells that lose Pax6 expression also downregulate Neurog2 (supplementary material Fig. S5F,H,J), but continue to express Atoh7lox/2 (supplementary material Fig. S5F,I,J). This suggests that Notch pathway suppression of Atoh7 may dominate over Pax6 activation, or that, once activated, the major mode of Atoh7 regulation becomes repression. Finally, co-labeling for Jag1 and Pax6 in E13.5 Rbpj conditional mutant sections verified their simultaneous dependence on Notch signaling within adjacent domains, and that Jag1 and Pax6 are oppositely regulated (Fig. 7K–L′, Fig. 8C). This study identifies Notch signaling as an integral component of wavefront propagation, acting ahead of the wave to fully prime cells to become neurons, but also to limit the proportions of each cell type produced.

**DISCUSSION**

In many species, bHLH transcription factors are key regulators of neurogenesis. As nervous system complexity increased, gene duplication and divergence allowed for additional tissue functionality. An excellent example is *Drosophila atonal* (*ato*), which encodes a bHLH protein that is highly related to the mammalian Atoh1, Neurog2 and *Neurod* protein families (Hassan and Belen, 2000; Bertrand et al., 2002). The *ato* semi-orthologues Atoh7 and Atoh1 are expressed in mutually exclusive regions of the mouse nervous system, subdividing *ato* sensory functions within the mammalian visual (Atoh7), auditory (both genes) and proprioceptive (Atoh1) systems (Jarmar et al., 1993, 1995; Helms et al., 2000; Hufnagel et al., 2007; Saul et al., 2008). Moreover, *ato* functions in the fly eye are further subdivided between mouse *ato7* and Neurog2 (Hufnagel et al., 2010). Here, RPCs require Atoh7 for specification of early fates, and Neurog2 for propagation of neurogenesis across the retina. In this study, we explored Notch pathway regulation of these two bHLH factors at the onset of retinal histogenesis. We found that Atoh7 is laterally inhibited by a Notch1-Rbpj-Hes1 cascade, but Neurog2 is more complex, undergoing both lateral inhibition and spatial restriction. In the latter case, Notch1 and Notch3 coordinately regulate Neurog2 patterning in the distal retina, which is mediated through Rbpj, but not Hes, repressors. In addition to clarifying the roles for Notch signaling during retinal proliferation and differentiation, we demonstrate a novel role, during the wave-like expansion of neurogenesis.

**Complexities of Notch regulation of bHLH factors**

In the fly eye, the consequences of removing *Notch* function on *ato* expression vary with time (Li and Baker, 2001). If *Notch* signaling is lost as *ato* becomes activated at the anterior furrow, too few R8 photoreceptor neurons form. By contrast, blocking *Notch* signaling later, produces too many R8 neurons, because *ato* is not laterally inhibited to produce a single R8 neuron. These phenotypes are spatially and temporally distinct, and are also defined by the genetic requirements for *Su(H)*, the fly orthologue of Rbpj (Furukawa et al., 1991). During early proneural enhancement, activated Notch binds to Su(H), presumably by relieving its association from a repressor complex (Baker et al., 1996; Li and Baker, 2001). This permits initial upregulation of *ato*, without invoking E(Spl) activity. In this case, *Su(H)* behaves as a genetic activator of *ato*. Conversely, during lateral inhibition, Notch-Su(H) complex formation initiates E(Spl) expression, which genetically suppresses *ato* (Ligoxygakis et al., 1998; Li and Baker, 2001). Intriguingly, these two modes of regulation are temporally separated by the onset of *ato*.
autoregulation within the furrow. In the mouse eye, neither mouse Atoh7 nor Neurog2 autoregulates their expression (Brown et al., 2001; Wang et al., 2001; Hutcheson et al., 2005; Kele et al., 2006; Hufnagel et al., 2007, 2010; Riesenberg et al., 2009a), but Neurog2 appears first along the advancing retinal wavefront, and directly activates Atoh7 transcription (Skowronska-Krawczyk et al., 2009; Hufnagel et al., 2010). Thus, Neurog2 crossregulation of Atoh7 is correlated with progression of neurogenesis. Given that Notch signaling differentially regulates ato expression in the fly, it is not surprising there are separate regulatory mechanisms for Atoh7 versus Neurog2 in mice.

Mammalian genomes contain four Notch genes, with Notch1 and Notch3 active during embryonic retinal neurogenesis (Jadhav et al., 2006b; Yaron et al., 2006; Riesenberg et al., 2009b). To distinguish the role of each receptor, we compared the individual and composite loss-of-function phenotypes with those of Rbpj. Notch1 mutants phenocopy Rbpj mutants with respect to the loss of RPC proliferation, expansion of multiple bHLH factor domains and neuronal differentiation (Austin et al., 1995; Henrique et al., 1995, 1997; Bao and Cepko, 1997; Jadhav et al., 2006b; Yaron et al., 2006). But loss of Notch1 also causes RGC apoptosis. Because Rbpj activity both blocks RGC differentiation and prevents RGC apoptosis (Riesenberg et al., 2009b; Zheng et al., 2009), we conclude that RGC genesis uniquely requires multiple Notch receptor inputs, especially during the initial wave of differentiation. Although Notch3 can promote progenitor cell proliferation and block RGC differentiation (Riesenberg et al., 2009b), it is relatively less important than Notch1. This raises the question of how can these receptors act alone rather than together in particular contexts? One possibility is that each receptor effectively cancels the other out during RGC genesis, with Notch3 suppressing differentiation and Notch1 promoting neuron survival. We do not favor this situation, as Notch1 exhibits the more ancestral functions, namely inhibiting other retinal neuron classes and promoting gliogenesis (Imayoshi, 2012). Instead, we propose that combined receptor regulation arose more recently in the mammalian retina, along with the appearance of Neurog2 during wavefront propagation. Intriguingly, the zebrafish genome has no Neurog2 gene, and here both RGC development and neurogenic wave progression depend upon Ath5/Atoh7 (Masai et al., 2000, 2005; Kay et al., 2001, 2005; Furlong and Graham, 2005). Deeper understanding of how Notch1 and Notch3 transduce both separate and combined signals can be determined only by understanding which retinal cells express full-length and activated isoforms of each receptor protein, the bHLH factors, and the fates they adopt.

**Notch signaling and neurogenic waves**

Neurogenesis typically begins in a small group of epithelial cells, and expands outward in a defined direction, creating a moving boundary between neurogenic and non-neurogenic cells. The developing retina is ideal for studying wavefront propagation. Although the mechanisms for this are best understood in the *Drosophila* eye, wave-like propagation of retinal neurogenesis have been explored in vertebrates (Hu and Easter, 1999; McCabe et al., 1999; Kay et al., 2005; Masai et al., 2005; Oron-Karni et al., 2008; Hufnagel et al., 2010). One conserved feature is that the first retinal neurons appear close to the boundary of the optic cup and stalk, then spread outwards (reviewed by Easter, 2000). The switch from a proliferative to a neurogenic state correlates with the position of RPCs within the optic cup. Rather than occurring stochastically, neurogenesis is synchronized and progressive, potentially mediated by non-neurogenic cells immediately ahead of the wavefront responding to signals from within and behind the moving boundary. During fly and zebrafish retinogenesis, nascent neurons secrete Shh, which signals ahead of the moving boundary. Moreover, in the fly, short-range Hh signaling also induces Dpp secretion (a BMP homologue), which further influences cells ahead of the furrow (reviewed by Kumar, 2012). In the vertebrate retina, less is known about signals emanating from behind the neurogenic wavefront, or how they are received and interpreted by non-neurogenic cells. There is no evidence in the mouse retina that Hh signaling drives this process. Instead, the relative distance ahead of the wavefront is one feature demarcating zones of non-neurogenic cells. Moreover, zone 1 distal retinal cells may be uniquely influenced by other signals originating from the adjacent presumptive ciliary body.

Our data support the idea that Notch signaling is a component of neuronal wavefront propagation (Fig. 8). There is precedent in vertebrates for Notch ligand-mediated coordination of neurogenesis among subsets of cells, in addition to canonical roles in controlling lateral inhibition. In the spinal cord, Jag1 and Dll1 have complementary, non-overlapping expression patterns. Separately, these ligands regulate the timing and rate of neurogenesis, but together maintain the boundaries between progenitor populations (Marklund et al., 2010; Ramos et al., 2010). In the mouse retina, Jag1 and Dll1 expression appears complementary, with Jag1 ahead of the wave and Dll1 expanding with the wave (Bettenhausen et al., 1995; Bao and Cepko, 1997; Le et al., 2009). Assuming this is correct, we propose that blocking Jag1 activity in non-neurogenic cells ahead of the wave could affect the rate of neurogenesis. This may not be apparent here, due to the constraints of the α-Cre driver used. One hint that the spatiotemporal kinetics of this Cre line influenced the phenotypes produced comes from the restricted derepression of Jag1 in Rbpj conditional mutants, versus its broader expansion in Hes1 germline mutants. Although more insight might be gained with a different Cre driver, demonstration that Jag1 is an integral component of the neurogenic wave may only be revealed in ligand double or triple mutants.

What constitutes the spatial architecture of the developing retina? Clearly, different zones of retinal cells have simultaneous and distinct requirements for Pax6 (Oron-Karni et al., 2008). Interestingly, we found that Notch signaling is correlated with high levels of Pax6 expression, but only in a subset of zone 2 cells, presumably adjacent to the advancing wavefront. This loss of Pax6 can account for the downregulation of Neurog2 expression, but paradoxically there was no analogous loss of Atoh7 expression. More work is needed to determine the regulatory relationships between Pax6 and Notch pathway genes, including understanding when and where their activities may dominate over another. It will be important to integrate the current genetic hierarchy (Fig. 8C), with RPC characteristics in different proximodistal locations across the optic cup. The proximal boundary may depend on the Cre driver employed or the progress of the neurogenic wave, yet on the distal side, the boundary with the presumptive ciliary body is fixed. This suggests that the boundary between zones 1 and 2 could be somewhat arbitrary, as it is based on gene expression differences. Nonetheless, previous subdivision of the adult frog ciliary marginal zone, via distinct patterns of gene expression, later led to identification of retinal stem cells (Perron et al., 1998; Perron and Harris, 2000; El Yakoubi et al., 2012). To understand mammalian retinal neurogenesis progression fully, new methods for following wave progression in real time and three dimensions will be needed.
Animals
Mouse lines used in this study were Atoh7<sup>pro-z</sup> (Atoh7<sup>neo</sup>/<sup>neo</sup>) (Brown et al., 2001) and Atoh7-GFP Tg (Math5.21-GFP) mice (Riesenberg et al., 2009a) both on a CD1 background; Deltalike (DII1) conditional (CKO) allele on a 129/SvJ background (Hozumi et al., 2004); Hes1 germline mutant mice on an ICR background (Ishibashi et al., 1995); Hes3;Hes5 double germline mutants on a CD1 background (Baek et al., 2006); Jagged1 (Jag1) CKO allele on a 129 background (Brooker et al., 2006); Notch1 CKO allele (Yang et al., 2004) and Rosa<sup>Notch1<sup>s</sup> flox-stop (Murtaugh et al., 2003) mice both on a mixed 129/B6/L6 background; Notch3 gene trap mutants on a C57BL/6 background (Leighton et al., 2001; Mitchell et al., 2001); Rbpj CKO allele on a 129/SvJ background (Han et al., 2002); and α-Cre transgenic (Marquardt et al., 2001) and Z/EG lineage tracing mice (Jackson Laboratory) (Novak et al., 2000) on a CD1 background. Our Z/EG mice no longer express β-geo (supplementary material Fig. S4), but retain Cre-mediated activation of GFP. The embryonic age was determined by timed matings, with the date of the vaginal plug being E0.5. A minimum of three embryos per age and genotype, from at least two litters were analyzed.

Immunofluorescence, in situ hybridization and X-gal staining
Embryos were fixed in 4% paraformaldehyde/PBS for 40-50 min at 4°C, cryoprotected in 5% and 15% sucrose/PBS, embedded in TissueTek OCT and 10 μm cryosections analyzed. Primary antibodies used were: rabbit anti-β-gal (ICN, 1:1000; 55976), rabbit anti-Crx (Cheryl Craft, 1:1000) (Zhu and Craft, 2000), chick or rabbit anti-GFP (Abcam, 1:1000; ab13970; Life Technologies, 1:1000; A21331), rabbit anti-Hes1 (1:1000) (Lee et al., 2005), goat anti-Jag1 (Santa Cruz Biotechnology, 1:1000; sc-6011), mouse anti-Neurog2 (David Anderson, CalTech, USA, 1:50; R&D Systems; 1:1000; MAB3314) (Lo et al., 2002), rabbit anti-Rxrg (Santa Cruz Biotechnology, 1:200; sc-555), rabbit or mouse anti-Pax6 (Covance, 1:1000; MAB3314) (Lo et al., 2002), rabbit anti-Rxrg (Santa Cruz Biotechnology, 1:200; sc-555), rabbit or mouse anti-Pax6 (Covance, 1:1000; PRB-278P; Santa Cruz Biotechnology, 1:50; sc-32766), goat anti-Pou4f (Santa Cruz Biotechnology, 1:50; sc-6026), and rat anti-Rbpj (CosmoBio USA, 1:100; SIM-2Z/RBP2). Secondary antibodies used were conjugated to Alexa Fluor 488, 594 or AMCA350 (Life Technologies, 1:500; A11034, A11037, A21074, A11058, A21034) (CosmoBio USA, 1:100; SIM-2ZRBP2). Secondary antibodies used were conjugated to streptavidin Alexa Fluor 488, 594 or AMCA350 (Jackson ImmunoResearch, 1:500; 712-066-150, 711-065-152) and sequentially labeled with streptavidin Alexa Fluor 488, 594 or AMCA350 (Jackson ImmunoResearch, 1:200, 016-150-084). Math5/Atoh7 in situ hybridization or X-gal staining to detect β-gal+ cells were performed as described previously (Brown et al., 1998, 2001). Imaging was performed on a Zeiss Apotome deconvolution microscope, and images adjusted for brightness and contrast, and pseudocolored using Axiovision (v6.0) and Adobe Photoshop Elements (v8.0) software.

Western blotting
Pooled E13.5 eyes/genotype were lysed in RIPA buffer with protease inhibitors (Complete, Roche), processed, electrophoresed (25% gels), transferred to nitrocellulose as described previously (Prasov et al., 2010). Blots were probed with rabbit anti-β-gal (ICN, 1:5000; 55976), blocked in 4% milk/0.1 M Tris (pH 7.4)/0.15 M NaCl/0.1% Tween20, and visualized with goat anti-rabbit HRP (Jackson ImmunoResearch, 1:5000; 711-035-152), followed by ECL reagents (SuperSignal West Pico, Thermo/Pierce) and GE Health Systems X-ray film.

Cell quantifications
Antibody-labeled tissue sections were quantified using the Axiovision (v6.0) Measurements module. Three or more animals were analyzed, per genotype and age, with at least two sections from each control or mutant littermate. Sections were judged to be of equivalent depth in the eye by anatomical landmarks, with only the nasal side imaged for consistency of mutant retinal phenotypes. To circumvent α-Cre mosaic expression, we incorporated the Z/EG transgene into our breeding scheme to mark and follow all Cre-derived cells, and score cell autonomy of all mutant phenotypes. Hes1<sup>t</sup>, Pou4f4<sup>s</sup>, Crx<sup>c</sup>, Pax6<sup>c</sup>, Atoh7<sup>pro-z</sup> (β-gal<sup>s</sup>), Neurog2<sup>c</sup> or βgal<sup>s</sup>Neurop2<sup>c</sup>. E13.5 distal retinal cells were quantified within a 200× field containing GFP-marked alpha-Cre lineage cells. The percentage of marker<sup>+</sup>GFP<sup>+</sup>GFP<sup>c</sup> cells (t.s.e.m.) was determined, with GFP reporting both IRES-GFP and/or Z/EG expression. A two-tailed Student’s t-test, plus Welch post-hoc test, were used to determine P-values for two genotype comparison; one-way ANOVA, plus Tukey’s post-hoc test, was used for four genotypes (Instat, v3).

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Competing interests
The authors declare no competing financial interests.

Author contributions
K.A.M., A.N.R. and N.L.B. developed the concepts, experimental approach and complex mouse stocks; K.A.M. and A.N.R. performed the experiments; K.A.M. and N.L.B. analyzed the data, and prepared and edited the manuscript prior to submission.

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