

## RESEARCH ARTICLE

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

Kate A. Maurer<sup>1</sup>, Amy N. Riesenber<sup>1</sup> and Nadean L. Brown<sup>1,2,\*</sup>**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 Lavail, 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 Ohsawa and Kageyama, 2008). As retinal histogenesis initiates, the two bHLH factors *Atoh7* (atonal 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, *ataonal* (*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; Riesenber<sup>1</sup> 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; Kopan and Ilagan, 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/mastermind. 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; Bao 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 forestalling retinal neurogenesis (Takatsuka et al., 2004; Jadhav et al., 2006b; Yaron et al., 2006; Riesenber<sup>1</sup> et al., 2009b; Rocha et al., 2009; Zheng et al., 2009), ensuring an adequate progenitor pool for all seven retinal 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 onset 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.

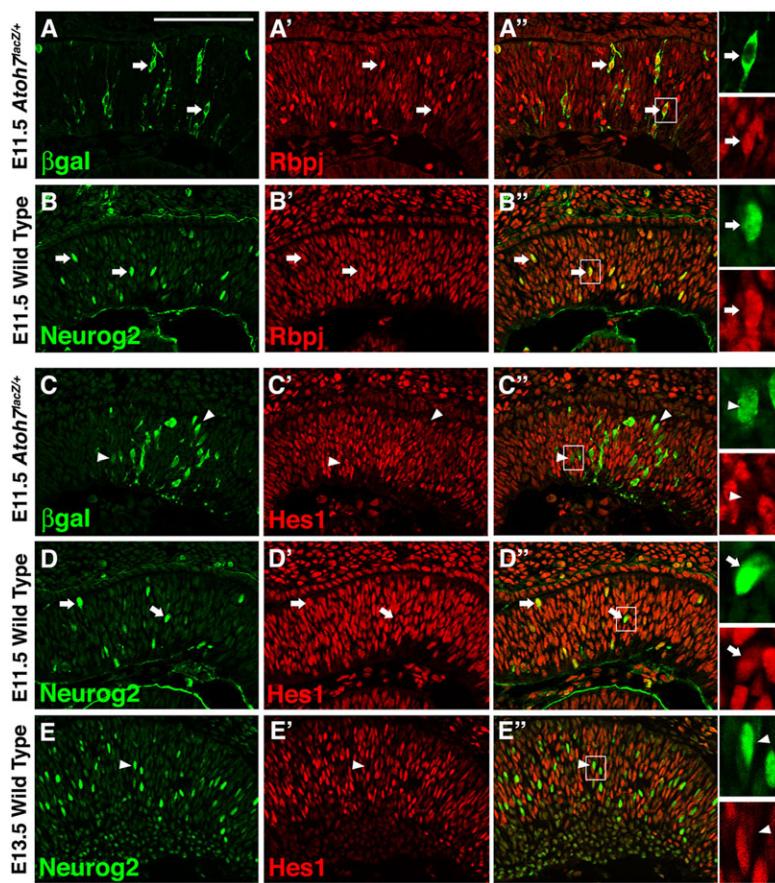
Here, we have examined the genetic requirements for Notch signaling in controlling *Atoh7* and *Neurog2* expression during early neurogenesis. We employed nine different germline or conditional mouse mutants, the α-Cre transgene, and the Z/EG lineage reporter to correlate phenotypic changes in *Atoh7* mRNA or *Atoh7*<sup>lacZ</sup> and *Neurog2* protein expression. Loss of *Notch1*; *Notch3* or *Rbpj* caused cell-autonomous derepression of both bHLH factors and unique mispatterning of *Neurog2*<sup>+</sup> cells. These changes in number and location of *Neurog2*<sup>+</sup> 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 *Atoh7*<sup>lacZ</sup> 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 *Atoh7*<sup>lacZ</sup>, *Neurog2*, *Rbpj* and *Hes1* retinal expression.** (A–A'') In *Atoh7*<sup>lacZ/+</sup> 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; Brzinski 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*

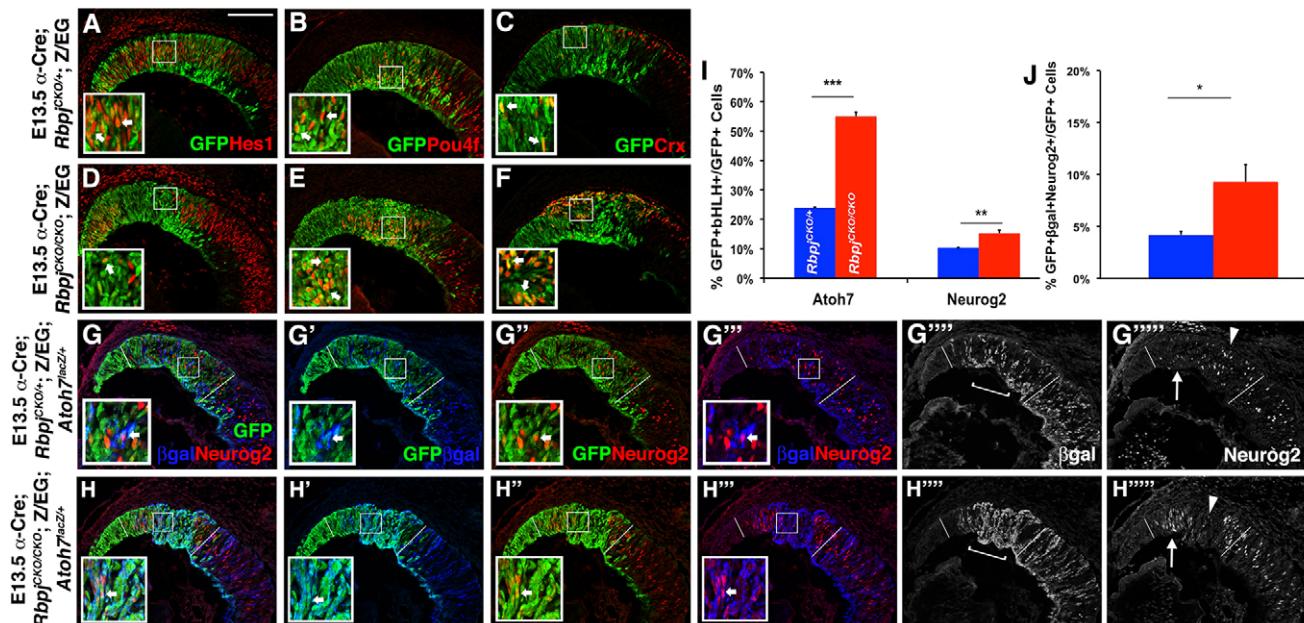
To understand how *Atoh7* and *Neurog2* retinal expression globally depends on Notch signaling, we conditionally deleted *Rbpj*, as it complexes with the intracellular domain of all Notch receptors, and is required for all Notch-mediated transcriptional regulation. For this we used the  $\alpha$ -Cre driver, which initiates Cre recombinase and IRES-GFP expression in the E10.5 distal optic cup (Marquardt et al., 2001), and *Rbpj*<sup>CKO</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 mispatterning and apoptosis in older mutant retinas, made quantifications of bHLH factors and cell type markers difficult (Jadhav et al., 2006b; Yaron et al., 2006; Riesenbergs 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* (Riesenbergs et al., 2009b). To interpret *Atoh7* or *Neurog2* expression within this context, E13.5 *Rbpj* control and mutant retinal sections were

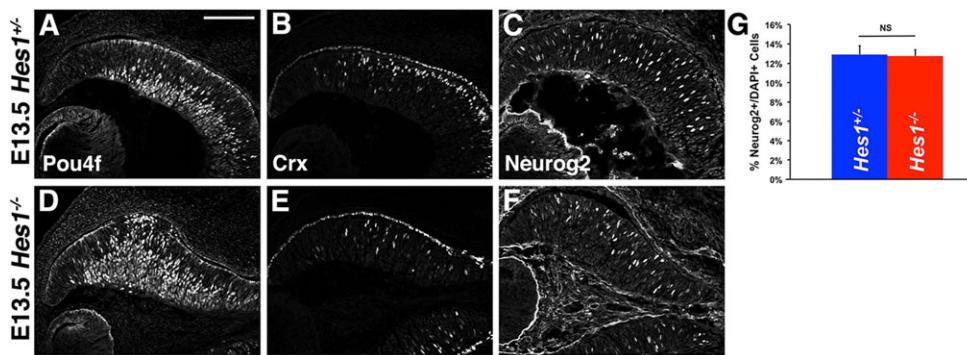
scrutinized with the same markers, ensuring continuity among multiple studies (Fig. 2A–F). Interestingly, in  $\alpha$ -Cre-lineage cells, triple-labeled for GFP,  $\beta$ -gal (*Atoh7*<sup>lacZ</sup>) and Neurog2, there were unique changes in the expression of each bHLH factor (Fig. 2G–H'). We observed a cell-autonomous and uniform expansion of  $\beta$ -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 mispattered. 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 requires *Atoh7* (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) (Riesenbergs 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



**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  $\alpha$ -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- $\beta$ -gal) and Neurog2 expression in the  $\alpha$ -Cre (GFP+) lineage. Arrow and arrowhead in G''', G'''''. (H–H''') In the absence of *Rbpj*, *Atoh7*<sup>lacZ</sup> + cells are increased (bracket in H'''). Although Neurog2+ and the  $\beta$ -gal+Neurog2+ double-positive cohorts increased (J), Neurog2 expression was also mispattered (H–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,  $\beta$ -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  $\mu$ m; n $\geq$ 3 embryos per genotype; \*\*\*P $\leq$ 0.001; \*P=0.05; error bars represent s.e.m.

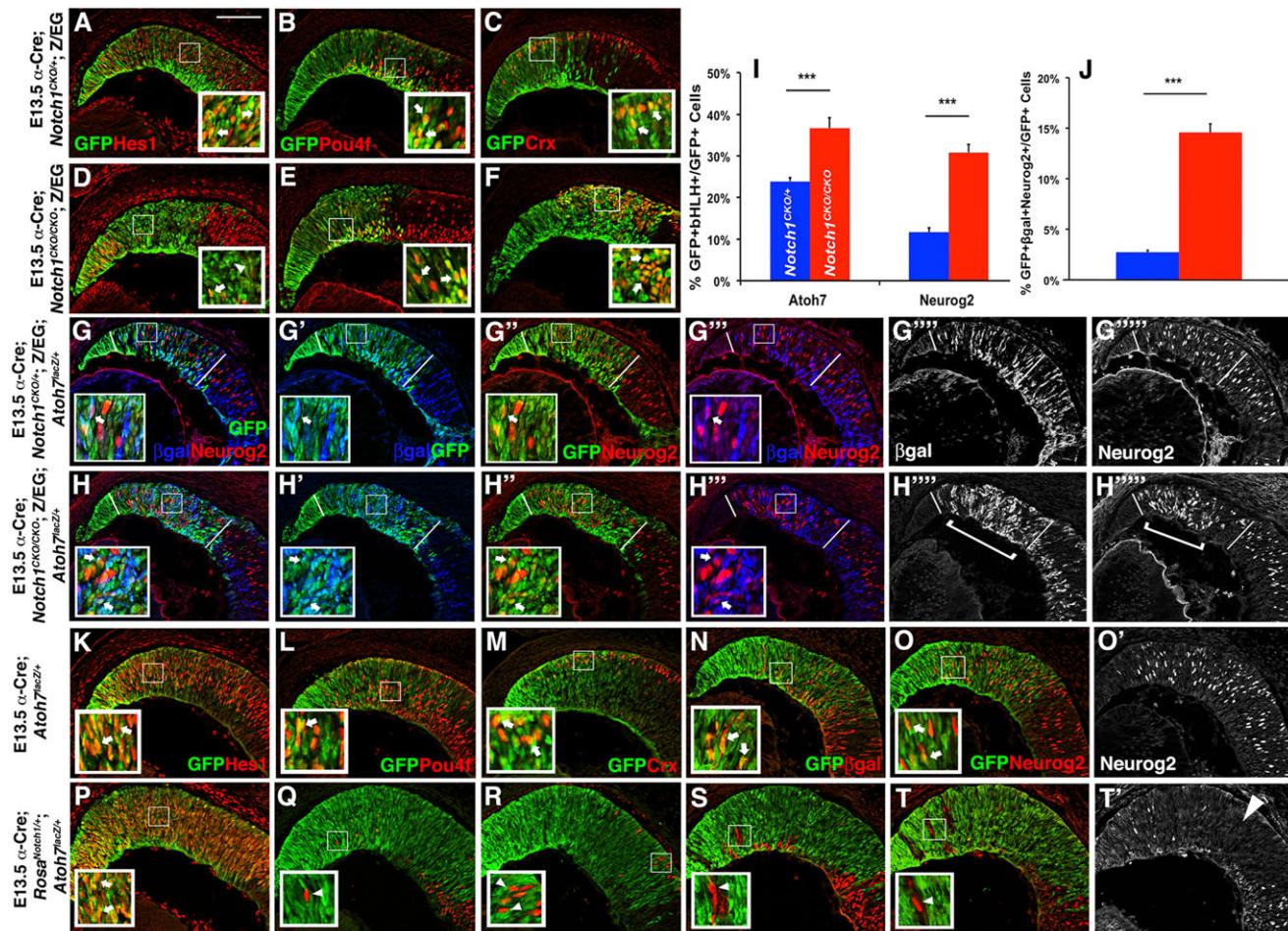


**Fig. 3. Hes1 does not regulate Neurog2 in the prenatal retina.** Pou4f<sup>+</sup> RGCs (A,D) are expanded and Crx<sup>+</sup> photoreceptor precursors are reduced (B,E) in E13.5 Hes1<sup>-/-</sup> retinas, consistent with previous reports (Takatsuka et al., 2004; Riesenbergs et al., 2009b). (C,F)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

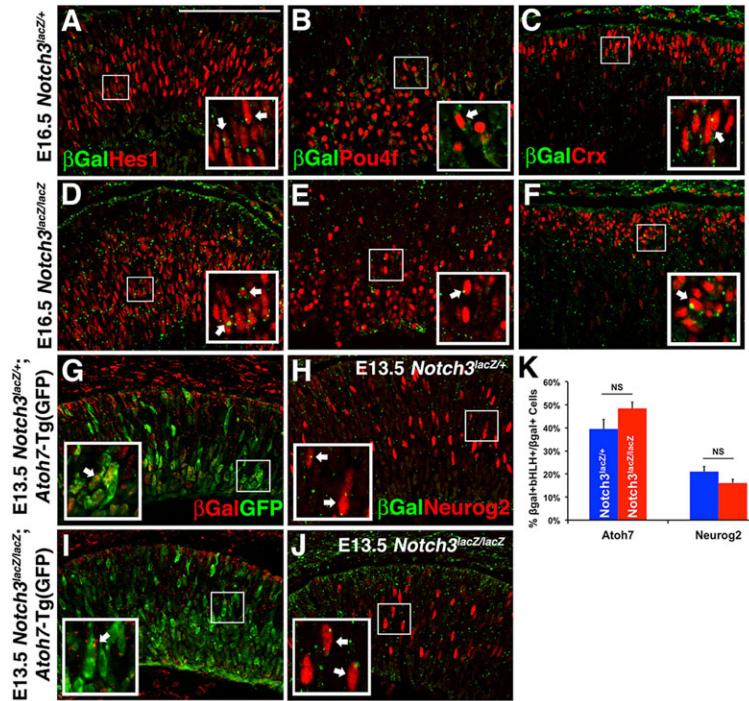
unlikely that *Rbpj* would regulate Neurog2 analogously (Fig. 2H). To verify this, we quantified the percentage of Neurog2<sup>+</sup> cells in E13.5 control and *Hes1*<sup>-/-</sup> retinas, and found no significant difference between genotypes (Fig. 3C,F,G). We conclude that *Hes1* does not regulate *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*<sup>-/-</sup>; *Hes5*<sup>-/-</sup> retinas, and found no difference between genotypes (supplementary material Fig. S1D,H). Additionally, we observed no change in Hes1<sup>+</sup> RPCs, Pou4f<sup>+</sup> RGCs or Crx<sup>+</sup> bipotential precursors in *Hes3*<sup>-/-</sup>; *Hes5*<sup>-/-</sup> retinas, compared with double heterozygous controls (supplementary



**Fig. 4. Notch1 regulates Atoh7<sup>lacZ</sup> and Neurog2 identically.** (A–F) The loss of Notch1 autonomously reduced Hes1<sup>+</sup> RPCs (D) and expanded Crx<sup>+</sup> photoreceptor precursors (F), with no effect on Pou4f<sup>+</sup> RGCs (E) (Jadav et al., 2006b; Yaron et al., 2006). (G–G''') Highly overlapping domains for Atoh7<sup>lacZ</sup> ( $\beta$ -gal) and Neurog2, in  $\alpha$ -Cre; Notch1<sup>CKO/+</sup>; Z/EG; Atoh7<sup>lacZ/+</sup> retinas. (H–J) In  $\alpha$ -Cre; Notch1<sup>CKO/+</sup>; Z/EG; Atoh7<sup>lacZ/+</sup> retinas, however (H–H'''), the Atoh7<sup>lacZ</sup>- and Neurog2-expressing cells are autonomously increased (H''', H''''', brackets, quantified in I,J). (K–T') Notch1 ICD overexpression induced an autonomous increase of Hes1<sup>+</sup> RPCs, and nearly abolished Pou4f<sup>+</sup> RGCs (P) and Crx<sup>+</sup> photoreceptor precursors (R). Both  $\beta$ -gal<sup>+</sup> (S) and Neurog2<sup>+</sup> cell populations (T, T') were dramatically reduced (arrowhead). Scale bar: 100  $\mu$ m; insets show boxed areas at higher magnification. Arrows indicate  $n \geq 3$  embryos per genotype; errors bars show s.e.m.; \*\*\* $P \leq 0.001$ .



**Fig. 5. Notch3 is not required for Atoh7 and Neurog2 retinal expression.** (A–F) At E16.5, the normal and germline mutant Notch3 lineages ( $\beta$ -gal+ vesicles) contain Hes1+ RPCs (A,D), Pou4f+ RGCs (B,E) and Crx+ photoreceptor precursors (C,F). To monitor Atoh7 expression in Notch3<sup>lacZ/+</sup> (G) and Notch3<sup>lacZ/lacZ</sup> (I) retinas, an Atoh7<sup>GFP</sup> Tg was crossed into the Notch3 background, while anti-Neurog2 labeling identified the Neurog2+ population. (G–K) Loss of Notch3 had no cell-autonomous effect on Atoh7-GFP+ or Neurog2+ cells, quantified in K. Scale bar: 100  $\mu$ m; boxed areas are at higher magnification in insets; arrows indicate  $\beta$ -gal+marker+ cells.  $n \geq 3$  embryos per age and genotype, error bars show s.e.m.

material Fig. S1A–C,E–G). Therefore, *Hes3* and *Hes5* also do not regulate Neurog2 expression in this context.

#### Loss of either Jag1 or Dll1 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* (*Jag1*), *Deltalike1* (*Dll1*) and *Deltalike4* (*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,  $\alpha$ -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*<sup>lacZ</sup> expression was unaffected in  $\alpha$ -Cre; *Dll1*<sup>CKO/+</sup>; Z/EG; *Atoh7*<sup>lacZ/+</sup> control and  $\alpha$ -Cre; *Dll1*<sup>CKO/CKO</sup>; Z/EG; *Atoh7*<sup>lacZ/+</sup> mutant eyes (supplementary material Fig. S3G–I). Additionally, the proportion and patterning of Neurog2+GFP+ and Neurog2+ $\beta$ gal+GFP+ cells were unchanged in the absence of *Dll1* (supplementary material Fig. S3G–J). 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; Riesenbergs et al., 2009b). 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  $\alpha$ -Cre; *Notch1*<sup>CKO/CKO</sup> distal retinal cells, suggesting that *Notch1* may be sufficient to suppress bHLH factor expression. To compare changes in bHLH factor expression between *Notch1* and *Rbpj* mutants (Fig. 2), we created E13.5  $\alpha$ -Cre; *Notch1*<sup>CKO/CKO</sup>; Z/EG; *Atoh7*<sup>lacZ/+</sup> and  $\alpha$ -Cre; *Notch1*<sup>CKO/+</sup>; Z/EG; *Atoh7*<sup>lacZ/+</sup> mice, and quantified  $\beta$ -gal+ and/or Neurog2+ cells within the GFP-marked  $\alpha$ -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 *Atoh7*<sup>lacZ/+</sup> ( $\beta$ -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 *Atoh7*<sup>lacZ/+</sup> 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 (*Notch1*<sup>ICD</sup>) 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  $\alpha$ -Cre-induced activation of a flox-stop *Rosa*<sup>Notch1/+</sup> IRES-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; *Rosa*<sup>Notch1/+</sup> retinas, we scrutinized Hes1+ RPCs, Pou4f+ RGCs and Crx+ photoreceptor precursors in  $\alpha$ -Cre; *Rosa*<sup>Notch1/+</sup>; *Atoh7*<sup>lacZ/+</sup> mutant and  $\alpha$ -Cre; *Atoh7*<sup>lacZ/+</sup> 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, *Atoh7*<sup>lacZ</sup> ( $\beta$ -gal+) cells in Fig. 4N,S) and Neurog2 (Fig. 4O,O',T,T') expression were autonomously missing from cells overexpressing the *Notch1*<sup>ICD</sup>. 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.

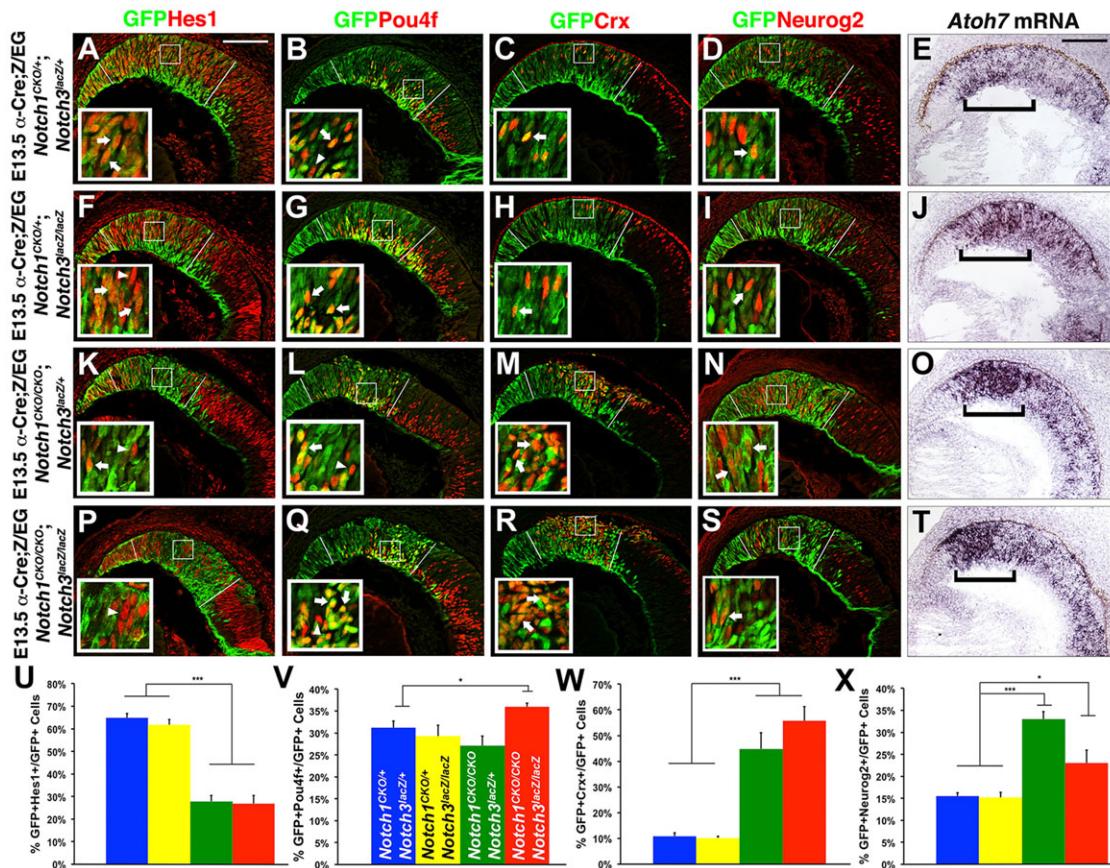
### **Notch3 is not required for Atoh7 or Neurog2 retinal expression**

We also explored *Notch3*-mediated regulation of RGC genesis and Neurog2 distal patterning, using a germline viable *Notch3<sup>lacZ</sup>* mutant mouse, in which a β-gal 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 (Riesenbergs 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 *Atoh7<sup>GFP</sup>* 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 *Notch3<sup>lacZ/+</sup>;Atoh7<sup>GFP</sup>* Tg control and *Notch3<sup>lacZ/lacZ</sup>;Atoh7<sup>GFP</sup>* 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**

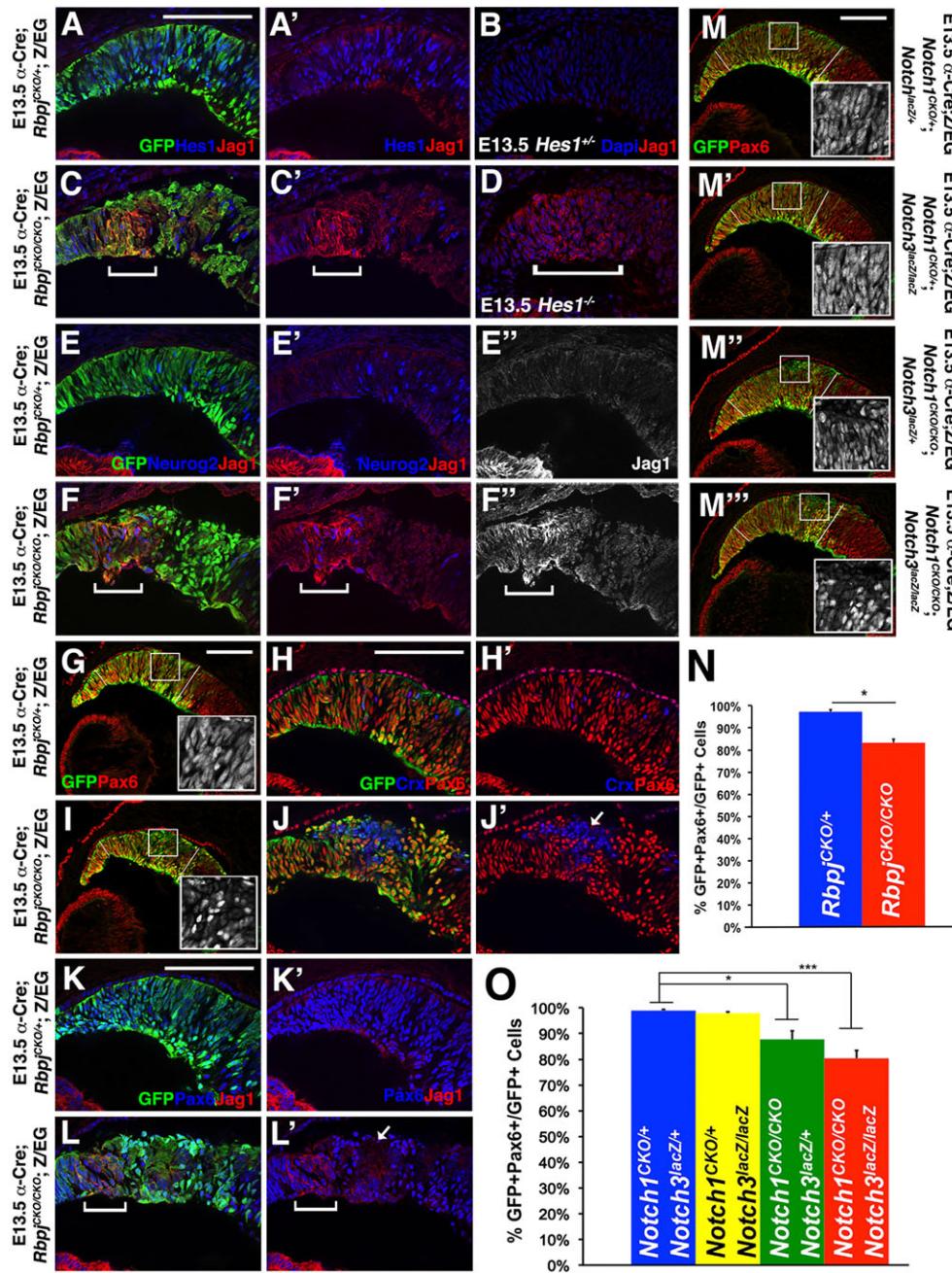
Next, we generated a *Notch1;Notch3* allelic series, consisting of α-Cre; *Notch1<sup>CKO/+</sup>;Z/EG;Notch3<sup>lacZ/+</sup>* (Fig. 6A–E), α-Cre; *Notch1<sup>CKO/+</sup>;Z/EG;Notch3<sup>lacZ/lacZ</sup>* (Fig. 6F–J), α-Cre; *Notch1<sup>CKO/CKO</sup>;Z/EG; Notch3<sup>lacZ/+</sup>* (Fig. 6K–O) and α-Cre; *Notch1<sup>CKO/CKO</sup>;Z/EG;Notch3<sup>lacZ/lacZ</sup>* (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



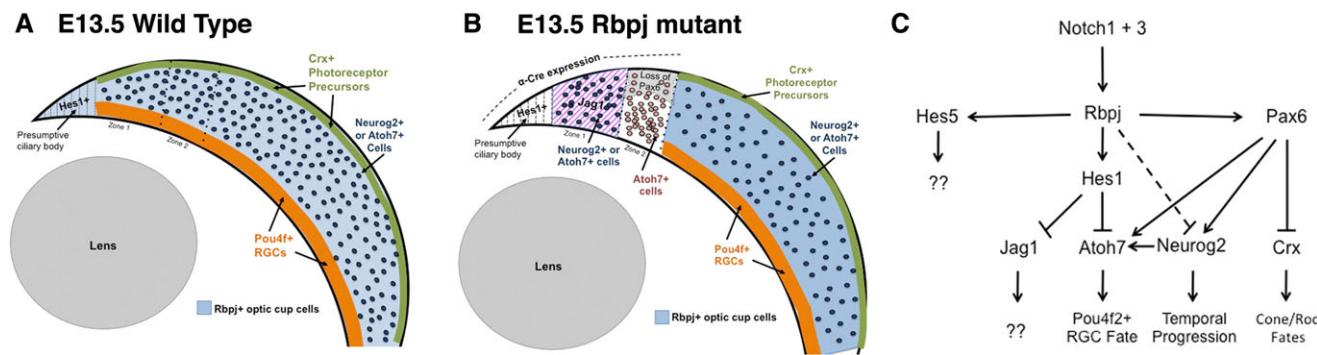
**Fig. 6. Notch1;Notch3 double mutants phenocopy the loss of *Rbpj*.** (A–C,F–H,K–M,P–R) *Notch1;Notch3* allelic series generated by conditionally deleting *Notch1* in the genetic background of *Notch3* germline mutants. Double heterozygous retinas (A–C α-Cre; *Notch1<sup>CKO/+</sup>;Z/EG; Notch3<sup>lacZ/+</sup>*) and those with one wild-type *Notch1* allele (F–H,U–W; α-Cre; *Notch1<sup>CKO/+</sup>;Z/EG; Notch3<sup>lacZ/lacZ</sup>*) had normal distribution of Hes1+ RPCs, Pou4f+ RGCs and Crx+ photoreceptor precursors, quantified in U–W. (K–M,U–W) α-Cre; *Notch1<sup>CKO/CKO</sup>;Z/EG; Notch3<sup>lacZ/+</sup>* retinas had reduced Hes1+ RPCs, increased Crx+ cells, but Pou4f+ RGCs were unaffected (green bars in U–W). (P–R,U–W) *Notch1;Notch3* double mutants had a loss of RPCs and increase in Crx+ cells (red bars in U–W). There was a unique derepression of nascent RGCs, quantified in U–W. (D,E,I,J,N,O,S,T) *Notch1;Notch3* double heterozygotes had no change in Neurog2 protein levels or in *Atoh7* mRNA expression (D,E), neither did retinas with one wild-type allele of *Notch1* (I,J). Both bHLH factors were derepressed when only the wild-type allele of *Notch3* remained (N,O). Receptor double mutants phenocopied *Rbpj* mutants, including Neurog2 mispatterning (S). (X) Quantification of Neurog2+ cells between white lines in D,I,N,S. Scale bars: 100 μm; boxed areas are at higher magnification in insets. Arrows indicate GFP+marker+ cells, arrowheads indicate marker+GFP-negative cells. Brackets in E,J,O,T define retinal zones 1+2 and *Atoh7* mRNA expression. \*P≤0.05, \*\*\*P≤0.001; n≥3 embryos per age and genotype; error bars indicate s.e.m.

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<sup>+</sup> RGCs (Fig. 6V), which phenocopies the loss of *Rbpj* (Fig. 2B,E) (Riesenbergs 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<sup>+</sup> 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.



**Fig. 7. Notch signaling maintains distal retinal patterning.** (A,A',C,C') Hes1 and Jag1 are normally not co-expressed in the E13.5 distal retina. Loss of *Rbpj* derepresses Jag1 in zone 1 (white bracket in C,C'). Ectopic Jag1 in the cell membrane does not colocalize with nuclear Hes1 (C'). (B,D) In E13.5 Hes1 mutants, Jag1 is broadly derepressed (white bracket in D). (E-F') Ectopic Jag1 and Neurog2 are co-expressed within zone 1 of *Rbpj* mutants. (G-J',N). In the absence of *Rbpj*, Pax6 expression is reduced only in zone 2, accompanied by ectopic Crx expression (J,J', arrow). (K-L') Co-labeling for Jag1 and Pax6 clearly shows zone-specific downregulation of Pax6 in *Rbpj* conditional mutants, with ectopic Jag1 (bracket in L,L') distal to Pax6-negative cells (arrow in L'). (M-M'') Significant loss of Pax6+ cells in *α-Cre;Notch1*<sup>CKO/CKO</sup>;Z/EG; *Notch3*<sup>lacZ/+</sup> (M'') and *α-Cre;Notch1*<sup>CKO/CKO</sup>;Z/EG; *Notch3*<sup>lacZ/lacZ</sup> (M''') retinas (quantified in O). Scale bars: 100 μm; boxed areas are at higher magnification in the insets. Brackets in C, F and L indicate distal zone 1, whereas in D the bracket denotes zones 1+2. White lines in G,I,M-M'' delineate area of quantification. \*P<0.05, \*\*\*P<0.001; n≥3 embryos per age and genotype; error bars indicate s.e.m.



**Fig. 8. Notch pathway regulation during early retinal neurogenesis.** (A) Atoh7 and Neurog2 ‘salt-and-pepper’ expression in the E13.5 retina. Pou4f+ RGCs and Crx+ bipotential photoreceptor precursor domains are indicated. (B) *Rbpj*<sup>−/−</sup> zone 1 cells have increased Jag1, Neurog2 and Atoh7<sup>lacZ</sup> expression. Mutant zone 2 has extra Atoh7<sup>lacZ</sup> cells, but fewer Neurog2 cells. Pax6 expression is also lost in cells that then ectopically express Crx. (C) Schematic of the regulatory relationship among the canonical Notch pathway genes *Pax6*, *Atoh7* and *Neurog2*.

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 bracket in 7C'). We also found that E13.5 *Hes1*<sup>−/−</sup> 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 Atoh7<sup>lacZ</sup> (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 *Atoh*, *Neurog* and *Neurod* protein families (Hassan and Bellen, 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 (Jarman 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 *Atoh7* 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; Riesenbergs 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; Riesenbergs 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 (Riesenbergs 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 (Riesenbergs 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 (Bettendorfhausen 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  $\alpha$ -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 one 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.

## MATERIALS AND METHODS

### Animals

Mouse lines used in this study were *Atoh7<sup>lacZ</sup>* (*Atoh7<sup>tm1G1a</sup>*) (Brown et al., 2001) and *Atoh7*-GFP Tg (Math52.1-GFP) mice (Riesenbergs et al., 2009a) both on a CD1 background; *Deltalike1* (*Dll1*) 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>* flox-stop (Murtaugh et al., 2003) mice both on a mixed 129/BL6 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  $\alpha$ -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  $\beta$ -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  $\mu$ m cryosections analyzed. Primary antibodies used were: rabbit anti- $\beta$ -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; 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-2ZRB2). Secondary antibodies used were conjugated to Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647 or Alexa Fluor 660 (Life Technologies, 1:500; A11034, A11037, A21074, A11058, A11039, A21209, A21135, A21241, A21125), or were biotinylated (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  $\beta$ -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  $\mu$ g/lane) and transferred to nitrocellulose as described previously (Prasov et al., 2010). Blots were probed with rabbit anti- $\beta$ -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  $\alpha$ -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*+, *Pou4f*+, *Crx*+, *Pax6*+, *Atoh7<sup>lacZ</sup>* ( $\beta$ gal+), *Neurog2*+ or  $\beta$ gal+*Neurog2*+ E13.5 distal retinal cells were quantified within a 200 $\times$

field containing GFP-marked alpha-Cre lineage cells. The percentage of marker+GFP+/GFP+ cells $\pm$ 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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### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.106245/-DC1>

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