Notch1 is required in newly postmitotic cells to inhibit the rod photoreceptor fate

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SUMMARY
Several models of cell fate determination can be invoked to explain how single retinal progenitor cells (RPCs) produce different cell types in a terminal division. To gain insight into this process, the effects of the removal of a cell fate regulator, Notch1, were studied in newly postmitotic cells using a conditional allele of Notch1 (N1-CKO) in mice. Almost all newly postmitotic N1-CKO cells became rod photoreceptors, whereas wild-type (WT) cells achieved a variety of fates. Single cell profiling of wild-type and N1-CKO retinal cells transitioning from progenitor to differentiated states revealed differential expression of inhibitor of DNA binding factors and Nrarp, a downstream target gene and inhibitor of the Notch signaling pathway, resulted in the overproduction of rod photoreceptors at the expense of Müller glial cells. These data demonstrate that cell fate decisions can be made in newly postmitotic retinal cells, and reveal some of the regulators downstream of Notch1 that influence the choice of rod and non-rod fates. Taken together, our results begin to address how different signals downstream from a common pathway lead to different fate outcomes.

KEY WORDS: Id, Notch, Nrarp, Cell fate, Postmitotic, Retina, Mouse

INTRODUCTION
The retina is an area of the central nervous system that offers excellent accessibility and a well-characterized anatomy. These attributes have allowed investigation of its development, including lineage analyses (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wets and Fraser, 1988) and birthdating studies (Rapaport et al., 2004; Young, 1985a). During development, retinal neurons arise in a conserved, temporal sequence from multipotent, cycling RPCs (reviewed by Livesey and Cepko, 2001). Retinal ganglion cells are born first, followed by horizontal cells, cone photoreceptors, amacrine cells, bipolar cells and Müller glia (Sidman, 1961; Wong and Rapaport, 2009; Young, 1985a).

Previous studies have not established how fate decisions are determined in retinal cells. One possibility is that a RPC might decide the fate of daughter cells and then pass on this decision via determinants. In support of this model, heterochronic mixing experiments showed that embryonic RPCs determine the fate of their amacrine cell daughters (Belliveau and Cepko, 1999). Furthermore, recent studies of chick and zebrafish retinal development revealed RPCs that make horizontal cells exclusively, suggesting the inheritance of the horizontal fate from a committed RPC (Godinho et al., 2007; Rompani and Cepko, 2008). In the cerebral cortex, the laminar fate of cortical cells was shown to be determined in the late S or G2 stage of a terminal cell cycle, and thus in the progenitor cell (McConnell and Kaznowski, 1991). Alternatively, even though newly postmitotic cells receive determinants from their progenitors, they may remain uncommitted. For example, when newly postmitotic cells fated to become rod photoreceptor cells were treated with ciliary neurotrophic factor, they began to express bipolar cell markers (Ezzeddine et al., 1997). This suggests that some postmitotic cells are plastic, or at least can change fate in response to signals after exiting cell cycle. It is possible that cell fate determination can occur at different stages in the continuum from RPC to postmitotic daughter, perhaps with different cell types choosing their fates at different points in this continuum.

During retinal development, the Notch signaling pathway regulates both cell cycle exit and cell fate specification (Jadhav et al., 2006; Yaron et al., 2006). Genetic removal of Notch1, or Notch downstream effectors such as Hes1 and RBP-J, during early and late stages of retinal development resulted in precocious cell cycle exit and the overproduction of cone and rod photoreceptors (Jadhav et al., 2006; Riesenberg et al., 2009; Tomita et al., 1996; Yaron et al., 2006; Zheng et al., 2009). Similar outcomes were observed when retinal explants were treated with DAPT, an inhibitor of the protease γ-secretase, which is required to activate Notch signaling (Nelson et al., 2007). These studies provide evidence that Notch signaling maintains RPCs in a cycling state, and determines cell type identity by inhibiting the photoreceptor fate. However, it is unknown whether the timing of Notch1 cell fate regulation is restricted to RPCs only, or whether postmitotic cells still require signal input to achieve non-rod fates. In order to investigate whether cell fate determination was dependent on Notch1 after cell cycle exit, the effects of removal of a Notch1 conditional allele in newly postmitotic cells were analyzed. In addition, single cell microarrays were performed to investigate gene expression changes that might lead to the acquisition of rod and non-rod fates (Mizeracka et al., 2013). Expression of Id1 and Id3, which may be direct targets of Notch signaling (Meier-Stiegen et al., 2010; Reynaud-Deonauth et al., 2002; Ruzinova and Benezra, 2003; Yokota, 2001), was found to be reduced. These factors have been shown to prevent...
differentiation during neurogenesis in the central nervous system (CNS) (Cai et al., 2000; Lyden et al., 1999). Another gene whose expression levels changed, Nrarf, is a known Notch downstream target gene (Krebs et al., 2001; Pirot et al., 2004) that serves as a feedback inhibitor of the Notch signaling pathway (Lamar et al., 2001; Yun and Bevan, 2003). Functional studies were carried out to assess the role of Id factors and Nrarp in the developing retina. These data begin to address how different fate outcomes arise from the same signaling pathway in RPCs and their newly postmitotic daughter cells.

MATERIALS AND METHODS

Animals

Notch1fl/fl were maintained as homozygotes (Radtke et al., 1999). CD-1 mice were obtained from Charles River Laboratories. All experiments were approved by the Institutional Animal Care and Use Committee at Harvard University.

Misexpression constructs

CAG:Id1 and CAG:Id3 were constructed by PCR amplification from full-length mouse cDNA clones (Matsuda and Cepko, 2004). Each construct was verified by sequencing. The full-length mouse cDNA sequence encoding Nrarp was cloned into the LIA vector at the NotI site (Bao and Cepko, 1997).

Electroporation and infection

In vivo injections of DNA constructs and viruses were performed as previously described, with the exception that an oocyte microinjector (Drummond) and pulled glass pipettes (DuMont/Drummond) were used to deliver 0.2 μl of 5 μg/μl DNA solution or 10^7 CFU/ml viral stock into the subretinal space of the postnatal mouse eye (Matsuda and Cepko, 2004). In vitro electroporations were performed as previously described (Matsuda and Cepko, 2004).

Viruses used include LIA, LIA-Cre (Bao and Cepko, 1997; JadHAV et al., 2006), BAG (Price et al., 1987), LIA-Id1-2A-Cre and LIA-NRARP. DNA constructs used include CAG:GFP, CAG:Cre, CALNL-GFP (Matsuda and Cepko, 2004), CRAbp:dsRed, Hes1:tdTomato (Matsuda and Cepko, 2007), Chx10:tdTomato (Kim et al., 2008) and CAG:Id1, CAG:Id3. Empty vectors were added to maintain equimolar ratios among DNAs that were co-electroporated. This allowed an assessment of the clone size and the types of cells normally found in the retina. Expression levels changed, Nrarf, is a known Notch downstream target gene (Krebs et al., 2001; Pirot et al., 2004) that serves as a feedback inhibitor of the Notch signaling pathway (Lamar et al., 2001; Yun and Bevan, 2003).

Microarray data

The raw and processed Affymetrix data files have been deposited in the NCBI Gene Expression Omnibus, Accession Number GSE35682.

RESULTS

Viral mediated loss of Notch1 reveals activity in newly postmitotic cells

In order to determine whether Notch1 signaling plays a role in cell fate specification in newly postmitotic cells, two independent strategies were undertaken. The first strategy takes advantage of the manner in which gammaretroviruses integrate the viral genome and express viral genes. Upon entering a host cell, viral reverse transcriptase creates only a single copy of the viral genome in the cytoplasm. The viral DNA in the pre-integration complex of a gammaretrovirus, which is the type used for lineage tracing, cannot penetrate the nuclear envelope. Thus, integration of the viral DNA into the host genome, which allows for stable marking of a clone, can occur only after the breakdown of the nuclear envelope during M phase (Roe et al., 1997). As the host genome will be 4N at this time, and there is a single copy of the viral genome, only one of the daughter cells of the initially infected cell will inherit the viral genome. In all subsequent cell cycles, the integrated viral genome will be replicated along with that of the host, and thus all of the progeny of the cell with the integrated viral genome will be marked (Fig. 1A,B). Clones can consist of one to thousands of cells following infection of the retina at various times (Fekete et al., 1994; Fields-Berry et al., 1992; Rompani and Cepko, 2008; Turner and Cepko, 1987; Turner et al., 1990). We focused our analysis on one-cell clones, in which viral transgene expression would initiate in a cell that exited the cell cycle, after being generated by the initially infected cell (Fig. 1B).

We wanted to remove Notch1 at a time point when the majority of clones produced normally would be one-cell clones. Proliferation wanes in the postnatal rodent retina (Alexiades and Cepko, 1996; Rapaport et al., 2004; Young, 1985a), and many small or one-cell clones have been observed following viral infection at these time points (Fields-Berry et al., 1992; Rompani and Cepko, 2008; Turner and Cepko, 1987; Turner et al., 1990). In order to deplete postmitotic cells of Notch1, postnatal day 3 (P3) was chosen as a time point for infection of Notch1fl/fl mice with a retrovirus encoding Cre and alkaline phosphatase. As a control, BAG, a virus encoding lacZ, but not Cre, was delivered at the same time to the same retina. This allowed an assessment of the clone size and the types of cells normally produced by P3 RPCs. Co-infection was unlikely, as the infection rates of both BAG and LIA-Cre were low.

BAG and LIA-Cre retroviruses were co-injected in vivo into the subretinal space of P3 Notch1fl/fl retinas. After the completion of retinal development (P21 or later), retinas were processed via

Trizol (Invitrogen) and stored at −80°C. Phenol-chloroform extractions were performed to isolate total RNA. cDNA was generated using Accurase High Fidelity (Agilent Technologies) according to manufacturer’s guidelines. Semi-quantitative real-time PCR was performed and gene expression was normalized according to actin expression in each sample. Primers used included: actin, ccaactggagacagctgagaa and tgaacgaggctatacagcggc; Id1, acatggacggtgctactca and tgggtcggcactgactc; Id3, actcagttagcugtggta, tcaagtggccaagctctct.

Statistical methods

For each condition, three or more retinas were analyzed. Data were grouped together according to category (i.e. percentage of electroporated cells of a particular cell type). A Student’s two-tailed t-test was used to compare differences between control and experimental values for statistical significance.
histochemical staining to detect BAG and LIA-Cre clones (Fig. 1C, schematized in D). As predicted by previous studies of retinal proliferation (Young, 1985a), 88.6±1.7% of BAG clones comprised only one cell. These data demonstrate that the vast majority of cells that integrated the viral genome did not re-enter the cell cycle, i.e. were in the process of exiting or were postmitotic when they initiated expression of viral genes (Fig. 1E). Among the one-cell BAG clones, 69.6±1.5% of cells were rods, 21.7±1.0% were bipolar cells and 8.7±1.3% were Müller glial cells (Fig. 1C,F). By contrast, the one-cell clones derived from infection with LIA-Cre resulted in 96.6±0.8% rods, 3.4±0.8% bipolar cells and 0±0% Müller glial cells (Fig. 1C,F). There also was an increase in the frequency of one-cell clones, to an average of 94.3±0.8% when compared with 88.6±1.7% (Fig. 1E). This is likely due to the loss of Notch1 in cells that would normally re-enter the cell cycle to produce two-cell clones. Although this effect on cell cycle likely occurred in RPCs, the number of RPCs affected was too small to create the increase in rods among the one-cell LIA-Cre clones. The overproduction of rods at the expense of other cell types is consistent with Notch1 being required for the acquisition of non-rod fates in newly postmitotic cells.

**Loss of Notch1 function in newly postmitotic electroporated N1-CKO cells**

An independent approach was undertaken to assess the role of Notch1 signaling in newly postmitotic cells. Newly postmitotic cells were identified among cells that had been electroporated with a plasmid encoding GFP, but which had not undergone an S-phase. This was accomplished by labeling with EdU, a thymidine analog that is incorporated during S phase (Salic and Mitchison, 2008). A plasmid encoding CAG:Cre, which uses the broadly active promoter CAG to drive Cre expression, and a plasmid, CALNL-GFP, which uses CAG to drive expression of a Cre-responsive GFP reporter (Matsuda and Cepko, 2007), were co-electroporated in vivo into wild-type and Notch1 fl/fl retinas at P1. Mitotic cells were labeled with three injections of EdU: immediately after electroporation, 8 hours after electroporation and 24 hours after electroporation. These EdU injections were timed according to the lengths of the cell cycle phases (16 hours for S, minimum of 2.6 hours for G2, 8.5 hours for G1 and 2.5 hours for M) and the overall cell cycle length at P1 (~30 hours) (Young, 1985b) to ensure that all cycling cells were labeled (Fig. 2A,B,C/H11032). As previous work suggested that mitotic cells and exiting cells are preferentially electroporated (Matsuda and Cepko, 2004), GFP+ EdU– cells were interpreted as...
cells that were near the scleral surface where DNA was injected, but were exiting or had recently exited cell cycle, and thus did not take up the EdU label. Retinas were harvested after P14 and the fate of GFP+ EdU– cells was assessed by location in the outer nuclear layer (where only photoreceptors reside) versus inner nuclear layer (where interneurons and Müller glia reside) (Fig. 2B,B′,B″,C,C′,C″). The majority, 90±3.2%, of GFP+ EdU– cells became photoreceptors in Notch1fl/fl retinas when compared with 69±3.0% in wild-type retinas, providing evidence that postmitotic precursors that lost Notch1 became rod photoreceptors at the expense of other cell types (Fig. 2D). These results are in accordance with the viral Cre experiments described above. In addition, this cell fate change did not appear to be due to excessive cell death, as N1-CKO retinas did not show significantly more TUNEL+ cells as compared with wild-type retinas (9.0±4.2 cells per 300×300 μm² electroporated area in N1-CKO versus 9.2±5.5 in wild type).

**Id1 and Id3 expression is reduced in the absence of Notch1**

Examination of microarray data from N1-CKO and wild-type cells transitioning from RPCs to newly postmitotic cells led to the discovery that Id1 and Id3 were almost completely absent in N1-CKO cells (Fig. 3A) (Mizeracka et al., 2013). This was confirmed by a semi-quantitative PCR assay on cDNA made from populations of N1-CKO and wild-type cells. Retinas of Notch1fl/fl P0 pups were electroporated in vivo at P1 with CAG:Cre and a Cre-responsive GFP reporter, CAG:CALNL-GFP. In order to assess the fate of electroporated newly postmitotic cells, all cells that underwent an S phase during or after the electroporation were labeled with EdU. Based on the length of the cell cycle (~30 hours) and S phase (~16 hours) (Young, 1985b), three EdU injections were performed. The first injection was performed at the time of electroporation to label cells in S phase (indicated by magenta nuclei). The second injection was 8 hours after electroporation to label any electroporated cells that had progressed into S phase. The third injection was 24 hours after electroporation to label any cells that had been in G2 at the time of electroporation and had re-entered S phase.

**Fig. 2. Depletion of Notch signaling in newly postmitotic cells.** (A) Wild-type and Notch1fl/fl retinas were electroporated in vivo at P1 with CAG:Cre and a Cre-responsive GFP reporter, CAG:CALNL-GFP. In order to assess the fate of electroporated newly postmitotic cells, all cells that underwent an S phase during or after the electroporation were labeled with EdU. Based on the length of the cell cycle (~30 hours) and S phase (~16 hours) (Young, 1985b), three EdU injections were performed. The first injection was performed at the time of electroporation to label cells in S phase (indicated by magenta nuclei). The second injection was 8 hours after electroporation to label any electroporated cells that had progressed into S phase. The third injection was 24 hours after electroporation to label any cells that had been in G2 at the time of electroporation and had re-entered S phase.

(B-C″) Electroporated cells that were not labeled by EdU, and thus had become postmitotic after electroporation, were identified as GFP+ EdU–. Retinas were harvested after P14, sectioned and stained for GFP (B,C), EdU (B,B′,C,C′) and DAPI. Examples of GFP+ EdU– cells are shown at higher magnification in insets. (B–C″) The fate of GFP+ EdU– cells was assessed by location either in the outer nuclear layer (rod photoreceptors) or in the inner nuclear layer (interneurons and Müller glia). (D) Percentage of GFP+ EdU– cells found in the ONL for wild-type or N1-CKO conditions. Scale bar: 50 μm. n=3 retinas per condition. **P<0.01. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

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Detection of Id1 and Id3 RNA by in situ hybridization showed that these genes are expressed throughout the progenitor layer at P1, with expression becoming more restricted to the inner neuroblastic layer, where newly postmitotic amacrine cells are found, and to the ganglion cell layer a few days later (supplementary material Fig. S1A,B,D,E). At P5, when proliferation has almost ceased in the central retina (Young, 1985a), Id1 and Id3 expression was localized to the inner nuclear layer, most likely in newly exited cells that are becoming Müller glial cells (supplementary material Fig. S1C,F). To confirm that Id factors are expressed in newly postmitotic cells, mitotic cells were labeled by three successive EdU injections. The EdU-labeled retinas were stained by anti-Id3 and examined for EdU and Id3 signals. Id3 staining was detected in both...
EdU+ and EdU– cells, demonstrating that this protein is expressed in both mitotic and postmitotic populations (supplementary material Fig. S2).

Functional analysis of Id1 and Id3 in the developing WT retina

In order to better understand Id function, retinas of wild-type P0 pups were electroporated in vivo with plasmids expressing the Id genes (CAG:Id1, CAG:Id3 or both plasmids) along with CAG:GFP. Retinas were harvested after P14, and the fates of the electroporated cells were assessed by morphology and molecular markers. Misexpression of CAG:Id1 and CAG:Id3 led to the overproduction of cells that exhibited features of both RPCs and Müller glial cells, when compared with wild-type control retinas (Fig. 4A-C). First, the morphology of the induced cells resembled that of RPCs or Müller glial cells, in that their cell bodies were located in the INL, with long processes that extended both into the photoreceptor layer and the GCL (Fig. 4B, schematized in C). Furthermore, these cells were positive for a marker of both RPCs and Müller glial cells, consistent with a reduction in RPC/Müller glial cells (Fig. 4F). Rescue of Notch1 loss-of-function phenotype by Id1 and Id3

Id1 and Id3 RNA levels were significantly lower in the absence of Notch1 (Jadhav et al., 2006), the Notch1fl/fl allele (Jadhav et al., 2005), the Notch1 floxed allele (Jadhav et al., 2006), and the Notch1–/– allele (Jadhav et al., 2006). The Affymetrix signal levels for Id1 and Id3 in each single cell (Fig. 3B). Notch1fl/fl retinas were electroporated at P0 in vitro with CAG:GFP to mark wild-type cells, or with CAG:Cre and CALNL-GFP, a Cre-responsive reporter, to mark N1-CKO cells. Electroporated retinas were cultured for 3 days and dissociated to single cells. GFP+ cells from wild-type or N1-CKO conditions were collected by FACS and used to prepare cDNA. Samples were subjected to semi-quantitative real-time PCR to detect differences in expression of Id1 and Id3 between N1-CKO and wild-type cells. All expression values were normalized to actin expression levels in each sample. *P<0.05. (C) Wild-type retinas were electroporated at P1 in vitro with CAG:GFP for controls, or with CAG:GFP and CAG:Notch1, and harvested for qPCR analysis 14 hours later. Differences in expression of Id1 and Id3 between wild-type and NICD cells, normalized to actin. n=3 retinas per condition. *P<0.05.

Two RNAi constructs, targeting either Id1 or Id3, were individually tested for efficacy (Fig. 4E), and then electroporated together with CAG:GFP and Hes1:tdTomato at P0 (Fig. 4F). For controls, GAPDH RNAi, which does not have an effect on retinal development (Matsuda and Cepko, 2004), was co-electroporated with CAG:GFP and Hes1:tdTomato. Co-expression of Id1 and Id3, along with CAG:GFP, and Hes1:tdTomato resulted in a robust increase of marker-positive cells, by 104.1%, when compared with wild type (Fig. 4D).
Notch1 inhibits rod fate

**Fig. 4. Misexpression of Id1 and Id3 in the wild-type postnatal retina.** (A,B) Wild-type retinas were electroporated in vivo at P0 with CAG:GFP for controls or CAG:GFP, CAG:Id1 and CAG:Id3. The fates of electroporated cells were analyzed in the mature retina (after P14) by assessment of their location and morphology in the retinal layers. Electroporation of CAG:GFP alone (WT) or CAG:GFP, CAG:Id1 and CAG:Id3 (Id1+Id3) labeled GFP+ photoreceptors, interneurons and Müller glial cells. Immunohistochemistry for p27 (red)-labeled Müller glial cells (arrowheads demarcate examples). Scale bar: 50 μm. (C) Schematic of cell types marked. (D) Combinations of plasmids encoding CAG:GFP, cell type-specific reporters (Cralbp:dsRed to mark Müller glial cells or Hes1:tdTomato to mark RPCs/Müller glial cells), and Id factors were co-electroporated in vitro into P0 wild-type retinas. Retinas were cultured for 11-12 days and dissociated to single cells. FACS was used to determine the percentage of GFP+ cells that were dsRed+ or tdTomato+ for each condition. Fold difference is the percentage of GFP+ marker+ cells induced by Id1+Id3 misexpression relative to wild type. n=5 retinas per condition. *P<0.05; n.s., P>0.05. (E) Wild-type retinas were electroporated at P0 in vitro with CAG:GFP and GAPDH:RNAi for controls, CAG:GFP and Id1 RNAi or CAG:GFP and Id3 RNAi. Retinas were harvested for qPCR analysis 3 days later to detect differences in expression of Id1 and Id3, normalized to actin expression. n=2 retinas per condition. *P<0.05. (F) Wild-type retinas were electroporated in vitro at P0 with CAG:GFP, Hes1:tdTomato, and GAPDH RNAi or Id1+3 RNAi. Retinas were cultured for 11-12 days and dissociated to single cells. FACS was used to determine the percentage of GFP+ cells that were tdTomato+ for each condition. Fold difference is the percentage of GFP+Hes1:tdTomato+ cells in Id1+3 RNAi conditions relative to GAPDH RNAi. n=5 retinas per condition. *P<0.05. r, rod; bp, bipolar cell; Mg, Müller glial cell; ac, amacrine cell.

As not all bipolar and Müller glial cells are labeled by the reporters used above, and to further assess the degree of maturation of rescued cells, a viral approach was undertaken to determine whether Id1 could rescue the cell fates lost when Notch1 was deleted in vivo. Because virally labeled cells can be identified readily by location and morphology, we found that this method was the most unambiguous for assessing retinal cell fates. Furthermore, we assessed whether the rescue occurred in postmitotic cells, as one-cell clones were assayed (as in experiments shown in Fig. 1). A retroviral vector that expressed three genes, Id1, Cre, and AP (Id1-2A-Cre-IRES-AP) was constructed (Fig. 5H). This virus was injected in vivo into the subretinal space of P3 Notch1ββ pups and retinas were harvested after retinal maturation (P21 or later). LIA-Id1-2A-Cre-IRES-AP-infected cells were detected by histochemical staining for AP activity. Quantification of the identity of cells in one-cell clones showed that Id1 could provide partial and statistically significant rescue of both bipolar cells and Müller glia in postmitotic cells (Fig. 5I).
Analysis of Nrarp function in the developing retina

Similar to Id1 and Id3, Nrarp RNA was reduced in N1-CKO cells assayed by microarray (Mizeracka et al., 2013). Nrarp expression was detected by in situ hybridization in the postnatal retina in areas where both mitotic and postmitotic cells are located (supplementary material Fig. S1G-I). To examine Nrarp function, the FACS assay described above was used to determine whether Nrarp misexpression affected the Hes1:tdTomato reporter. Overexpression of Nrarp resulted in a 72.5% decrease in Hes1 reporter expression, when compared with the reporter in wild type (Fig. 6A). These results suggest that Nrarp can inhibit Notch signaling in the developing retina, as has been shown in other developmental systems (Krebs et al., 2012; Yun and Bevan, 2003).

The role of Nrarp in specifying cell fates during postnatal retinal development was examined. Viruses expressing Nrarp and AP (LIA-Nrarp) or expressing AP alone (LIA) were injected separately into the subretinal space of P3 wild-type pups. Retinas were harvested after retinal maturation (P21 or later) and clonal composition was examined. Again, only one-cell clones were examined in order to
assess Nrarp function in postmitotic cells. Misexpression of Nrarp led to the increase of rod photoreceptors, no change in bipolar cells, a slight decrease in amacrine cells and a loss of Müller glial cells (Fig. 6B). These results provide evidence that Nrarp inhibits the production of Müller glial cells in postmitotic cells.

**DISCUSSION**

Numerous studies have determined a role for Notch signaling in regulating neural cell diversity in a variety of developmental systems (Cau and Blader, 2009; Yamamoto et al., 2006). Our study shows that Notch signaling in newly postmitotic cells is required for acquisition of non-rod fates. In the developing retina, diversity may already exist at the progenitor cell level, with molecularly distinct RPCs producing restricted types of postmitotic progeny that respond differentially to Notch signaling. This is similar to ganglion mother cells (GMCs) in the fly ventral nerve cord, which often undergo terminal divisions to give rise to two different cell types in a Notch-dependent manner (Skeath and Doe, 1998; Spana and Doe, 1996; Truman et al., 2010). Recently, we have identified RPCs that express Olig2 and behave like GMCs, in that they undergo terminal divisions and produce specific fates, either two rods or a rod and an amacrine cell in the postnatal period (Hafler et al., 2012). As both rod and amacrine fates are produced under N1-CKO conditions (Jadhav et al., 2006, this study), it is likely that the fates of the progeny of Olig2-expressing RPCs are Notch1 independent, as rods and amacrines are produced under N1-CKO conditions. Conversely, Olig2-negative RPCs divide to give rise to a rod and a bipolar cell or a rod and Müller glial cell. The progeny of these divisions are Notch1 dependent, as loss of Notch1, even after cell cycle exit, results in a loss of bipolar and Müller glial fates. We speculate that Notch signaling induces high levels of Id1 and Id3, which play a role in specifying the Müller glial fate. Conversely, high levels of Nrarp expression, an inhibitor of Notch signaling, promote the formation of rod photoreceptors and a loss of Müller glial cells. Commitment to the bipolar fate is proposed to require intermediate levels of Notch1 signaling. Mg, Müller glial cell; ac, amacrine cell.

Fig. 6. Analysis of Nrarp function in the developing retina. (A) Wild-type retinas were electroporated in vitro at P0 with CAG:GFP for controls, or CAG:Nrp and CAG:Narp, along with the Hes1:tdTomato reporter. Retinas were cultured for 3 days and dissociated to single cells. FACS was used to determine the percentage of GFP+ cells that were tdTomato+ for each condition. Fold difference is the percentage of GFP+marker+ cells induced by Nrarp misexpression relative to wild type. n=3 retinas per condition. n.s., P>0.05. (B) Wild-type retinas were infected in vivo at P3 with LIA and LIA-Nrar, and the fate of single cells was assessed after P21 by histochemical staining. Quantification of cell types found in one-cell clones infected with LIA or LIA-Nrar. n=3 retinas per condition, 718 LIA clones and 672 LIA-Nrar clones were scored. **P<0.01.

Fig. 7. A model of Notch regulation of cell fate decisions in the postnatal retina. Removal of Notch1 from RPCs or their postmitotic progeny results in almost all cells achieving the rod fate at the expense of bipolar and Müller glial cells (this study) (Jadhav et al., 2006; Nelson et al., 2007; Yaron et al., 2006). Postnatal RPCs frequently divide to give rise to two postmitotic cells (Turner and Cepko, 1987). These two daughter cells commonly consist of two rods, as well as a rod and an amacrine cell, a rod and a bipolar cell, and a rod and a Müller glial cell. Previous work has determined that Olig2+ RPCs divide to produce two rods or a rod and an amacrine in a terminal division (Hafler et al., 2012). We hypothesize that the progeny of postnatal Olig2-positive RPCs are Notch1 independent, as rods and amacrines are produced under N1-CKO conditions. Conversely, Olig2-negative RPCs divide to give rise to a rod and a bipolar cell or a rod and Müller glial cell. The progeny of these divisions are Notch1 dependent, as loss of Notch1, even after cell cycle exit, results in a loss of bipolar and Müller glial fates. We speculate that Notch signaling induces high levels of Id1 and Id3, which play a role in specifying the Müller glial fate. Conversely, high levels of Nrarp expression, an inhibitor of Notch signaling, promote the formation of rod photoreceptors and a loss of Müller glial cells. Commitment to the bipolar fate is proposed to require intermediate levels of Notch1 signaling. Mg, Müller glial cell; ac, amacrine cell.
The existence of distinct postnatal RPCs and progeny is consistent with the observation that not all cells respond to the addition of Id factors and Nrarp. The majority of cells take on the rod fate even when Id1 and Id3 are misexpressed. Differences in the intrinsic state of responding cells probably explain an intriguing aspect of development, which is that many common signaling pathways produce distinct effects at different times and places. Here, it appears that even though most RPCs express Notch and its downstream regulators (Trimarchi et al., 2008; Mizeracka et al., 2013), only a subset is responsive to these regulators, and they translate this responsiveness to varying extents.

**Ids act downstream of Notch to induce the Müller glial fate**
From microarray studies of single wild-type and NI-CKO cells (Mizeracka et al., 2013), we identified Id1 and Id3 as genes that were downregulated in the absence of Notch1. We found that misexpression of these factors in the postnatal wild-type retina resulted in an overproduction of cells that resemble RPC/Müller glial cells. Interestingly, expression of the activated form of the Notch receptor, the NICD, in the postnatal retina resulted in cells that also had characteristics of RPCs and Müller glial cells, with almost all transduced cells taking on this phenotype (Bao and Cepko, 1997; Furukawa et al., 2000). The difference in the penetrance of this effect is likely due to NICD being a more potent regulator of gene expression, and/or only certain types of newly postmitotic cells being sensitive to Id factor expression.

We found that Id factors could partially rescue the Notch loss-of-function phenotype, with the robustness of the rescue dependent upon the assay. The FACS-based assay, which used a readout of Hes1 expression and function phenotype, with the robustness of the rescue dependent upon the assay. The FACS-based assay, which used a readout the activation of the Crlpufs:dsRed reporter, showed that a large number of cells could respond to the addition of the Ids. The viral misexpression assay, which used more stringent morphological criteria for the assessment of fate induction, showed that only a small percentage of cells could take on the Müller glial fate following addition of an Id. Moreover, the viral misexpression assay showed that the induction occurred in newly postmitotic cells and that Ids are sufficient for induction of the Müller glial and bipolar cell fates, even in the absence of other Notch regulated genes. Taken together, these results suggest that Id genes are not only involved in maintaining cells in the progenitor state, but are also directly involved in cell fate specification.

Notch signaling activates expression of Hes family members, which can transcriptionally repress pro-neurogenic bHLH factors (Kageyama et al., 2007). Misexpression of Hes genes can also induce a RPC/Müller glial state and repress the rod and bipolar fates (Furukawa et al., 2000). Similarly, Id factors are also known to maintain cells in an undifferentiated state. They function as dominant negatives by binding pro-neurogenic bHLH transcription factors via a HLH binding domain, thus preventing these factors from activating downstream target genes (Benezra et al., 1990). In addition, Id factors can sustain the expression of Hes1, which also delays the onset of differentiation (Bai et al., 2007). From our microarray studies, we found that NeuroD1, a rod-inducing bHLH transcription factor (Akagi et al., 2004; Cherry et al., 2011; Morrow et al., 1999), was upregulated in the absence of Notch1 (Mizeracka et al., 2013). Potentially, in a postmitotic cell, Notch signal transduction leads to the expression of Hes and Id factors, which then prevent the activity of factors such as NeuroD1 at the level of RNA expression and protein function. We speculate that Id factors only inactivate bHLH transcription factors (and perhaps some non-bHLH transcription factors) that are specific to rod induction.

**Nrrap activity during retinal development**
Nrrap is a known downstream target and inhibitor of Notch signaling (Lamar et al., 2001; Pirot et al., 2004; Yun and Bevan, 2003). As the role of this protein had not been studied in the mammalian CNS, and as it is one of the genes reduced in expression level following removal of Notch1, we analyzed its role in the developing retina. We found that overexpression of Nrrap resulted in reduced expression of a Notch activity reporter, in keeping with its role as a feedback inhibitor of Notch signaling. Consistent with this role, the effects of Nrrap misexpression resulted in induction of rods and inhibition of Müller glial cell production in newly postmitotic cells. These results suggest that under wild-type conditions, Nrrap expression must be low in order to generate Müller glial cells, whereas high expression may be part of the normal process of rod genesis. Nrrap is thus part of a network that includes bHLH, Hes and Id genes, which regulate the number of Müller glia and rods in the postnatal retina.

**Model for Notch activity in the postnatal retina**
Our findings demonstrate a role for Notch1 in specifying cell fate in newly postmitotic cells, similar to its activity described during Drosophila neurogenesis. We have identified factors downstream of Notch that mediate this function. We propose that the cells with the highest levels of Notch activity also express high levels of Hes and Id factors, and take on the fate of Müller glial cells, while those with lower levels take on the bipolar fate. Cells with low or no Notch signal, but high Nrrap expression, take on the rod fate (Fig. 7). Notch signaling in newly postmitotic cells may allow those cells to stay in a plastic state, or give them time to execute the non-rod fate specification program(s) inherited from their progenitor cell.

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Author contributions
K.M. and C.L.C. conceived the project. K.M. and C.R.D. performed the experiments. K.M. and C.L.C. wrote the manuscript.

Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/suppl offering detailed information.

References
Notch1 inhibits rod fate


