Development 133, 1367-1378 (2006) doi:10.1242/dev.02311

Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina

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Notch receptor-mediated cell-cell signaling is known to negatively regulate neurogenesis in both vertebrate and invertebrate species, while being implicated in promoting the acquisition of glial fates. We studied Notch1 function directly during retinal neurogenesis by selective Cre/*loxP*-triggered *Notch1* gene inactivation in peripheral retinal progenitor cells (RPCs) prior to the onset of cell differentiation. Consistent with its previously established role, *Notch1* inactivation led to dramatic alteration in the expression profile of multiple basic helix-loop-helix transcription factors, consequently prompting premature cell-cycle exit and neuronal specification. Surprisingly, however, *Notch1* inactivation led to a striking change in retinal cell composition, with cone-photoreceptor precursors expanding at the expense of other early- as well as late-born cell fates. Intriguingly, the *Notch1*-deficient precursors adhered to the normal chronological sequence of the cone-photoreceptor differentiation program. Together, these findings reveal an unexpected role of Notch signaling in directly controlling neuronal cell-type composition, and suggest a model by which, during normal retinogenesis, Notch1 functions to suppress cone-photoreceptor fate, allowing for the specification of the diversity of retinal cell types.

KEY WORDS: Notch1, Cre/*loxP*, Lineage tracing, Retinal progenitor cells, Photoreceptors, Mouse, Notch1, Retina development

INTRODUCTION

The developing mammalian retina is an excellent model system for studying the molecular events that control neurogenesis in the CNS. The multipotent retinal progenitor cells (RPCs) residing in the inner layer of the optic cup differentiate into the six major classes of retinal neurons, as well as Müller glia, following a defined chronological sequence. Retinal ganglion, cone photoreceptor and horizontal cells are formed first, followed in overlapping phases by amacrine and rod photoreceptor cells, with bipolar and Müller cells appearing last (Young, 1985). Despite vast differences in the total time scale, the relative histogenic birth order of the different retinal cell types is largely conserved among vertebrate species, suggesting that the same principal mechanisms mediate the differentiation program for the different retinal cell types.

Cell-lineage studies in vertebrates have revealed that RPCs are multipotent progenitors, as each progenitor may give rise to each of the retinal cell types (Turner and Cepko, 1987; Wetts and Fraser, 1988). However, heterochronic culturing experiments suggest that the differentiation potential of the progenitors is dynamic and changes as development progresses (Cepko et al., 1996; Livesey and Cepko, 2001; Watanabe and Raff, 1990). It has been proposed that the differentiation competence of the progenitors is determined by a combination of intrinsic and extrinsic factors (Livesey and Cepko, 2001; Marquardt, 2003). The intrinsic signals include a combination of factors, such as bHLH- and homeodomain-containing transcription regulators (Kageyama et al., 1997; Levine et al., 2000; Marquardt, 2003). Studies have indicated that several secreted factors, including sonic hedgehog, GDF11, retinoic acid (RA) and taurine, play a role in cell-type specification in the retina (Kim et al., 2005; Levine et al., 2000; Neumann and Nuesslein-Volhard, 2000). In addition to the influence of secreted factors, another important developmental cue is cell-cell contact-dependent mechanisms, such as those mediated by Notch signaling. The Notch pathway has been shown to function in a variety of processes regulating tissue growth, patterning and morphogenesis in different organisms, including insects and mammals (Artavanis-Tsakonas et al., 1999; Kopan and Cagan, 1997).

The Notch family members are transmembrane receptors with large extracellular and intracellular domains connected by a single membrane-spanning domain (del Amo et al., 1993). The current model for Notch signaling suggests that upon binding of the Notch receptor to its membrane-linked ligands, the receptor is proteolyzed and the released intercellular domain operates as a transcription regulator through interaction with nuclear mediators such as RBP-jk (Lai, 2004). Activation of the Notch receptor upregulates the expression of target genes, such as *Hes1/Hes5* in mammals (Ohtsuka et al., 1999). These genes negatively regulate the expression of proneural bHLH transcription factors that are known to affect cell-fate specification of progenitors (Kageyama et al., 1997). Thus, in cells in which Notch signaling has been initiated, differentiation is generally inhibited.

Current studies on flies and vertebrates suggest that the role of Notch is not uniform but depends on the developmental and cellular context of the tissue (Bray, 1998; Perron and Harris, 2000). In vertebrate neurogenesis, Notch1 and its ligand Delta1 have been implicated in maintaining a pool of uncommitted precursors, while release from Notch1/Delta1 inhibition allows the cells to differentiate (Chitnis et al., 1995; Henrique et al., 1995; Henrique et al., 1997; Perron and Harris, 2000). In addition to this generalized role in preventing the premature depletion of the progenitor pool, several studies suggest that Notch-mediated lateral inhibition might inhibit the acquisition of specific cell fates more selectively, in particular that of the ganglion cells. This was suggested, for example, following antisense treatment of chick retina, which resulted in an increased number of ganglion cells (Austin et al.,

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Accepted 6 February 2006

1995; Waid and McLoon, 1998). Such an effect on cell fate due to Notch1 reduction is more pronounced at early stages and seems to be gradually lost during later stages of retinal development (Silva et al., 2003), indicating that differences in the intrinsic makeup of the progenitors determine their eventual response to Notch1 reduction. The most direct evidence for the role of Notch in cell-type specification in the retina was obtained from a study of mice deficient in one of the Notch1 effectors, Hes1. Inactivation of Hes1 resulted in premature differentiation into rods while bipolar cells failed to survive (Tomita et al., 1996). Other studies, however, support the role of Notch1 in vertebrate gliogenesis. In particular, Notch1 and its effectors, Hes1 and Hes5, seem to promote certain glial fates, such as Müller glia in the retina (Chambers et al., 2001; Dorsky et al., 1995; Furukawa et al., 2000; Gaiano and Fishell, 2002; Hojo et al., 2000; Scheer et al., 2001). By contrast, Notch1 has been found to inhibit gliogenesis in the optic nerve (Wang et al., 1998) and spinal cord (Genoud et al., 2002). These seemingly conflicting observations may reflect a significant influence on Notch1 activity of cell-intrinsic changes that depend on the respective cellular and developmental context.

The direct in vivo study of the role of Notch1 in mice was previously precluded owing to the early embryonic lethality of Notch1-deficient embryos just when neuronal differentiation is initiated (Conlon et al., 1995; Swiatek et al., 1994). Thus, the precise requirement of Notch1 activity during different stages of vertebrate neurogenesis still remains largely elusive. Here we used the Cre/*loxP* approach to inactivate the *Notch1* gene exclusively in retinal progenitor cells (RPCs) prior to the onset of their differentiation. This somatic inactivation resulted in abnormal retinal morphology and a reduction in eye size. Similar to the known activity of Notch signaling in invertebrates, Notch1 inactivation in RPCs resulted in the dramatic downregulation of Hes5 and Hes1, and upregulation of several proneural transcription factors, eventually triggering premature differentiation of the RPCs. Surprisingly, a dramatic increase in the number of cone-photoreceptor precursors was detected in the *Notch1*$^{-}$ retina at the expense of other early- and late-born retinal cell types. These *Notch1*$^{-}$ photoreceptor precursors followed the pattern of cone-photoreceptor differentiation and developed to cone photoreceptors postnatally. Thus, in addition to its well-established role as a generic inhibitor of neuronal differentiation, our results demonstrate an essential requirement for Notch1 activity in facilitating the diversification of retinal cell types by selectively suppressing the acquisition of the cone-photoreceptor cell fate.

MATERIALS AND METHODS

The mouse lines

Two transgenic lines were employed in this study for obtaining somatic inactivation of *Notch1* exclusively in the RPCs: *Notch1*$^{f/f}$ mice and the *α-Cre* transgenic line (Fig. 1) (Radtke et al., 1999; Marquardt et al., 2001). For cell-lineage tracing of Cre expressing cells the *Z/AP* reporter line was employed (Fig. 1) (Lobe et al., 1999).

Histology, immunofluorescent-analysis, BrdU and TUNEL assays

Tissues were fixed for 2 hours or overnight in 4% paraformaldehyde (PFA) at 4°C, dehydrated and embedded in paraffin wax. Paraffin sections (5-10 μm) were stained with Hematoxylin and Eosin (H&E) using standard procedures. Human placental alkaline phosphatase (AP) staining was performed on sections as previously described (Lobe et al., 1999). Immunofluorescent analysis was performed on dewaxed paraffin sections as previously described (Ashery-Padan et al., 2000). The primary antibodies were: rabbit anti-Pax6 (1:1000, Chemicon), goat anti-Brn3b (1:100, Santa Cruz), goat anti-AP (1:100, Santa Cruz), mouse anti-Nf165 (1:500, Hybridoma Bank), rabbit anti-PKCα (1:1000, Santa Cruz), rabbit anti-CycD3 (1:100, Santa Cruz), mouse anti-Syntaxin (1:600, Sigma), rabbit anti-recoverin (1:750, kind gift from C. Koch), rabbit anti-S-opsin (1:1000), mouse anti-rhodopsin (1:1000) (Applebury et al., 2000) and rabbit anti-Hes1 (1:200) (Lee et al., 2005). Secondary antibodies conjugated to rhodamine red-X or Cy2 were from Jackson Immuno Research Laboratories. For detection of cones, peanut agglutinin (PNA) conjugated to fluorescein (1:200, Vector) was employed (Blanks and Johnson, 1983). For detection of cells in the S phase, BrdU (10 μl/g of 14 mg/ml) was injected 1.5 hours before the sacrifice. Antibody staining using mouse anti-BrdU (1:100, Chemicon) was performed as described (Marquardt et al., 2001). TUNEL assay was performed using the in situ Cell Death Detection Kit (Roche). Slides were viewed with an Olympus BX61 fluorescent microscope or laser-scanning confocal microscope CLSM 410 (Zeiss) and images were analyzed using the image analysis system 'AnalySIS'.

The relative proportion of Brn3b, TUNEL, BrdU, Crx, cone-arrestin, Gnat1 and recoverin positive cells was calculated from the total nuclear (DAPI^{+}) area. The areas were measured using the color-threshold function of 'AnalySIS' software on the same predefined region of the peripheral optic cup for the *Notch1*$^{f/f}$*;α-Cre* and the corresponding control eyes. The measurements were conducted on serial sections (~50 μm apart) with well-preserved morphology and that were central based on the detection of the lens. For each eye, an average value for the presented parameter was calculated from all sections. The presented ratio values are the averages obtained from all eyes (n) with the same genotype.

Analysis of Brn3 expression and AP detection on dissociated embryonic retinas

E14 retinas were dissected and incubated for 1 hour at room temperature in dissociation solution (125 mM NaCl, 2 mM KCl, 1.2 mM EDTA, 5 mM HEPES pH 7.4), then triturated to a single-cell suspension and fixed 15 minutes on poly-D-lysine-coated glass slides with 4% PFA. The cells were subjected to immunofluorescent-analysis (as above) using Brn3b specific antibody for the detection of ganglion cells. After documenting Brn3b expression in random fields, AP activity was detected on the same slides as described (Lobe et al., 1999). The AP images were superimposed on the images of the Brn3 antibody staining and the ratio of Brn3b^{+} to AP^{+} cells was calculated.

In situ hybridization

In situ hybridization analysis was performed on dewaxed paraffin sections. Hybridization was conducted overnight at 55°C with digoxigenin-labeled probes (3 μg/ml). The slides were then treated with RNaseA, washed, blocked with 10% normal goat serum (NGS) and incubated with sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (1:2500, Roche) in PBST with 1% NGS overnight at 4°C, washed and incubated in BM Purple (Roche). The probes used in this study were as follows: Notch1 (Schroder and Gossler, 2002), Hes5 (Ohtsuka et al., 1999), Neurod1 (Lee et al., 1995), Ngn2 (Sommer et al., 1996), Math5 (Brown et al., 1998), Mash1 (Lo et al., 1997), Math3 (Akagi et al., 2004), Nr2e3 (Chen et al., 2005), Otx2 (Nishida et al., 2003), Crx (Furukawa et al., 1997) and Thrβ2 (Ng et al., 2001; Wood et al., 1994).

RESULTS

Somatic inactivation of Notch1 in the distal optic cup prior to neuronal cell differentiation

To address the in vivo functions of *Notch1* in the complex spatial-temporal process of retinogenesis, we inactivated the Notch1 receptor exclusively in the RPCs, using the *Notch1*^{f} allele and the *α-Cre* mouse lines (Fig. 1A,B) (Marquardt et al., 2001; Radtke et al., 1999). *Notch1*^{f} has been shown to render Notch1 inactive following Cre-mediated recombination (Fig. 1A) (Radtke et al., 1999), while in the *α-Cre* transgenic line, Cre expression is restricted to the RPCs residing in the temporal and nasal parts of the peripheral optic cup (Kammandel et al., 1999; Marquardt et al., 2001). To trace Cre activity in vivo, we employed the *Z/AP* reporter mouse line (Lobe et al., 1999). In this line, the Cre-mediated recombination enables the expression of the *AP* reporter gene (Fig. 1C, Fig. 2I) (Lobe et al., 1999).

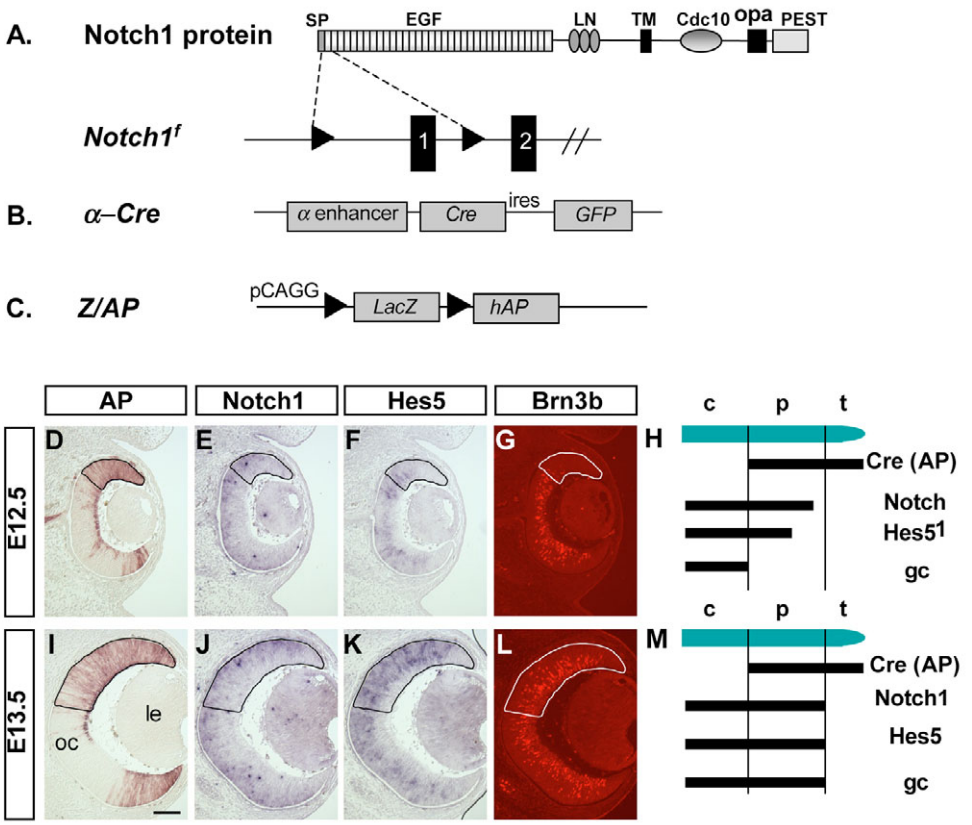

Fig. 1. Notch1 inactivation in retinal progenitor cells. (**A**) Notch1 protein includes a signal peptide (SP), 36 epidermal growth factor-like repeats (EGF), three lin repeats (LN), a transmembrane domain (TM), six Cdc10 ankyrin-like repeats, a polyglutamine stretch (opa), and a proline, serine and threonine stretch (PEST) (del Amo et al., 1993). The *Notch1*^{f} allele includes two *loxP* sites (black arrowheads) around the first exon encoding *Notch1* signal peptide (Radtke et al., 1999). (**B**) *α-Cre* transgene drives the expression of Cre recombinase and a downstream GFP reporter under a *Pax6* α-enhancer in RPCs of the distal retina (Marquardt et al., 2001). (**C**) The *Z/AP* transgene construct used for lineage tracing of Cre-expressing cells. Cre-mediated recombination of the *Z/AP* transgene eliminates the *lacZ* cassette, enabling expression of the reporter gene human placental alkaline phosphatase (*AP*). (**D-L**) Serial sections of E12.5 (D-G) and E13.5 (I-L) *α-Cre;Z/AP* control eyes characterized for AP activity (D,I), *Notch1* (E,J) and *Hes5* (F,K) expression by in situ hybridization, and for Brn3b (G,L) detected by indirect immunofluorescent analysis. (**H,M**) Summary of the expression patterns along the central (c), peripheral (p) and the non-neuronal tip (t) of the optic cup is presented for E12.5 (H) and E13.5 (M). *Notch1* and *Hes5* expression already partially overlaps with AP at E12.5, while Brn3b expression seems to overlap with the AP^{+} domain at E13.5. le, lens; oc, optic cup; gc, ganglion cells. Scale bar: 100 μm.

To define the developmental time window in which *α-Cre* inactivates Notch1, we monitored AP activity (Fig. 1D,I) in *α-Cre;Z/AP* control retina sections at E12.5 (Fig. 1D-G) and E13.5 (Fig. 1I-L). In addition, the expression patterns of *Notch1* (Fig. 1E,J), its downstream target *Hes5* (Fig. 1F,K) and the earliest ganglion cell marker Brn3b (Fig. 1G,L) were monitored on adjacent sections by in situ hybridization (Fig. 1E,F,J,K) or antibody labeling (Fig. 1G,L). The results are summarized schematically (Fig. 1H,M) and demonstrate that at E12.5, *Notch1* (Fig. 1E) and *Hes5* (Fig. 1F) transcripts are already present in most of the optic cup, including a part in the peripheral retina overlapping the domain of *α-Cre* activity (Fig. 1H). At this stage, the expression of *Notch1* and *Hes5* seems to precede the central to peripheral differentiation wave, as Brn3b expression is more central to the *Notch1, Hes5* and AP-expressing cells (Fig. 1G,H). At E13.5 (Fig. 1I-M), *Notch1*, *Hes5* and Brn3b expression overlaps with AP in the peripheral retina, excluding the most peripheral tip of the optic cup where only AP is detected and later gives rise to the non-neuronal ocular tissues. This analysis suggests that the α-Cre-mediated recombination will delete *Notch1*^{f} in the RPCs located in the peripheral optic cup prior to the onset of cell differentiation.

Notch1 inactivation results in microphthalmia and distorted retina morphology

Microphthalmia was observed in the *Notch1*$^{f/f}$*;α-Cre* mice (P60, Fig. 2E). To characterize the morphology of the *Notch1*$^{f/f}$*;α-Cre* eyes, detailed histological analysis was conducted by Hematoxylin-Eosin (H&E) staining of eye sections from control littermate controls (Fig. 2B-D) and *Notch1*$^{f/f}$*;α-Cre* mutants (Fig. 2F-H). In the control eyes at P15, the cellular and synaptic layers of the retina have formed (Fig. 2B). In the *Notch1*$^{f/f}$*;α-Cre* eyes, however, distortion of the retinal morphology, including large rosettes and disruption of the retinal layers, was observed (Fig. 2F), and this aberrant morphology was already prominent at E17.5 (Fig. 2G). Further histological analysis revealed that the rosettes are first apparent at E13.5 in the peripheral retina of *Notch1*$^{f/f}$*;α-Cre;Z/AP* embryos (Fig. 2H). Importantly, we could not detect any noticeable phenotype in the retina of the *Notch1*$^{f/+}$*;α-Cre* and *Notch1*$^{f/+}$*;α-Cre;Z/AP* eyes (Fig. 2A-D,J-M), suggesting that proper Notch1 activity is not susceptible to haploinsufficiency and that the combination with Cre or AP expression alone does not disrupt retinal development.

To characterize the cell-autonomous functions of Notch1, the cells in which Cre was active were identified by monitoring AP activity in control (*Notch1*$^{f/+}$*;α-Cre;Z/AP*) and *Notch1* mutant (*Notch1*$^{f/f}$*;α-Cre;Z/AP*) eyes (Fig. 2I-Q). Corresponding with the early activity of α-Cre in most RPCs of the embryonic peripheral optic cup (E13.5; Fig. 2M), AP was detected in the postnatal eyes in all cellular layers of the peripheral retina (P15, P0; Fig. 2J-L). In the *Notch1*$^{f/f}$*;α-Cre;Z/AP* eyes at E13.5, the distribution of AP was similar to that of the control and seemed to encompass most of the peripheral retina, including the rosettes (Fig. 2Q), suggesting that the rosettes are formed initially from *Notch1*$^{-}$ cells. However, at P0 and later at P15, extensive reduction in the AP^{+} region was detected in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* retina (Fig. 2N-P), as compared with the size of the AP^{+} domain detected in the *Notch1*$^{f/+}$*;α-Cre;Z/AP* (Fig. 2J-L)

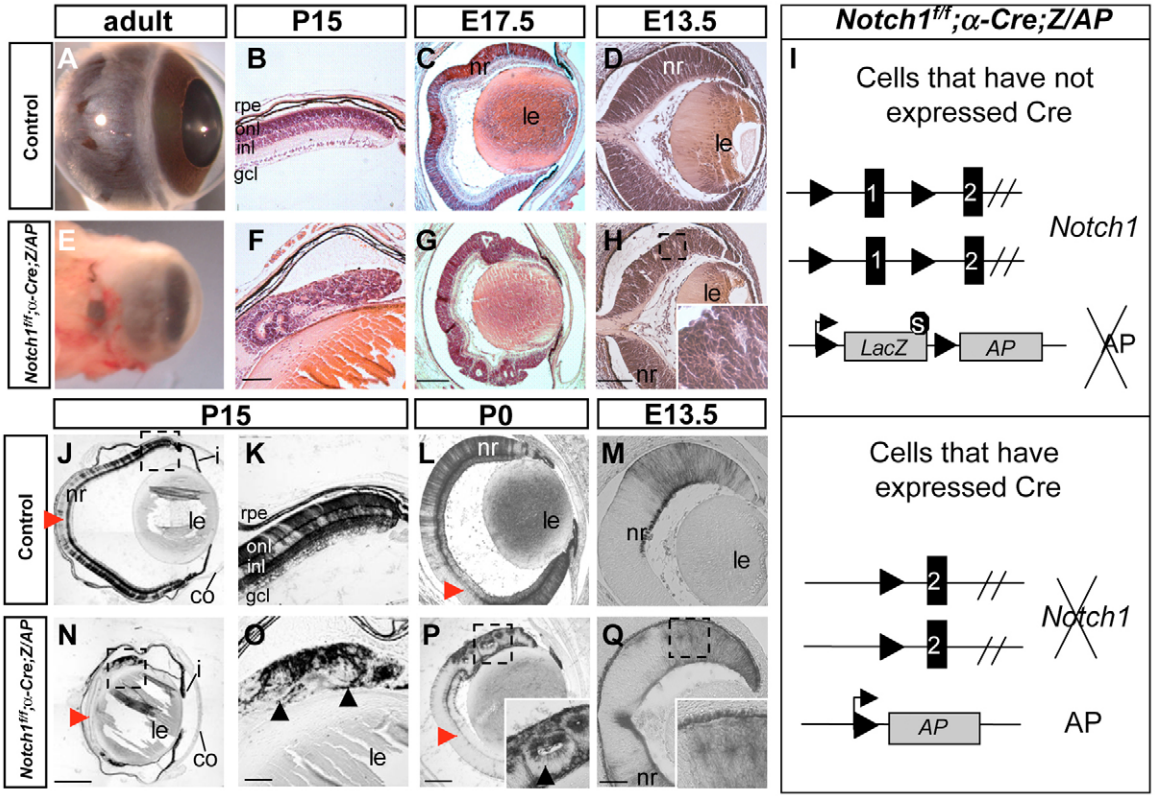

Fig. 2. Microphthalmia and disrupted retinal morphology following Notch1 inactivation in the RPCs. (**A**) Normal eye size is observed in the control, while microphthalmia is evident in the *Notch1*$^{f/f}$*;α-Cre* mice (**E**). (**B-D**,**F-H**) H&E staining of eye sections from P15 (B,F), E17.5 (C,G) and E13.5 (D,H) embryos demonstrating the normal retinal morphology in the control eyes (B-D), while in the *Notch1*$^{f/f}$*;α-Cre* eyes rosettes are detected in the retina (F-H) as early as E13.5 (H). (**I**) Diagram illustrating the lineage tracing of the *Notch1*$^{-}$ cells employing the *Notch1*$^{f/f}$*;α-Cre;Z/AP* mice. In these mice, *Notch1* gene remains intact and AP is not expressed in cells that have not expressed Cre. *Notch1*, however, is deleted and AP activity detected in cells that expressed Cre and in their progeny. (**J-Q**) AP activity was monitored on sections from control (J-M) and *Notch1*$^{f/f}$*;α-Cre;Z/AP* (N-Q) eyes. At P15 (J,K) and P0 (L), AP is detected throughout the peripheral *α-Cre;Z/AP* retina as expected from the early activity of Cre observed in most RPCs in this region (M). In the *Notch1*$^{f/f}$*;α-Cre;Z/AP* P15 eyes, however, AP^{+} (*Notch1*$^{-}$) cells are dramatically reduced in number thus most of the peripheral retina is missing (N,O). The iris maintains its normal structure despite loss of retinal tissue (compare J with N). At P0 (P), fewer AP^{+} cells are detected in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* retina when compared with the wide distribution of AP^{+} cells in the control (L). The rosettes at P0 are composed of a mixture of AP^{+} (*Notch1*$^{-}$) and AP^{-} (*Notch1*$^{+}$) cells. However, at E13.5 (Q), when rosettes first appear in the *Notch1*$^{f/f}$*;α-Cre;Z/AP*, the distribution of AP is similar to its distribution in the controls (M), encompassing most of the peripheral retina and the rosettes seem to be mostly composed of AP^{+} cells (Q, inset). The red arrowheads in J,N,L,P indicate the AP^{+} amacrine precursors that seem to be unaffected following Notch1 inactivation. Control genotypes: *Notch1*$^{f/+}$*;α-Cre* (A-D); *Notch1*$^{f/+}$*;α-Cre;Z/AP* (B-D,J-M). co, cornea; gc, ganglion cells; inl, inner nuclear layer; i, iris; le, lens; nr, neuroretina; onl, outer nuclear layer; rpe, retinal pigmented epithelium. Scale bars: 100 μm in F,H,O,Q; 200 μm in G,P; 500 μm in N.

eyes. Furthermore, the rosettes at P0 seemed to contain a mixture of both AP^{-} and AP^{+} cells (Fig. 2P inset, black arrowhead). Along with the observed reduction of AP^{+} cells, the size of the retina of *Notch1*$^{f/f}$*;α-Cre* embryos was extensively reduced to about 73% of that of control littermates at E15.5 (n=6 for each genotype, P<0.001).

In addition to the AP^{+} cells detected in the peripheral RPCs, we observed AP activity starting from E14.5 in few cells within the central optic cup. These AP^{+} cells are primarily destined to become amacrine cells, according to the pattern of AP activity in the central retina of *α-Cre;Z/AP* at postnatal stages (Fig. 2J,N,L,P, red arrowheads) (Ashery-Padan, 2002). These AP^{+} cells within the central retina were detected in the postnatal *Notch1*$^{f/f}$*;α-Cre*;Z/AP eyes (Fig. 2N, red arrowhead), thus suggesting that Notch1 activity is redundant for the survival and normal morphology of cells already committed to the amacrine cell fate at the time of Notch1 inactivation.

Inactivation of Notch1 alters the expression profile of bHLH transcription factors

Notch1 activity in mammals, as in invertebrates, has been shown to inhibit cell differentiation by upregulation of the E(Spl)-related Hes transcription factors, which in turn repress the expression of proneural bHLH genes (de la Pompa et al., 1997; Hatakeyama and Kageyama, 2004; Ohtsuka et al., 1999). Thus, downregulation of Hes gene expression and a change in the expression profile of proneural bHLH factors is expected to follow Notch1 receptor inactivation.

Cre-mediated recombination of the *Notch1*^{f} allele has been shown to inactivate Notch1 in the immune system and the cerebellum (Fig. 1A) (Lutolf et al., 2002; Radtke et al., 1999). To validate the functional inactivation of Notch1 in the *Notch1*$^{f/f}$*;α-Cre* retina, we monitored the expression of *Hes5* and *Hes1* in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* and control *Notch1*$^{f+}$*;α-Cre;Z/AP* retinas of E13.5 embryos, when the initial morphological abnormality

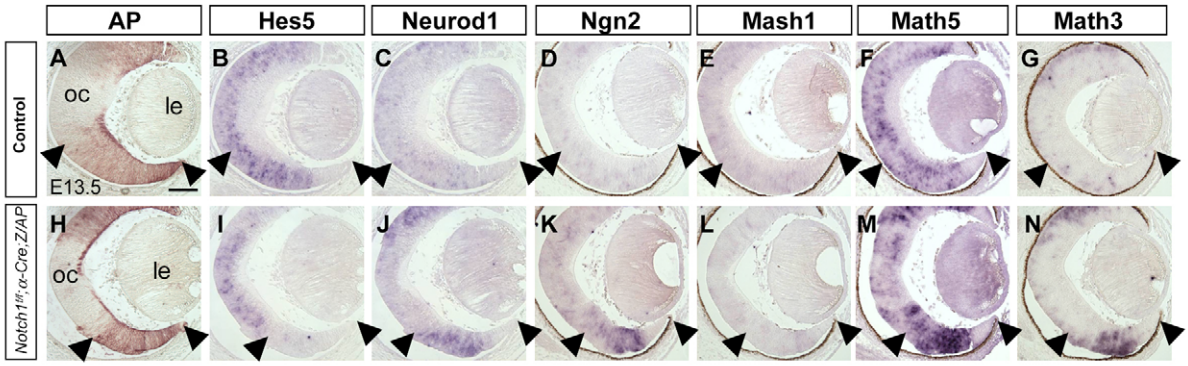

Fig. 3. The expression profile of proneural bHLH genes is altered in the *Notch1*$^{-}$ retina. To visualize the domain of *α-Cre*-mediated recombination, AP activity was monitored on sections from E13.5 *α-Cre;Z/AP* control (**A**) and *Notch1*$^{f/f}$*;α-Cre;Z/AP* mutants (**H**). The black arrowheads indicate the peripheral retina where Cre activity is detected. The expression of *Hes5* (B,I), Neurod1 (C,J), Ngn2 (D,K), Mash1 (E,L), Math5 (F,M) and Math3 (G,N) were determined by in situ hybridization. In control eyes (**B-G**), the normal pattern of bHLH gene expression was observed, while a change in the expression profile of these factors was detected in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* eyes (**I-N**). The expression of Hes5 was reduced (compare I with B), the expression of Neurod1, Ngn2, Math5 and Math3 was enhanced (compare J,K,M,N with C,D,F,G), and the expression of Mash1 appears unchanged (compare L with E) in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* when compared with *α-Cre;Z/AP* controls. le, lens; oc, optic cup. Scale bar: 100 μm.

becomes evident (Fig. 2, see Fig. S1 in the supplementary material). In the *Notch1*$^{f/+}$*;α-Cre;Z/AP* embryos, *Hes5* and *Hes1* expression was detected in the peripheral optic cup overlapping with AP expression (Fig. 3B, see Fig. S1B in the supplementary material). In the peripheral retina of the *Notch1*$^{f/f}$*;α-Cre;Z/AP*, however, Hes5 and Hes1 expression was dramatically downregulated in the AP^{+} region, while in the central (AP^{-}) retina *Hes5/1* expression was comparable with normal (Fig. 3I, see Fig. S1D in the supplementary material). The downregulation of *Hes5/1* in the *Notch1*$^{f/f}$*;α-Cre* embryos validates the notion that *α-Cre*-mediated deletion of the *Notch1*^{f} allele results in functional inactivation of the Notch1 receptor in the peripheral optic cup.

Notch1$^{-/-}$ mouse mutants have been reported to exhibit upregulation of several proneural genes, including Mash1 (Ascl1 – Mouse Genome Informatics), Ngn2 (Neurog2 – Mouse Genome Informatics) and Neurod1 (de la Pompa et al., 1997). The Notch1-null embryos die before the eye is formed, thus precluding the study of Notch1-dependent bHLH expression in the retina (Conlon et al., 1995; Swiatek et al., 1994). We therefore used in situ hybridization to compare the expression profile of the proneural genes *Neurod1*, *Ngn2*, *Mash1*, *Math5* (*Atoh7* – Mouse Genome Informatics) and *Math3* (*Neurod4* – Mouse Genome Informatics) in the *Notch1*$^{f/f}$*;α-Cre* retinas (Fig. 3J-N) to their expression in control *Notch1*$^{f/f}$ retinas at E13.5 (Fig. 3A-G). In *Notch1*$^{f/f}$ control retinas, the expression of *Neurod1*, *Ngn2*, *Mash1*, *Math5* and *Math3* (Fig. 3C-G, respectively) was detected in subtypes of RPCs corresponding with their intrinsic heterogeneity (Akagi et al., 2004). In *Notch1*$^{f/f}$*;α-Cre* E13.5 peripheral retina however, increased expression of *Neurod1*, *Ngn2*, *Math5* and *Math3* was detected in the distal retina (Fig. 3J,K,M,N, respectively, demarcated with black arrowheads). No apparent change was detected in the expression of these genes at E12.5, prior to the onset of distinguishable morphological phenotypes (data not shown). In contrast to previous observations (de la Pompa et al., 1997), *Mash1* expression seemed unchanged in the *Notch1*$^{f/f}$*;α-Cre* retina when compared with the control (Fig. 3E,F respectively). bHLH factors have been shown to cross-regulate each other's expression (Akagi et al., 2004; Mu et al., 2005). For example, *Mash1* expression is upregulated in *Ngn2*$^{-/-}$ and *Math3*$^{-/-}$ retinas (Akagi et al., 2004), thus suggesting possible inhibition of Mash1 expression by Ngn2 and Math3. It is therefore possible that upregulation of Ngn2 and Math3 in the *Notch1*$^{-}$ optic cup inhibits Mash1 upregulation in *Notch1*$^{f/f}$*;α-Cre* retinas.

Reduced proliferation and decreased proportion of ganglion and horizontal cells in the *Notch1*$^{-}$ retina

Notch activity has been linked by several studies to cell-cycle regulation with effects that are context specific (Bao and Cepko, 1997; Dorsky et al., 1995; Ohnuma et al., 2002; Scheer et al., 2001). The consequence of Notch1 inactivation on the mitotic index was therefore examined in the embryonic retina (E14.5) by BrdU assay. The proportion of BrdU^{+} area to total nuclear area (DAPI^{+} area) in the optic cup was calculated (Fig. 4A-C, Materials and methods). A significant reduction (of 21.3%) in proliferating cells was detected in the *Notch1*$^{f/f}$*;α-Cre* E14.5 retina, where only 25.8% of the DAPI^{+} area was BrdU^{+}, compared with the *α-Cre* control retina, where 32.7% of the DAPI^{+} area was BrdU^{+} (Fig. 4C). This suggests that due to premature differentiation of the *Notch1*$^{-}$ cells, cell proliferation is compromised resulting in the depletion of the RPC pool.

The involvement of Notch1 in cell survival has been demonstrated in several cases (Lutolf et al., 2002; Oishi et al., 2004). Hence, the reduction in retinal size following Notch1 inactivation could also be attributed to apoptosis. We therefore tested for programmed cell death by TUNEL assay on sections from E14.5 and E16.5 *Notch1*$^{f/f}$*;α-Cre* and control peripheral optic cup. In the control embryos, TUNEL^{+} cells were detected in only 0.054% of DAPI^{+} area at E14.5 (s.d.=0.018%, *n*=4 eyes) and 0.035% of DAPI^{+} area at E16.5 (s.d.=0.014%, *n*=3 eyes). A significant increase (P<0.02) in the number of TUNEL^{+} cells was observed in the *Notch1*$^{f/f}$*;α-Cre* retinas to 0.325% of DAPI^{+} area at E14.5 (s.d.=0.044%, *n*=4 eyes) and 0.28% of DAPI^{+} area at E16.5 (s.d.=0.1%, *n*=4 eyes). Thus, although a six- (at E14.5) to eightfold (at E16.5) increase in apoptosis is observed following Notch1 inactivation in the RPCs, the proportion of TUNEL^{+} cells to total retinal tissue is very low and therefore could not solely account for the extensive reduction in retinal tissue detected in the *Notch1*$^{f/f}$*;α-Cre* retina.

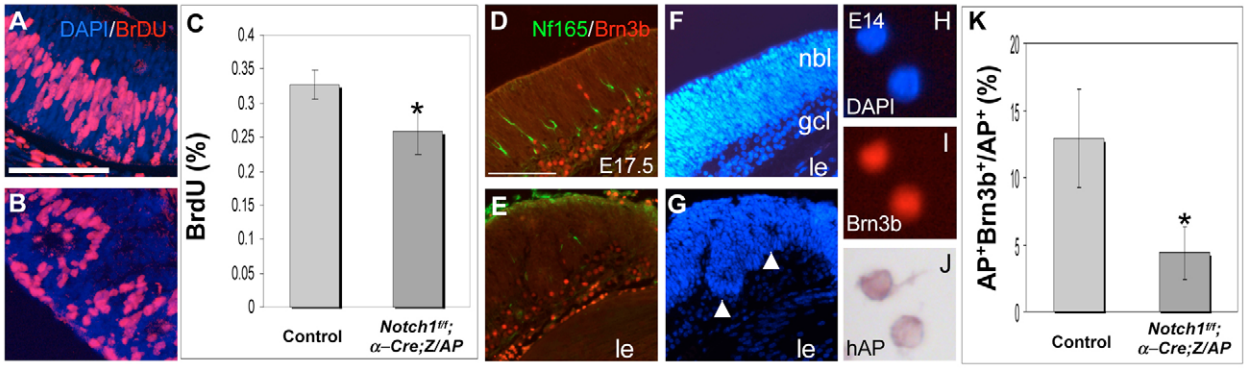

Fig. 4. Reduction in the mitotic index and the number of ganglion and horizontal cell types in the *Notch1*$^{-}$ retina. (**A-C**) Fewer BrdU^{+} nuclei (BrdU^{+} in red, DAPI in blue; A,B) are detected in the *Notch1*$^{f/f}$*;αCre* (B) when compared with the control *Notch1*$^{f/+}$*;α-Cre* (A) retina. (C) The proportion of BrdU^{+}/DAPI^{+} area was 32.7% in the *Notch1*$^{f/+}$*;α-Cre* eyes (s.d.=2 %, *n*=4), whereas only 25.8% BrdU^{+}/DAPI^{+} area was detected in the *Notch1*$^{f/f}$*;α-Cre* retina (s.d.=3.4%, *n*=4). Thus, the mitotic index in the *Notch1*$^{f/f}$*;α-Cre* retina is significantly reduced, to about 78% of the control (**t*-test, *P*<0.02). (**D**,**E**) The ganglion cells (Brn3b, red) and horizontal cells (Nf165, green) are detected in the peripheral retina of E17.5 control *Notch1*$^{f/+}$*;α–Cre;Z/AP* (D) eyes. Reduction in the number of Brn3b^{+} and almost complete loss of Nf165^{+} cells is observed at this stage in the *Notch1*$^{f/f}$*;αCre;Z/AP* peripheral retina (E). (**F**,**G**) Counterstaining with DAPI (blue) reveals the abnormal retinal morphology and rosettes (white arrowheads, G) in the *Notch1*$^{-}$ but not in the *Notch1*$^{+}$ peripheral retina (F). (**H-K**) Quantitative evaluation of the reduction in Brn3b^{+} cells in the *Notch1*$^{-}$ RPCs was conducted by dissociation of E14 retinas from control *Notch1*$^{f/+}$*;α-Cre;Z/AP* or mutant *Notch1*$^{f/f}$*;α-Cre;Z/AP* embryos. The cell nuclei were visualized by counterstaining with DAPI (blue, H). The Brn3b^{+} cells (red, I) were detected by indirect immunofluorescent analysis and documented. On the same slide, AP activity was monitored (purple, J). The proportion of Brn3^{+};AP^{+} was calculated from the total number of AP^{+} cells (K). A significant reduction of 35% (K, *t*-test, **P*<0.02) in the proportion of Brn3b^{+};AP^{+} positive cells from AP^{+} cells was observed in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* dissociated retina (average=4.4±2%, *n*=6 embryos) when compared with the *Notch1*$^{f/+}$*;α-Cre;Z/AP* control retinas (average=12.9±3.7%, *n*=6 embryos). gcl, ganglion cell layer; le, lens; nbl, neuroblastic layer. Scale bars: 100 μm.

Notch1 activity has been implicated in the inhibition of ganglion cell fate. This is based on the observation that ganglion cell production increases following antisense treatment of chick retinas with *CNotch-1* or its ligand *CDelta-1* (Ahmad et al., 1997; Austin et al., 1995; Silva et al., 2003). In contrast to these observations, analysis of the phenotype of Hes1 mutant embryos revealed premature differentiation of precursors into rod and horizontal cells (Tomita et al., 1996). To examine the role of Notch1 directly in the specification of RPCs to ganglion, horizontal and photoreceptor cell fates, we characterized the expression of markers specific to these cell types in the normal and *Notch1*$^{f/f}$*;α-Cre* retina (Figs 4, 5). Brn3b is the earliest known marker for ganglion cell precursors and is required for their terminal differentiation and survival (Gan et al., 1996; Gan et al., 1999; Wang et al., 2000). Surprisingly, at E17.5, Brn3b expression seemed to be reduced in *Notch1*$^{f/f}$*;α-Cre* when compared with the control retina (compare Fig. 4E to 4D) and a significant reduction (*t*-test *P*<0.05) in the in the proportion of Brn3b^{+} to DAPI^{+} area was detected peripheral retina of *Notch1*$^{f/f}$*;α-Cre* E16.5 embryos: from an average of 13.9% in the control (*Notch1*$^{f/+}$*;α-Cre*; s.d.=1.7%, *n*=3 eyes) to an average of 8.6% (*Notch1*$^{f/f}$*;α-Cre*; s.d.=0.3%, *n*=3 eyes). For quantitative evaluation exclusively of the *Notch1*$^{-}$ cellular phenotype, we calculated the proportion of Brn3b^{+} cells from the AP^{+} population in dissociated retinas from *Notch1*$^{f/f}$*;α-Cre;Z/AP* or *Notch1*$^{f/+}$*;α-Cre;Z/AP* E14 age-matched embryos (Fig. 4E-K). The average proportion of Brn3b^{+};AP^{+} from total AP^{+} in the *Notch1*$^{f/+}$*;α-Cre;Z/AP* retinas was found to be 13% (Fig. 4K). By contrast, the proportion of Brn3b^{+};AP^{+} from total AP^{+} was only 4.4% in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* retinas (Fig. 4K). Thus, Notch1 inactivation at this stage does not seem to inhibit ganglion cell genesis; rather, Notch1 appears to be required for their production in normal numbers.

To detect differentiating horizontal cells, we characterized the expression of Nf165 in the *Notch1*$^{f/f}$*;α-Cre* and *α-Cre* control embryos at E17.5, when the expression of Nf165 reaches the peripheral region of the optic cup. As early as E17.5 (Fig. 4), as well as at postnatal stages (data not shown), Nf165^{+} cells seem to be virtually excluded from the *Notch1*$^{f/f}$*;α-Cre* peripheral retina. Interestingly, however, in the *Hes1*$^{-/-}$ retina, premature differentiation into horizontal cells has been previously reported (Tomita et al., 1996). The different phenotypes of the Hes1 and Notch1 retina mutants reflect the fact that Hes1 is only one of many factors that mediate Notch1 activity (see Discussion).

Increased numbers of photoreceptor precursors following Notch1 inactivation in RPCs

We next evaluated the acquisition of photoreceptor cell fate by the *Notch1*$^{-}$ RPCs. To identify the photoreceptor precursors in the embryonic retina, we characterized the expression of Otx2 and Crx, members of the *otd/Otx* homeobox gene family, by in situ hybridization. Otx2 is essential for photoreceptor specification, while Crx is important for their terminal differentiation (Chen et al., 1997; Furukawa et al., 1997; Furukawa et al., 1999; Nishida et al., 2003). In the *Notch1*$^{f/f}$ E13.5 and E16.5 retinas, Otx2 (Fig. 5A,C) and Crx (Fig. 5B,D) were weakly detected in the outer neuroblastic layer (NBL) (Nishida et al., 2003). In contrast to this normal pattern of expression, as early as E13.5, both Otx2 and Crx expression were detected in the outer and inner layers of the peripheral *Notch1*$^{f/f}$*;α-Cre;Z/AP* retina (Fig. 5F-I). This suggests that Notch1 functions to inhibit progenitors from adopting a photoreceptor fate by repressing the expression of two key mediators of photoreceptor cell fate: Otx2 and Crx.

We next quantified the proportion of photoreceptor precursors in the control and *Notch1*$^{f/f}$*;α-Cre* embryonic retinas by calculating the ratio of Crx^{+} area from the peripheral retinal area at E16.5. In the

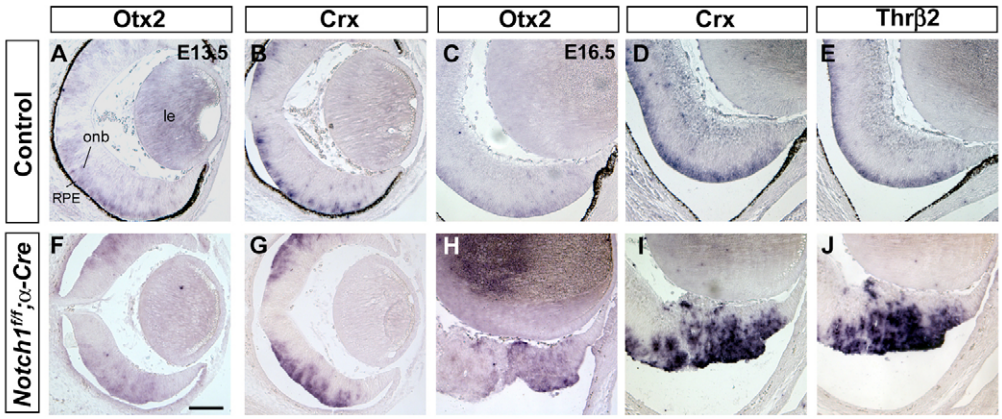

Fig. 5. *Notch1*⁻ RPC differentiation into precursors of photoreceptors. The expression of factors involved in photoreceptor differentiation Otx2 (A,C,F,H), Crx (B,D,G,I) and Thrβ2 (E,J) was analyzed by in situ hybridization on control (A-E) and *Notch1*$^{f/f}$*;α-Cre* (F-J) eyes. In the control E13.5 (**A**,**B**) and E16.5 (**C-E**) retinas, the expression of Otx2 (A,C), Crx (B,D) and Thrβ2 (E) is detected in the outer neuroblastic layer (onb). In the *Notch1*$^{f/f}$*;α-Cre* retinas, the expression of Otx2 (**F**,**H**), Crx (**G**,**I**) and Thrβ2 (**J**) is detected in the different layers of the peripheral retina. le, lens; onb, outer neuroblastic layer; RPE, retinal pigmented epithelium. Scale bar: 100 μm.

control eyes, Crx expression is detected in 14.2% (s.d.=0.93%, n=4 eyes) of the peripheral retina. By contrast, in the *Notch1*$^{f/f}$*;α-Cre* eyes, Crx$^+$ cells are detected in 56.6% (s.d.=9.8%, n=4 eyes) of the distal retina (P<0.001). This fourfold increase in the proportion of Crx$^+$ cells in the *Notch1*$^{f/f}$*;α-Cre* mice, when compared with the control littermates at E16.5, demonstrates the depletion of the RPCs available for the formation of other cell fates.

The thyroid hormone receptor β2 (Thrβ2) isoform is detected in cone-photoreceptor precursors and based on the knock-out phenotype, it is essential for proper cone-photoreceptor patterning (Ng et al., 2001). Peak expression is reported for Thrβ2 at mid-embryogenesis (E13.5-E17.5) (Jones et al., 2003; Ng et al., 2001). We therefore characterized Thrβ2 expression by in situ analysis at E16.5. Corresponding with previous reports, Thrβ2 expression was restricted to the outer nuclear layer of the *Notch1*$^{f/+}$*;α-Cre* retina at E16.5 (Fig. 5E). In the *Notch1*$^{f/f}$*;α-Cre* retina, however, Thrβ2 expression was found to be upregulated in the outer and inner layers of the peripheral optic cup in a pattern resembling Crx distribution identified on adjacent sections (Fig. 5J). The enhanced expression of Otx2, Crx and Thrβ2, as well as the early cell-cycle exit following Notch1 inactivation in the RPCs, indicated premature differentiation of the *Notch1*⁻ RPCs to photoreceptor precursors.

To test if these precursors differentiate to mature photoreceptors prematurely, we analyzed in the embryonic (E16.5) and early post-natal (P5) *Notch1*$^{f/f}$*;α-Cre* and control retinas the expression of several markers that are normally expressed in mature photoreceptors. In the embryonic retina (E16.5) the expression of recoverin, a photoreceptor-specific Ca^{2+}-binding protein (Haverkamp and Wassle, 2000; Sharma et al., 2003), S-cone opsin, Nr2e3 a rod-specific nuclear receptor important for the development of photoreceptors (Chen et al., 2005; Cheng et al., 2004; Peng et al., 2005) and rhodopsin were tested. Furthermore, the expression of cone-arrestin (Arr3) for the detection of cones and the α-subunit of rod transducin (Gnat1) for the detection of rods were tested in P5 eyes. Expression of none of these markers was detected in the control or *Notch1*$^{f/f}$*;α-Cre* retinas (data not shown). This suggests that the *Notch1*⁻ photoreceptor precursors do not complete their differentiation into photoreceptors prematurely.

The *Notch1*⁻ photoreceptor precursors differentiate primarily into cone-photoreceptors

The cellular phenotype acquired by the *Notch1*⁻ precursors was characterized after all retinal cell types had been born (P15) (Young, 1985), by detecting markers specific to the retinal cell types using antibody labeling or in situ hybridization (Fig. 6). The analysis was conducted on *Notch*$^{f/+}$*;α-Cre;Z/AP* controls and *Notch1*$^{f/f}$*;α-Cre;Z/AP* mice in order to distinguish between *Notch1*⁻ (AP$^+$) and *Notch1*$^+$ (AP⁻) cells (Fig. 1). AP enzymatic activity was detected either on adjacent sections (Fig. 6) or when the antibody reaction protocol permitted, by double-immunolabeling with antibodies specific to the different retinal cell types (see Fig. S2 in the supplementary material).

First, we analyzed the expression of recoverin, which is expressed in differentiated photoreceptors (Haverkamp and Wassle, 2000; Sharma et al., 2003). In the *Notch*$^{f/+}$*;α-Cre;Z/AP* control retina, AP expression was detected in all of the cellular layers of the peripheral retina (Fig. 6A) while recoverin expression was detected exclusively in the photoreceptor layer (Fig. 6B). In the *Notch1*$^{f/f}$*;α-Cre;Z/AP* peripheral retina, however, recoverin expression (Fig. 6E) was detected in almost all of the AP$^+$ cells (Fig. 6D). Quantitative analysis of the proportion of recoverin from AP expressing cells in the peripheral retina of the control and the *Notch1*$^{f/f}$*;α-Cre;Z/AP* mice was performed (see Material and methods, Fig. 6M). In the control peripheral retina (AP$^+$), recoverin was detected in 56.7% of the nuclear (DAPI$^+$) area (Fig. 6M). By contrast, in the AP$^+$ regions of the *Notch1*$^{f/f}$*;α-Cre;Z/AP* retinas recoverin was detected in 96.7% of the DAPI$^+$ area (Fig. 6M). Thus, consistent with the increased number of photoreceptor precursors observed during embryogenesis following Notch1 inactivation, a dramatic increase in the proportion of differentiated photoreceptors is observed in the postnatal *Notch1*⁻ retina. In agreement with the detection of recoverin in most of the AP$^+$ cells of the peripheral retina of the *Notch1*$^{f/f}$*;α-Cre;Z/AP* mice, co-expression of AP with syntaxin (amacrine cells), PKCα (bipolar cells) and cyclin D3 (Müller glia cells) was not observed in the peripheral retina of *Notch1*$^{f/f}$*;α-Cre;Z/AP* mice, while these cell-specific markers were co-localized with AP-expression in the *Notch*$^{f/+}$*;α-Cre;Z/AP* controls (see Fig. S2 in the supplementary material).

To further characterize the photoreceptor cell types produced from the *Notch1*$^{-}$ RPCs, we identified cone-photoreceptors by labeling sections with cone-specific peanut agglutinin (PNA) conjugated to fluorescein (Blanks and Johnson, 1983). Rods were identified by immunofluorescent analysis for detection of rhodopsin in the control and the *Notch1*$^{f/f}$*;α-Cre;Z/AP* eyes (Fig. 6C,F). AP activity was monitored on adjacent sections (Fig. 6A,D). In the control *Notch*$^{f/+}$*;α-Cre;Z/AP* retina, rhodopsin expression and PNA labeling were confined to the outer segments of the photoreceptor (Fig. 6C) and only a few cells were labeled with PNA, corresponding to the low number of cones present in the mouse retina (Young, 1985). By contrast, in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* peripheral retina, a dramatic increase in the number of PNA^{+} cells was detected in AP^{+} regions (identified on adjacent section Fig. 6D), while rhodopsin^{+} cells were identified mostly in AP^{-} regions (Fig. 6F). To further validate cone-fate acquisition, the expression of cone arrestin (Arr3) for the detection of cones and of the α-subunit of rod transducin (Gnat1) for the detection of rods was characterized in the control and *Notch1*$^{f/f}$*;α-Cre;Z/AP* retina by in situ hybridization (Fig. 6G-L). The proportion of the retinal area expressing each of these markers in the AP^{+} regions was calculated (measurements were conducted on the area that was AP^{+} detected on adjacent sections, Fig. 6M). Consistent with the normal differentiation pattern observed in the *Notch*$^{f/+}$*;α-Cre;Z/AP* control retina, Arr3 (cones) was detected in few cells of the outer nuclear layer while Gnat1 (rods) was detected in most of the outer nuclear layer (Fig. 6H,I). In the peripheral retina (AP^{+}) of the control mice Arr3 was detected in 2% of the DAPI^{+} area, while Gnat1 was detected in 66.7% of the DAPI^{+} area (Fig. 6M). However, in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* peripheral retina (AP^{+}; demarcated with broken white line Fig. 6J-K), Arr3 was detected in 89.1% of the DAPI^{+} area, while Gnat1 was detected in 10.1% of the DAPI^{+} area (Fig. 6M). This demonstrates that the *Notch1*$^{-}$ RPCs differentiate predominantly into cone-photoreceptors.

DISCUSSION

Notch1 activity is required for the multipotency of RPCs

In this study, we employed a Cre/*loxP* approach to address directly the in vivo requirement of Notch1 in RPCs during the crucial early stages of mouse retinogenesis. According to the prevailing model, which relies mostly on misexpression studies, release from Notch1 inhibition provides a generic permissive cue for RPCs to differentiate (Ahmad et al., 1997; Austin et al., 1995; Bernardos et

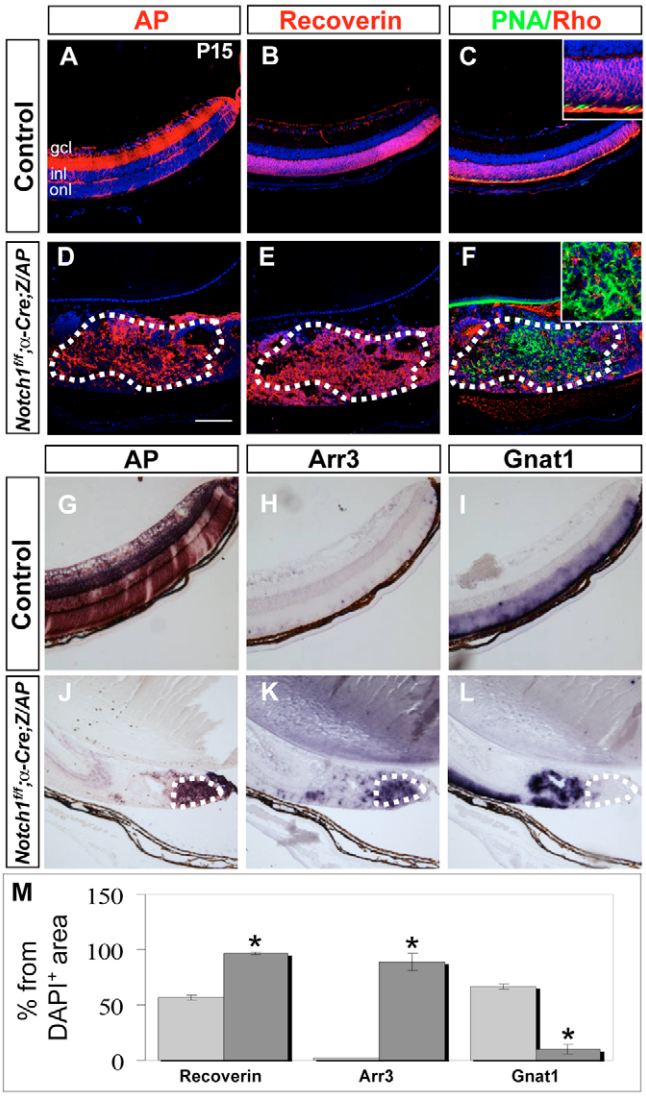

Fig. 6. *Notch1*$^{-}$ RPCs differentiate predominantly to cone photoreceptors. In the P15 retina of *Notch1*$^{f/+}$*;α-Cre;Z/AP* (**A-C**,**G-I**) and *Notch1*$^{f/f}$*;α-Cre;Z/AP* (**D-F**,**J-L**) mice, the expression of factors specific for photoreceptor cell types were analyzed using specific antibodies for the detection of recoverin (photoreceptors, B,E) and rhodopsin (rods, C,F), or by labeling with PNA for the detection of cone photoreceptors (C,F) and by detection of transcript using in situ hybridization for Arr3 (cones, H,K) and Gnat1 (rods, I,L). (A-F) Nuclei were visualized by DAPI counterstaining. The distribution of these markers was correlated with AP^{+} activity (A,D,G,J) monitored on adjacent sections (A is adjacent to B,C; D is adjacent to E,F; G is adjacent to H,I and J is adjacent to K,L). In the retina of *Notch1*$^{f/+}$*;α-Cre;Z/AP*, recoverin labels the onl (B), while in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* peripheral retina, the laminar organization is lost and most of the cells are recoverin^{+} (E), demonstrating their differentiation into photoreceptors. Next, analysis of the distribution of cone and rod photoreceptors was performed in correlation with AP distribution. In the normal retina, cones were detected in a few cells in the outer nuclear layer (PNA in C and Arr3 in H), while rods are detected in most of the photoreceptor layer of the control *Notch1*$^{f/+}$*;α-Cre;Z/AP* retina (rhodopsin in C and Gnat1 in I). In the peripheral retina of the *Notch1*$^{f/f}$*;α-Cre;Z/AP* mice, the regions that are AP^{+} (indicated by a broken white line) are mostly populated by PNA^{+};Arr3^{+} cells (F,K), while the AP^{-} regions (G,J) are occupied mostly by rod photoreceptors (F,L). (**M**) Quantitative analysis of the ratios of recoverin, Arr3 and Gnat1 to DAPI^{+} areas was conducted on the AP^{+} regions (indicated by the white line; example shown in J-L) detected in the peripheral optic cup. This analysis revealed significant (*P<0.005) increase in the number of photoreceptors (recoverin^{+}) in the *Notch1*$^{-}$ retina from 56.6% in the *Notch1*$^{f/+}$*;α-Cre;Z/AP* (s.d.=2.63%, n=3) to 97% (s.d.=1.63%, n=3) in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* mice. Dramatic increase (*P<0.005) in the number of cone-photoreceptors (Arr3) was detected in the *Notch1*$^{-}$ retina from 2% (s.d.=0.2%, n=3) in the *Notch1*$^{f/+}$*;α-Cre;Z/AP* to 89% (s.d.=7.6%, n=3) in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* mice. The number of rods was significantly reduced (*P<0.005) in the *Notch1*$^{-}$ retina from 66.8% (s.d.=2.56%, n=3) in the *Notch1*$^{f/+}$*;α-Cre;Z/AP* to 10.1% (s.d.=4.81%, n=3) in the *Notch1*$^{f/f}$*;α-Cre;Z/AP* mice. gcl, ganglion cell layer; inl, inner nuclear layer; le, lens; onl, outer nuclear layer. Scale bar: 100 μm.

al., 2005; Dorsky et al., 1997; Furukawa et al., 2000; Perron and Harris, 2000; Rapaport and Dorsky, 1998; Silva et al., 2003). In this case, it follows that premature loss of Notch1 activity in the RPCs of the peripheral retina during early embryogenesis (as in the *Notch1$^{f/f}$;α-Cre* mice) would result in the excessive differentiation of *Notch1*$^{-}$ RPCs into cell types normally born during the corresponding early stages of retinogenesis: ganglion, cone, horizontal and amacrine cells (Fig. 7) (Young, 1985). Consistent with its proposed role as an inhibitor of cell differentiation, we indeed observed that the selective targeted deletion of Notch1 in early RPCs leads to their premature terminal exit from the cell cycle, and their differentiation and accumulation within rosette-like structures. Surprisingly, however, the premature loss of Notch1 in RPCs leads to the acquisition of primarily the cone-photoreceptor cell fate. Intriguingly, these *Notch1*$^{-}$ RPC-derived cone precursors appear to be specified at the expense of the other early cell types.

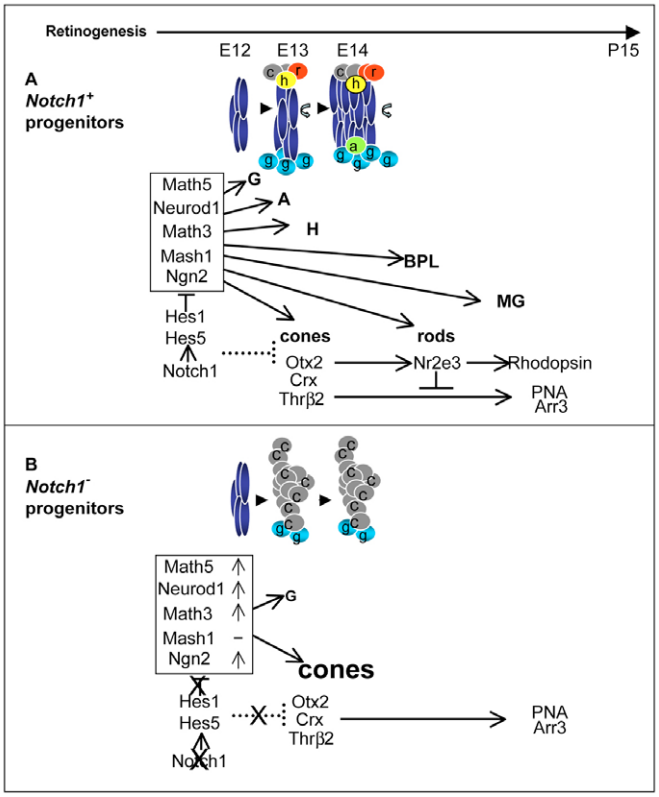

Fig. 7. A scheme summarizing the possible functions of Notch1 in maintaining the multipotency of retinal progenitor cells and in photoreceptor cell differentiation. During normal retinal development (**A**), Notch1 is expressed in RPCs (blue) and is required for the expression of Hes1 and Hes5, which function to repress proneural gene expression and thus to inhibit cell differentiation. Release from Notch1 inhibition occurs gradually, in only a few cells at a time, and these cells will differentiate into the different retinal cell types (G, ganglion; C, cone; A, amacrine; H, horizontal; BPL, bipolar; MG, Mueller glia). Inactivation of *Notch1* in the RPCs during early stages of retinogenesis does not result in premature differentiation into the different early cell types (**B**). The premature loss of Notch1 activity resulted in differentiation of most RPCs into cone-photoreceptor precursors expressing Otx2, Crx and the early cone marker Thrβ2. These cone precursors differentiate to mature cones during postnatal stages, thus following the normal temporal order of the differentiation of photoreceptors.

A possible explanation for the observed increase in cone precursors in the *Notch1*$^{-}$ retina is that Notch1 is required for the survival of all RPCs except nascent cones. Indeed the involvement of Notch1 in the survival of neural precursor cells has been demonstrated in several cases (Lutolf et al., 2002; Oishi et al., 2004). Furthermore, the aberrant retinal morphology is expected to result in cell death. The findings presented in this study, however, seem to rule out selective cell death as the primary explanation for reduced retinal size and the higher number of cone precursors in the *Notch1$^{f/f}$;α-Cre* retina. Although we observed enhanced apoptosis at E14.5 and E16.5 in the *Notch1$^{f/f}$;α-Cre* when compared with control littermates, this cell death was not extensive (covering only 0.32% at E14.5 or 0.28% at E16.5 of *Notch1$^{f/f}$;α-Cre* retina tissue). This suggests that cell death does not account for the massive reduction in retina size or for the fourfold increase in Crx^{+} cells in the *Notch1$^{f/f}$;α-Cre* retina. Although the above findings do not exclude selective cell death of a specific low-abundance progenitor cell type, it appears that the reduced retinal tissue and the abnormal cellular composition is primarily due to the precocious specification of *Notch1*$^{-}$ progenitors to cone precursors, which results in the depletion of the RPCs destined to other cell fates.

Notch1 functions to inhibit cone-photoreceptor cell fate specification during early retinal neurogenesis

The current model for retinal development suggests that retinal precursors consist of mixed subsets of progenitors with distinct competence states that are subject to changes over time, presumably driven by the shifting presence of external and internal cues (Livesey and Cepko, 2001; Watanabe and Raff, 1990). Cell-birth studies reveal that during early retinogenesis, the ganglion, cone, horizontal, amacrine and rod cells are born from the pool of RPCs (between E12 and E13) (Seidman, 1961; Young, 1985). Thus, one might expect that premature differentiation during early retinogenesis will result in early differentiation of the progenitors into these early cell types. Our finding that the *Notch1*$^{-}$ RPCs acquire predominantly the cone fate raises two non-mutually exclusive models for explaining the mechanisms controlling cell-type diversification in the retina: first, these findings may suggest that there is an underlying bias toward the cone-photoreceptor fate of early RPCs. The forced early differentiation imposed by the inactivation of Notch1 in the *Notch1$^{f/f}$;α-Cre* merely exposes this dominant bias to the cone-photoreceptor fate. However, this possibility seems less likely as the cone precursors are never the predominant cell type (Young, 1985). Another explanation for the observed *Notch1$^{f/f}$;α-Cre* retinal phenotype is that Notch1 maintains the multipotency of RPCs by selectively suppressing acquisition of the cone-photoreceptor fate. Consistently, premature loss of Notch1 activity in early RPCs leads to excessive production of cone precursors, thereby depleting the pool of RPCs available for formation of the other retinal cell types.

Involvement of Notch1 pathway in acquisition of retinal cell fates

Several intrinsic and extrinsic factors appear to regulate the onset of cell differentiation and cell-type specification by influencing the expression of bHLH proneural factors. In *Hes1*-null mutant mice, rod photoreceptor and horizontal cells were reported to appear prematurely during retinogenesis (Tomita et al., 1996), suggesting that loss of Hes1 alone leads to a late-onset phenotype compared with that observed for the Notch1-deficient retina. These phenotypic differences are consistent with the notion that Notch1 acts through several pathways to control retinal neurogenesis, in addition to a

late-acting Hes1-dependent pathway. For example, in the Hes1-null mutant retina, persistent expression of other Hes genes, such as Hes5, could potentially lead to a rescue of earlier aspects of neurogenesis. In this regard, the earlier and more dramatic phenotype observed in our Notch1-deficient retinal model may be at least partially explained by the loss of expression of both Hes1 and Hes5.

Another key regulator of eye development known to be essential for the multipotency of RPCs is Pax6 transcription factor. The inactivation of Pax6 in RPCs resulted in downregulation of Math5, Ngn2 and Mash1, while Neurod1 expression seemed unchanged. This change in expression profile was associated with the RPCs acquiring the amacrine cell fate (Marquardt et al., 2001). The different expression profile of the proneural bHLH factors in the Pax6 when compared with Notch1 mutants is consistent with the difference in eventual cell fate acquired by the cells; amacrine fate by *Pax6*$^{-}$ RPCs and cone-photoreceptor fate by the *Notch1*$^{-}$ RPCs. Recently, the secreted factor GDF11 has been suggested to control cell-type specification of progenitor cells in the developing retina by regulation of Math5, Mash1 and Neurod1 expression (Kim et al., 2005). Together, these studies suggest that several factors and pathways intersect in terms of regulating the expression of the bHLH transcription factor profile in the progenitor cells. In future studies, it would be intriguing to discover the interaction between these different pathways at the onset of differentiation and in mediating the specification of multiple retinal cell types.

The development of photoreceptors has been studied extensively as a model for understanding neuronal specification and differentiation (Cepko et al., 1996). Photoreceptor differentiation has been shown to occur in two phases. Cones are one of the first cells to appear (between E11 and E13), together with the other early-born cell types: ganglion, horizontal and amacrine. The second phase of photoreceptor differentiation is marked by the production of rods that peaks around birth (Sidman, 1961; Young, 1985). The transcription factors Otx2 and Crx are the main factors currently known to regulate the early events of photoreceptor specification and differentiation (Chen et al., 1997; Furukawa et al., 1997; Nishida et al., 2003). Using the Cre/*loxP* approach, Otx2 was found to be essential for the formation of photoreceptor precursors, while its target, Crx, is required for their normal differentiation (Chen et al., 1997; Furukawa et al., 1997; Nishida et al., 2003). The expression and function of Otx2 in the eye, however, seems not to be restricted to photoreceptor precursors and thus cannot be the only mediator of Crx expression (Martinez-Morales et al., 2001).

The results presented here suggest that Notch1 is functionally upstream of *Otx2* and *Crx*, and it acts to inhibit the expression of both genes in early RPCs (Fig. 7). Future studies will have to address the identity of the mediators that regulate *Otx2* and *Crx* expression downstream of Notch1 activation. Following Notch1 inactivation in the RPCs, we observed down-regulation of *Hes5* and *Hes1* and enhanced expression of a number of proneural genes, such as *Neurod1*, *Math5*, *Math3* and *Ngn2*. This radically altered combination of bHLH factors expressed in the *Notch1*$^{-}$ cells could therefore underlie the observed change in cell fate toward cone-photoreceptor precursors (Fig. 7). Furthermore, in previous studies using gain- and loss-of-function approaches, the rod-photoreceptor cell fate has been shown to be affected by combinations of several bHLH factors (Akagi et al., 2004; Ma et al., 2004; Morrow et al., 1999; Pennesi et al., 2003; Yan and Wang, 1998). It would therefore be interesting in future studies to evaluate the regulation of Otx2 and Crx by the combination of bHLH proneural factors and to assess more carefully the acquisition of the cone cell fate in bHLH mouse mutants.

Although many of the photoreceptor precursors are generated during embryogenesis, the differentiation into functional photoreceptors is completed mostly at postnatal stages (Morrow et al., 1998b). This long interval between photoreceptor birth and onset of visual pigment expression has been observed in many different species (Adler et al., 2001; Knight and Raymond, 1990; Morrow et al., 1998a). We observed that the cone precursors originating from Notch1-deficient RPCs seem to obey the normal cone-photoreceptor developmental program. Similar to normal cone precursors, the expression of recoverin, S-opsin, Arr3 and PNA labeling was detected only after birth. Together, the presented findings demonstrate an important role for the Notch1 pathway in actively preventing RPCs from choosing the cone-photoreceptor fate, a role that appears, however, to be independent of the later temporal process that leads to the maturation of committed cone-photoreceptor precursors.

The authors thank Dr Freddy Radtke for the *Notch1*^{flox} line, Dr Peter Gruss for the α-*Cre* mice, and Drs Corrinne Lobe and Andras Nagy for the *Z/AP* reporter line. For the in situ probes, we thank Drs Ryoichiro Kageyama, Thomas A. Reh, Takahisa Furukawa, Jichao Chen and Jeremy Nathans. Antibodies were generous gifts from Karl W. Koch (recoverin) and Nadean Brown (Hes1). We are grateful for the assistance of Leonid Mittleman for confocal analysis and for Noa Davis-Silberman's comments on the manuscript. R.A.-P.'s research is supported by the Israeli Academy of Sciences, Israeli Ministry of Health, the German Israeli Foundation, Recanati Foundation, ADAMS Center for Brain Studies. O.Y. is supported by a Fred Shaul Scholarship. M.L.A.'s research is supported by NIH Grants, Macular Foundation/Ronald McDonald House Charities and the Massachusetts Lions Eye Research Fund.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/7/1367/DC1

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