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Notch 1 inhibits photoreceptor production in the developing mammalian retina

Ashutosh P. Jadhav[1], Heather A. Mason[2] and Constance L. Cepko[1,*]

The transmembrane receptor Notch1 plays a role in development and homeostasis in vertebrates and invertebrates. The mammalian retina is an excellent tissue in which to dissect the precise role of Notch signaling in regulating cell fate and proliferation. However, a systematic analysis has been limited by the early embryonic lethality of *Notch1*-null mice. Here, *Notch1* was conditionally removed from the murine retina either early or late in development. Removal of *Notch1* early led to a reduction in the size of the retina as well as aberrant morphology. A decrease in the number of progenitor cells and premature neurogenesis accounted for the reduction in size. Unexpectedly, ablation of *Notch1* in early progenitor cells led to enhanced cone photoreceptor production, and ablation of *Notch1* at later points led to an almost exclusive production of rod photoreceptor cells. These data suggest that Notch1 not only maintains the progenitor state, but is required to inhibit the photoreceptor fate. These cone enriched mutant mice should prove to be a valuable resource for the study of this relatively rare mammalian photoreceptor cell type.

KEY WORDS: Neural development, Retina, Cell fate, Notch, Photoreceptor

INTRODUCTION

Studies conducted in vertebrates and invertebrates over the last decade have led to the realization that a few families of signaling molecules are used repeatedly to regulate multiple aspects of development. The Notch transmembrane receptor is one such molecule. Notch was first discovered in *Drosophila*, and was subsequently shown to be crucial for proper development in vertebrates as well (Artavanis-Tsakonas et al., 1999). However, the precise roles played by Notch in vertebrates have not yet been elucidated. One of the outstanding issues concerns its role in regulating cell fate decisions. Introduction of an activated allele of Notch into the vertebrate nervous system has led to the suggestion that it controls the decision of a cell to become a neuron or a glial cell (Furukawa et al., 2000; Gaiano et al., 2000; Morrison et al., 2000). However, loss-of-function studies have not clearly demonstrated this role. In part, this is due to the fact that mice with a null allele of *Notch1* die at E10, prior to gliogenesis (de la Pompa et al., 1997). Clarification of the role of Notch in the neuron versus glial cell fate decision, or of the potential role of Notch in regulating which type of neuron might be made, will require the use of a system where one can readily quantify the various neuronal cell fates, as well as exert temporal and spatial control over the loss of Notch.

The retina offers many advantages for the dissection of the roles of a molecule such as Notch. Retinal development is relatively well characterized. The vertebrate retina is an exquisitely light-sensitive tissue capable of transducing the signal from a single photon into a neural stimulus. Moreover, it performs sophisticated information transformations that begin the process of vision. To perform these functions, it requires the correct stochiometric production of seven major cell types during development. Retinal ganglion cells, horizontal cells, rod and cone photoreceptor cells, amacrine cells, bipolar cells, and Muller glial cells arise from multipotent RPCs in a conserved order during development (reviewed by Altshuler et al., 1991). Birthdating studies in the mouse have shown that retinal ganglion cells are the first-born cell type, whereas rods, bipolar interneurons and Muller glial cells are the late-born cell types (Sidman, 1961; Young, 1985).

Although, there is considerable overlap in the generation of the various cell types, there are a limited number of cell fates available to the daughter of an RPC at any given time. For example, while extrinsic factors can alter the ratio of the various cell types produced at one time point (reviewed by Levine et al., 2000), heterochronic mixing experiments have established that progenitor cells from a particular time in development cannot be induced to generate temporally inappropriate cell types (Belliveau and Cepko, 1999; Belliveau et al., 2000; Rapaport et al., 2001). The logic of these observations can be accommodated by the competence model of retinal development (Cepko et al., 1996; Livesey and Cepko, 2001). This model proposes that progenitor cells progress through temporal states; in any particular state, the progenitor is competent to produce only a subset of retinal cell types. A predicted consequence of the competence model is that the failure to maintain RPCs would result in the generation of early born cell types, but not the later born cell types, as progenitor cells would be prematurely depleted and unavailable for the production of later born cell types.

Several gain- and loss-of-function studies of the Notch pathway in the developing retina have suggested a crucial role for this pathway in controlling progenitor multipotency as well as proliferation and apoptosis (Ahmad et al., 1997; Ahmad et al., 1995; Austin et al., 1995; Bao and Cepko, 1997; Dorsky et al., 1997; Dorsky et al., 1995; Furukawa et al., 2000; Henrique et al., 1997; Lindsell et al., 1996; Scheer et al., 2001; Silva et al., 2003; Waid and McLoon, 1998). Expression analysis have further established that *Notch1* and *Notch3* are both expressed in the developing central retina, whereas *Notch2* is expressed in the peripheral retina and the retinal pigmented epithelium (Bao and Cepko, 1997; Lindsell et al., 1996). In chick embryos, reduction of Notch1 levels in the early retina enhanced production of the first born retinal cell type – the retinal ganglion cell. In that study, it was also determined that a high

[1]Department of Genetics and Howard Hughes Medical Institute, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA. [2]Developmental Genetics Program and the Department of Cell Biology, The Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York, NY 10016, USA.

*Author for correspondence (e-mail: cepko@genetics.med.harvard.edu)

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percentage of retinal progenitors were competent to adopt the retinal ganglion cell fate, and that Notch signaling was limiting in permitting ganglion cell production (Austin et al., 1995). In complementary studies, the Notch pathway was constitutively activated, which led cells to adopt an undifferentiated progenitor-like state (Dorsky et al., 1995). In fish and rodent, these progenitor-like cells have glial properties, suggesting that Notch1 also may be regulating the glial versus neuronal cell fate decision (Furukawa et al., 2000; Scheer et al., 2001).

Some of the downstream effectors of Notch signaling have been described; the most notable of which is the Hes gene family (Iso et al., 2003). This class of basic helix-loop-helix (bHLH) genes are homologous to *Drosophila hairy* and *Enhancer of split E(spl)*. In both vertebrates and invertebrates, these transcription factors are known to mediate at least a subset of Notch signaling activities. Evidence for the relationship between Notch1, Hes1 and Hes5 in the retina is supported by misexpression experiments in which these negative bHLH proteins are sufficient to promote the Muller glial/late progenitor-like fate at the expense of neurons (Furukawa et al., 2000; Hojo et al., 2000; Takatsuka et al., 2004). Interestingly, these negative bHLHs do not re-capitulate all of the effects observed with constitutive Notch activation. It was found in the rat retina that activated Notch led to glial formation, concomitant with hyperproliferation, while Hes1 misexpression directed glial formation without promoting proliferation (Furukawa et al., 2000). These data suggest that Notch1 activation engages as yet unidentified downstream effectors, in addition to Hes gene family members.

Null alleles in *Hes1* and *Hes5* have been generated in mice and analyzed for retinal defects. *Hes5*-null retinae have normal retinal size and morphology, but display a 30-40% reduction in Muller glial cell production relative to wild-type littermates (Hojo et al., 2000). *Hes1*-null mice are more severely affected (Tomita et al., 1996). These retinae are reduced in size and full of abnormal rosettes. Cell fates in these retinae were dramatically altered as there was an increase in early born cell types, such as retinal ganglion cells, horizontal cells and amacrine cells, and a decrease in the later born Muller glial cells (Takatsuka et al., 2004; Tomita et al., 1996). Furthermore, horizontal and photoreceptor cell markers were prematurely expressed. Together, the loss-of-function experiments suggest that Notch-Hes signaling is important for the maintenance of a pool of progenitor cells and the acquisition of the glial fate.

The above data support a model in which *Notch1* is expressed by RPCs of each competence state. Notch signaling allows cells to remain undifferentiated and continue proliferating, and progenitor cells downregulate Notch activity in order to produce a postmitotic cell that becomes a neuron. We directly tested this model by conditionally inactivating *Notch1* at different times in murine retinal development. Notch1 was found to be crucial for multiple aspects of development, including regulating organ size, morphogenesis and cell fate decisions. Consistent with the competence model, ablating *Notch1* early led to enhanced production of an early-born cell type, whereas eliminating *Notch1* later led to production of a later born cell type. Surprisingly, progenitors in which *Notch1* is deleted do not simply produce all neuronal fates available in their competence state; they predominantly produce the rod or cone photoreceptor fates. These unexpected results demonstrate a crucial role for Notch signaling in inhibiting the photoreceptor fate, and suggest a broader role for Notch signaling in not only inhibiting the neuronal fate, but also in selectively biasing production of one neuronal cell type over another.

MATERIALS AND METHODS

Animals

Mice carrying the *Chx10-CRE BAC* transgene (Rowan and Cepko, 2004), *Foxg1-CRE* knock-in allele (Hebert and McConnell, 2000), *R26R* allele (Soriano, 1999) or *Notch1* floxed allele (Radtke et al., 1999) were crossed to generate mice hemizygous for *R26R* allele, hemizygous for *Chx10-CRE* or hemizygous for *Foxg1-CRE* and homozygous or hemizygous for the *Notch1* floxed allele. *Notch1* flox/+; *CHX10-CRE* mice were phenotypically indistinguishable from wild-type mice and used as controls. Mice were genotyped for the *Notch1* floxed allele, *Chx10-CRE* and *R26R* using DNA extracted from tail preps and PCR amplification as described (Radtke et al., 1999; Rowan and Cepko, 2004; Soriano, 1999). Longwood Medical Area's Institutional Animal Care and Use Committee (IACUC) approved the animal experiments.

In vitro and in vivo retroviral infection and BrdU incorporation

In vitro infection was performed on dissected retinae grown as explants on top of polycarbonate filters in 12-well culture plates with media. The media consisted of 45% DMEM, 45% F12 medium and 10% fetal calf serum (FCS). The retina was immediately infected with virus diluted in a 5 μl droplet of media. In vivo infection was performed by injecting 0.5 μl of highly concentrated virus into the subretinal space of P0 or P3 mice. Mice were allowed to develop for 2-4 weeks before processing. Freshly dissected retinae were cultured with media containing BrdU for 1 hour at 37°C. Labeled cells were detected according to the manufacturer's instructions (Roche).

Replication incompetent retrovirus production

Myc-tagged cre recombinase (T. Matsuda and C.L.C., unpublished) was cloned directly into the retroviral expression construct pNIN (Dyer and Cepko, 2001a). Virus was produced and concentrated as described (Cepko et al., 1998).

Microarray hybridization and analysis

RNA was purified from mouse retinal tissue by Trizol extraction (GIBCO). cDNA was prepared from RNA essentially as described (Tietjen et al., 2003). In brief, DNA was reverse transcribed from RNA with reverse transcriptase (GIBCO) and a polyT primer (TATAGAATTCGCGG-CCGCTCGCGAT(24)) (Oligos etc.). PolyA tailing was achieved with terminal transferase (TdT, Roche). DNA was subsequently amplified by PCR using the polyT primer and LA-Taq DNA polymerase (Takara). Between 16 and 22 cycles of PCR amplification was used to obtain the necessary amount of DNA still within the linear range of amplification. For microarray hybridization, cDNAs were labeled by incorporation of Cy3- or Cy5-dCTP (Amersham-Pharmacia). Pairs of labeled probes were hybridized at 42°C overnight microarrays consisting of clones from the Brain Molecular Anatomy Project clone set (kind gift of Dr Bento Soares) and additional clones of interest from the laboratory (Livesey et al., 2004). Slides were washed extensively (Young and Cepko, 2004) before scanning in an Axon GenePix 4000B Scanner (Axon Instruments). Data images were retrieved and analyzed using the GenePix software package (Axon Instruments).

In situ hybridization and immunohistochemistry

All tissue sections were prepared by equilibrating retinae in 30% sucrose/PBS at 4°C, followed by equilibration in a 1:1 mix of 30% sucrose/PBS and OCT and finally embedded in OCT. All sections were 20 μm. For immunostaining, sections were hydrated in PBS and then blocked in 5% goat serum in 0.1% Triton X-100/PBS (PBST). Primary antibody was applied for 2 hours at room temperature, followed by three PBST washes and secondary antibody incubation at room temperature for 2 hours. Antibodies used were: anti-β-tubulin III (mouse monoclonal, 1:200, Upstate), anti-RMO 270.7 directed against low-molecular weight neurofilament (Carden et al., 1987) and goat anti-mouse or goat anti-rabbit Cy3 or Cy5 (Jackson Immunoresearch Laboratory 1:200). Cells were counterstained with DAPI and washed several times. For alkaline phosphatase staining, retinal tissue was harvested and fixed with 4% PFA/PBS for 15 minutes at room temperature. After extensive washing in PBS, endogenous alkaline phosphatase activity was heat inactivated by

placing the tissue at 65°C for 1 hour. Retinae were then stained as whole mounts for 1-4 hours at room temperature (Fekete and Cepko, 1993). For X-gal staining, retinal tissue was harvested and fixed with a 1% PFA/0.5% glutaraldehyde mix as described (Kwan et al., 2001). Retinae were stained as whole mounts overnight at 37°C. Section in situ hybridization was performed on retinal cryosections as described (Chen and Cepko, 2002). The following probes were used: Crx (Furukawa et al., 1997), Otx2 (Ang et al., 1994), NeuroD1 (Morrow et al., 1999), S-opsin and M-opsin (Corbo and Cepko, 2005), and genes listed below in the GenBank Accession Numbers section. All images were taken on a Nikon Eclipse E1000 microscope using a Leica DC200 digital camera. For further descriptions see also http://www.stjude.org/faculty/0,2512,407_2030_10417,00.html.

GenBank Accession Numbers

PNR, BC017521; *NRL*, BF464350; islet 1, AI845893; *NF-L*, BE953485; *Hes1*, BI557608; *Hes5*, BE952148; *Hey1*, AI851652; *Chx10*, BF461223; Delta-like 1, AW047187; *Math3*, AI846749; clusterin, BE996359; *p57*, BF464158; *Pax6*, BE953199; *Notch1*, BE981557.

RESULTS

Notch1 is essential for proper retinal morphology and size

The requirement for Notch1 in early retinal development was investigated by breeding floxed *Notch1* mice (Radtke et al., 1999) to mice expressing cre recombinase under the *Chx10* promoter (Rowan and Cepko, 2004) (see Fig. 2A). This strategy circumvented the embryonic lethality observed in Notch1-null mice (de la Pompa et al., 1997). Chx10 is a relatively retinal specific transcription factor that is expressed in progenitor cells starting at ~E10. The *Chx10-CRE* line has been shown to have cre activity beginning around the onset of *Chx10* expression (Rowan and Cepko, 2004). Floxed *Notch1*; *Chx10-CRE* mice had wild-type viability and survived to adulthood, consistent with the finding that *Chx10* expression is relatively specific to the retina. Most notably, reduced eye size was observed in all *Notch1* conditional knockout mice (CKO) relative to their wild-type littermates, as early as E13.5. Hematoxylin and Eosin staining of wild-type and mutant littermates was performed at P21 when retinal development is complete. Relative to retinae from wild-type littermates, retinae from Notch1 mutant mice displayed severe morphological defects and reduced optic nerve thickness (Fig. 1A-B and not shown). Cells in the outer nuclear layer (ONL) of the mutant mice were aberrantly organized into rosette-like structures. The number of cells in the inner nuclear layer (INL) was severely reduced in the mutant retinae compared with the wild-type retinae.

As Notch signaling is known to have cell-autonomous and cell non-autonomous effects on cell proliferation and apoptosis (Artavanis-Tsakonas et al., 1999), it was unclear whether the reduction in retinal size was due to a cell autonomous reduction in Notch1 in proliferating progenitors or to a cell non-autonomous effect. To distinguish between these possibilities, *Notch1* flox/flox retinae were explanted at E14.5 and infected with a low titer replication incompetent retrovirus encoding nuclear β-galactosidase without (NIN) or with cre recombinase (NIN-CRE). Retinae were allowed to grow as explants for 10 days and then processed for β-galactosidase histochemical detection using X-gal. Single clones were easily identified as patches of X-gal+ cells, which were assayed for clone size (Fig. 1C). NIN-infected control clones varied from 1-52 cells per clone, with an average of 5.9±1.3 cells per clone. By contrast, NIN-CRE infected clones ranged from 1-28 cells per clone, with an average of 3.7±0.6 cells per clone. There was a slight increase in 1-3 and 4-6 cell clones, and a slight decrease in 7-9 and greater than 10 cell clones, in NIN-CRE versus NIN clones (Fig. 1D). The overall reduction in clone size in the *Notch1*-deleted progenitors suggests that cell-autonomous effects account for at least some aspects of progenitor cell proliferation.

Notch1 deficient progenitors differentiate prematurely

To better understand the early events that may have contributed to the abnormal retinal morphology and size observed in *Notch1*-null adult mice, *Notch1*-deficient mice were examined at embryonic timepoints. In the course of using the *Chx10-CRE* transgenic mice, variable levels of CRE activity have been observed (Rowan and Cepko, 2004). As a result, variable degrees of the mutant phenotype were observed depending on the percentage of cells with deletion of Notch1. To track the degree of cre recombinase activity more precisely and fate map the individual cells in which *Notch1* was probably deleted, the *R26R* cre reporter allele (Soriano, 1999) was crossed into the *Chx10-CRE*; *Notch1* flox/flox mice (Fig. 2A). Retinae from mutant mice with a very high percentage of X-gal+ cells were consistently very small in size and

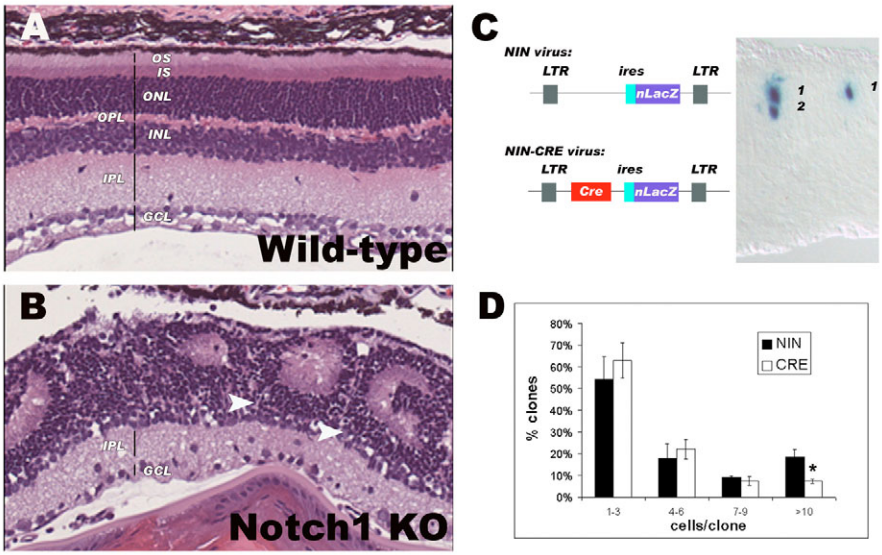


Fig. 1. Morphology of *Notch1* CKO retinae. (**A**,**B**) Hematoxylin and Eosin staining of retinae from 3-week old wild-type (A) and *Notch1* CKO (B) mice. The white arrowheads indicate rosette-like structures. (**C**) Schematic illustration of NIN and NIN-CRE constructs. Representative two- and one-cell clones from NIN infection. (**D**) Quantitation of clone sizes resulting from NIN or NIN-CRE infections on *Notch1* flox/flox retinae. Two independent retinae totaling 300-350 clones were analyzed for each type of virus. Change in clone size in the NIN-CRE-infected retinae compared with control (NIN-infected retinae) is statistically significant (*P<0.05, two-tailed t-test assuming equal variances). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment.

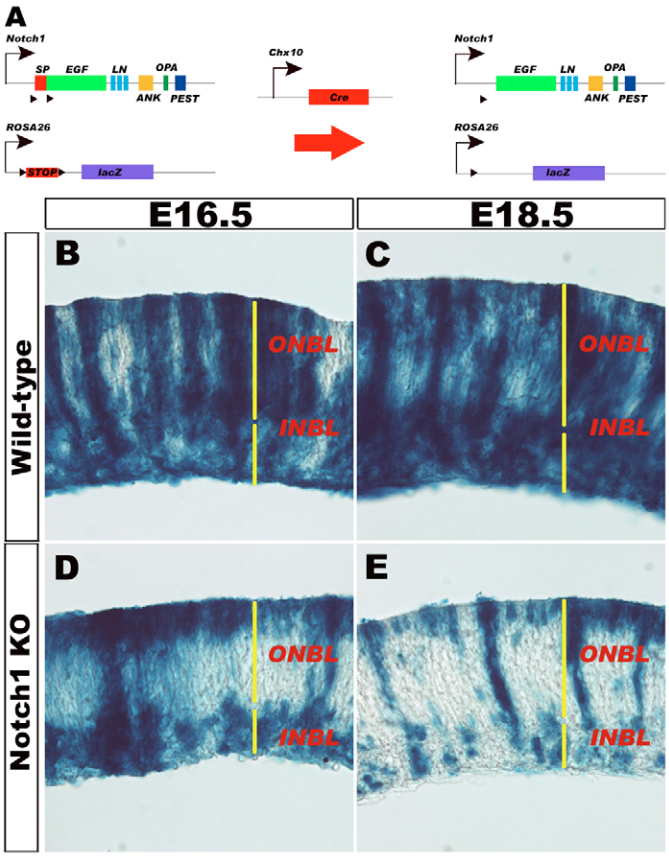


Fig. 2. Fate mapping of *Notch1* ablated retinal cells at embryonic timepoints. (**A**) Schematic illustration of transgenic constructs. The floxed *Notch1* allele was generated by flanking the first coding exon with LoxP sequences. Removal of exon1 of *Notch1* with cre recombinase removes the exon encoding the signal peptide and leads to the generation of a null allele. *ROSA26-R (R26R)* is a cre recombinase reporter comprising a LoxP flanked stop codon preceding a β-galactosidase coding region (*lacZ*). The *Chx10-CRE* mice contain a BAC transgene consisting of a cre-GFP fusion knocked into the *Chx10* promoter. *Notch1*-ablated retinae were generated by crossing the *Chx10-CRE* allele into *Notch1* flox/flox mice. Fate mapping of recombined cells was possible by X-gal staining in mice additionally containing the *R26R* allele. (**B-E**) Fate mapping of wild-type (B,C) and *Notch1* ablated (D,E) retinal progenitor cells at E16.5 (B,D) and E18.5 (C,E), as detected by X-gal staining. INBL, inner neuroblastic layer; ONBL, outer neuroblastic layer.

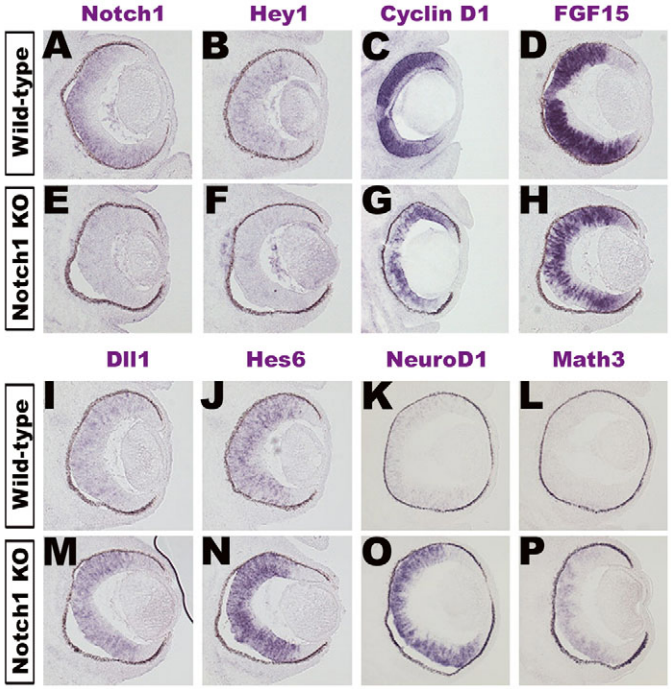


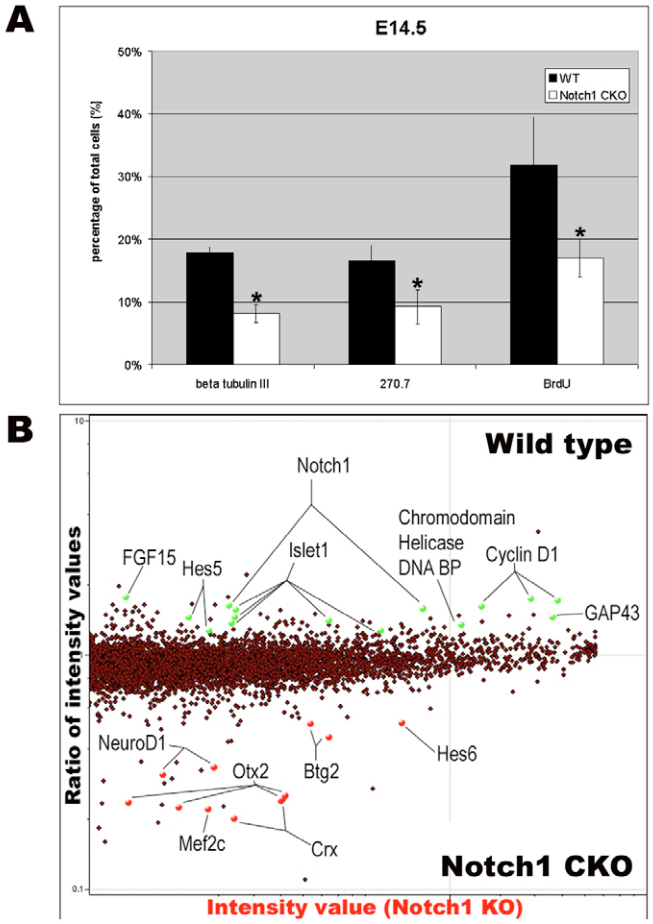
Fig. 3. Expression of markers of RPCs and enhanced neurogenesis in Notch1 deficient retinae. Section in situ hybridization on wild type (**A-D**,**I-L**) and *Notch1* CKO (**E-H**,**M-P**) retinae at E13.5. (A,E) *Notch1*, (B,F) *Hey1*, (C,G) cyclin D1, (D,H) *Fgf15*, (I,M) *Dll1*, (J,N) *Hes6*, (K,O) *Neurod1*, (L,P) *Math3*.

highly rosetted as early as E13.5. Mutant retinae with a much lower percentage of X-gal+ cells were more wild type in size and displayed normal lamination.

Fate mapping of wild-type littermates at E16.5 and E18.5 by X-gal staining revealed cells with morphology consistent with progenitor cells in that they had radial processes that spanned the retina. In addition, there were X-gal+ cells in the outer neuroblastic layer (ONBL) and in the inner neuroblastic layer (INBL) (Fig. 2B,C). These data are consistent with previous experiments that examined the morphology of individual X-gal+ retinal cells in *Chx10-CRE*/+; *R26R*/+ mice. That study demonstrated the potential of *Chx10*-expressing progenitor cells to produce all retinal cell types (Rowan and Cepko, 2004). In mutant littermates with a relatively low percentage of X-gal+ cells, very few of the X-gal+ cells exhibited radial or progenitor-like morphology. There was an increase in more compact X-gal+ cells lining the scleral surface of the ONBL and in the INBL (Fig. 2D,E). The locations and morphologies are suggestive of cells that have exited cell cycle and begun to differentiate prematurely.

Markers of RPCs were examined in Notch1-deficient retinae at E13.5. *Fgf15* and cyclin D1 are normally expressed by RPCs at this age (Blackshaw et al., 2004; Sicinski et al., 1995). These genes were markedly decreased in the Notch1 CKO (Fig. 3C,D,G,H). Previous studies in invertebrates and vertebrates have demonstrated that at least a subset of Notch activities are mediated by members of the E(Spl)-related Hes genes (Iso et al., 2003). To address the consequence of deleting Notch1 on these canonical downstream effectors, in situ hybridization for *Notch1*, *Hey1*, *Hes1* and *Hes5* at E13.5 was performed. Both *Notch1* and *Hey1* were noticeably reduced (Fig. 3A,B,E,F), but changes in *Hes1* or *Hes5* expression were not seen (data not shown). Interestingly, *Hes5*, along with *Notch1* and *Hey1*, was found to be lower by microarray expression profiling (see below).

A decrease in Notch signaling, as well as in expression of progenitor cell markers, might be due to a depletion of RPCs resulting from cell death or precocious neurogenesis. To distinguish between these possibilities, expression of markers of neuronal differentiation, as well as proneuronal genes, were examined. The Notch ligand, Delta-like 1 (*Dll1*) (Fig. 3I,M), and proneuronal bHLHs *Hes6*, *Neurod1* and *Math3* were significantly upregulated in the CKO retinae (Fig. 3J-P). Interestingly, expression profiling and in situ hybridization (data not shown) failed to detect a change in *Math5*, a proneuronal bHLH that is essential for ganglion cell production. To examine cell death, TUNEL staining was performed on CKO versus wild-type retinae at early stages (E12.5 and E13.5) and later stages (P4 and P10). No differences in TUNEL labeling were observed (data not shown). Together, these data are consistent



C

GENBANK	GENENAME	Average	Std Dev
BE954943	Cyclin D1	2.04	0.94
BE952133	Notch gene homolog 1 (Drosophila)	1.87	0.52
BE952148	hairy and enhancer of split 5 (Drosophila)	1.80	0.68
Lab clone	secreted frizzled-related sequence protein 2	1.79	0.47
BE953485	neurofilament, light polypeptide	1.73	0.72
BE951606	ISL1 transcription factor, LIM/homeodomain (islet 1)	1.74	0.72
AI841303	growth associated protein 43	1.65	0.65
AI851652	Hey1	1.61	0.16
BE952015	fibroblast growth factor 15	1.56	0.13

D

GENBANK	GENENAME	Average	Std Dev
Lab clone	cone-rod homeobox containing gene	4.30	0.87
Lab clone	Orthodenticle homolog 2 (Drosophila)	3.55	0.66
AI846749	Math3	3.53	1.20
Lab clone	Guanine nucleotide binding protein, alpha transducing 2	3.30	2.07
BE995557	thyroid hormone receptor beta	2.64	1.15
Lab clone	NeuroD1	2.59	0.55

Fig. 4. Gene changes in Notch1 ablated retinae at E13.5. Retinae from wild-type and *Notch1* CKO littermates were harvested at E14.5, pulsed with BrdU in vitro for 1 hour and dissociated. (**A**) DAPI-positive cells were scored for immunoreactivity for β-tubulin III, 270.7 or BrdU. Two-thousand to 3000 cells were scored from two or three independent retinae for each genotype. Change in immunoreactive cells in the *Notch1* CKO retinae compared with control (wild type) is statistically significant (*P<0.05, two-tailed t-test assuming equal variances). Retinae from four wild-type and *Notch1* CKO littermates were harvested at E13.5 and processed for RNA isolation and cDNA preparation. cDNA was amplified, labeled and hybridized to cDNA microarrays. (**B**) Scatterplot representing the ratio of gene expression in wild-type versus mutant samples (y axis) plotted against the intensity value in the mutant sample (x axis). Each spot corresponds to one gene. Any spot that lies above 1 on the y axis represents a gene that is expressed at a higher level in the wild-type tissue; conversely, any spot that lies below 1 on the x axis represents a gene that is higher in the Notch1 CKO tissue. (**C**,**D**) Selected genes expressed at a lower (C) or higher (D) level in the *Notch1* CKO are summarized with gene name, Accession Number, average fold change and s.d. in a dye swap experiment. *Some spots on array that correspond to laboratory clones, sequences are available upon request. The complete set of microarray results are included in Data S1 in the supplementary material.

with an increase in neurogenesis in Notch1-deficient retinae, probably as a consequence of progenitor cells producing a higher ratio of postmitotic to mitotic progeny, relative to control retinae.

Notch1 deficient progenitors initiate the photoreceptor transcriptional program

Previously, it was observed that reduction of *Notch1* levels early during chick retinal development with antisense oligonucleotide-mediated knockdown led to an enhancement of ganglion cell production (Austin et al., 1995). As deletion of *Notch1* in the embryonic mouse retina led to the precocious onset of neuronal markers, it was next determined whether completely removing *Notch1* results in a similar increase in retinal ganglion cell production. In addition, it was of interest to determine if removal of *Notch1* led to a change in proliferation at this age. Retinae were harvested from wild-type and *Notch1* CKO mice at E14.5 and pulsed with BrdU for 1 hour. Retinae were dissociated and then immunostained with anti-BrdU and two antibodies that recognize retinal ganglion cells, anti-β-tubulin III and anti-neurofilament (antibody #270.7) (Fig. 4A). The percentage of DAPI-positive cells that incorporated BrdU was 32±8% in the wild-type retinae when compared with 17±2% in the *Notch1* CKO retinae. This reduction in BrdU-positive cells in the *Notch1* CKO is consistent with mitotic cells either prematurely exiting cell cycle and producing neurons or an alteration of cell cycle kinetics. Surprisingly, the percentage of DAPI-positive cells expressing β-tubulin III and NF was also reduced in *Notch1* CKO retinae relative to wild-type retinae. The percentage of β-tubulin III-positive cells in the wild-type retinae was 18±1%, whereas in the *Notch1* CKO retinae it was 8±2%. Similarly, the percentage of NF-positive cells was reduced in the mutant retinae from 17±1% cells in the wild-type retinae to 9±3% cells in the *Notch1* CKO retinae.

Microarray analysis was carried out on CKO versus wild-type E13.5 mouse retinae to determine more broadly the molecular changes that resulted from deleting Notch1 (Fig. 4B). Consistent with in situ hybridization analysis, components of the Notch pathway (*Notch1*, *Hey1*) as well as progenitor markers (cyclin D1, *Fgf15*, *Sfrp2*) were lower in mutant retinae (Fig. 4C). In addition, higher levels of the proneuronal bHLHs *Neurod1* and *Math3* were seen. Consistent with the dissociated cell immunostaining analysis, there were lower levels of the retinal ganglion cell markers, islet 1 and *Gap43*. By contrast, higher levels of the photoreceptor transcription factors *Crx* and *Otx2*, and of the photoreceptor specific gene cone transducin α were observed (Fig. 4D).

To confirm these results, in situ hybridization was carried out on E13.5 *Notch1* mutant retinae with the photoreceptor genes, *Otx2* and *Crx* (Fig. 5A,B,E,F), and retinal ganglion cell markers, neurofilament light (NF-L; *Nefl* – Mouse Genome Informatics) and islet 1 (Fig. 5C,D,G,H). As predicted by the microarray results, a significant increase in photoreceptor markers concomitant with a decrease in retinal ganglion cell markers were observed. Thyroid hormone receptor β 2 and retinoid x receptor gene γ are two additional genes involved in early photoreceptor development (Forrest et al., 2002; Hoover et al., 1998). Both of these genes were also observed to be upregulated in *Notch1* CKO

retinae at E13.5 by in situ hybridization (data not shown). These data suggest that enhanced neurogenesis in E13.5 *Notch1*-deficient retinae included an increased production of photoreceptors. Interestingly, not all early-born neurons were increased; in fact, there was a decrease in expression of retinal ganglion cell markers.

As previous data in other species had suggested that reduction of the Notch pathway would result in enhanced production of neuronal cell types, and in particular of the first-born retinal ganglion cell (Austin et al., 1995), the early requirement for Notch1 in the mouse was further determined by breeding floxed *Notch1* mice to mice expressing cre recombinase under the *Foxg1* promoter (Hebert and McConnell, 2000). *Foxg1* is a winged helix transcription factor that proceeds *Chx10* expression in the retina. Fate-mapping experiments have determined that *Foxg1* descendants are uniformly labeled in the nasal embryonic retina (Hebert and McConnell, 2000; Pratt et al., 2004). Deletion of *Notch1* in *Foxg1-CRE* expressing cells therefore should exclude the timing or variable expression of *Chx10-CRE* as a potential explanation for why photoreceptor production is enhanced, while ganglion cell production is reduced.

To confirm that the loss of *Notch1* reduces progenitor status and enhances neurogenesis, in situ hybridization was carried out on E13.5 *Notch1* flox/flox; Foxg1-CRE mutant retinae for cyclin D1 and *Dll1*. As predicted, cyclin D1 expression (Fig. 5I,M) was reduced, whereas *Dll1* expression was enhanced (data not shown). In situ hybridization was next performed for the photoreceptor genes *Otx2* and *Crx* and retinal ganglion cell markers, *Nefl* and islet 1. Both photoreceptor genes were upregulated (Fig. 5J,N and not shown), whereas both ganglion cell genes were downregulated (Fig. 5K,L,O,P). These data further corroborate a requirement for Notch1 in the production of retinal ganglion cells. The *Notch1* flox/flox; *Foxg1-CRE* mice are perinatal lethal (Mason et al., 2005) and so later developmental aspects of Notch signaling were examined only in the *Notch1* flox/flox; *Chx10-CRE* mice.

Retinal progenitors lacking Notch1 develop predominantly into cone photoreceptors at the expense of all other cell types

Ablation studies of Notch1 in other regions of the nervous system have demonstrated a requirement for Notch1 in the completion of neuronal differentiation. Premature differentiation of *Notch1*-deleted cells in the cerebellum led to an upregulation of proneuronal bHLH proteins at embryonic stages, but fate mapping of these mutant cells into the adult stages revealed a decrease in cell survival. As a result, many *Notch1*-deficient neurons were eliminated by apoptosis and very few of them were detected in the mature cerebellum (Lutolf et al., 2002).

To test for a similar requirement for Notch1 in the retina, the fates of X-gal+, presumed *Notch1*$^{-/-}$, cells in the fully formed P15 retina were determined. The wild-type littermate retinae revealed X-gal+ cells contributing to all of the cellular layers (Fig. 6A,D). By contrast, X-gal+ cells contributed only to a subset of the mature retinal cell layers in the *Notch1* mutants (Fig. 6B,E). In CKO retinae with a very high level of X-gal+ cells, abnormal morphology precluded proper identification of the cell types that were fate mapped (Fig. 6C,F). By contrast, a lower expression of cre recombinase in some *Notch1* mutants led to a more normal retinal morphology and size (Fig. 6B,E). In these mice, X-gal staining was most prominent in the scleral region of the outer nuclear layer. Staining was observed also in the scleral portion of the inner nuclear layer, and/or possibly in the outer plexiform layer, and in a subset of cells in the ganglion cell layer. The staining in the scleral region of the outer nuclear layer is most consistent with cone photoreceptor

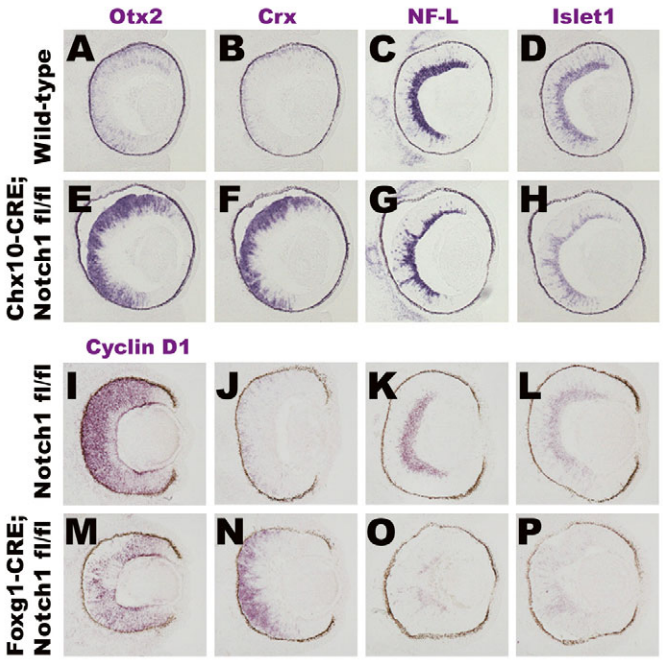


Fig. 5. Gene expression in Notch1 ablated retinae at E13.5. Gene changes were examined by section in situ hybridization on wild-type (**A-D**), *Notch1* flox/flox; *Chx10-CRE* (**E-H**), *Notch1* flox/flox (**I-L**), and *Notch1* flox/flox; *Foxg1-CRE* (**M-P**) retinae at E13.5. (A,E) *Otx2*, (B,F,J,N) *Crx*, (C,G,K,O) *Nefl*, (D,H,L,P) islet 1, (I,M) cyclin D1.

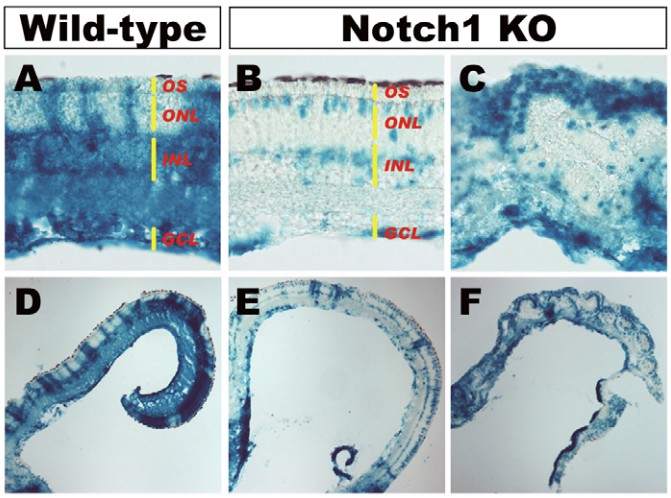


Fig. 6. Fate mapping and gene expression in mature *Notch1* ablated retinae. Fate mapping of wild-type (**A**,**D**) and *Notch1*-ablated (**B**,**C**,**E**,**F**) retinal progenitor cells at P14 as detected by X-gal staining at high (A-C) and low (D-F) magnification. INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segment.

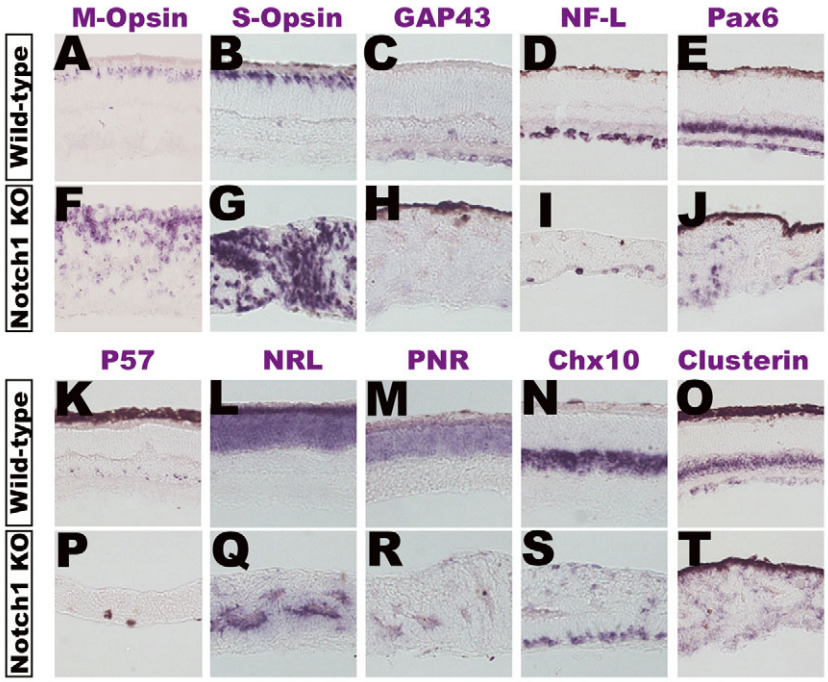


Fig. 7. Gene expression in mature *Notch1* ablated retinae. Section in situ hybridization on wild-type (A-E,K-O) and *Notch1* CKO (F-J,P-T) retinae at P15. (**A**,**F**) *M-Opsin*, (**B**,**G**) *S-Opsin*, (**C**,**H**) *Gap43*, (**D**,**I**) *Nefl*, (**E**,**J**) *Pax6*, (**K**,**P**) *p57*, (**L**,**Q**) *Nrl*, (**M**,**R**) *Pnr*, (**N**,**S**) *Chx10*, (**O**,**T**) clusterin,

cells. These data suggest that *Notch1* deficient progenitors predominantly give rise to cone photoreceptor cells at the expense of all other cell types.

To further clarify the adult cell composition of *Notch1*-deficient retinae, in situ hybridization was performed with markers on P15 retinae. Markers of cone photoreceptor cells (*M-opsin, S-opsin, Pde6h*) were significantly higher in *Notch1*-deficient retinae (Fig. 7A,B,F,G and data not shown). Markers of other early born retinal neurons, including *Gap43* and *Nefl* (retinal ganglion cells), *Pax6* (amacrines, retinal ganglion cells, horizontals) and *p57* (amacrines) (Dyer and Cepko, 2001b) were significantly lower (Fig. 7C-E,H-K,P). Markers of late-born cell types also were lower. The rod photoreceptor specific transcription factors, *Nrl* and *Pnr*, were downregulated (Fig. 7L,M,Q,R) as were *Chx10* (mature bipolar marker) and clusterin (Muller glial cells) (Fig. 7N,O,S,T).

Deletion of Notch1 in postnatal retinal progenitors promotes rod photoreceptor production

To probe whether late RPCs require Notch1 for proper cell fate determination, *Notch1* was clonally inactivated in postnatal day 0 (P0) and postnatal day 3 (P3) *Notch1* flox/flox mice. Newborn or 3 day old mice were injected with a retrovirus encoding alkaline phosphatase without (LIA) or with cre recombinase (LIA-CRE). Retinae were harvested and processed for alkaline phosphatase staining after two or more weeks of development. At these stages, retinal cell fate decisions are complete and the fates of infected cells can be readily identified by alkaline phosphatase staining (Fig. 8A) (Fields-Berry et al., 1992; Turner and Cepko, 1987).

When P0 retinae were infected with LIA virus, the cells labeled were 80% rod photoreceptor cells, 2% amacrine cells, 13% bipolar interneurons and 5% Muller glial cells. By contrast, infection with LIA-CRE virus resulted in 93% rod photoreceptor cells, 2% amacrine cells, 5% bipolar interneurons and 0% Muller glial cells (Fig. 8B). Similarly, infection of the P3 retina with LIA-CRE led to enhanced rod photoreceptor production from 78% to 93%. Again, this occurred at the expense of other cell types, as bipolar interneurons decreased from 11% to 3% and Muller glial cell production was reduced from 10% to 1% (Fig. 8C). Furthermore, the percentage of clones with more than one cell consisting of only rods increased from 37% (LIA) to 76% (LIA-CRE) in clones initiated at P0 and from 32% (LIA) to 69% (LIA-CRE) in clones initiated at P3. Clones of more than one cell are those in which viral integration occurred in a cell that went on to divide at least once more; these clones tend to be more diverse in terms of cell type, and thus are a more sensitive indicator of an effect of loss of Notch1 on cell fate decisions.

DISCUSSION

In this study, the cellular and molecular consequences of inactivation of Notch1 in the retina at early and late developmental time points were analyzed. Notch1 was found to be essential for proper proliferation, morphology and cellular diversification through inhibition of the photoreceptor fate.

Chx10 driven expression of cre recombinase most probably eliminates *Notch1* between E11 and E12 (Rowan and Cepko, 2004), coincident with the first *Notch1* expression observed by in situ hybridization in the mouse retina (data not shown). By E13.5, in situ hybridization and microarray analysis showed a reduction in early progenitor markers, such as *Fgf15* and cyclin D1, and BrdU incorporation at E14.5 revealed a decrease in proliferating cells. Understanding which genes are directly sensitive to Notch signaling will be crucial to furthering our understanding of how Notch influences progenitor cells. Clonal inactivation of Notch1 revealed that cell-autonomous effects account for at least some of the retinal size defects. The known Notch targets, *Hes1*, *Hes5* or *Hey1*, may account partly for the reduction in size, as well as the morphological defects. *Hes1* null mice have severely reduced retinal size and form rosettes (Tomita et al., 1996). However, we were unable to detect a reduction in *Hes1* mRNA in the *Notch1*-deficient retinae at E13.5 by in situ hybridization and microarray analysis. By contrast, both *Hey1* and *Hes5* were reduced in the microarray analysis, and these genes also may be partially responsible for the reduction in size. Further clarification may require compound inactivation of both Notch1 and

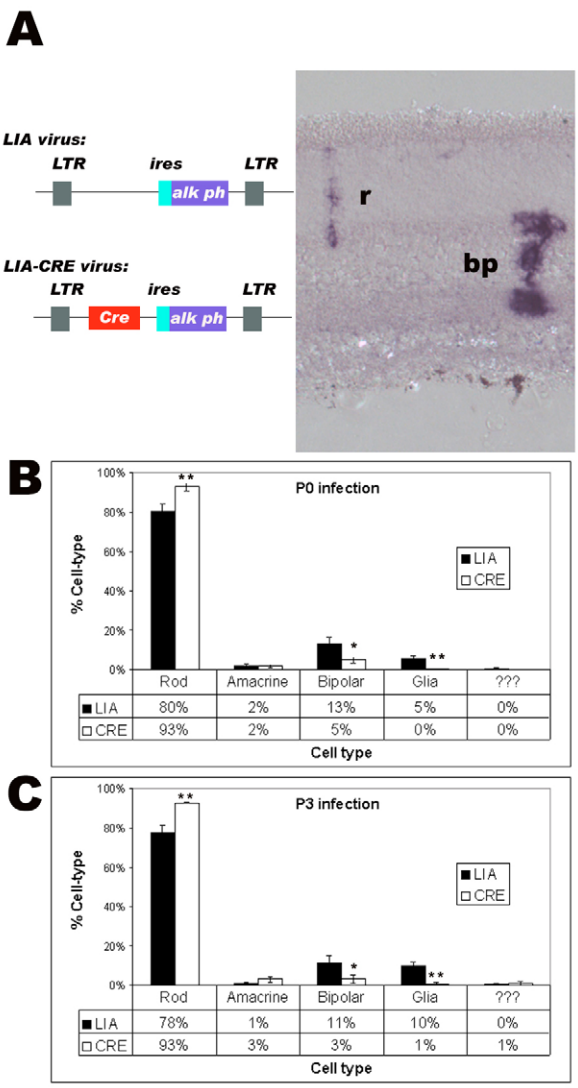


Fig. 8. Clonal inactivation of *Notch1* in postnatal RPCs. P0 or P3 *Notch1* flox/flox mice retinae were infected in vivo with a replication incompetent retrovirus encoding alkaline phosphatase without or with cre recombinase (pLIA versus pLIA-CRE). (**A**) Representative clones from a LIA infection including one single rod clone and one bipolar clone. (**B**,**C**) Quantitation of cell types resulting from LIA or LIA-CRE infections on *Notch1* flox/flox retinae at P0 (B) and P3 (C). Three independent retinae totaling 400-800 clones were analyzed for each timepoint and type of virus. Change in cell types in the LIA-CRE-infected compared with control (LIA-infected retinae) is statistically significant (*P<0.05, **P<0.01, two-tailed t-test assuming equal variances).

Hes family members. Interestingly, previous experiments examining the status of Hes genes in *Notch1*-deficient mice also have shown that some Hes genes are sensitive to Notch signaling whereas others are not (de la Pompa et al., 1997; Lutolf et al., 2002). For example, *En1-CRE* mediated excision of *Notch1* led to a reduction of *Hes5*, but not *Hes1*, in the ventral cerebellum (Lutolf et al., 2002).

Although Notch1 loss-of-function studies in other parts of the central nervous system have also demonstrated an enhanced initiation of the neurogenesis program, these studies did not specifically address a potential role for Notch activity in determining particular neuronal cell fates (de la Pompa et al., 1997; Lutolf et al., 2002). Previously, our laboratory showed that knockdown of *Notch1* in the developing chicken retina led to increased production of retinal ganglion cells (Austin et al., 1995). Although other cell types were not assayed, these data were consistent with the hypothesis that Notch1 blocks cell differentiation and release from this signal allows enhanced production of the early-born cell types. Consistent with our previous results, we found that ablation of *Notch1* in the early mouse retina also leads to enhanced production of early-born neurons.

Surprisingly, we found that not all early-born cell fates were enhanced in the mature retina at P15. In fact, markers for all retinal cell types were dramatically reduced, except for the markers of cone photoreceptor cells. As precociously produced neurons can be eliminated by apoptosis, we checked for early neuronal markers at E13.5 by gene profiling and in situ hybridization. These approaches showed that both the proneuronal bHLH genes, *Math3* and *Neurod1* as well as early photoreceptor genes, *Otx2* and *Crx*, were upregulated. By contrast, the early ganglion cell markers, *Gap43*, islet 1 and *Nefl*, were downregulated. Similar in situ hybridization results were observed when *Notch1* was deleted by cre driven by the earlier expressed and uniformly acting *Foxg1* promoter. Furthermore, TUNEL staining revealed no differences in cell death in the wild-type and CKO retinae at E12.5 and E13.5. A reduction of β-tubulin III and *Nfel*-positive cells on dissociated E14.5 CKO retinae further confirmed a loss of retinal ganglion cells and other non-photoreceptor neuronal cells. Together, these data suggest that ganglion cell production is significantly reduced in the absence of Notch1.

The reasons for overproduction of ganglion cells in the chick following *Notch1* knockdown, versus cone photoreceptor cell overproduction in the mouse following removal of *Notch1*, are not clear. However, overproduction of photoreceptor cells was also observed in *Xenopus* when Delta1 was misexpressed at early and late timepoints (Dorsky et al., 1997). Presumably, cells overexpressing *Delta1*, which were surrounded by wild-type cells, escaped Notch inhibition through negative feedback. Interestingly, the *Delta1*-expressing cells predominantly became or produced photoreceptor cells, even though other cell fates were available at both timepoints tested. When Delta1 was misexpressed early (16-cell stage), retinal ganglion cell production increased slightly, whereas cone photoreceptor production increased dramatically from ~7% to ~61% at the expense of other cell types, including the early-born amacrine and horizontal cells. When Delta1 was introduced at stage 18, rod and cone photoreceptor production increased dramatically from ~26% to greater than 50% at the expense of both bipolar and Muller glial cells (Dorsky et al., 1997). Similarly, addition of the γ secretase inhibitor DAPT in the early developing chick retina led to a significant increase in visinin positive photoreceptor cells (Kubo et al., 2005). By contrast, introduction of a dominant negative form of Delta1 led to enhanced production of ganglion and amacrine cells at the expense of photoreceptor cells in both Xenopus and chick (Dorsky et al., 1997; Henrique et al., 1997; Kubo et al., 2005). The relative levels of Notch activity in DAPT treated or Delta1- and dnDelta1-misexpressing cells is unclear. However, one interpretation consistent with all of the data from chick, mouse and *Xenopus* is that the level of Notch signal is crucial for cell fate determination (Fig. 9). Very low levels of Notch would favor photoreceptor production, probably through induction of Otx2 (Nishida et al., 2003), while slightly higher levels of Notch would favor production of other neuronal cell types (Hatakeyama and Kageyama, 2004). Very high levels of Notch would prohibit exit from cell cycle and inhibit production of any postmitotic cell type (Dorsky et al., 1995) (A.P.J. and C.L.C., unpublished).

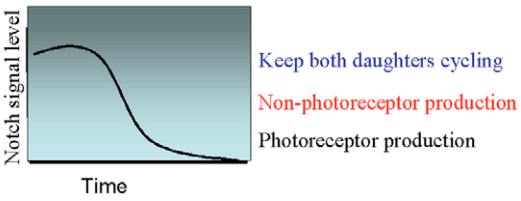


Fig. 9. Model of Notch activity in retinal development. Notch is expressed in cycling retinal progenitor cells, where it can be proteolytically processed to form an intracellular fragment, NICD, following its interaction with a Notch ligand. The level of NICD-mediated signal that is finally transduced is read by a progenitor cell to determine the mitotic fate of the daughter cells. If the signal is below a threshold for cycling, at least one postmitotic daughter will be made. If the signal is above physiological levels for cycling, early progenitor cells do not continue to cycle, but they also do not make neurons (Dorsky et al., 1995) (A.P.J. and C.L.C., unpublished). If a postmitotic daughter is made, the level of Notch signaling is a determinate of the fate of the postmitotic daughter. If the Notch signal level is very low, as is the case in a complete loss of function, a photoreceptor is made, probably through activation of Otx2 (Nishida et al., 2003). If the level is intermediate, a non-photoreceptor neuron is made. The type of non-photoreceptor neuron is determined by the combination of homeobox and bHLH proteins present in the progenitor and/or newly postmitotic cell (Hatakeyama and Kageyama, 2004).

By viral transduction of cre recombinase, *Notch1* was inactivated in clones initiated at P0 and P3. If removing *Notch1* simply forces cells to exit and differentiate, it might be expected that ablation of *Notch1* would promote generation of the types of neurons peaking in production at the relevant time point. Birthdating studies in mouse have established that at P0, rod photoreceptor production peaks, whereas at P3, bipolar interneuron production peaks (Young, 1985). Surprisingly, *Notch1* deleted progenitor cells almost exclusively produced rod photoreceptors at both P0 and P3. We conclude from these studies that Notch1 is required for progenitor cells and/or newly postmitotic cells to sort out the photoreceptor versus non-photoreceptor cell fate. As predicted by the birthdating curve, ablation of *Notch1* early in development promotes the first born photoreceptor cell type, the cone photoreceptor, while ablation of *Notch1* later in development promotes the later born photoreceptor cell type, the rod photoreceptor.

Hes1-null mutants show precocious and enhanced development of many early born cell types, including retinal ganglion cells, horizontal cells and amacrine cells, and a decrease in the later born Muller glial cells. *Hes5*-null mice are also deficient in Muller glial cells, but the production of neuronal cell types is unaffected. As neither *Hes1*- nor *Hes5*-null mice exhibit a bias in producing the photoreceptor versus non-photoreceptor cell fate (Hojo et al., 2000; Takatsuka et al., 2004; Tomita et al., 1996), this aspect of Notch activity is most likely Hes independent. Single or compound mutations in various positive bHLH transcription factors also lead to a relatively specific loss of particular non-photoreceptor cell fates. Null mutations in *Math5* lead to loss of ganglion cells (Brown et al., 2001; Wang et al., 2001), mice lacking both *Mash1* and *Math3* fail to produce bipolar interneurons (Hatakeyama et al., 2001), while mice lacking both *Neurod1* and *Math3* are missing amacrine cells (Inoue et al., 2002). The loss of non-photoreceptor cell fates in the *Notch1* CKO mice is most probably not due to the loss of specific bHLH transcription factors, as a decrease in *Math5* expression could not be appreciated by gene profiling or in situ hybridization (data not shown). Furthermore, Math3 and Neurod1 are both required for the amacrine and bipolar fates, and expression of both these genes was significantly upregulated in the *Notch1* CKO retinae. Another gene significantly increased in E13.5 Notch1 ablated retinae was *Otx2*, a transcription factor recently shown to be expressed in newly postmitotic neurons and essential for photoreceptor differentiation (Nishida et al., 2003). In that study, misexpression of Otx2 in postnatal day 0 rat increased the production of rod photoreceptor-only clones from 80.7% to 95% (Nishida et al., 2003). Our results suggest that Notch signaling directly or indirectly regulates the level of *Otx2*, which is crucial for ensuring the proper ratio of photoreceptor versus non-photoreceptor fates produced. High levels of *Otx2* might be dominant to the activities of bHLH and other genes that can induce non-photoreceptor neuronal fates.

In the developing *Drosophila* retina, Notch signaling is recruited for multiple activities including: (1) setting up a dorsal/ventral boundary, (2) establishing planar polarity, (3) upregulating early atonal levels to promote neurogenesis and (4) antagonizing late atonal activity to inhibit neurogenesis (Frankfort and Mardon, 2002). This study and previous reports suggest similarly diverse roles for Notch activity in the vertebrate retina. First the early developing vertebrate retina expresses *Notch1* initially only in the central region of the retina (Lindsell et al., 1996) (data not shown), suggesting that Notch-Delta signaling is necessary only when neurogenesis begins. As ablation of *Notch1* dramatically influences organ size and introducing activated Notch can lead to hyperproliferation (Bao and Cepko, 1997), Notch1 most probably engages the cell cycle machinery. Interestingly, when *Notch1* was deleted in E14.5 retinal progenitor cells, smaller clones were observed, but a total loss of more than three cell clones was not observed. The modest effect of loss of Notch on clone size is in keeping with data from *Drosophila*. Loss of Notch from the entire eye disc leads to a dramatic reduction in size, whereas loss of Notch in clones does not dramatically reduce clone size. A non-autonomous ligand, unpaired, that is activated by Notch is thought to mediate these effects (Chao et al., 2004). In vertebrates, it is not known if there is such a non-autonomous ligand. Nonetheless, in mice, Notch appears to be one regulator of cell proliferation, but it is not essential for cell cycle progression. It is, however, sufficient for at least some period of time, as sustaining Notch activity in late retinal progenitor cells leads to formation of very large clones (Furukawa et al., 2000). Notch activation also leads to a block in neuronal fates and an increase in cells with some glial characteristics. The data presented here also clearly indicate a cell fate role for Notch1 in the vertebrate retina. The specific promotion of the photoreceptor fate at the expense of both glial and other neuronal cell fates following deletion of Notch1 reveals that it normally represses the photoreceptor fate. If photoreceptors were the initial cell type when the retina first evolved, one speculative interpretation of these data is that Notch was recruited to allow preservation of a pool of RPCs for later cellular diversification

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/5/913/DC1

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