Notch Activity Is Downregulated Just prior to Retinal Ganglion Cell Differentiation

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Abstract
The Notch signaling pathway is important at several stages of retinal development including the differentiation of retinal ganglion cells and Müller glia. The downstream effectors of Notch signaling, Hes1 and Hes5, have been shown to be critical in the retina as well. While Notch activity directly regulates Hes1 and Hes5 elsewhere in the nervous system, it has been unclear whether Hes1 and/or Hes5 are directly regulated by Notch activity in the developing retina. Here, we report that both Hes1 and Hes5 are directly regulated by Notch activity during retinal development. Using fluorescence-based Hes1 and Hes5 reporter constructs, we can monitor Notch activity in progenitor cells in the intact retina, and we find that Notch activity is downregulated just prior to retinal ganglion cell differentiation.

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Introduction
The cellular and molecular mechanisms by which neurons in the central nervous system (CNS) form ordered mosaic arrays are largely unknown. The retina is a good example of a highly mosaic CNS tissue, where mature retinal neurons are patterned in well-organized arrays, an arrangement that is critical to their function in high-acuity vision. The retina has also served as an excellent model system for exploring both molecular function and cellular interactions during neuronal development. Most of the factors that have been shown to regulate aspects of retinal neurogenesis have also been shown to be key regulators of neurogenesis in other regions of the CNS, and much is known about the type and timing of cells generated during retinogenesis [reviewed by Reh and Levine, 1998; Cepko et al., 1998; Hartenstein and Reh, 2002].

In all species that have been studied, retinal ganglion cells (RGCs) are the first neurons born in the retina [Hartenstein and Reh, 2002]. The process of lateral inhibition, whereby one cell in a field of cells prevents its adjacent neighboring cells from choosing the same fate, has been shown to regulate the total number of RGCs that arise during development [Reh and Klijavin, 1989; Adler and Hatlee; 1989; Altshuler and Cepko, 1992]. Dissociation of early embryonic retinas, which primarily contain progenitor cells, into single cells promotes their differentiation into RGCs. The process of lateral inhibition in the retina is mediated primarily by the Notch signaling pathway [Dorsky et al., 1995, 1997; Austin et al., 1995; Henrique et al., 1997; Bao and Cepko, 1997; Rapaport and Dorsky, 1998; Furukawa et al., 2000; Silva et al., 2003]. Widespread activation of Notch signaling prevents progenitor cells from differentiating and instead, maintains them in the progenitor state [Henrique at al., 1997; Bao...
and Cepko, 1997], while downregulation of the Notch signaling pathway promotes neuronal differentiation [Austin et al., 1995; Silva et al., 2003].

These studies and others have led to the general hypothesis that cells expressing higher levels of Notch activity are inhibited from differentiating, while cells with lower levels of Notch pathway activation differentiate. Thus, the Notch signaling pathway could generate a regular mosaic whereby every other cell would express a Notch ligand or the Notch receptor. Computational modeling studies support this hypothesis by revealing that lateral inhibition mediated by the Notch signaling pathway is sufficient to generate an initial regular mosaic pattern of two different cell types in the retina, which could later be refined by cell death and tangential migration to create a mature pattern [Eglen and Willshaw, 2002].

To investigate whether the Notch pathway is indeed active during ganglion cell differentiation, we developed an assay to monitor the activity of Notch signaling under conditions that maintain normal in vivo intercellular interactions. We show that Notch activity regulates both Hes1 and Hes5 expression in the developing retina. Using fluorescence-based versions of the mouse Hes1 and Hes5 promoters [Takebayashi et al., 1994, 1995; gift of R. Kageyama], we reveal endogenous spatial patterns of active Notch signaling during RGC differentiation. We confirm that the Hes1 and Hes5 reporters are specifically active in progenitor cells and not RGCs. These studies demonstrate that active Notch signaling is inhibited just prior to RGC differentiation. Thus, fluorescence-based versions of the Hes reporters are powerful new molecular tools that will allow spatial and temporal studies of active Notch signaling.

Methods

Embryos and Notch Inhibition

Fertilized white leghorns were obtained (Hyline) and incubated to desired stages [Hamburger and Hamilton, 1951]. All embryos were treated in accordance with IACUC regulations. Embryos were incubated to stage 20/embryonic day E3 or stage 25/E4.5 and pairs of eyes were collected in HBSS+ (Gibco/BRL). Extraocular tissues and the pigmented epithelium were removed, leaving the retina and lens intact. Matched pairs of retinal explants were transferred to a 24-well non-tissue culture-treated plate and cultured [as described in McCabe et al., 1999] for 2–4 days at 37°C with nutation. The combined cytomegalovirus immediate early enhancer (CMV IE) and chicken beta-actin promoter drives enhanced green fluorescent protein (eGFP) in the CAGGS/CAX eukaryotic expression vector [Niwa et al., 1991]. The far-red fluorescent protein from Heteractis crispa (RFP; Clontech) was subcloned into the pMes vector (pCax with IRES2:eGFP) [Swartz et al., 2001; gift of C. Krull] to remove the IRES2:eGFP sequence have been deposited into GenBank (Accession AY916777). Multiple protein alignments were shaded in BOXSHADE.

Cloning and Sequence Analysis of Chicken Hes5

ChEST382121 corresponding to a previously reported chick expressed sequence tag (ChEST) from the BBSRC Chick EST Database with similarity to mouse Hes5 [Bylund et al., 2003] was obtained (MRC GeneService), sequenced, and assembled with Sequencher3.0. This ChEST represents an approximately 1.188-kb insert with an internal 474-bp open reading frame that translates into a 157AA peptide. This translated peptide sequence was used for ClustalX multiple protein alignment with mouse Hes5 (MM-Hes5; GenBank Protein Accession NP_034549), M. musculus Hes1 (MM-Hes1; GenBank Protein Accession NP_032261), Homo sapiens Hes5 (HS-Hes5; GenBank Protein Accession NP_001019926), H. sapiens Hes1 (HS-Hes1; GenBank Protein Accession NP_005515), and Gallus gallus c-Hairy1 (GG-Hes1; GenBank Protein Accession O57337). GG-Hes5 DNA and peptide sequence have been deposited into GenBank (Accession AY916777). Multiple protein alignments were shaded in BOXSHADE.

Quantitative Polymerase Chain Reaction Analysis

Quantitative polymerase chain reaction (QPCR) was performed essentially as described [Kubota et al., 2004; Nelson et al., 2004]. Three pairs of E4.5 retinal explants were prepared as above; 10 μM DAPT was added to one retina while an equal volume of DMSO was added to the sister retina from each pair, and cultured for 2 days as above. Afterwards, the lens was removed and tissue was extracted for total RNA with Trizol (Invitrogen). Genomic DNA was then digested with DNase (Invitrogen) for 1 h at 37°C and further purified on an RNasey column (Qiagen). Samples were split and prepared for oligo-dT primed cDNA synthesis reactions. SuperScript II reverse transcriptase (RT; Invitrogen) was added to the positive reaction only, creating an RT-minus control reaction for each sample. Primers (Invitrogen) were designed against the available DNA sequence for chick glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank Accession NM_204305) and GG-Hes1 (GenBank accession AF032966) and GG-Hes5 (GenBank submission in progress). Primer sets: Hes1 forward 5'-GAA-GTCCTTCAAAACCATCA-3', reverse 5'-AGGTTGCTTACCGTG- CATCCT-3'; Hes5 forward 5'-CCAGACAGACACACAAC-3', reverse 5'-CAGAGCTTCTTT GGAGCCAC-3'; GAPDH forward 5'-CATCCAAGAGTGAGCCAAG, reverse 5'-TGAGGAAATTTGGAGGA.

Expression Plasmids

The combined cytomegalovirus immediate early enhancer (CMV IE) and chicken beta-actin promoter drives enhanced green fluorescent protein (eGFP) in the CAGGS/CAX eukaryotic expression vector [Niwa et al., 1991]. The far-red fluorescent protein from the reef coral Heteractis crispa (RFP; Clontech) was subcloned into the pCax expression vector as follows: pHcRed1 (Clontech) was cut with HI, blunt-ended as before, and then cut with BamHI. The pMes vector (pCax with IRES2:eGFP) [Swartz et al., 2001; gift of C. Krull] was cut with BgII, blunt-ended as before, and then cut with BamHI to remove the IRES2:eGFP sequence, leaving the pCax vector backbone for ligation of the HcRed1 sequence (pRFP). Hes1 and Hes5 cis-regulatory elements have been described [Takebayashi et al., 1994, 1995]; Hes1 and Hes5 cis-regulatory elements driving destabilized eGFP (approx.
and cultured for 1–4 days at 37 °C with nutation. Explants were transferred to a new 24-well plate with 1 ml of media and applied with a square-wave electroporator (BTX). Transfected explants were transferred to a new 24-well plate with 1 ml of media and cultured for 1–4 days at 37°C with nutation.

To determine if the Hes reporters were sensitive to DAPT inhibition of Notch signaling, explants were cotransfected with a mixture of GFP/RFP, Hes1GFP/RFP, or Hes5GFP/RFP (six explants per mixture). DAPT was added to a final concentration of 10 μM to half of the explants, while an equal volume of DMSO was added to the other half as a negative control, and cultured for 2 days as above.

Immunocytochemistry and in situ Hybridizations

Explants were prepared for cryosectioning by fixing in 4% paraformaldehyde for 15–30 min at room temperature, and cryoprotected through a sucrose gradient before being embedded in OCT (Tissue-Tek). Sections were rinsed in PBS and blocked in 10% goat serum PBS-0.1% Triton X-100. For BrdU detection, slides were first treated with 4 N HCl for 7 min, rinsed 4 times with PBS and then blocked. Primary antibodies include rabbit antineurofilament M (145 kDa; 1:1,000 dilution, Chemicon), rat anti-BrdU (1:80 dilution; Accurate Chemical), and mouse anti-Islet1 (1:5, Developmental Hybridoma Studies Bank). Secondary antibodies were species-specific AlexaFluor 350, 488, or 568 nm, depending on the desired wavelength (1:500, Molecular Probes). Sections were mounted in Fluoromount-G (Southern Biotechnology Associates). DIG-labeled antisense riboprobes were prepared for in situ hybridization analysis for endogenous chicken Hes1 and Hes5 expression, and were combined with immunolabeling for BrdU incorporation and neurofilament detection as described [Nelson et al., 2004].

Imaging

Explants were imaged with a fluorescent stereo dissecting scope (Zeiss and/or a Nikon, GFP/Texas Red filter set, equipped with a Spot Camera) and/or a 2-photon laser scanning confocal microscope (Leica SP1/MP). Images of whole-mount DAPT-treated transfected explants were acquired by first optimizing the RFP signal settings (which was held constant for all images). The GFP signal was optimized for control GFP, Hes1GFP, and Hes5GFP transfactions, respectively, and then was held constant and used to image the DAPT-treated explants. Explants were also flatmounted in 75% glycerol/PBS for 2-photon laser scanning confocal microscopy (2P/LSCM). Acquired optical series were analyzed with Velocity Imaging Software (Improvision). Stacks were rendered and analyzed in 3 dimensions, which allowed the detection of double- and triple-labeled cells in flatmounted explants and sections. All images were assembled in Photoshop (Adobe).

LSCM was also used to quantify the effect of NICD on Hes1GFP and Hes5GFP reporters. Imaging conditions were established for each Hes1GFP/RFP and Hes5GFP/RFP control groups, respectively, by optimizing the laser and detector settings to maximize signal and minimize saturation for both RFP and GFP signals. Once optimal imaging conditions were established, the remaining Hes1GFP/RFP and Hes5GFP/RFP/NICD explants, and Hes5GFP/RFP and Hes5GFP/RFP/NICD explants were imaged, respectively. Histograms plotting the absolute frequency of the number of pixels at a given signal strength (0–250 relative intensity units) were acquired from each image. The sum of pixels weighted according to their signal strength (with a cutoff threshold of 10) for each respective channel was acquired and used to calculate the ratio of GFP to RFP levels. The mean ratios and standard deviation for each control and NICD condition (n = 3 explants per condition) were calculated, and a one-way Student’s t test was used to determine significance between control and experimental groups. This optical expression assay is likely to reflect a conservative estimate of changes in reporter expression due to the fact that pixels with intensities greater than 250 are not measured as such and are only weighted according to the maximum intensity value of 250.

Results

Loss of Notch Signaling Inhibits Retinal Growth and induces Neuronal Differentiation

To determine the effects of the loss of active Notch signaling during early retinal development, we used the soluble gamma-secretase inhibitor DAPT to prevent the gamma-secretase-dependent S3 cleavage event that generates active Notch intracellular domain (NICD) [Geling et al., 2002]. This cleavage step is necessary to allow Notch ligand-mediated binding and release of the Notch extracellular domain and subsequent cleavage of the

Fig. 1. Inhibition of Notch signaling prevents retinal growth and proliferation by inducing neuronal differentiation. DAPT, a gamma-secretase inhibitor [Geling et al., 2002], was used in a dose-response assay to block active Notch signaling. Pairs of stage 20/E3.0 retinas were collected. DAPT was added to one retina at 0.1, 1.0, 10, and 100 μM (final concentration), while an equal volume of DMSO was added to the sister retina for control, and cultured for 2 days: results are shown for 1.0 and 10 μM DAPT experiments. Notch inhibition prevents retinal growth (compare a, b). Explants were pulsed with BrdU for 2 h prior to fixation, sectioned, and immunolabeled with antibodies to BrdU to identify progenitor cells, or Islet1 to identify neurons. DAPT inhibition of retinal growth was due to a reduction of proliferation (BrdU+ progenitor cells, compare d–f) and induction of neuronal differentiation (Islet1+ neurons, compare g–i; g’–i’ are propidium iodide (PI) counterstained images to visualize all nuclei) in a dose-dependent manner. Scale bar equals 1 mm (a–c), 100 μm (d–f), and 45 μm (g–i).
NICD, which is then free to translocate to the nucleus, form a complex with CSL/RBPj-kappa, and mediate downstream transcriptional events [reviewed by Mumm and Kopan, 2000; Selkoe and Kopan, 2003]. We performed a dose-response experiment to assay the effects of Notch signal inhibition by DAPT (fig. 1). Pairs of E3 retinas were collected and treated with DAPT at 0.1, 1.0, 10, or 100 μM. An equal volume of DMSO vehicle was added to the sister retina to serve as a control, and retinas were cultured for 2 days. Comparison of overall retinal growth demonstrated that increasing DAPT concentration inversely correlated with explant size: concentrations of DAPT 0.1–10 μM completely inhibited growth (fig. 1a–c), while concentrations of DAPT <1.0 μM had negligible effects (data not shown). To further characterize the effects of DAPT on retinal growth, we analyzed cell proliferation with BrdU; 1.0 μM DAPT did not have a noticeable effect on proliferation, although DAPT-treated retinas were still smaller than their control sister retinas, while 10 μM DAPT almost completely blocked proliferation (fig. 1d–f). To determine whether the growth inhibition of DAPT was due to a premature induction of differentiation, we analyzed sections of the explants for ganglion cells, with Islet1 antibody. 1.0 μM DAPT was somewhat effective at inducing neuronal differentiation, while 10 μM DAPT induced virtually complete neuronal differentiation (fig. 1g–i). We conclude from these data that DAPT is effective at inducing premature neuronal differentiation in retinal explants, consistent with its activity in other systems.

Fig. 2. Characterization of chicken Hes5. A chicken EST corresponding to a previously reported sequence with similarity to mouse Hes5 [Bylund et al., 2003] was obtained, sequenced, and assembled using Sequencher. Open reading frame analysis indicated a sequence with a start and stop codon that coded for a 157AA peptide. This translated chick Hes5 (GG-Hes5) peptide sequence was used for multiple peptide sequence alignment with mouse and human Hes5 and Hes1 (MM-Hes5 and MM-Hes1, HS-Hes5 and HS-Hes1) and chick Hes1 (GG-Hes1). a Comparison with mouse and human Hes5 indicates that chicken Hes5 contains a highly similar bHLH motif in conjunction with an orange domain and conserved C-terminus. b Overall protein sequence comparisons indicate that chick Hes5 is 66% and 68% identical to mouse and human Hes5, respectively. Thus, this EST represents the chicken homolog of mammalian Hes5; DNA and peptide sequences have been submitted to GenBank (AY916777).
Cloning and Analysis of Chicken Hes5

To investigate the downstream effectors of Notch signaling during chick retinal development, we took advantage of a previously reported chicken EST with sequence similarity to mouse Hes5 [Bylund et al., 2003]. DNA and protein sequence analysis indicates that this clone had a 1.188-kb cDNA insert with a 474-bp open reading frame that coded for a 157AA peptide containing a basic DNA-binding region in conjunction with a helix-loop-helix motif (bHLH), an orange domain, and conserved WRPW C-terminal tail (fig. 2a). Peptide sequence comparisons with mouse and human Hes5 and chick, mouse, and human Hes1 indicate that chick Hes5 is 66% and 68% identical to human and mouse Hes5 proteins, respectively (fig. 2b). Thus, we conclude that this EST represents the chick-en homologue of mouse and human Hes5.

Notch Signaling Regulates Endogenous Hes1 and Hes5 Expression

Previous studies have implicated the Notch pathway in the regulation of the differentiation of RGCs, but it is not known whether this involves Hes1, Hes5, or both [reviewed by Vetter and Moore, 2001]. To determine whether the inhibition of Notch signaling affected its downstream mediators, Hes1 and Hes5, we analyzed pairs of retinal explants treated with DAPT or control DMSO for changes in Hes1 and Hes5 expression levels by RT-QPCR. Three pairs of E4.5 retinas were prepared and treated with 10 μM DAPT as above, and cultured for 2 days. QPCR was separately performed on each pair. Addition of DAPT shifted the amplification profile, indicating that an increased number of cycles are required due to decreased transcript levels; representative experiments are depicted, each run in triplicate. Quantitation of DAPT effects indicates that inhibition of Notch signaling activity shows a significant approx. 2-fold repression of Hes1 and approx. 10-fold repression of Hes5 transcript levels (error bars indicate standard deviation, three independent experiments).

Notch Signaling Can Be Visualized with Hes Reporters in Developing Retina

To investigate active Notch signaling during RGC differentiation, we monitored expression of destabilized GFP under control of cis-regulatory elements of Hes1 and Hes5. We first used GFP driven by the constitutively active dual CMV IE and chick beta-actin promoter, along with an RFP construct with the same dual ubiquitous promoter system, to monitor cotransfection efficiency.

Fig. 3. Inhibition of Notch signaling represses Hes1 and Hes5 expression. Three pairs of E4.5 eyes were cultured as before (2 days), 10 μM DAPT was added to one of the eyes from the pair, while DMSO was added to the other as a control. Lenses were removed and total RNA was isolated and converted to cDNA, and used as template for QPCR analysis of Hes1 and Hes5 transcript levels. a, b QPCR results for Hes1 and Hes5, respectively: note that addition of DAPT shifts the amplification curve, indicating that an increased number of cycles are required due to decreased transcript levels; representative experiments are depicted, each run in triplicate. c Quantitation of DAPT effects indicates that inhibition of Notch signaling activity shows a significant approx. 2-fold repression of Hes1 and approx. 10-fold repression of Hes5 transcript levels (error bars indicate standard deviation, three independent experiments).
(fig. 4a). For retinal explant transfections, E3–E4.5 chick retinas were prepared as above, and both GFP and RFP control constructs were transfected into the mitotic surface of the retina via electroporation (fig. 4b). The explants were cultured 1–4 days and then analyzed as whole explants, flatmounted retinas, and/or sections (fig. 4c, d). Cotransfection of GFP and RFP demonstrates that >90% of cells express detectable GFP and RFP, although the relative levels may vary somewhat within the same cell (fig. 4e, f). Thus, this method proved quite efficient and reproducible at yielding large numbers of transfected retinal explants under conditions that allow normal intercellular interactions to be maintained.

To visualize Notch activity during RGC differentiation, we used the mouse Hes1 and Hes5 promoters [Takebayashi et al., 1994, 1995; gift of R. Kageyama], which drive d2eGFP (fig. 4a). PEST sequences added to d2eGFP allow greater temporal precision of reporting compared to stable eGFP and RFP. To first test that the Hes reporters were active in response to Notch signaling, we performed loss of Notch and gain of Notch function experiments in conjunction with Hes1 and Hes5 reporter transfections (fig. 5). For loss of Notch function, we added DAPT (10 μM) to GFP/RFP, Hes1GFP/RFP, and Hes5GFP/RFP cotransfected retinas (E4.5 retinal explants, 6 explants per transfection mix: 3 treated with DAPT, 3 with DMSO as negative control) and allowed them to develop in culture for 2 days. Inhibition of Notch activity had no effect on reporting from control GFP and RFP plasmids (fig. 5a, b). However, DAPT treatment completely prevented expression of d2eGFP from the Hes1 and Hes5 reporters com-

![Diagram](image)

**Fig. 4.** Strategy to assay Notch1 signaling pathway activity. **a** Schematic of expression vectors used in this study. eGFP and *H. crispa* RFP (Clontech) are driven by the combined CMV IE and chicken beta-actin promoters. d2eGFP is under the control of approx. 2.5 kb of upstream cis-regulatory sequence of the mouse Hes1 promoter, Hes1::d2eGFP (Hes1GFP) [Takebayashi et al., 1994], and approx. 0.76 kb of upstream cis-regulatory sequence of the mouse Hes5 promoter, Hes5::d2eGFP (Hes5GFP) [Takebayashi et al., 1995; both gifts from R. Kageyama, not drawn to scale]. **b** Schematic of embryonic chick retinal explant transfection method (E3.5 chick) [adapted from Hamburger and Hamilton, 1951]. **c–d** Transfection of GFP demonstrates the ability to target transfection to a discrete region (**c**, brightfield image; **c’**, corresponding GFP fluorescent signal from separate channel as indicated), and reproducibility of explant electroporation technique (**d**: three separately transfected explants exhibiting similar amounts of GFP fluorescence). **e, f** Cotransfection of GFP and RFP into the peripheral eye demonstrates that >90% of cells are double-labeled (**f**, LSCM, image from **e**). Scale bar equals approx. 1 mm (**c–e**), and 45 μm (**f**).
**Fig. 5.** Notch activity regulates Hes1 and Hes5 reporter expression. We performed loss of Notch function (a–f) and gain of Notch function experiments (g–k) to determine whether Notch activity regulates Hes reporter expression during retinal development. a–f For loss of Notch function, E4.5 pairs of retinal explants were cotransfected with GFP/RFP (a, b), Hes1GFP/RFP (c, d), or Hes5GFP/RFP (e, f), 3 pairs of retinas per transfection mix. DAPT (10 μM) was added to 3 explants per transfection mix (b, d, f), while DMSO was added to the corresponding sister explants for control (a, c, e), and cultured for 2 days. Loss of active Notch signaling does not prevent GFP or RFP expression (a–a', b–b'), but does prevent Hes1GFP (c–c', d–d') and Hes5GFP (e–e', f–f') reporter expression (whole-mount view of explants imaged via fluorescent stereo dissecting scope). g–k For gain of Notch function, we used a constitutively expressed NICD (gift of R. Kopan). E4.5 retinal explants were co-transfected with GFP/RFP (data not shown), Hes1GFP/RFP (g–g'), or Hes5GFP/RFP (i–i') (3 retinas per condition) or GFP/RFP/NICD (data not shown), Hes1GFP/RFP/NICD (h), and Hes5GFP/RFP/NICD (j) (3 retinas per condition). A constitutively active Notch signal upregulates both Hes1GFP (h–h') and Hes5GFP (j–j') reporter expression (single LSCM optical sections of flatmounted explants). Hyphenated letters represent corresponding fluorescent signals from separate channels as indicated in each panel. k Quantification of the ratio of GFP-to-RFP expression levels for each condition measured by LSCM demonstrates that NICD significantly increased Hes1GFP (approx. 9- to 10-fold) and Hes5GFP (approx. 2- to 3-fold) reporter expression (Hes1GFP * p < 0.003, Hes5GFP ** p < 0.002, one-way Student’s t test). Scale bars 1 mm (a–f), and 45 μm (g–j).
pared to control RFP (fig. 5c–f). For gain of Notch function experiments, we cotransfected a constitutively active form of Notch (NICD, gift of R. Kopan) along with GFP/RFP, Hes1GFP/RFP, and Hes5GFP/RFP constructs. Addition of NICD to Hes1GFP/RFP and Hes5GFP/RFP transfection mixes resulted in a significant increase in the expression from both Hes1 and Hes5 reporters (fig. 5g–k). Thus, our data together with the results of previous reports [Nishimura et al., 1998; Ohtsuka et al., 1999] indicate that the Hes1 and Hes5 cis-regulatory elements are specifically active in neural progenitor cells due to active Notch signaling, and can be used to monitor Notch signaling activity during retinal development.

Notch Signaling Is Active in Progenitor Cells and Not Ganglion Cells

We noticed that Hes1 and Hes5 reporter constructs were active only in a subset of RFP-transfected cells. Differences in expression levels of d2eGFP were also observed within the subset of Hes1 and Hes5 transfected cells. The highest levels of Hes1GFP reporting were most often observed in cells that extended the entire thickness of the retina, compared to much lower to no reporting in cells located at the mitotic surface (fig. 6a). Hes5GFP/RFP cotransfections closer to the region of initial RGC differentiation also revealed that Hes5GFP had more limited expression within a subset of RFP-transfected cells (fig. 6b, c), although Hes5GFP consistently seemed to report in a greater number of cells and more robustly than Hes1GFP. Differences in expression of d2eGFP were again observed within a subset of Hes5 transfected cells, such that the brightest cells also extended the entire thickness of the retina. While the brightest GFP+ cells appear green in these rendered images, they also express RFP at a cotransfection rate >90% as determined above, which can be observed in single-channel images. In general, the cells with the highest levels of d2eGFP, driven from either the Hes1 or Hes5 reporter constructs, typically had a bipolar morphology and extended across the thickness of the retina, consistent with a progenitor phenotype. In contrast, the cells with the lowest levels of d2eGFP driven from either reporter were located at the ganglion cell surface or at the ventricular surface. In the cotransfections with either the Hes1 or the Hes5 reporter constructs, the red cells in the ganglion cell layer had the morphology of ganglion cells, and often had axons extending some distance to the optic nerve head (fig. 6c).

To determine the identity of Hes1 and Hes5 transfected cells, we immunolabeled sections of Hes1GFP/RFP and Hes5GFP/RFP cotransfected explants with antibodies to neurofilament to label newborn and differentiating ganglion cells [McCabe et al., 1999], rather than Islet1, which does not label newborn ganglion cells, and BrdU to label progenitor cells (fig. 6d, e). 2P/LSCM was used to acquire high-resolution optical series that were rendered and visualized in 3 dimensions to allow unambiguous detection of label overlap. In both Hes1 and Hes5 reporter transfections, d2eGFP was most highly expressed in BrdU+ progenitor cells. However, it is interesting to note that not all RFP+/BrdU+ cells were active for Hes1GFP or Hes5GFP, which could be part of the approx. 10% of cells not transfected with both constructs, or perhaps that not all progenitor cells mediate Notch activity through Hes1 or Hes5. Additionally, not all Hes1GFP+/RFP+ or Hes5GFP+/RFP+ cells were BrdU+, which could be due to not incorporating BrdU in the short time period. In contrast to progenitor cells, d2eGFP was not detected in neurofilament-positive newborn, migrating, or maturing RGC extending axonal projections. These data demonstrate that the Hes1GFP and Hes5GFP reporters are specifically expressed in retinal progenitor cells and not newborn RGCs, and that RFP expression can serve to identify RGCs.

To further confirm that Hes1 and Hes5 reporters are active in progenitor cells, we analyzed endogenous Hes1 and Hes5 expression by in situ hybridization (fig. 7). Both Hes1 and Hes5 were expressed within the developing neural retina. Hes5 was consistently more strongly expressed than Hes1 in the retina, while Hes1 was more strongly expressed in the peripheral retina and presumptive ciliary epithelium (data not shown). DAPI counterstaining and/or immunolabeling for BrdU incorporation (progenitors) combined with neurofilament detection (RGCs) after in situ hybridization revealed that both Hes1 and Hes5 were expressed in progenitor cells and not in differentiating RGCs. It is interesting to note that subtle differences were observed with respect to Hes1 and Hes5 expression in progenitor cells. While Hes5 was expressed in progenitor cells throughout the ventricular zone, from S-phase to M-phase, Hes1 was primarily restricted to progenitor cells in the upper part of the ventricular zone, marking primarily those cells in S-phase, and excluded from mitotic cells.

**Fig. 6.** Notch signaling is active in progenitor cells but not newborn RGCs. Hes1GFP/RFP and Hes5GFP/RFP were cotransfected into E4.5 retinal explants, cultured for 2 days, fixed and flatmounted for high-resolution LSCM. Optical stacks were viewed and rendered with Volocity imaging software. a View from the mitotic surface of...
Hes1GFP/RFP cotransfection into peripheral retina reveals a subset of cells reporting active Notch1 signaling through Hes1GFP; arrowheads and asterisk denote Hes1GFP+ cells; view in a’ is rotated 90° and tilted backwards; note the simple radial morphology of Hes1GFP transfected cells (asterisk). b, c View from ganglion cell surface of Hes5GFP/RFP cotransfection near the wave front of initial RGC differentiation (b) and the central limit of the transfection zone (c) reveals high levels of Notch1 signaling through Hes5GFP; note that maintained RFP serves to identify nascent RGCs (arrowheads) and clearly labels ganglion cell neurites (arrows, c) navigating towards the optic nerve; also note that no Hes5GFP+ or Hes1GFP+ neurites are observed. To confirm that Hes1GFP and Hes5GFP reporters are active in progenitor cells and not in RGCs, explants were pulsed with BrdU for 2 h prior to fixation, processed for cryosectioning and immunolabeled for BrdU incorporation into progenitor cells or neurofilament (NF); GFP, RFP, and BrdU or NF (blue) triple-labeled sections were analyzed via 2P/LSCM, rendered and visualized with Volocity as before. d–d” Hes1GFP reporter is not active in NF+ cells (arrowheads mark Hes1GFP+/RFP+/NF– cells), while RFP is active in differentiating ganglion cells (arrows mark Hes1GFP–/RFP+/NF+ cells); similar results are observed for Hes5GFP reporter (data not shown). e–e” Hes5GFP reporter is active in BrdU+ progenitor cells (arrows mark Hes5GFP+/RFP+/BrdU+ progenitor cells); note that Hes5GFP is not active in all RFP+/BrdU+ progenitor cells (arrowheads); similar results are observed for Hes1GFP reporter (data not shown). Hyphenated letters represent corresponding fluorescent signals from each channel as indicated in each panel.
Fig. 7. Hes1 and Hes5 are expressed in progenitor cells but not newborn RGCs. Paraffin sections from chick embryos incubated to stage 25/E4.5 were hybridized with DIG-labeled antisense riboprobes to Hes1 (a–a’’, b–b’’ or Hes5 (c–c’’, d–d’’): all panels are oriented with anterior retina to the left and central to the right, mitotic layer to the top and ganglion cell layer to the bottom; hyphenated letters represent corresponding Nomarski and/or fluorescent signals as indicated in each panel. Analysis of Hes1 expression within the neural retina near the front of initial RGC differentiation (a) and more centrally (b) demonstrates that Hes1 expression is complementary to differentiating neurofilament-positive (NF+) RGCs (arrows), and primarily restricted to nondividing progenitors, identified by DAPI counterstaining and visualization of mitotic spindles (arrowheads). Analysis of Hes5 expression within the neural retina near the front RGC differentiation (c) and more centrally (d) also demonstrates that Hes5 expression is detected in BrdU+ progenitor cells (arrowheads), and complementary to differentiating NF+ RGCs (arrows). Scale bar 15 μm.
Thus, these data demonstrate that endogenous Hes1 and Hes5 are normally expressed in progenitor cells, confirming the Notch-dependent activity of Hes1 and Hes5 reporters within retinal progenitors.

**Discussion**

We have investigated the role of Notch activity in the regulation of its downstream mediators Hes1 and Hes5, and during RGC differentiation. We first showed that inhibition of Notch activity prevented retinal growth and proliferation by inducing neuronal differentiation. To demonstrate that Notch activity regulates both Hes1 and Hes5 during early retinogenesis, we obtained and characterized the chicken homolog of mammalian Hes5. Next we showed that inhibiting endogenous Notch signaling downregulated Hes1 and Hes5 gene expression. We then developed an assay to monitor activity of the Notch signaling pathway in an intact embryonic retina using fluorescence-based Hes1 and Hes5 reporter constructs. We confirmed that Hes reporters are specifically active in progenitor cells and not newborn, differentiating ganglion cells. We further showed that endogenous Hes1 and Hes5 are expressed in progenitor cells and not ganglion cells. These studies demonstrate that active Notch signaling is downregulated just prior to RGC differentiation.

**DAPT Is an Effective Pharmacological Inhibitor of Notch Activity**

Previous studies have demonstrated the requirement of Notch signaling for retinal development using misexpression of a dominant-negative Notch construct (Notch receptor without NICD) [Bao and Cepko, 1997], a truncated Notch-ligand (Delta with a mutated C-terminus) [Henrique et al., 1997], or an antisense oligo-mediated block on Notch protein translation [Austin et al., 1995; Silva et al., 2003] to generate an inhibition of Notch activity. Small molecule gamma-secretase inhibitors have been developed primarily as a method for inhibiting amyloid precursor protein processing. However, gamma-secretase activity is also necessary for generating an active Notch signal [Selkoe and Kopan, 2003]. Hence, inhibiting gamma-secretase during early development generates a phenotype indistinguishable from Notch loss of function phenotypes [Geling et al., 2002]. Here we demonstrate that DAPT blocks proliferation, induces neuronal differentiation, and prevents expression from Notch activity-dependent reporter constructs. Thus, in the appropriate developmental context, gamma-secretase inhibitors are useful reagents for specifically inhibiting Notch activity without the need to misexpress dominant-negative constructs or to use antisense oligo approaches.

**Retinal Explant Transfections Allow Rapid Analysis of cis-Regulatory Element Activity**

Chicken embryos are a classic embryological system and offer several advantages for developing a rapid assay to characterize the ability of cis-regulatory elements to promote transcription in specific cell types during development. For example, the avian embryonic eye is large enough to observe gross eye structure formation such as the ciliary body [Kubota et al., 2004] in addition to smaller-scale intercellular patterning events such as RGC differentiation [McCabe et al., 1999]. The adult avian retina also harbors a resident population of progenitor cells at the margin and exhibits a capacity for limited regeneration in response to growth factors and/or damage [reviewed in Fischer and Reh, 2003; Moshiri et al., 2004]. Furthermore, chick retinas are easily obtained and manipulated, and develop appropriately when cultured as short-term explants. Using this assay, we showed that fluorescence-based Hes reporters specifically reported in retinal progenitor cells, exhibiting a discrete spatial pattern of expression that is dependent on Notch activity. Thus fluorescence-based Hes reporters are useful new molecular tools.

**Downregulation of Notch Activity Drives Early Progenitor Cells to Differentiate**

We have found that, although all newborn RGCs at the mitotic surface express Notch [Nelson and Reh, unpubl. obs.], the loss of Hes gene expression and reporter activities indicate that Notch activity is inhibited at some point prior to this final division. The loss of Notch signaling in differentiating neurons is consistent with the known role for this pathway in maintaining the undifferentiated state of the progenitor (see Introduction). Several mechanisms could account for the loss of Notch signaling in these cells, including (1) cis-inactivation from one of the Notch ligands, (2) inhibition of the Notch signal by numb, numb-like, and/or neuralized in one of the daughters of an asymmetric mitotic division, (3) differential expression of mastermind [Wu et al., 2004], a transcriptional coactivator with Notch/RBP-J-gamma complex that is necessary for Hes expression, and/or (4) restriction of senseless or MyT1 to the developing RGCs and inhibition of Notch signaling in a cell-autonomous manner [Bellefroid et al., 1996].

The first possibility, that Notch is cell-autonomously inactivated by coexpression of a Notch ligand, is supported by data from several other developing systems, ob-
tained primarily through overexpression studies [Jacob-

sen et al., 1998; Sakamoto et al., 2002], and by the obser-

vation that newborn RGCs might express the Notch ligand

Delta1 [Henrique et al., 1997; Bao and Cepko, 1997].

However, the applicability of cis-inactivation to eye
development has recently been questioned. Li and Baker
[2004] have found that cell-autonomous cis-inactivation of
Notch occurs in Drosophila ommatidial development
only when a Notch ligand is expressed at very high, prob-
ably nonphysiological, levels. The second possibility, that
Notch activity is inherited asymmetrically by the cell that
will differentiate into the RGC, is attractive, since numb,
numb-like, and neuralized are important regulators of the
Notch pathway in both vertebrates and invertebrates, and
can be asymmetrically inherited themselves [Rhyu et al.,
1994; Petersen et al., 2002; Li et al., 2003; Yeh et al.,
2001]. However, this model is also problematic in the ret-
ina; while there have been some reports of regularities in
the mitotic cleavage plane that predict future RGC dif-
erentiation, these studies taken together have failed to
find such a consistent correlation [for a review, see Ro-
egiers and Jan, 2004]. Third, it is possible that the newly
generated RGCs do not express mastermind genes, and
hence lack a critical coactivator of Hes expression. Con-
istent with this possibility is the observation that the Xen-
opus mastermind homolog Xmam1 is expressed in the
developing retina [Katada and Kinoshita, 2003]. As for
the last alternative, in situ expression data indicate that
the MyT1 isoforms are expressed in newly generated neu-
rons throughout the CNS, including RGCs. Thus, the reg-
ulation of MyT1 expression could then allow newly gener-
ated cells to escape from Notch inhibition [Matsushita et
al., 2002]. Further experimentation will be necessary to
determine the respective contributions of these mech-
анизms in mediating the inhibition of Notch activity in dif-
ferentiating ganglion cells.

Heterogeneity of Notch Signaling Activity within
Subsets of Retinal Progenitor Cells

Examination of endogenous Hes1 and Hes5 expression
patterns within the developing retina suggests that each
Hes gene may be expressed in separate or partially overlap-
ning subsets of retinal progenitor cells. Additionally, com-
parison of d2eGFP expression from Hes1 and Hes5 re-
porter constructs also supports the possibility that Notch
activity is differentially mediated in subsets of retinal pro-
genitor cells. In fact, we have begun to test this hypothesis
by creating new versions of these constructs to report as
spectrally distinguishable fluorescent readouts, and pre-
liminary data indicate that Hes1 and Hes5 are likely active
in partially overlapping subpopulations of progenitor cells
[Nelson and Reh, unpubl. obs.]. The significance of this
level of Notch activity regulation is not yet clear, but sim-
ilar manipulations of Hes1 and Hes5 during retinal devel-
opment do not always yield the same effect. For example,
loss of Hes1 and Hes5 function resulted in an increase in
the rate of retinal cell differentiation and a decrease in
Müller glia [Tomita et al., 1996; Furukawa et al., 2000;
Hojo et al., 2000]. Conversely, overexpression of Hes5 pro-
moted Müller glia differentiation at the expense of other
later-born cell types [Hojo et al., 2000], while overexpres-
sion of Hes1 generally inhibited neuronal and glial differ-
enting, yielding a population of undifferentiated ‘Müll-
er glia-like’ cells in the postnatal retina [Takatsu et al.,
2004]. The effects of Hes5 loss and gain of function ex-
periments demonstrate that Hes5 can only partially con-
tribute to later developmental events such as Müller glia
specification, while a role for Hes5 in the early embryonic
retina remains unknown. Thus, Hes1 and Hes5 may have
unique roles within subsets of retinal progenitor cells.

In conclusion, we have demonstrated that Notch activ-
ity regulates both Hes1 and Hes5 during retinal develop-
ment, and that Notch activity is downregulated just prior
to RGC differentiation. The possibility that subpopu-
lations of retinal progenitor cells may interpret and mediate
different aspects of Notch activation is an intriguing hy-
pothesis. It will also be interesting to determine how pat-
terns of Notch activity contribute to the pattern of new-
born RGCs during their initial differentiation. Neverthe-
less, regulation of Notch signaling activity is a key step
in the decision to differentiate or to remain as a pro-
genitor.

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Note Added in Proof

Since submission of this manuscript, Fior and Henrique (2005)
have described 3 chicken Hes5-like genes, Hes5-1, Hes5-2 and
Hes5-3; the Hes5 gene described in our study is the Hes5-1 gene
from Fior and Henrique (2005). Additionally, Kohyama et al.
(2005) have also developed a Notch reporter assay based on a min-
imal mouse Hes1 promoter.
References


