A Positive Feedback Loop between ATOH7 and a Notch Effector Regulates Cell-Cycle Progression and Neurogenesis in the Retina

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Abstract

The HES proteins are known Notch effectors and have long been recognized as important in inhibiting neuronal differentiation. However, the roles that they play in the specification of neuronal fate remain largely unknown. Here, we show that in the differentiating retinal epithelium, the proneural protein ATOH7 (ATH5) is required for the activation of the transcription of the Hes5.3 gene before the penultimate mitosis of progenitor cells. We further show that the HES5.3 protein slows down the cell-cycle progression of Atoh7-expressing cells, thereby establishing conditions for Atoh7 to reach a high level of expression in S phase and induce neuronal differentiation prior to the ultimate mitosis. Our study uncovers how a proneural protein recruits a protein known to be a component of the Notch signaling pathway in order to regulate the transition between an initial phase of selection among uncommitted progenitors and a later phase committing the selected progenitors to neuronal differentiation.

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SUMMARY

The HES proteins are known Notch effectors and have long been recognized as important in inhibiting neuronal differentiation. However, the roles that they play in the specification of neuronal fate remain largely unknown. Here, we show that in the differentiating retinal epithelium, the proneural protein ATOH7 (ATH5) is required for the activation of the transcription of the Hes5.3 gene before the penultimate mitosis of progenitor cells. We further show that the HES5.3 protein slows down the cell-cycle progression of Atoh7-expressing cells, thereby establishing conditions for Atoh7 to reach a high level of expression in S phase and induce neuronal differentiation prior to the ultimate mitosis. Our study uncovers how a proneural protein recruits a protein known to be a component of the Notch signaling pathway in order to regulate the transition between an initial phase of selection among uncommitted progenitors and a later phase committing the selected progenitors to neuronal differentiation.

INTRODUCTION

The early development of the vertebrate CNS critically depends on the timely generation of particular classes of neurons at distinct positions and in appropriate numbers. Although the plan of the retina is well conserved across vertebrate species, there are considerable variations in cell-type diversity and cell-type number as well as in the organization and properties of the tissue. Because high visual acuity requires a high ratio of retinal ganglion cells (RGCs) to photoreceptors, the proportion of progenitor cells recruited to produce RGCs in the developing retina contributes to the functional characteristics of the adult eye. ATOH7 activates neurogenesis and is required for the production of RGCs (Brown et al., 2001; Kanekar et al., 1997; Kay et al., 2001; Liu et al., 2001; Matter-Sadzinski et al., 2001; Wang et al., 2001). In our previous studies, we have outlined the genetic program that operates during the two main phases of ATOH7 expression, coordinating the selection of RGC precursors and the induction of RGC-specific traits with cell-cycle exit. Initially, cross-regulatory interactions among the ATOH7, NGN2, and HES1 proteins allow the expansion of pools of proliferating progenitors while maintaining ATOH7 expression below the level required for inducing RGC differentiation. A second phase is initiated when the ATOH7 protein upregulates its own expression and promotes the transcription of genes that contribute to the RGC phenotype (Matter-Sadzinski et al., 2005).

The fact that RGCs are specified at the time precursor cells exit the cell cycle (Livesey and Cepko, 2001; Matter-Sadzinski et al., 2005; Matter et al., 1995; Ohnuma et al., 2002; Poggi et al., 2005) raises the question whether ATOH7 has a role in coordinating cell-cycle exit and the onset of neuronal differentiation. To address this issue, we searched for genes regulated by ATOH7 in proliferating progenitors. A chromatin immunoprecipitation (ChIP) coupled with DNA microarray (chip) screen identified the avian homologs of hairy and enhancer of split (Hes5.1, Hes5.2, Hes5.3) as early targets of ATOH7. The HES5 proteins are known to be components of the Notch signaling pathway (Fior and Henrique, 2005). We now show that ATOH7 is required to activate transcription of the Hes5.3 gene before the penultimate mitosis. The HES5.3 protein, in turn, regulates the cell-cycle length and sets conditions for Atoh7 to upregulate, thereby activating genes involved in RGC differentiation. This positive feedback loop underlies the conversion of progenitors to neurons. Live imaging of Atoh7-expressing cells enabled us to establish the schedule of this transition.
RESULTS

The Hes5 Genes Are Targets of ATOH7 during Retina Ontogenesis

To identify targets of ATOH7 in retinal progenitors, we performed ChIP-on-chip screens at two developmental stages: embryonic day 3.5 (HH22-23 according to Hamburger and Hamilton, 1951) and embryonic day 6 (HH29-30), thus covering retinal progenitor proliferation, neuroepithelium patterning, RGC specification, cell-cycle exit, and early neuronal differentiation (Figure S1A). For each stage, five independent ChIP-on-chips were performed using antibodies against chicken ATOH7, NGN2, and NEUROM (NEUROD4). Among ATOH7-bound putative targets, the ChIP-on-chip screen identified noncoding DNA sequences located upstream of the Hes5.2 and Hes5.3 genes. These sequences consistently bound ATOH7 alone or in combination with NGN2 but did not bind NEUROM (Figures S1B and S1C); moreover they were not immunoprecipitated from HH29-30 optic tectum, a tissue expressing NGN2 but not ATOH7. The binding of ATOH7 to sequences upstream of Hes5.2 and Hes5.3 was verified by standard ChIP in HH22-23 and HH29-30 retinas (Figure S1D). In chicken, the three Hes5 genes are clustered on chromosome 21 within a 20 kb region of DNA (Fior and Henrique, 2005). Although the sequences upstream of the Hes5.1 gene (ENSGALG00000001141) were not included in the screen, a standard ChIP assay revealed that ATOH7 was bound to sequences upstream of Hes5.1 in HH29-30 retina but not in HH22-23 retina.

We monitored the expression of the Hes5 genes by quantitative PCR (qRT-PCR) during retina development (Figure S1E): Hes5.1 and Hes5.2 transcript levels were low at HH22-23 and peaked at HH30, coinciding with the kinetics of Atoh7 expression (Matter-Sadzinski et al., 2005). The expression of Hes5.3 also peaked around HH30, but the difference in the relative accumulation of transcripts between HH22-23 and HH30 was less pronounced. The binding and expression profiles both indicate that Hes5.3 might be one of the earliest targets of the ATOH7 protein during the ontogenesis of the chick retina.

Hes5.3 Is Activated at the Onset of Atoh7 Expression

We next sought to determine the onset date of Hes5.3 expression in the developing retina. Accumulation of Hes5.3 transcripts was detected by in situ hybridization in the central retina at stage 17 (HH17; Figure S2B), coinciding with the onset of Atoh7 expression (Matter-Sadzinski et al., 2005). The strong radioactive signal detected in HH17 to HH28 retinas revealed a robust expression of Hes5.3 at these stages (Figure S2A). The overlapping Hes5.3 and Atoh7 expression domains expanded to the periphery as development proceeded (Figures S2C–S2E).

To investigate the requirement for ATOH7 in the regulation of Hes5.3, we examined the regulatory potential and cell-type specificity of the ATOH7-bound regions of the Hes5.3 gene. The sequence encompassing ATOH7-binding sites and extending ~2 kb upstream from the first exon in Hes5.3 was fused to the GFP or DsRed (RFP) reporter genes. To determine whether this sequence displayed promoter activity in HESS.3-expressing cells, HH22-23 retinas were electroporated with a Hes5.3-GFP plasmid and the central domains were microdissected 16 hr later. Fluorescence-activated cell sorting (FACS) analysis of dissociated retina fragments revealed a population of cells, comprising ~20% of the total, that express green fluorescent protein (GFP). RNA was isolated from GFP+ and GFP− cells and processed for qRT-PCR. Both the Hes5.3 and Atoh7 transcripts were detected in GFP+ cells (Figure S2F). We conclude that the cloned Hes5.3 promoter is activated in cells that coexpress Hes5.3 and Atoh7. To test for the spatial activity of the Hes5.3 promoter, Hes5.3-RFP and the ubiquitously expressed CMV-GFP plasmid were electroporated into HH22 chick retina and fluorescent cells were detected 24 hr later. While GFP+ cells were detected in both the central and peripheral retina, no red fluorescent protein (RFP)-positive cells were detected at the periphery, whereas they were abundant in the central region (Figure 1A). In HH26 retina, we noticed that the promoter activity and accumulation of Hes5.3 transcripts were both stronger at the periphery of the expanding Hes5.3 domain (Figure 1B; Figure S2G). Real-time imaging of the promoter activity revealed that as development proceeds, the number of Hes5.3-RFP-labeled cells augmented at the periphery of the expanding domain while it diminished in the central retina (data not shown). To determine whether Hes5.3 promoter activity was related to the developmental status of Atoh7-expressing cells, Hes5.3-RFP and Atoh7-GFP plasmids (Skowronska-Krawczyk et al., 2009) were electroporated in HH22, HH24, and HH26 retinas. Whereas all RFP+ cells coexpressed GFP at those three stages (Figures 1C, 1D, and 1F), a subset of GFP+ cells did not express RFP. Interestingly, we noticed variations in the spatiotemporal distribution of these cells: at the periphery of the Hes5.3 domain, all GFP+ cells coexpressed RFP, whereas in the central retina, the proportion of GFP+ cells that did not express RFP increased between HH22 and HH26 (Figure 1F). To define the developmental status of Atoh7+ expressing cells that did not activate the Hes5.3 promoter, Hes5.3-GFP and Atoh7-RFP plasmids were coelectroporated into HH24 and HH26 retinas. The Atoh7-RFP and Atoh7-Lac plasmids both label cells that have upregulated Atoh7 (~20% of Atoh7+ expressing cells at HH23-24; Figures S4A [right] and S4C; Skowronska-Krawczyk et al., 2009) and these cells are committed to becoming RGCs (see below). We found that less than 5% of the RFP+ cells coexpressed GFP (Figures 1E and 1F); a similarly low proportion of the Lac+ cells accumulated Hes5.3 transcripts (Figures S2H–S2K). Taken together, our data indicate that the expression of Hes5.3 coincides with the leading edge of onset for Atoh7 expression; it is transient and restricted to cells expressing Atoh7 at a low level.

ATOH7 Is Required for Activation of Hes5.3

To determine whether ATOH7 activates transcription of the Hes5.3 gene, we carried out RNA interference-mediated inhibition of Atoh7 and monitored Hes5.3 promoter activity. The Hes5.3-RFP and CMV-GFP plasmids were mixed with either Atoh7 small interfering RNAs (siRNAs) or nontargeting siRNAs and electroporated into HH23-24 retinas, which were scored for fluorescent cells 24 hr later. Atoh7 siRNAs markedly...
Figure 1. Hes5.3 Is Activated by ATOH7 at the Leading Edge of Atoh7 Expression

(A–F) Spatiotemporal activation of the Hes5.3 promoter. (A) HH22 retina coelectroporated with Hes5.3-RFP and CMV-GFP plasmids. GFP+ cells are abundant at the periphery (p). RFP+ cells are restricted to the central retina (c). (B) HH26 retina electroporated with HES5.3-RFP. The density of RFP+ cells is high at the periphery and low in the central retina. (C–F) The Hes5.3 promoter is activated in Atoh7-expressing cells in a stage-dependent manner. HH22 to HH26 retinas were electroporated with plasmids as follows: Hes5.3-RFP and Atoh7-GFP (C, D, and F), Hes5.3-GFP and Atoh7-RFP (E and F). (C) At the periphery, all cells are double labeled. (D) In the central retina, all RFP+ cells coexpress GFP. A subset of GFP+ cells does not express RFP. (E) RFP+ cells do not express GFP. (F) Relative numbers of RFP+ and GFP+ cells in the peripheral and central Hes5.3 domains. The yellow fill represents the fraction of double-labeled cells.

(legend continued on next page)
decreased the density of RFP+ cells at the periphery of the Hes5.3 domain (Figures 1G–1K). The downregulation of the Hes5.3 promoter activity by Atoh7 siRNAs at the expanding domain border indicates that ATOH7 activates transcription of the Hes5.3 gene.

We reasoned that if ATOH7 is necessary to activate the expression of Hes5.3, then the genes regulated by HESS.3 must be a subset of those regulated by ATOH7. To test this idea, HH23–24 retinas were electroporated with Hes5.3 siRNAs, Atoh7 siRNAs or nontargeting siRNAs, and a Hes5.3-RFP plasmid. Retina fragments encompassing the edge of the expanding Hes5.3 domain (Figures 1G and 1H, frame p) were microdissected 16 or 36 hr later, RNA was isolated and processed for qRT-PCR and Affymetrix gene chip analysis. Strikingly, the set of 427 genes regulated by ATOH7 included 41 out of the 43 genes regulated by HESS.3 (Figure S3A). Moreover, the expression of genes affected by Hes5.3 siRNAs and Atoh7 siRNAs displayed similar fold changes (Table S2). Hes5.1, Hes5.2, Hes5.3 were downregulated by Atoh7 siRNAs and Hes5.1, Hes5.2 were downregulated by Hes5.3 siRNAs (Figure 1L; Figure S3B). Taken together, our ChIP, siRNA, and expression data reveal that Hes5.3 is a downstream target of ATOH7 and that ATOH7 is required to activate transcription of the Hes5.3 gene. They also indicate that HESS.3 positively regulates the expression of Hes5.1 and Hes5.2.

**Autostimulation of Hes5.3**

The inhibitory effects of the Atoh7 siRNAs upon Hes5.3 expression and of the Hes5.3 siRNAs upon Hes5.1 and Hes5.2 expression were stronger at 16 hr than at 36 hr after electroporation (Figure 1L). We wondered whether these differences were due to HES5.3 stimulating the transcription of its own gene (Fior and Henrique, 2003). To address this issue, we carried out RNA interference-mediated inhibition of HES5.3 and monitored promoter activity. The Hes5.3-RFP and Hes5.3-GFP plasmids were mixed with Hes5.3 or nontargeting siRNAs and electroporated into HH23 retinas (Figure S3C). In early retina, the Hes5.3 promoter is much stronger than the Atoh7 promoter (Figure S4A, left). Despite the low intrinsic fluorescence of the DsRed (RFP) chromophore, ≥95% of cells were double labeled in the control retinas (Figure S3D). Hes5.3 siRNAs markedly decreased the fraction of double-labeled cells at the periphery of the domain, indicating that the specific siRNAs downregulated Hes5.3 promoter activity at the expanding domain border (Figure S3D). By contrast, overexpression of the HES5.3 protein in HH23–24 retinas consistently enhanced activity of the Hes5.3 promoter (Figure S4B). We conclude that a positive feedback contributes to the elevated expression of HES5.3 in cells expressing ATOH7 at a low level.

**Time-Lapse Imaging of Hes5.3- and Atoh7-Expressing Progenitor Cells**

Proliferating retinal cells form columns of progenitors possessing both apical and basal processes that span the neuroepithelium. The cell shape, the position of nuclei, and interkinetic nuclear migration (INM) along the apicobasal axis are indicative of their status (Sauer, 1935). To ascertain the status of Hes5.3- and Atoh7-expressing progenitor cells, we monitored their progression through the cell cycle by time-lapse imaging. Hes5.3-RFP and Atoh7-GFP plasmids were coelectroporated into HH24–25 retinas and INM was recorded at 20 min intervals (Figure 2; Movie S1). At this stage of development, the peripheral edge of the expanding Hes5.3 and Atoh7 domains are well positioned for optical sectioning and INM recording. Following mitosis at the apical surface, the daughter cell nuclei migrated toward the basal surface for approximately 7 hr. The majority of tracked nuclei reached the basal half of the retina, although the distances they migrated within that half were highly variable. Once there, the nuclei moved back and forth along their apicobasal axis for 8 to 20 hr. At high temporal resolution, the direction of movement changed frequently, apparently at random intervals (Movie S1). The trajectories of daughter nuclei appeared independent: they may contact during short periods but most of the time they stay apart. The fact that no stereotopic trajectory pattern was identified on tracking ≥100 nuclei suggests that the basal oscillations are largely stochastic (Norden et al., 2009). The majority of these cells are in S phase, as evidenced by the accumulation of bromodeoxyuridine (BrdU) pulse-labeled nuclei in the basal half of HH24 to HH29 retinas (Matter-Sadzinski et al., 2005).

Later on, as their nuclei move back to the apical side, cells enter G2. The tracked cells went through a process that appears to be stereotopic: the two daughter cells were entrained for 1–2 hr in a single column spanning the neuroepithelium before direct and rapid movements transported the nuclei to the apical surface (Figure 2A: 17 hr). Following mitosis M0, the two new daughter cells, together with the remaining M cell, were again assembled in a single column spanning the neuroepithelium during the 1–2 hr period preceding M0. The nuclei of the four daughter cells then remained grouped and displayed complex rotary and sliding motions (Figure 2A; Movie S1). The cell-cycle length of double-labeled cells that we were able to track from mitosis to mitosis was variable and lasted 17–32 hr (Figure 2B). The 6–7 hr period of basally directed INM following the mitosis M1, the 1–3 hr of direct apical translocation prior to M0, and the 1–2 hr period that separates M0 and M0′ were approximately constant, whereas the duration of stochastic movements was highly variable.

The identification of double-labeled cells going from mitosis to mitosis demonstrated that Atoh7 and Hes5.3 are activated in
progenitors before they enter the last cell cycle. This conclusion is consistent with the presence of the ATOH7 protein in a fraction of BrdU pulse-labeled cells (Figures S3E–S3H) and with previous reports showing the accumulation of Atoh7 transcripts in chick and mouse retinal cells pulse labeled with BrdU or [3H]-thymidine (Matter-Sadzinski et al., 2001; Matter-Sadzinski et al., 2005; Trimarchi et al., 2008b).

**Hes5.3 Inhibition Accelerates the Cell Cycle**

Having shown that Hes5.3 expression was transient and restricted to progenitor cells expressing Atoh7 at low level and that the upregulation of Atoh7 correlated with the downregulation of Hes5.3, we wondered how these activities are related to cell-cycle progression. Within the population of Atoh7-expressing progenitors, the cell-cycle length is variable (Figure 2B) and we know that about one-fifth of these cells upregulate Atoh7 (Skowronska-Krawczyk et al., 2009). To determine whether this upregulation correlated with cell-cycle lengthening, the Atoh7-RFP and Atoh7-GFP plasmids were coelectroporated in HH24 retina and INM was recorded in the red and green channels at 20 min intervals from 24 hr and up to 72 hr after electroporation. Whereas the cell cycle of Atoh7-expressing cells that did not upregulate Atoh7 (GFP+, RFP/C0 cells) lasted 19.6 ± 2.6 hr, the cell-cycle length of double-labeled cells was significantly increased to 27.7 ± 3.5 hr (Figure 3A; Movie S2), indicating that the upregulation of Atoh7 is associated with the lengthening of the cell cycle.

HES5.3 regulates genes that might be involved in INM (e.g., myosin regulatory light chain; Table S2). To determine whether HES5.3 influences cell-cycle kinetics, we carried out RNA interference-mediated inhibition of Hes5.3 and monitored INM of Atoh7-expressing cells. The Atoh7-GFP plasmid was mixed with either Hes5.3 siRNAs or nontargeting siRNAs and electroporated into HH24 retinas, and INM was recorded at 20 min intervals beginning 24 hr later. We found that the inhibition of Hes5.3 had two related effects: it shortened the cell cycle to 13.7 ± 1.8 hr (Figures 3A–3C) and it increased the proportion of

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**Figure 2. Real-Time Imaging of Progenitor Cells Coexpressing Hes5.3 and Atoh7**

(A and B) HH24-25 retinas were coelectroporated with Hes5.3-RFP and Atoh7-GFP plasmids. (A, upper) Stills taken from a movie in the red channel. The stills show the INM of daughter cells after mitosis M. (A, lower left) Uncoordinated motion profiles of daughter cells from the 5th to the 16th hr at 20 min intervals. (A, lower right) High contrast stills of daughter cells and of their progeny. Note the coordinated apical translocations prior to M’ (18th hr) and prior to M” (19th hr). The four sibling cells stayed grouped after M”. The white dots mark the peaks of fluorescence intensity. (B) Motion profiles of progenitor cells from mitosis to mitosis. The curves map the position of nuclei along the basoapical axis (y axis). The contact points of the curves with the x axis mark mitosis. The INM of one daughter cell in each pair is shown.

See also Movie S1.
**Figure 3. Monitoring the Effects of Hes5.3 Inhibition on Cell-Cycle Length**

(A) Box plots illustrating cell-cycle lengths of 31 GFP+ RFP+ cells (green dots), 31 GFP+ RFP+ cells (red dots), and 30 GFP+ Hes5.3 siRNAs cells (blue dots) measured from movies in the green and red channels (e.g., Movie S2). HH24-25 retinas were coelectroporated with plasmids and siRNAs as follows: Atoh7-GFP and Atoh7-RFP (green and red plots), Atoh7-GFP plus Hes5.3 siRNAs (blue plot). The top and bottom of each box indicate upper and lower quartiles, respectively, and the horizontal line represents the median.

(B, cells 1–6) Representative INM profiles from sibling cells coelectroporated with an Atoh7-GFP plasmid and Hes5.3 siRNAs and tracked from mitosis to mitosis.

(C) Stills from a movie of cells coelectroporated with an Atoh7-GFP plasmid and Hes5.3 siRNAs and tracked from mitosis to mitosis.

(D) Fraction of GFP+ cells going through mitosis in 24 hr counted on maximal projection snapshot (/C24) at 20 min intervals from INM profiles of cells coelectroporated with an Atoh7-GFP plasmid plus Hes5.3 siRNAs or nontargeting siRNAs. Data are presented as mean ± SD; **p < 0.01 (Welch’s t test).

See also Movie S2.

**Atoh7-expressing cells going through mitosis within the observation period (24 hr; Figure 3D).**

**Onset of RGC Differentiation in the S Phase of the Last Cell Cycle**

To determine how the upregulation of Atoh7 and the lengthening of the cell cycle are related to the onset of neuronal differentiation and cell-cycle exit, we tracked cells that upregulate Atoh7 and activate the beta 3 nicotinic acetylcholine receptor (Chrb3) gene, a direct target of ATOH7 and an early marker of RGCs. When Atoh7-RFP and Chrb3-GFP reporter plasmids are coelectroporated in HH25 retina, ≥95% of RFP+ cells coexpress GFP (Figures S4D–S4G). We surmise that the GFP+ RFP+ cells are differentiated RGCs that have reached the basal aspect (Figure 4, cell 3; Movie S3). About 30% of retinal cells express Atoh7 between HH22 and HH30, 20%–30% of which upregulate Atoh7 between HH25 and HH30 (i.e., at the peak of RGC production) (Matter-Sadzinski et al., 2005; Skowronska-Krawczyk et al., 2009). The fact that the binding of ATOH7 to the Chrb3 promoter is undetectable in chromatin isolated from HH22 retinas but is detected at HH29-30 (Figure S1D; Skowronska-Krawczyk et al., 2004) when the Chrb3 promoter is most active (Matter-Sadzinski et al., 2001; Skowronska-Krawczyk et al., 2005) argues that the activation of the Chrb3 gene is restricted to cells expressing ATOH7 at high level.
Figure 4. Atoh7 Is Upregulated and Chrnb3 Is Activated in Retinal Cells that Exit the Cell Cycle

(A) HH25 retinas coelectroporated with Atoh7-RFP and Chrnb3-GFP plasmids. Stills spanning 18.3 hr from a movie in the red and green channels. INM, mitosis (M), and cell migration of three cells are shown. Cells 1 and 2 are double labeled, and cell 3 is a RGC reaching the basal aspect and does not express Atoh7. An apical process (green arrowheads), a growing axon at the basal pole (yellow arrowheads), and dendrites (white arrowheads) are visible.

(B) HH25 retinas coelectroporated with Atoh7-RFP plus Chrnb3-GFP or Atoh7-GFP plasmids. (B, left) INM profiles of Atoh7-RFP labeled cells prior to and after the last mitosis. (B, right) Migration of newborn RGCs. The curves map the positions of the somas migrating from the apical (0 hr) toward the basal surface. (B, inset) Newborn RGCs reside on the apical surface before they migrate toward the basal surface. Sections from HH29-30 retina were immunostained with an antibody against ATOH7. The ATOH7 protein accumulates in cells predominantly located on the apical side.

(legend continued on next page)
bipolar with a short apical process and an outgrowing axon at the basal pole (Figure 4A, cell 2 at 4.6 and 5.6 hr). When the growth cones reached the basal surface, they turned at right angle and navigated along the basal surface toward the head of the optic nerve (Figures 4A, cell 2 at 6.6 hr; Movies S2 and S3). Finally, the RGC somas slowly and steadily migrated toward the basal surface (Figure 4A, cell 2 at 7.6–18.3 hr and cell 3 at 0–7 hr; Figure 4B, right; Movies S2 and S3). The apical process of these cells shortened (Figure 4A, cell 2 at 5.6–7.6 hr) and began to extend dendrites as the cells lost connections to the limiting membrane and reached the basal side (Figure 4A, cell 2 at 14 and 18.3 hr). The cell transit from mitosis to its final location on the basal surface lasted at least 25 hr (Figure 4B; Movie S3). Atoh7 was downregulated as RGCs reached the basal side (Figure 4A, cell 3; Movie S2). Taken together, our data suggest that the upregulation of Atoh7 is associated with the onset of RGC differentiation prior to the terminal mitosis.

**Hes5.3 Inhibition Represses the Upregulation of Atoh7 and the Activation of RGC Genes**

Having shown that inhibiting Hes5.3 accelerated the cell cycle and that Atoh7 was upregulated in cells whose cell cycle was longest, we next sought to determine whether shorter cell cycle affects the proportion of cells that upregulate Atoh7, a prerequisite for the RGC lineage. The Atoh7-GFP and Atoh7-RFP plasmids were mixed with either Hes5.3 siRNAs or nontargeting siRNAs, electroporated into HH23 retinas, and scored for fluorescent cells 48 hr later (Figures 5A–5G). Whereas Hes5.3 inhibition did not influence the total proportion of cells expressing GFP, it markedly decreased the fraction of cells expressing RFP, consistent with this finding, Atoh7 was downregulated by Hes5.3 siRNAs (Figure 5I). To confirm whether this reflected a decrease in the fraction of cells that have upregulated Atoh7, the retina fragments electroporated with Hes5.3 siRNAs were processed for gene chip analysis. Strikingly, the downregulation of Stmn2, Snap25, Robo2, and Ptn by Hes5.3 siRNAs was revealed both by Affymetrix gene-chip and by qRT-PCR analysis (Figures 5H and S1). The genes Stmn2, Snap25 are direct targets of ATOH7 and Stmn2, Snap25, Robo2, and Ptn are regulated by ATOH7 in a dose-dependent manner (Skowronska-Krawczyk et al., 2009). Stmn2, Snap25, and Robo2 are known to participate in the development of dendritic arbors and axons in newborn RGCs and other neurons (Campbell et al., 2007; Delgado-Martinez et al., 2007; Greka et al., 2003; Grenningloh et al., 2004). We conclude that Hes5.3 inhibition represses the upregulation of Atoh7 and the activation of genes that participate in RGC differentiation.

**DISCUSSION**

In this study, we show how the proneural protein ATOH7 regulates the conversion of progenitor cells into newborn RGCs. We find that ATOH7 is necessary to activate the expression of Hes5.3 before the penultimate mitosis. HES5.3, in turn, lengthens the cycle. The HES5.3-mediated upregulation of Atoh7 in the last S phase sets conditions for ATOH7 to promote cell-cycle exit and activate those RGC genes that are regulated by a higher level of ATOH7 protein. We suggest that this positive feedback loop between ATOH7 and HES5.3 marks the transition from competent progenitors to cells committed to the RGC lineage.

The NICD-Rbpj complex, a component of the Notch signaling pathway, is known to induce the expression of genes such as Hes1 and Hes5, which repress proneural gene expression and thereby inhibit neuronal differentiation and maintain the neural stem cell population (reviewed in Bertrand et al., 2002; Kageyama et al., 2008). Our finding that Hes5.1, Hes5.2, and Hes5.3 are targets of ATOH7 and that ATOH7 activates expression of these genes reveals a hierarchical relationship between a proneural protein and Notch effectors in the specification of neuronal fate. The Hes5 cluster is well conserved from teleosts to avians (Fior and Henrique, 2005). In mammals, however, there is a single Hes5 gene whose closest homolog is Hes5.1. Despite the fact that in birds, as in mammals, the HES5 proteins are components of the Notch signaling pathway (Fior and Henrique, 2005; Nelson et al., 2006), one can argue that the positive feedback loop between ATOH7 and HES5.3 underlies a mechanism for the production of RGCs in birds and lower vertebrate, which is not conserved in mammals. Yet, the fact that both Hes5 and Atoh7 (Math5) are prematurely upregulated in E9.5 retina of Hes1−/− embryonic mice compared to the wild-type littermate (Hatakeyama et al., 2004; Lee et al., 2005) suggests that ATOH7 may have a positive effect upon Hes5 expression in mammals. Consistent with this idea, the overexpression of ATOH7 in human fetal retinal progenitors led to the upregulation of Hes5 (D.S.K., unpublished data). In general, the ratio of RGCs to photoreceptors is considerably smaller in mammals than in birds (~0.01 in mouse versus ~0.30 in chicken). We surmise that the alternate regulatory pathways put to use in mammals to produce RGCs do not fully compensate for the absence of Hes5.3. Despite causing lower visual acuity, the loss of Hes5.3 may increase sensitivity and thus may have a beneficial influence on adaptation of mammals to night vision.

Although ATOH7 is required to produce RGCs (Vetter and Brown, 2001), only a fraction of ATOH7-expressing cells are selected to enter the RGC lineage (Matter-Sadzinski et al., 2005; Prasov and Glaser, 2012b; Yang et al., 2003). Because of cross-inhibitory interactions (Fior and Henrique, 2005; Takebayashi et al., 1994), the Hes genes are often expressed in nonoverlapping domains (Hatakeyama et al., 2004). In the chick, a robust accumulation of Hes1 transcripts takes place throughout the peripheral retina in a domain that expresses neither Hes5.3 nor Atoh7. Yet, cells express Hes5 at low levels in the Atoh7-domain and ~80% of Atoh7-expressing cells coexpress Hes1 in early retina (Matter-Sadzinski et al., 2005).

(C) The transition from Atoh7-expressing progenitor cells to newborn RGCs. Live imaging of retinal cells expressing Atoh7, Hes5.3, and Chrnb3 reveals the schedule for the conversion of progenitor cells to newborn RGCs. The average timeline for producing RGCs is representative of ≥50 movies. Scale bar represents 20 μm.

See also Movie S3 and Figures S3E–S3H and S4D–S4G.
Likewise, a large subset of Atoh7-expressing precursors may coexpress Hes1 and Hes5.3. NGN2 activates transcription of the Atoh7 gene (Hufnagel et al., 2010; Matter-Sadzinski et al., 2005), but this positive effect is moderated by the negative effect the HES1 protein exerts upon the Atoh7 promoter (Lee et al., 2005; Matter-Sadzinski et al., 2005), thus keeping the rate of Atoh7 transcription at a low level in progenitor cells. Here, we show that the ATOH7-bound promoter region regulating transcription of the Hes5.3 gene has some quite remarkable properties: first, it is exclusively activated in progenitor cells that express Atoh7 at a low level; and second, a very efficient and stage-selective positive feedback mediates its peak activity. The fact that Atoh7 and Hes5.3 are activated before the penultimate mitosis suggests that expression of these genes is of itself not sufficient for cell-cycle exit. We suppose that highly dynamic ATOH7- and HES-mediated positive and negative cross-regulations may select, in a stochastic manner, a subset of progenitor cells to enter the RGC lineage. Our study shows that HES5.3 influences the length of the cell cycle, and we suggest that the selected progenitors are the ones that delay cell-cycle exit.

![Graph and figure](image)

**Figure 5. Monitoring the Effects of Hes5.3 Inhibition on Atoh7 Upregulation and Activation of RGC Genes**

(A–G) Hes5.3 siRNAs decreased the proportion of Atoh7-expressing cells that upregulate Atoh7 (i.e., RFP*). A mixture of Atoh7-GFP and Atoh7-RFP plasmids plus Hes5.3 or nontargeting (nt) siRNAs were electroporated into HH23-24 retinas (A–F); fluorescent cells were detected 48 hr later. (G) Ratio of RFP* to GFP* cells in the presence or absence of Hes5.3 siRNAs. Data are presented as mean ± SD. (H and I) Hes5.3 siRNAs and Atoh7 siRNAs affect the expression of Atoh7 and RGC genes at the periphery of the expanding Atoh7 and Hes5.3 domains. (H) The microarray analysis was run in quadruple for each species of siRNAs. (I) qRT-PCR data were obtained in four independent experiments and they are presented as the mean ± SD. Dot plot (H) and qRT-PCR (I) show the downregulation of Atoh7, Stmn2, Snap25, Robo2, and Ptn by Hes5.3 siRNAs and the downregulation of Stmn2, Snap25, Robo2, and Ptn by Atoh7 siRNAs. ***p < 0.001; **p < 0.01; *p < 0.05 (Welch’s t test). Scale bar represents 20 μm. See also Figure S4C.
progression long enough for ATOH7 to upregulate and thereby trigger RGC differentiation and cell-cycle exit. Cells coexpressing ATOH7 and HESS.3 but that do not upregulate ATOH7 and do not enter the late phase might be directed toward other cell fates. This idea is consistent with the fact that their cell cycle is slower than the cycle induced by the downregulation of HESS.3 by Hes5.3 siRNA. In zebrafish retina, the increase in cell-cycle time is more often associated with the symmetrical terminal differentiative divisions than with the asymmetrical proliferative and asymmetrical divisions (He et al., 2012).

Time-lapse imaging revealed that Atoh7 reached a high level of expression in S/G2 9–18 hr prior to the last mitosis. In zebrafish retina, expression of Atoh7 (Ath5) was likewise detected in G2 (Poggi et al., 2005). In mouse retina, Atoh7 expression in proliferating progenitors was reported (Trimbarchi et al., 2008b), but until recently the commonly held view was that Atoh7 is expressed after the terminal M phase (Ghiasvand et al., 2011; Hufnagel et al., 2010; Mu et al., 2005; Yang et al., 2003). We suppose that, because of the low expression of Atoh7 in mouse (Skowronska-Krawczyk et al., 2009), the enhanced Atoh7 expression in late S or early G2 could have been taken for the onset of gene expression and interpreted as evidence for a postmitotic event. This controversy was finally settled with the publication of data showing expression of Atoh7 and of RGC markers in mouse retinal progenitors (Brzezinski et al., 2012; Prasov and Glaser, 2012a), thus suggesting that the kinetics of Atoh7 expression prior to the last cell division is to some extent conserved across vertebrate evolution.

The control of progenitor fate commitment by proneural genes has long been viewed as a two-step process (reviewed in Bertrand et al., 2002). In agreement with this idea, we found that ATOH7 is expressed in two phases (Figure 4C): an initial phase of selection of progenitors, where ATOH7 is expressed at a low level and progenitors are not yet committed to differentiation, and a later phase committing progenitors to differentiation where ATOH7 has reached a high level of expression. Here, we show that ATOH7 controls distinct gene regulatory networks in the initial and late phases and that HESS.3 is required for transit to the late phase. Real-time imaging indicated that cells enter the initial phase before the penultimate mitosis and that the HESS.3-mediated lengthening of the cell cycle is a prerequisite for cells to enter the late phase. It will be fascinating to determine how the genes regulated by HESS.3 affect INM in relation with ATOH7-regulated cell-cycle progression and the commitment of progenitors to differentiation.

EXPERIMENTAL PROCEDURES

Animals

Chick embryos from a White Leghorn strain were staged according to Hamburger and Hamilton (1951).

ChIP, ChIP-on-chip Array Hybridization

ChIP was done at HH22-23 (E3.5) and HH29-30 (E6) using chicken anti-AOTOH7, anti-NGN2, and anti-NEUROM antibodies (Skowronska-Krawczyk et al., 2004). All ChIPs with anti-AOTOH7 antibodies were done in triplicate. To identify ChIP-enriched fragments, dye-labeled DNA from immunoprecipitated chromatin was hybridized to custom-designed promoter arrays of 497 noncoding sequences. Candidate selection was based on literature and bioinformatics (GALA, Genome Alignment and Annotation database). Arrays included numerous genes expected to be involved in RGC specification/differentiation, signaling and cell-cycle pathways, Notch and Shh pathways, cytoskeleton, and cell adhesion. The arrayed sequences encompassed 2 kb upstream of the initiation codons. These selected genomic regions were tiled at 100 bp intervals using variable-length (65–75 bases) polynucleotides to keep a constant target Tm of 76°C. Sample labeling, array manufacture, sample hybridization, array scanning, image gridding, and data extraction were performed under contract with NimbleGen (SeqWright). ChIP assays (primers are listed in Table S1) were performed essentially as described in Skowronska-Krawczyk et al. (2004). ChIP DNA sequences were quantified by qRT-PCR.

Microarray Analysis and Quantitative PCR

RNA extraction and qRT-PCR were done as described in Skowronska-Krawczyk et al. (2009). All primers listed in Table S1 were tested for efficiency. The microarray analysis was designed to test the effects of Hes5.3 siRNAs, Atoh7 siRNAs, and nontargeting siRNAs on gene expression at the periphery of the expanding HES5.3 domain. HH24 retinas were electroporated with Hes5.3 siRNAs, Atoh7 siRNAs, or nontargeting siRNA and a Hes5.3-RFP plasmid. Retina fragments encompassing the peripheral fluorescent domain were microdissected under a stereoscopic microscope 16 or 36 hr later. For each condition, we did four independent experiments. RNA was isolated from fragments dissected from four retinas (~260 ng per fragment) and processed for gene chip and/or qRT-PCR analysis. Complimentary RNAs (one round of amplification) were hybridized to Affymetrix GeneChip Chicken Genome arrays. The array contains comprehensive coverage of 32,773 transcripts corresponding to over 28,000 chicken genes. The signal intensities were analyzed using Partek Genomics suites (Partek, St. Louis, MI, USA). The data were normalized using RMA (Bolstad et al., 2003). The messenger RNA expression levels of genes (ProbeSet) of Hes5.3, Atoh7, and nontargeting siRNAs were compared using a one-way ANOVA model (Eisenhart, 1947) and contrast (Tamhane and Dunlop, 2000). The selections were based on the fold-change intensities (1.5×) and corrected for multitestting using the Benjamini/Hochberg procedure with a false discovery rate of 5%.

DNA Constructs

To construct the Hes5.3-GFP, Hes5.3-RFP, and Hes5.3-CAT reporter plasmids, the wild-type upstream sequence, 1,960 bp in length, was subcloned in the vectors pEGFP-C1, pDsRed2-N1 (Clontech), and pDsRed-CAT (Hernandez et al., 2007). The Atoh7 reporter plasmids were previously described. The wild-type upstream sequence, 2,220 bp in length and bounded by EcoRI and BstXI restriction sites, was subcloned in the vectors pEgfp-C1 (Hernandez et al., 2007), p00-lac, p00-CAT, and pDsRed2-N1 (Skowronska-Krawczyk et al., 2009). For detection, the DsRed protein must accumulate at higher levels than enhanced GFP because the photomultipliers are less sensitive to long wavelengths and the chromophore maturation process is more complex (Figure S4C; Strongin et al., 2007). In the Chnmb3-GFP reporter plasmid, the wild-type upstream sequence, 143 bp in length (Matter-Sadzinski et al., 2001), was subcloned in the vector pEGFP. The beta actin–Hes5.3 GFP expression vector (Fior and Henrique, 2005) was a gift from D. Henrique.

siRNAs

We designed the Atoh7 and Hes5.3 siRNAs (Table S1). siRNA synthesis was performed under contract with Thermo Scientific Dharmacon. siSTABLE nontargeting siRNAs are from Thermo Scientific Dharmacon.

Eye Electroporation

Retina electroporations were as described in Matter-Sadzinski et al. (2005). Embryonic eyes were positioned in an electroporation chamber (BT 640, BTX, Genetronics) filled with 0.1 ml PBS (pH 7.4) containing reporter plasmids (0.1–0.5 μg/μl each) with or without siRNAs (0.1 μg/μl each). Electroporation consisted in five 12.5 V/cm pulses of 50 ms duration spaced 1 second apart.

FACS

The posterior domains of eight HH24 retinas were electroporated with a Hes5.3-GFP plasmid. Retina fragments encompassing the Hes5.3 domain were microdissected 16 hr later and dissociated as described in...
Matter-Sadzinski et al. (2005). Freshly dissociated cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM) without phenol red and sieved immediately prior to cell sorting into 70 μm mesh strainer collection tubes (BD Biosciences). Cell sorting was performed using a BD FACS Vantage SE (Flow Cytometry Service, CMU, University of Geneva) to detect GFP fluorescence. RNA was immediately isolated from the GFP+ (≈1.5 × 10^6 cells) and GFP− (≈0.8 × 10^6 cells) fractions and processed for qRT-PCR.

**Time-Lapse Imaging and Data Processing**

The electroporated eye was placed on a 35 mm glass bottom dish (Peico, Wilico Wells) and covered with 0.8 ml collagen prepared as follows: 100 mg of rat tail collagen type VI (Sigma C8897) was dissolved in 6 ml of 16.7 mM acetic acid overnight at 4°C. Then, 18 ml of double-distilled water was added and the solution was dialyzed overnight against 4.2 mM acetic acid at 4°C. Finally, 550 μl of this reconstituted collagen, 80 μl of 4.2 mM acetic acid, 100 μl of DMEM 10x (Amimed) without phenol red, and 100 μl of 0.28 M sodium bicarbonate were mixed. The dish was incubated in a CO₂-incubator at 37°C for 30 min. After polymerization of the collagen, 2 ml of DMEM 1x without phenol red supplemented with 10% fetal bovine serum (GIBCO) and 1% of penicillin-streptomycin (Invitrogen) was added and the dish was maintained in a CO₂-incubator at 37°C for ~12 hr. The dish was positioned on an inverted fluorescence Leica widefield microscope AF6000LX in a temperature-controlled chamber and incubated in presence of 5% CO₂ at 37°C. The retina was viewed using a Leica x40 NA 0.55 dry objective and fluorescent cells were imaged in the green and red channels. Stacks 40 to 60 μm thick, composed of optical Z sections separated by 1 μm, were taken every 20 or 30 min for 20 to 72 hr. 3 to 6 fields were captured at each time point. Images were acquired with a Cascade B 512 × 512 CCD camera (Roper Scientific). All data were saved in uncompressed .avi files. Merging of the green and red channels was done using Imagej (v1.4). Further analysis of time-lapse movies was done using Imagej (v1.4) and a set of Imagej plugins (available on request) that we developed for INM analysis.

**Confocal Microscopy**

For confocal microscopy, retinas were fixed for 20 min in 4% paraformaldehyde, rinsed 24 hr in PBS, and mounted in PBS containing 43% glycerol. 21 mM 1,4-diazabicyclo [2.2.2] octane (Sigma). Red and green fluorescent cells were imaged with a confocal laser scanning microscope (LEICA SP2 AOBS) using a x20 NA 0.7 oil objective (Leica). An argon/krypton (Ar/Kr) laser (488, 543 nm lines) was used for both GFP and RFP excitation. Optical sections of 0.8 μm were taken through a volume of the retina up to 70 μm in depth. Image data were acquired and stored as TIFF files using confocal software (Leica). Pictures were processed in Imagej and cells were counted in 375 μm × 375 μm microscope fields. Cells were counted in at least ten (n ≥ 10) fields per retina. The sum of GFP+ cells, RFP+ cells, and double-labeled cells was set arbitrarily at 100% and the fractions of GFP+ cells, RFP+ cells, and double-labeled cells are given relative to this value. At least five (n ≥ 5) electroporated retinas were analyzed per condition.

For further details, please see the Extended Experimental Procedures.

**ACCESSION NUMBERS**

The complete microarray data set has been deposited in the public data repository of the European Bioinformatics Institute (ArrayExpress) under the accession number E-MTAB-1430.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, four figures, two tables, and three movies and can be found with this article online at [http://dx.doi.org/10.1016/j.celrep.2013.01.035](http://dx.doi.org/10.1016/j.celrep.2013.01.035)

**LICENSING INFORMATION**

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