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RESEARCH ARTICLE

Selection of cell fate in the organ of Corti involves the integration of Hes/Hey signaling at the *Atoh1* promoter

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ABSTRACT

Determination of cell fate within the prosensory domain of the developing cochlear duct relies on the temporal and spatial regulation of the bHLH transcription factor *Atoh1*. Auditory hair cells and supporting cells arise in a wave of differentiation that patterns them into discrete rows mediated by Notch-dependent lateral inhibition. However, the mechanism responsible for selecting sensory cells from within the prosensory competence domain remains poorly understood. We show in mice that rather than being upregulated in rows of cells, *Atoh1* is subject to transcriptional activation in groups of prosensory cells, and that highly conserved sites for Hes/Hey repressor binding in the *Atoh1* promoter are needed to select the hair cell and supporting cell fate. During perinatal supporting cell transdifferentiation, which is a model of hair cell regeneration, we show that derepression is sufficient to induce *Atoh1* expression, suggesting a mechanism for priming the 3′ *Atoh1* autoregulatory enhancer needed for hair cell expression.

KEY WORDS: Cochlear development, Organ of Corti, Hair cell regeneration, Transdifferentiation, Atoh1, Hes5, Mouse

INTRODUCTION

The auditory sensory epithelium in mammals, the organ of Corti, is composed of a linear array of inner and outer sensory hair cells surrounded by a variety of supporting cells that lie in discrete rows on a basilar membrane within the mature cochlear duct. These cells first differentiate from a postmitotic prosensory domain that forms within the cochlear duct between approximately embryonic day (E) 12.5 and E14.5 in the mouse (Ruben, 1967; Lee et al., 2006). Cells within the prosensory region are competent to form either sensory hair cells or the supporting cells that surround them (Woods et al., 2004; Driver et al., 2013). Patterning of the prosensory domain occurs under the influence of the basic helix-loop-helix (bHLH) transcription factor ATOH1, which is first observed in nascent hair cells near the base of the cochlea and drives differentiation in a wave towards the apex over a period of ∼4 days from E13.5 to E17.5 or later (Bermingham et al., 1999; Lanford et al., 2000; Chen et al., 2002; Woods et al., 2004). In the current model, and by analogy with *Drosophila* sensory organ precursor selection, once *Atoh1* expression is established in nascent hair cells, Notch-mediated lateral inhibition stimulates the activity of the Hairy/Enhancer of Split families of transcriptional repressors (Hes/Hey) in the adjacent prosensory cells (reviewed by Jarman and Groves, 2013). This inhibits further differentiation of prosensory cells to hair cells (Lanford et al., 1999; Kiernan et al., 2005), leading them to differentiate as supporting cells (Woods et al., 2004). Although a number of factors are known to influence *Atoh1* expression (reviewed by Jarman and Groves, 2013), primarily through interaction with a previously characterized 3′ enhancer (Helms et al., 2000), the mechanism by which prosensory cells are selected for *Atoh1* upregulation has not been identified.

The Hes and Hey families of bHLH transcriptional repressors function throughout development to mediate Notch signaling (Jarriault et al., 1995; Nishimura et al., 1998; Ohtsuka et al., 1999). In the developing nervous system, HES1 and HES5 are important for maintaining the neural progenitor status, and their loss leads to premature cell cycle exit and neuronal differentiation (Kageyama et al., 2000; Nakamura et al., 2000; Ohtsuka et al., 2001). Previous reports suggest that repression is accomplished by the recruitment of co-repressors, such as those encoded by Groucho-related genes (*Grg1-4*; also known as *Tle1-4*) in the case of the Hes family, which in turn are able to recruit histone deacetylases (HDACs) to mediate gene inactivation (Fisher et al., 1996; Grbavec and Stifani, 1996; Winkler et al., 2010). In the organ of Corti, Hes/Hey genes are expressed in a complex combinatorial pattern (Doetzlhofer et al., 2009; Tateya et al., 2011) and are needed to maintain supporting cell fate (Zine et al., 2001; Zine and de Ribaupierre, 2002; Hayashi et al., 2008; Li et al., 2008), but the molecular mechanism by which this is accomplished has not been investigated.

The model in which *Atoh1* is selectively upregulated in nascent hair cells has recently come into question following reports that *Atoh1* may be initially upregulated in progenitors that go on to form not only all the hair cells but also some of the supporting cells (Matei et al., 2005; Yang et al., 2010; Cai et al., 2013; Driver et al., 2013). This suggests a second model in which the final configuration of hair cells does not rely on the positive selection by *Atoh1* of specific cells within the prosensory domain, but rather on the complex regulation of repressive mechanisms that shut down *Atoh1* expression in those cells that are to become the different supporting cell types. In this paper, we show that this second model is likely to be correct. We identify a set of highly evolutionarily conserved Hes/Hey binding sites in the promoter of the *Atoh1* gene that are needed for timely repression of *Atoh1* within the cells of the prosensory domain destined to be supporting cells. In functional studies in neural progenitors we show that Hes/Hey factors bind directly to these sites and, in the case of HES5, actively repress *Atoh1* expression by recruiting the Groucho co-repressor in a Notch-dependent manner. We show that in FACS-purified supporting cells, inhibition of Notch leads to an increase in histone H3K9 acetylation (H3K9ac) at the *Atoh1* promoter, commensurate with

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increased expression, and that inhibition of acetylation limits Notch-mediated *Atoh1* expression during transdifferentiation. We also show that the transcriptional silence of *Atoh1* in supporting cells at postnatal day (P) 1, and thus the maintenance of the supporting cell fate, although dependent on active repression by Hes/Hey factors, is reinforced by activator insufficiency and a switch-like behavior of the *Atoh1* 3′ autoregulatory enhancer. We suggest that derepression of the *Atoh1* promoter is sufficient to stimulate *Atoh1* expression and thus prime the autoregulatory function of this enhancer during transdifferentiation of supporting cells.

RESULTS

Derepression of *Atoh1* is sufficient to induce *Atoh1* transcription

In non-mammalian vertebrates, hair cell loss leads to *Atoh1* upregulation in surrounding supporting cells and their direct transdifferentiation, followed later by a wave of proliferation and additional transdifferentiation (Roberson et al., 2004; Cafaro et al., 2007; Stone and Cotanche, 2007). By contrast, in mammals, hair cells in the cochlea fail to regenerate after damage (Chardin and Romand, 1995; Brigande and Heller, 2009). However, we and others have shown that blocking Notch signaling in the perinatal mouse organ of Corti leads to transdifferentiation of supporting cells to a sensory hair cell-like state (Yamamoto et al., 2006; Doetzlhofer et al., 2009). To confirm this result, we treated cochlear cultures with and without DAPT (a γ-secretase inhibitor) for 72 h. Supporting cell transdifferentiation was visualized using a well-characterized transgenic mouse line that expresses GFP in hair cells under the control of the *Atoh1* 3′ autoregulatory enhancer (Atoh1 enhancer/β-globin promoter/GFP), which is sufficient to direct accurate expression during organ of Corti development (although a weak expression in inner phalangeal cells is occasionally noted) (Lumpkin et al., 2003; see below). Inhibition of Notch signaling by DAPT increased the number of GFP-positive hair cell-like cells and reduced the number of supporting cells labeled with PROX1, an early marker of supporting cell differentiation, suggesting their direct transdifferentiation (Fig. 1A). Using a different reporter line carrying a p27/GFP BAC transgene that is expressed in supporting cells (Lee et al., 2006; White et al., 2006), we FACS purified supporting cells from DAPT-treated and DMSO-treated organ cultures. qPCR analysis showed that, 24 h after inhibition of Notch signaling, *Atoh1* expression increased more than 10-fold, whereas the expression of *Hey1* and *Hes5* decreased ∼70% and 90%, respectively ($P<0.005$; Fig. 1B).

In other systems, the Hes/Hey family members HES1 and HES7 have been shown to have short protein half-lives (Hirata et al., 2002, 2004). Transfecting flag-tagged HES5 into HEK 293 cells indicated that its protein half-life is ∼2.5 h, and that selective inhibition of the proteasome pathway significantly increased HES5 stability (Fig. S1). Within the Hes/Hey family, *Hes5* is expressed strongly in Deiters' cells, a subpopulation of supporting cells that readily transdifferentiate into hair cells when Notch signaling is blocked. *Hes5* appears to be the most highly expressed Hes/Hey family member in supporting cells (Tateya et al., 2011), and appears to be the most sensitive to loss of Notch signaling (Fig. 1B) (Doetzlhofer et al., 2009). We hypothesized that if HES5 is needed to directly silence constitutive *Atoh1* transcriptional activity, inhibition of protein synthesis might derepress *Atoh1* expression as a consequence of HES5 protein turnover in supporting cells and, similarly, stabilizing HES5 protein would prevent *Atoh1* upregulation in supporting cells in response to blocking Notch signaling. To test this hypothesis in supporting cells, we treated P1 organ cultures for 6 h with cycloheximide (CHX) to block protein synthesis, and then FACS purified the supporting cells. qPCR analysis indicated a more than 9-fold increase in *Atoh1* expression, showing that derepression is sufficient to drive endogenous *Atoh1* expression (Fig. 1C). If upregulation requires *Atoh1* derepression through HES5 turnover, then blocking HES5 degradation should inhibit *Atoh1* upregulation. To test this we simultaneously blocked Notch signaling to induce *Atoh1*, and inhibited HES5 turnover by blocking the activity of the proteasome pathway with MG132 (Hirata et al., 2002). Analysis of FACS-purified supporting cells indicated that *Atoh1* failed to be upregulated following inhibition of Notch when proteasome activity was simultaneously inhibited (Fig. 1D).

The *Atoh1* 3′ enhancer contains a conserved E-box, which has been shown to bind ATOH1 (Helms et al., 2000), upon which the enhancer's autoregulatory function and *Atoh1* expression depend (Helms et al., 2000). In perinatal supporting cells, the *Atoh1* transgene containing the 3′ autoregulatory enhancer (Atoh1 enhancer/β-globin promoter/GFP) is silent (Chen et al., 2002; Lumpkin et al., 2003) (Fig. 1A), suggesting that lateral inhibition might repress *Atoh1* through sites in the enhancer. Indeed, the E-box site is overlapped by a consensus N-box, a potential site of Hes/Hey binding (Helms et al., 2000). Interestingly, although blocking protein synthesis with CHX stimulated endogenous *Atoh1* expression, it failed to stimulate expression from the *Atoh1* enhancer transgene (Fig. 1E), suggesting that the repressive effects of HES5 are mediated through elements that are not in the enhancer but elsewhere at the *Atoh1* locus. The reason for enhancer inactivity might be activator insufficiency (see below). These results also show that the enhancer sequence alone is not sufficient to initiate expression, even when repression is relieved.

HES5 targets the *Atoh1* promoter region for repression

bHLH proteins of the Hes/Hey family have been shown to bind a canonical N-box (CACNAG) or C-site (CACGNG) in their target genes (Liu et al., 2006; Grogan et al., 2008; Zheng et al., 2011). In addition to the N-box and a C-site in the 3′ enhancer, the *Atoh1* promoter contains four highly evolutionarily conserved sites within 226 bp of the transcription start site (Fig. 2A), suggesting that HES5 might directly regulate *Atoh1* expression through the promoter. To test the functional significance of the HES5 binding sites in the *Atoh1* promoter and enhancer region, we compared the ability of HES5, transfected into HEK 293 cells, to repress a co-transfected *Atoh1* promoter-reporter construct driving GFP, relative to the heterologous β-globin promoter, which does not contain C-sites, driving mCherry. The percentage of cells expressing GFP and mCherry was determined by flow cytometry (Fig. 2B). The reporter with the β-globin minimal promoter (mCherry) was unaffected by the expression of HES5, whereas the *Atoh1* promoter containing four C-sites (GFP) was strongly inhibited (>60%).

To determine if the predicted Hes/Hey binding sites in the *Atoh1* promoter region are required for HES5-mediated repression of *Atoh1*, we mutated the C-sites in the *Atoh1* reporter construct (Fig. 2C) and again tested them in HEK 293 cells. In the absence of HES5 expression, the mutation of C-sites had no effect on the expression of GFP (data not shown). However, mutation of the C-sites in the *Atoh1* promoter had an additive effect: the more sites that were mutated, the greater the loss of HES5 inhibitory activity on the *Atoh1* promoter (Fig. 2C).

HEY2, which is also expressed in the organ of Corti with other Hes/Hey family members (Li et al., 2008; Doetzlhofer et al., 2009), was tested using the same reporter constructs co-transfected into HEK 293 cells. Similar to HES5, HEY2 represses expression in an *Atoh1* promoter-dependent manner, although site usage varied from that seen with HES5 (Fig. S2).

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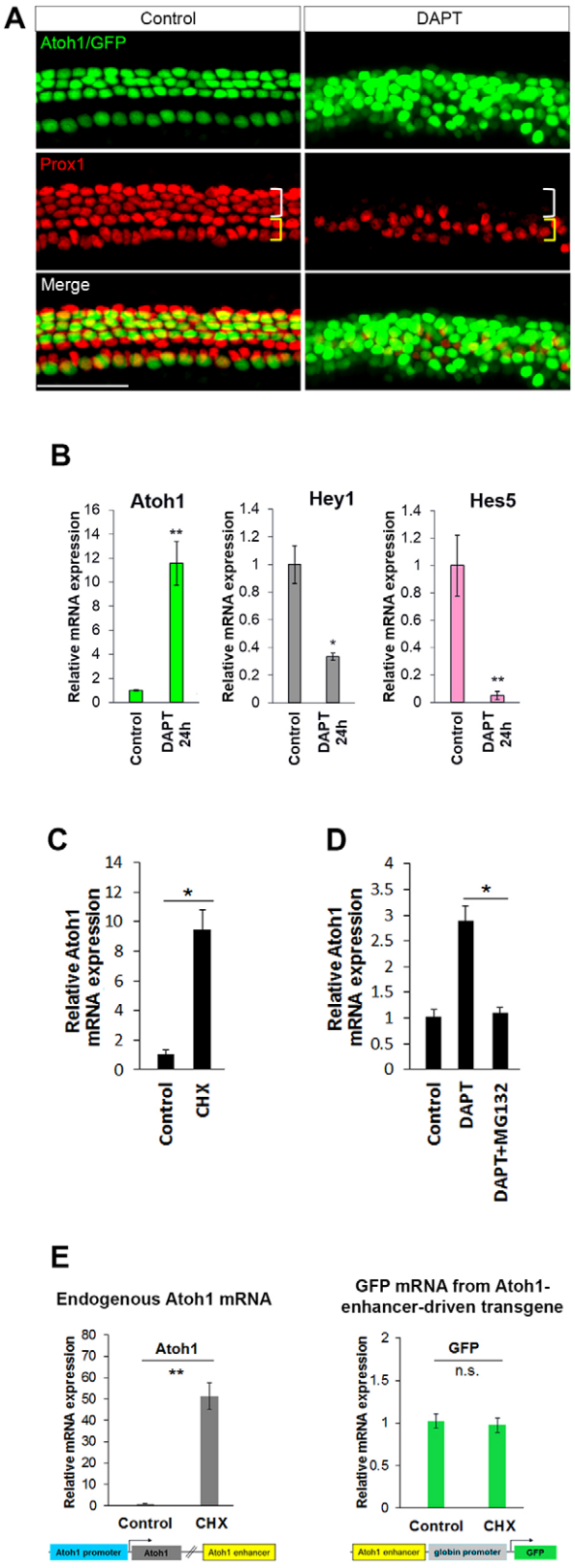

Fig. 1. Derepression of *Atoh1* in supporting cells is sufficient to drive expression and occurs through promoter elements, not through the 3′ autoregulatory enhancer. (A) P1 Atoh1 enhancer/β-globin promoter/GFP transgenic organ of Corti was cultured for 72 h in DMSO (control) or DAPT (γ-secretase inhibitor) and immunostained with anti-PROX1 antibody to label supporting cells (Deiters' and pillar cells). The appearance of ectopic GFP^+ hair cell-like cells in DAPT is accompanied by loss of $PROX1^+$ supporting cells (white bracket). (B-E) Changes in mRNA expression level were examined by real-time quantitative PCR (qPCR). (B) The observed transdifferentiation of supporting cells in A correlates with upregulation of *Atoh1* and downregulation of Hes/Hey factors mRNA in FACS-purified supporting cells ($p27/GFP^+$) after 24 h treatment with DAPT. n=4. (C) Inhibition of protein synthesis with cycloheximide (CHX) for 6 h induces *Atoh1* expression in FACS-purified supporting cells ($p27/GFP^+$). n=3. (D) Inhibition of protein degradation by MG132 prevents DAPT-induced upregulation of *Atoh1* in FACS-purified supporting cells ($p27/GFP^+$) treated for 12 h. n=3. (E) CHX induces endogenous *Atoh1*, but not the Atoh1 enhancer/β-globin promoter/GFP transgene. P1 cochlear organ cultures from Atoh1 enhancer/β-globin promoter/GFP mice were incubated without and with CHX for 6 h, after which organ cultures were dissociated and FACS sorted to eliminate hair cells. The GFP^- population from these transgenic organs includes supporting cells, cells from the greater epithelial ridge and lesser epithelial ridge. *Atoh1* and GFP levels were measured in GFP^- cells (non-hair cells of the cochlear epithelia). n=4. All values are mean±s.e.m. *P<0.05, **P<0.005; n.s., not statistically significant. Scale bar: 100 µm. See also Fig. S1.

Atoh1 is upregulated in prosensory progenitors, not just in nascent hair cells

The mutational analysis of the *Atoh1* promoter-reporter *in vitro* demonstrated the importance of the C-sites for *Atoh1* repression in HEK 293 cells. To better understand the mechanism of HES-mediated lateral inhibition *in vivo*, we generated a double-transgenic mouse line carrying both the wild-type and mutated *Atoh1* promoter-reporter constructs. During organ of Corti development, *Atoh1* is upregulated in nascent hair cells in a basal-to-apical wave (Chen et al., 2002). Evidence suggests that lateral inhibition is responsible for repressing *Atoh1* in the prosensory progenitors surrounding the nascent hair cells, allowing their differentiation as supporting cells (Kiernan et al., 2005; Yamamoto et al., 2006; Doetzlhofer et al., 2009). We hypothesized that if *Atoh1* is initially upregulated in groups of prosensory progenitors, followed by rapid repression in those selected progenitors that will become supporting cells, the mutant promoter-reporter (GFP) would be deficient in this process and would report the presence of GFP in the nascent progenitors destined to be supporting cells, as well as the nascent hair cells. By contrast, the wild-type promoter-reporter (tdTomato) would accurately identify only the nascent hair cells. As hypothesized, at E16.5 in the middle region of the cochlear duct, the expression of the wild-type promoter-tdTomato was observed only in inner and outer hair cells, whereas GFP was strongly expressed in the hair cell region as well as being misexpressed in the differentiating supporting cell layer (Fig. 3B, arrowheads). In a slightly more apical and thus less differentiated position along the cochlear duct, only nascent inner hair cells expressing the wild-type tdTomato transgene were observed, with a commensurate increase in the number of progenitors expressing GFP from the mutant promoter. The appearance of only the inner hair cells is consistent with the well documented earlier differentiation of inner, relative to outer, hair cells (Sher, 1971; Lim and Anniko, 1985; Chen et al., 2002). Together, these results indicate that *Atoh1* is upregulated in groups of prosensory progenitors, not just in nascent hair cells, and that the promoter-proximal Hes/Hey binding motifs are crucial for the silencing of *Atoh1* in these prosensory progenitors.

At P1, *Atoh1* mRNA expression is localized to the inner and outer hair cells (Lanford et al., 1999; Woods et al., 2004), as is expression of both the wild-type and mutant promoter transgenic constructs (Fig. 4A). The lack of expression of the mutant promoter-reporter, which is unable to undergo Hes/Hey-mediated repression, indicates that active repression is unlikely to be responsible for the silence of this construct in supporting cells. Rather, it suggests that Hes/Hey

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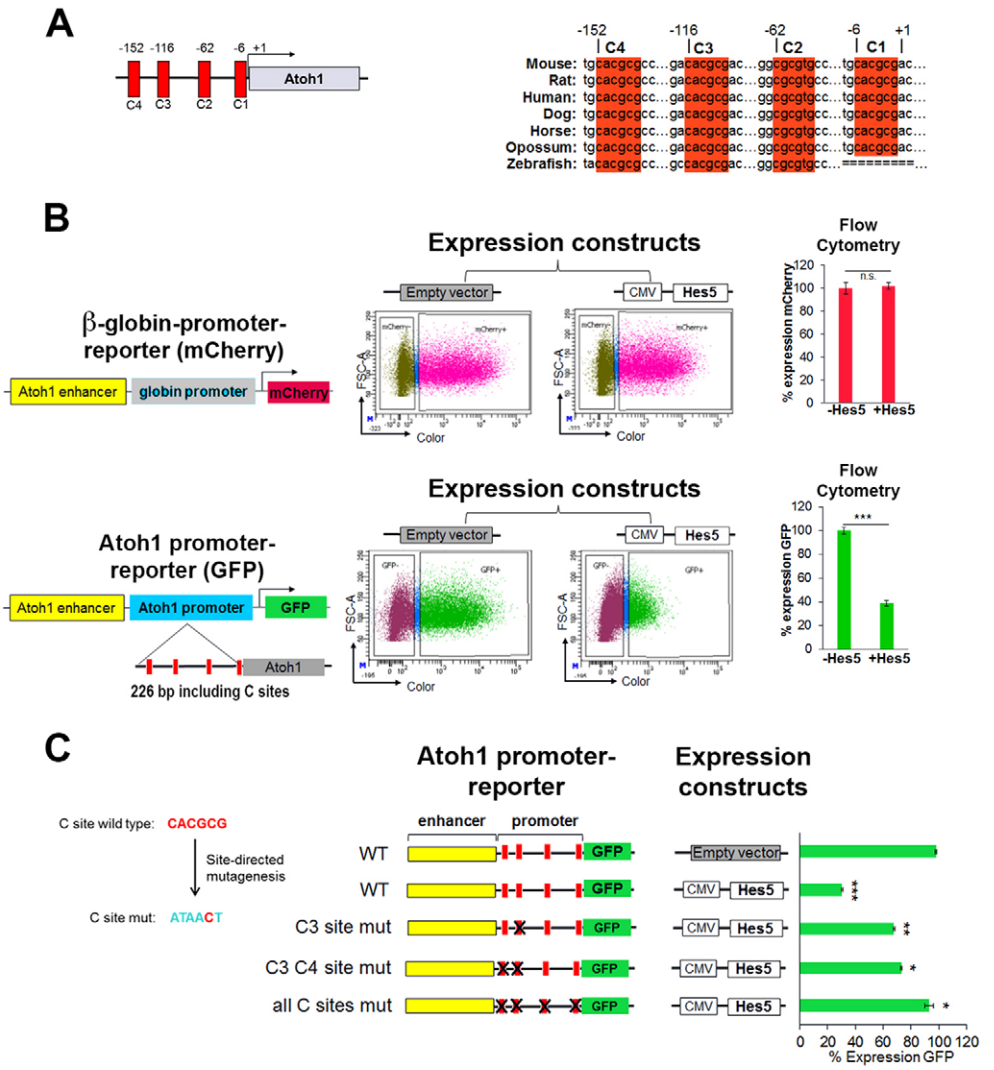

Fig. 2. HES5 functions to repress *Atoh1* through highly conserved binding sites in the promoter region. (A) Schematic of the murine *Atoh1* promoter indicating the C-sites and their conservation among vertebrates. Numbers are relative to the transcription start site (+1) in mouse. (B) Ectopic *Hes5* expression requires the *Atoh1* promoter to repress *Atoh1* expression in heterologous HEK 293 cells [compare β-globin promoter (top) with *Atoh1* promoter (bottom)]. Reporters and expression constructs were transiently transfected and expression was quantified by flow cytometry. (Top) Reporter contains the *Atoh1* enhancer and β-globin basal promoter driving the expression of mCherry. (Bottom) Reporter contains the *Atoh1* enhancer and 226 bp of the *Atoh1* promoter (containing the predicted C-sites) driving the expression of GFP. Reporters were cotransfected with a control (empty vector) or a plasmid expressing *Hes5*. The number of cells expressing GFP or mCherry was determined by flow cytometry after 48 h. Values are mean±s.e.m.; n=6. ***$P<1\times10^{-7}$. (C) Mutational analysis of C-site function. Five out of six nucleotides of each C-site were mutated. Quantification was performed as in B with the indicated plasmids reported as percentage of cells expressing GFP relative to the CMV-RFP co-transfected control (not shown) set to 100% in the absence of *Hes5* expression (empty vector). Values are mean±s.e.m.; n=4. *$P<0.0003$, **$P<2\times10^{-5}$, ***$P<4\times10^{-7}$. See also Fig. S2.

repression of endogenous *Atoh1* mRNA is sufficient to keep the expression of endogenous ATOH1 protein below the threshold needed to trigger the *Atoh1* enhancer *in vivo* – an example of activator insufficiency (Barolo and Posakony, 2002; see below).

To test the importance of C-site repression in the maintenance of supporting cell fate at P1, we treated cochlear explants with DAPT for 18 h to inhibit Notch signaling and induce the transdifferentiation of supporting cells (Fig. 1) and, by doing so, also induce expression of the two transgenic reporters, which contain identical copies of the 3′ autoregulatory enhancer (Fig. 4B). We hypothesized that, upon washing out DAPT after 18 h, lateral inhibition would return and repression of *Atoh1* expression would be re-established at the endogenous promoter and the wild-type transgene, but that the mutant promoter would be unable to re-establish repression. As expected, expression of the wild-type promoter-reporter (tdTomato) transgene and the endogenous *Atoh1* mRNA were partially re-repressed at 6 h following DAPT washout, but expression from the promoter-reporter mutant (GFP) transgene lagged significantly behind.

HES5 recruits the co-repressor GRG to the *Atoh1* promoter in a Notch-dependent fashion

Hes proteins have been reported to recruit GRG co-repressors (GRG1-4) through their conserved C-terminal WRPW motif (Grbavec and Stifani, 1996; Grbavec et al., 1998). Our *in vitro* results suggested that loss of the HES5 WRPW motif severely reduces its ability to inhibit the *Atoh1* reporter, and that HES5 and GRG interact through the WRPW motif of HES5 (Fig. S3). To test whether the interaction of HES5 and GRG co-repressor could be responsible for mediating the repression of *Atoh1* we used neural progenitor cells (NPCs) as a model (Fig. S4), since the paucity of supporting cells in the organ of Corti makes reliable transcription factor ChIP currently impractical. NPCs, like supporting cells, rely on active Notch signaling for maintenance of their progenitor state (Ohtsuka et al., 2001; Hatakeyama et al., 2004; Crawford and Roelink, 2007; Borghese et al., 2010). Consistent with this, we observed more than 5-fold (P<0.005) upregulation of *Atoh1* and a complete loss of *Hes5* mRNA in NPCs following DAPT treatment (Fig. 5A). Also like supporting cells, NPCs express GRG4 at higher levels than the other GRG factors (Fig. S5).

To test whether HES5 directly interacts with the *Atoh1* promoter region, NPCs were transfected with a flag-tagged HES5 expression plasmid, and ChIP-qPCR indicated that HES5 interacts with the *Atoh1* promoter region, but not the enhancer, confirming the reporter assay findings (Fig. 5B). Similarly, ChIP-qPCR in untransfected NPCs with a pan-GRG antibody showed that endogenous GRG is also normally enriched at the *Atoh1* promoter region (Fig. 5C). Interestingly, the association of endogenous GRG

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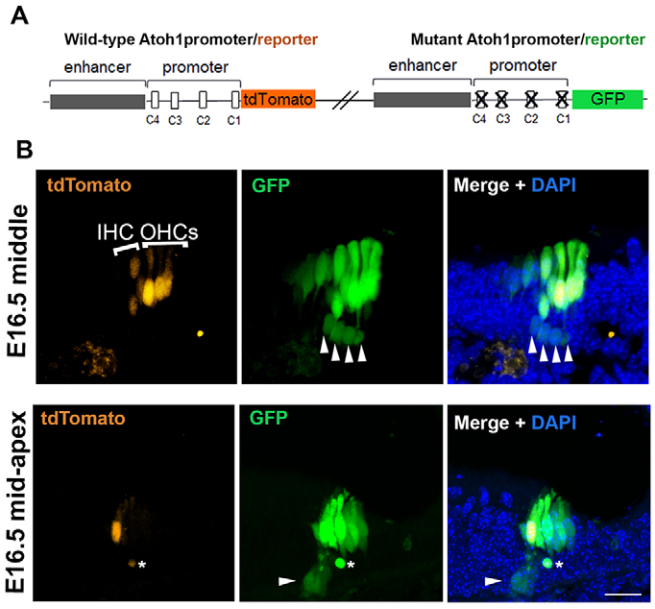

Fig. 3. The misexpression of a mutant promoter transgene *in vivo* shows that Hes/Hey binding sites in the *Atoh1* promoter are required for the proper silencing of *Atoh1* in supporting cells. (A) Schematics showing wild-type and mutant constructs used to generate the double-transgenic mouse (see Materials and Methods). (B) The expression pattern of the wild-type and mutant transgenes at E16.5 is shown in transverse section at two positions (middle and mid-apex) of the cochlear duct. The GFP from the mutant promoter transgene is misexpressed in the supporting cell layer (white arrowheads; asterisk indicates debris), in addition to the expected expression in the hair cells, indicating the need for active repression for supporting cell silencing. tdTomato from the wild-type transgene is absent from the supporting cell layer. IHC, inner hair cell; OHCs, outer hair cells. Scale bar: 20 µm.

with the *Atoh1* promoter region was Notch dependent (Fig. 5C), suggesting that it is the interaction of HES5 with this co-repressor in NPCs that maintains them in an undifferentiated state.

Inhibition of Notch induces histone acetylation at the *Atoh1* promoter in supporting cells

GRG-mediated repression has been shown to be partially dependent on HDAC recruitment in *Drosophila* (Winkler et al., 2010). In neonatal supporting cells purified from cochlear cultures treated with the HDAC inhibitor trichostatin A (TSA) for 6 h, *Atoh1* was upregulated ($P<0.05$; Fig. 6A), suggesting that the repression of *Atoh1* in supporting cells is dependent on maintenance of the deacetylated state of histones at the *Atoh1* locus. Similar experiments in *Hes5*-transfected HEK 293 cells suggested a similar conclusion (Fig. S6). Furthermore, inhibition of histone acetyltransferase (HAT) activity by curcumin partially blocks the DAPT-induced accumulation of *Atoh1* mRNA, further indicating a need for ongoing histone acetylation to induce *Atoh1* (Fig. 6B). Curcumin, either on its own or in conjunction with DAPT, had no effect on the expression level of the Notch effectors *Hes5* and *Hey1* (data not shown). This prompted us to ask if blocking Notch signaling in supporting cells brings about changes in the acetylation status of histones at the *Atoh1* locus. We performed ChIP-qPCR with FACS-purified DMSO-treated and DAPT-treated supporting cells for H3K9ac, a histone modification highly correlated with actively transcribed genes (Wang et al., 2008). Inhibition of Notch signaling for 24 h increased the H3K9ac level at the promoter region in transdifferentiating supporting cells (Fig. 6C). This result strongly suggests that the upregulation of *Atoh1* in supporting cells is accompanied by epigenetic changes that render the chromatin more permissive at the *Atoh1* locus.

DISCUSSION

Our data help explain several important aspects of *Atoh1* regulation that underlie embryonic sensory epithelia differentiation and patterning, including the identification of the sites of active Hes/Hey-mediated repression of *Atoh1*, the sufficiency of derepression to stimulate *Atoh1* expression, and the role of activator insufficiency in reinforcing the transcriptional silence of *Atoh1* in prosensory and supporting cells. These results suggest a model of *Atoh1* regulation during embryonic development that emphasizes the importance of the repressive activity of Hes/Hey factors to select the rows of hair cells and supporting cells, as opposed to the selective upregulation of *Atoh1* in prosensory populations destined to be hair cells. These issues are discussed below and the results presented in a model of *Atoh1* regulation (Fig. 7).

Atoh1 repression is mediated through highly conserved Hes/Hey binding motifs in the *Atoh1* promoter

Our results show that HES5 represses *Atoh1* expression by directly binding to the promoter region of *Atoh1* (Fig. 2B, Fig. 5B) and that this binding is dependent on four highly conserved C-sites located within the proximal 226 bp of the transcription start site (Figs 2–4). Mutation in all four C-sites is required to abolish the inhibitory effect of ectopically expressed HES5 on promoter function (Fig. 2C), indicating a synergistic role in this context. However, a comparison of the effects of HES5 and HEY2 on the repression of *Atoh1* revealed differences in strength of inhibition and binding (Fig. S2), suggesting the possibility that different members of the Hes/Hey families could play independent roles in *Atoh1* regulation, as previously shown (Zine et al., 2001; Li et al., 2008; Doetzlhofer et al., 2009). While Hes/Hey repressor sequences are present in regulatory elements of *atonal* homologs separated by hundreds of millions of years of evolution, there are fewer recognizable Hes/Hey motifs associated with proneural genes in non-vertebrate lineages, just as there are fewer homologs of Hes/Hey genes (Rebeiz et al., 2005), suggesting that duplication of the binding sites in the *Atoh1* promoter might have paralleled the expansion of the Hes/Hey family of genes, leading to specialization rather than redundancy in mammals. The complex combinatorial pattern of Hes/Hey gene expression in the organ of Corti (Doetzlhofer et al., 2009) and the differing patterning phenotypes of the Hes/Hey mutant mice suggest a more subtle role for these sites in sculpting organ of Corti structure.

Atoh1 is upregulated in groups of prosensory progenitors

Earlier observations using a transgenic reporter (Atoh1 enhancer/β-globin promoter/GFP), and by antibody staining, suggested that *Atoh1* is upregulated only in nascent hair cells during the embryonic patterning of the organ of Corti (Chen et al., 2002). However, recent evidence suggests an alternate model in which *Atoh1* transcriptional activity may begin in groups of prosensory cells and then be rapidly refined to just the nascent hair cells. First, loss of Notch signaling through the conditional mutation of *Rbpj* leads to the broader expression of *Atoh1* within the prosensory domain (Basch et al., 2011; Yamamoto et al., 2011). Second, lineage-tracing experiments indicated that significant numbers of supporting cells derive from *Atoh1-cre*-expressing prosensory cells (Yang et al., 2010; Driver et al., 2013). Third, Cai et al. (2013) report that, in an *Atoh1-GFP* knock-in mouse, low levels of GFP are detectable in multiple rows of cells at the leading edge of hair cell differentiation (Cai et al.,

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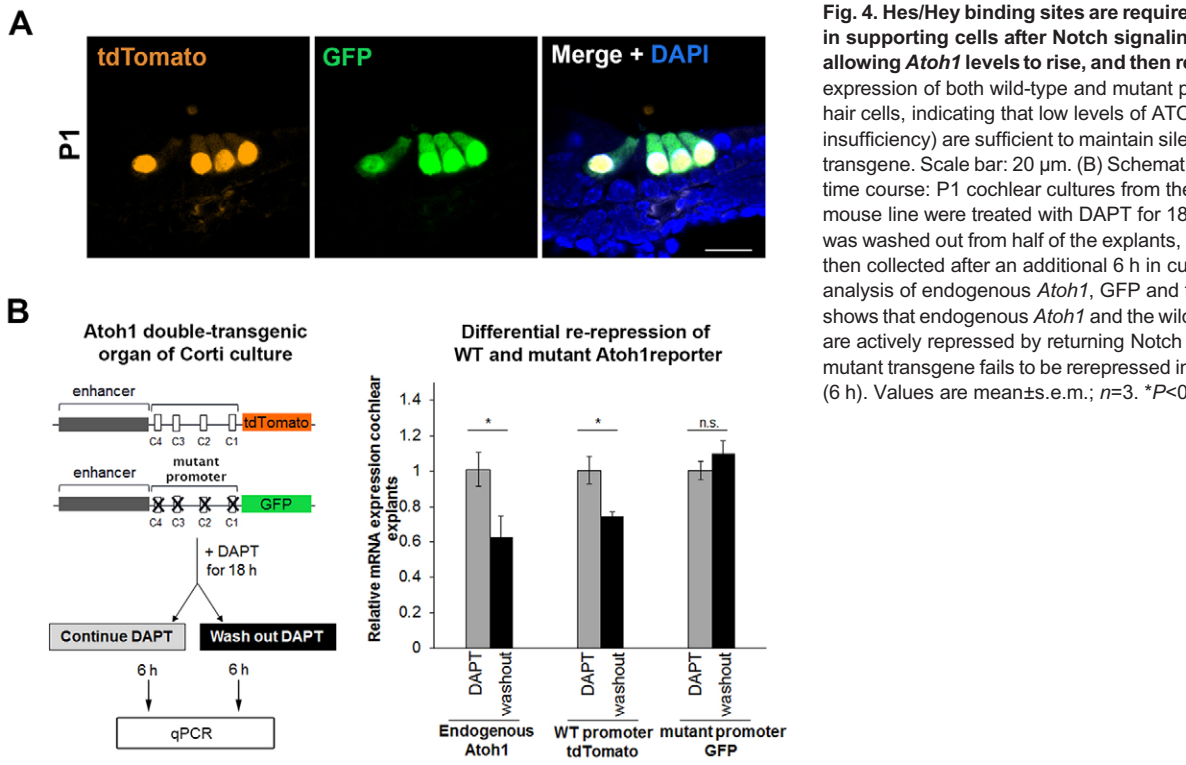

Fig. 4. Hes/Hey binding sites are required to rerepress *Atoh1* in supporting cells after Notch signaling is first inhibited, allowing *Atoh1* levels to rise, and then restored. (A) At P1, the expression of both wild-type and mutant promoters is limited to hair cells, indicating that low levels of ATOH1 (activator insufficiency) are sufficient to maintain silencing from the mutant transgene. Scale bar: 20 µm. (B) Schematic shows experimental time course: P1 cochlear cultures from the double-transgenic mouse line were treated with DAPT for 18 h, after which DAPT was washed out from half of the explants, and the explants were then collected after an additional 6 h in culture. Expression analysis of endogenous *Atoh1*, GFP and tdTomato by qPCR shows that endogenous *Atoh1* and the wild-type (WT) transgene are actively repressed by returning Notch signaling, but the mutant transgene fails to be rerepressed in the same time period (6 h). Values are mean±s.e.m.; n=3. *P<0.05.

2013). Our current transgene data support these observations by showing that the mutant *Atoh1* promoter allows expression in both nascent hair cell types, as well as in additional prosensory cells, which are likely destined to become supporting cells (Fig. 3B).

This upregulation of *Atoh1* in groups of cells within the prosensory domain is reminiscent of recent observations regarding the selection of the sensory organ precursors (SOP) that go on to form the micro- and macrochaete, the sensory bristles in the

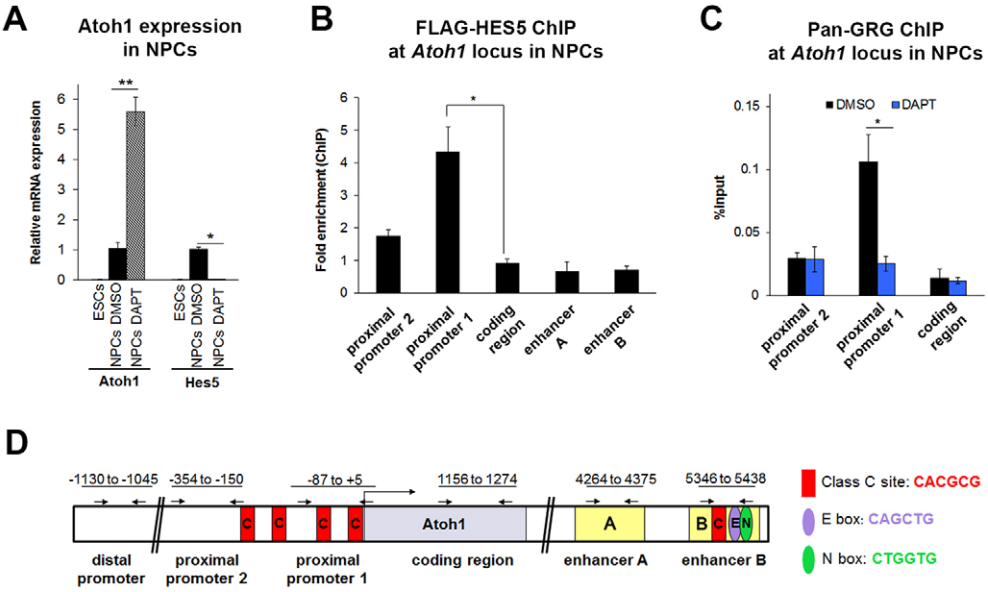

Fig. 5. GRG localizes to the *Atoh1* locus in a Notch-dependent manner in neural progenitor cells. Neural progenitor cells (NPCs) were differentiated from ESCs and FACS purified (Sox1-GFP$^+$). (A) *Atoh1* and *Hes5* mRNA expression levels in 46C ESCs and NPCs that were treated with control (DMSO) or γ-secretase inhibitor (DAPT) for 48 h. Similar to supporting cells, inhibition of Notch signaling by DAPT induces the expression of *Atoh1* and represses the expression of *Hes5* in NPCs. n=4. (B) HES5 localizes to the proximal promoter and not the enhancer by ChIP-qPCR of NPCs transfected with CMV-FLAG-Hes5 plasmid (or CMV-RFP control). Results are reported as fold enrichment (FLAG-Hes5 transfected percentage input/RFP transfected percentage input). n=3. (C) GRG localizes to the *Atoh1* promoter in a Notch-dependent manner. ChIP-qPCR with anti-pan-GRG antibody and primers scanning the *Atoh1* locus in NPCs treated with control (DMSO) or γ-secretase inhibitor (DAPT) for 24 h after 7 days of differentiation. n=3. (D) Schematic of the *Atoh1* locus in mouse showing the promoter and the autoregulatory enhancer regions. Numbers refer to the regions amplified in ChIP-qPCR. The primers used to measure the enrichment after ChIP are shown by arrows. All values are mean±s.e.m. *P<0.05, **P<0.005. See also Figs S3-S5.

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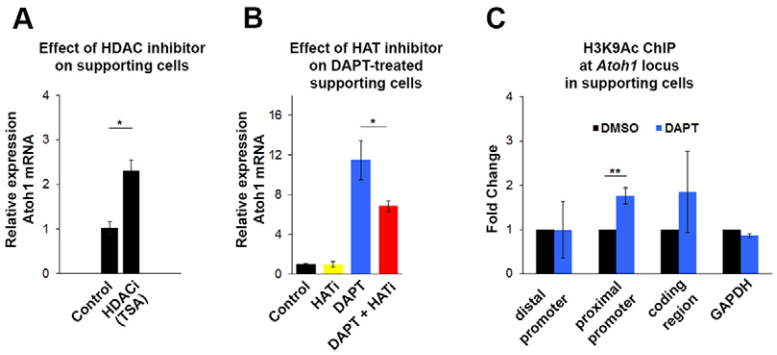

Fig. 6. Inhibition of Notch leads to increased histone acetylation at the *Atoh1* promoter in supporting cells. (A) Inhibition of HDAC activity by trichostatin A (TSA) for 6 h induces *Atoh1* expression in FACS-purified supporting cells (p27/GFP$^+$). Values are mean± s.e.m.; n=3. (B) Inhibition of HAT activity by curcumin (Balasubramanyam et al., 2004) for 24 h blocks DAPT-induced *Atoh1* activation in FACS-purified supporting cells (Lfng/GFP$^+$). n=3. (C) Inhibition of Notch signaling (DAPT for 24 h) increases H3K9ac, as analyzed by ChIP-qPCR at the *Atoh1* promoter in FACS-purified supporting cells (p27/GFP$^+$). Values are mean± s.e.m.; n=3. *P<0.05, **P<0.005. See also Fig. S6.

Drosophila notum (Troost et al., 2015). Here, the standard theory holds that small stochastic differences in proneural gene expression in single cells within a proneural cluster are amplified by strong Notch-mediated feedback to select them as SOPs (reviewed by Fortini, 2009). However, in their re-evaluation of this system, Troost et al. (2015) discovered that, similar to the situation in the cochlea, the proneural genes *achaete* and *scute*, which are involved in SOP selection, are first upregulated in a band of cells that crosses the proneural domain and are subsequently repressed in all but the SOP. Thus, although the mechanism by which SOPs are selected remains unknown, this re-evaluation recognizes that it is unlikely to be strictly stochastic.

Atoh1 silencing in supporting cells is dependent on active repression and reinforced by 'activator insufficiency'

Our early observation that the transgenic *Atoh1* 3′ enhancer linked to the β-globin minimal promoter (Chen et al., 2002; Lumpkin et al., 2003) is not expressed in supporting cells led us to the initial hypothesis that the site of Notch-mediated repression of *Atoh1* is likely to be in the enhancer. However, our current results indicate that this is not the case, as shown by the failure to repress *Atoh1* enhancer-mediated expression in our co-transfection assay with HES5 (Fig. 2B) and HEY2 (Fig. S2B), and by the role of the *Atoh1* promoter in active repression (Fig. 2). This, in turn, raises the question of why the *Atoh1* 3′ enhancer transgene, linked to the β-globin minimal promoter, is silent in supporting cells. We hypothesize that this silencing is an example of 'activator insufficiency' (Barolo and Posakony, 2002), in which active repression of endogenous *Atoh1* through the promoter maintains the ATOH1 protein below a threshold needed to stimulate the autoregulatory enhancer activity. In this context, the *Atoh1* enhancer acts in a switch-like manner, so that once the threshold is crossed *Atoh1* is robustly stimulated (Fig. S7). Activator insufficiency is further confirmed by the absence of expression of this transgene in the neural tube or the ear of the *Atoh1* knockout mouse (Helms et al., 2000; Raft et al., 2007), and demonstrated by the failure of the *Atoh1* enhancer alone (Atoh1 enhancer/β-globin promoter/GFP) to initiate transcriptional upregulation when protein synthesis is blocked, whereas the endogenous *Atoh1* gene is upregulated (Fig. 1E). This later observation strongly indicates the presence of positively acting transcriptional mechanisms that are engaged at the endogenous *Atoh1* promoter in the perinatal supporting cells, but held in check by active repression and activator insufficiency.

Our data also suggest that the reported low level of *Atoh1* transcription in the prosensory domain prior to E13.5 (Lanford et al., 2000; Woods et al., 2004), before the overt upregulation of *Atoh1* in the nascent hair cells (Chen et al., 2002; Lumpkin et al., 2003), does not rise to the threshold needed to trigger enhancer autoregulation (Fig. 7, activator insufficiency). This begs the question of what is responsible for the onset of *Atoh1* expression. This could occur either through a derepression signal, such as the downregulation of Hey factors, as recently suggested (Benito-Gonzalez and Doetzlhofer, 2014), or by the active stimulation of *Atoh1* transcription through other binding motifs in the 226 bp promoter.

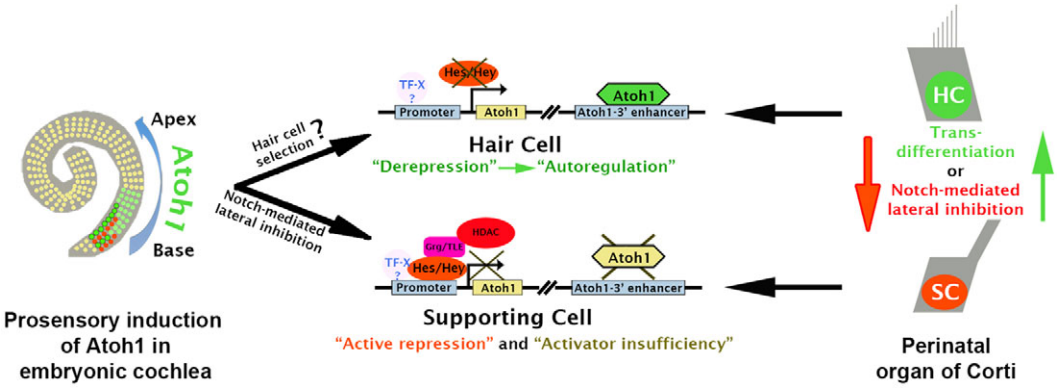

Fig. 7. Model of *Atoh1* regulation during organ of Corti development. Upregulation of *Atoh1* in groups of cells within the embryonic prosensory domain occurs in a basal-to-apical wave. Once the *Atoh1* autoregulatory threshold is achieved in selected nascent hair cells, Notch-mediated active repression is triggered in the surrounding prosensory cells to stimulate the silencing of *Atoh1* through Hes/Hey binding, leading to the onset of supporting cell differentiation and the patterning of the cellular mosaic of the organ of Corti. At P1, cell fate is maintained by continuing Notch-mediated repression through recruitment of GRG/HDACs. Loss of Notch activity leads to derepression of the *Atoh1* promoter and increased *Atoh1* expression through hypothetical positive transcriptional activity (TF-X), leading to autoregulation and transdifferentiation of supporting cells (SC) to a hair cell-like state (HC).

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Repression and derepression through the *Atoh1* promoter regulates *Atoh1* expression

Notch signaling has been shown to prevent the recruitment of the HAT p300 and the acetylation of histones (Krishnamoorthy et al., 2015), and transcriptional repression by Hes/Hey proteins has been linked to interaction with HDACs (Iso et al., 2001; Takata and Ishikawa, 2003; Weber et al., 2015). Our results indicate that HES5-dependent repression of the *Atoh1* promoter is mediated by the recruitment of GRG co-repressors, and that the presence of HDAC activity is Notch dependent (Figs 5, 6, S3 and S5). Further studies are required to identify which HDAC(s) are specifically involved in *Atoh1* repression in supporting cells.

We show that blocking *de novo* protein synthesis with CHX is sufficient to rapidly stimulate *Atoh1* expression, and leads to the rapid loss of HES5, not unlike blocking Notch signaling activity with DAPT in supporting cells (Fig. 1C, Fig. S1). Similarly, HES1 protein has previously been shown to have a short half-life (Hirata et al., 2002; Kobayashi et al., 2009) and loss of HES1 expression leads to selective derepression and transcriptional stimulation of target genes (Riccio et al., 2008).

Conclusion

Together, these data suggest that *Atoh1* levels transiently rise in groups of cells in the prosensory domain at the onset of differentiation, followed by a rapid repression in those cells not destined to be hair cells. It seems likely that this initial upregulation is sufficient to stimulate the production of Notch ligands, but is likely to just skirt the threshold needed to stimulate the autoregulatory function of the *Atoh1* enhancer. Active repression integrated at the *Atoh1* promoter is required to inhibit this process of transcriptional activation in nascent supporting cells. Our results argue for a model in which the complex pattern of Hes/Hey expression, interacting with multiple binding motifs in the *Atoh1* promoter, sculpts the precise hair cell and supporting cell mosaic required for organ of Corti function.

MATERIALS AND METHODS

Experimental animals, generation of transgenic mouse lines and genotyping

The experimental procedures were approved by the Animal Care and Use Committees of the University of Southern California and the House Research Institute. p27/GFP BAC transgenic (Lee et al., 2006; White et al., 2006), Atoh1 enhancer/β-globin promoter/GFP transgenic (Lumpkin et al., 2003) and Lfng/GFP BAC transgenic (Korrapati et al., 2013) mice were described previously. For generating the *Atoh1* reporter double-transgenic line, Atoh1 enhancer/Atoh1 promoter^{wt}/tdTomato and Atoh1 enhancer/Atoh1 promoter^{mut}/GFP plasmids were linearized, and founder animals were generated by pronuclear co-injection (UCI transgenic mouse facility; FVB background). Positive founders were identified by PCR genotyping using tail DNA and the primers listed in Table S3. Postnatal transgenic mice were identified by direct observation of GFP or tdTomato fluorescence in the cerebellum. Animals were put together in the evening, and the next morning was designated as E0.5.

Cell culture

HEK 293 cells (ATCC) and OC1 (organ of Corti 1) cells (generous gift from Federico Kalinec, UCLA) were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; GIBCO), 2 mM L-glutamine, and 10 mM HEPES maintained in a 5% CO_2 incubator at 37°C and 33°C, respectively. Mouse embryonic stem cell (ESC) line 46C, a Sox1-GFP knock-in line, was a generous gift from Qi-Long Ying (University of Southern California, USA). ESCs were cultured and differentiated to NPCs as reported (Ying and Smith, 2003); for details see the supplementary Materials and Methods.

Cochlear explant culture

Cochlea of P1 pups were dissected and cochlear explants were cultured on SPI black membranes as previously described (Doetzlhofer et al., 2009), except that B27 supplement was not added to the media. All cultures were incubated in a 5% CO_2, 5% O_2 humidified incubator (Forma Scientific).

Immunohistochemistry and imaging

Cochlear explants, HEK 293 cells and NPCs were fixed in 4% paraformaldehyde in PBS and kept in PBS at 4°C until the staining was performed. E16.5 whole heads or P1 inner ears were prepared for immunohistological analysis as previously reported (White et al., 2006). Anti-nestin (1:500; Developmental Studies Hybridoma Bank) and anti-PROX1 (1:500; Novus Biologicals, NBP1-18605) primary antibodies with species-specific Alexa Fluor secondary antibodies (1:1000; Life Technologies) were used. Confocal images were taken on a Zeiss LSM 780 scanning confocal microscope. Fluorescent imaging of NPCs was performed with a Zeiss Axio Observer.A1.

Plasmid construction and site-directed mutagenesis

A description of the plasmid constructs used is provided in the supplementary Materials and Methods. Detailed cloning and mutagenesis procedures are available upon request. Point mutations in the *Atoh1* reporter plasmids and deletion in the *Hes5* expression plasmid were introduced with the aid of the Finnzyme Phusion site-directed mutagenesis kit following the manufacturer's instructions.

Small-molecule inhibitors

The inhibitors/chemicals used in this study and their final concentrations were as follows: 10 µM γ-secretase inhibitor DAPT (Calbiochem, EMD Millipore); 100 µM proteasome inhibitor MG132 (Sigma); 100 µg/ml protein synthesis inhibitor cycloheximide (Sigma); 1 mM protease inhibitor PMSF (Sigma); 10 µM proteasome inhibitor lactacystin (Sigma); 30 µM HAT inhibitor curcumin (Enzo Life Sciences); and 200 nM HDAC inhibitor trichostatin A (Sigma).

FACS purification

All cells were sorted on a BD FACSAria II cell sorter with lasers at 488 nm and 561 nm wavelength and 100 µm nozzle. For RNA extraction, cells were sorted directly into RNA lysis buffer (Zymo Research) and kept at −80°C until the RNA isolation was performed. Preparation of cochlear samples for FACS was undertaken as described (White et al., 2006). FACS-purified supporting cell samples (GFP$^+$) were analyzed by qPCR and compared with the GFP$^-$ population. Only samples that showed more than 40-fold enrichment for *Hes5* mRNA and less than 0.8-fold enrichment for *Atoh1* mRNA were used for further analysis. NPCs transfected with pCS2-CMV-FLAG-Hes5 and/or pCS2-CMV-H2BmRFP (control) were collected 48 h post transfection, and RFP$^+$ cells were FACS purified.

RNA extraction and real-time quantitative PCR (qPCR)

Total RNA was isolated using the Zymo Research Quick-RNA MicroPrep Kit, with DNase digestion (Qiagen). RNA from supporting cells (8000 cells), NPCs (10,000 cells) and non-hair cells (100,000 cells) was reverse transcribed using the Quanta qScript cDNA Synthesis Kit. qPCR was performed with Power SYBR Master Mix (Applied Biosystems) on a ViiA 7 real-time PCR system with fast 96-well block (Applied Biosystems). Relative quantification of gene expression was performed by the comparative ΔΔCt method (Livak and Schmittgen, 2001) with *Gapdh* as the endogenous reference. Two-tailed Student's *t*-test was used to determine statistical significance. $P<0.05$ was considered statistically significant. The primers used in qPCR are listed in Table S1.

Western blotting

Western blot analysis of proteins extracted from transfected HEK 293 cells is described in the supplementary Materials and Methods.

Proximity ligation assay

To visualize GRG/TLE:FLAG-HES5 interaction, the proximity ligation assay was performed as described in the supplementary Materials and Methods.

Chromatin immunoprecipitation (ChIP)-qPCR

For HES5 ChIP, NPCs in each well of a 6-well culture dish were transfected with 15 µg pCS2-CMV-FLAG-Hes5 and/or pCS2-CMV-H2BmRFP (control) using Lipofectamine LTX (Invitrogen). NPCs were FACS purified into DMEM containing 2% FBS, 1 mM PMSF (Sigma) and Protease Inhibitor Cocktail (Calbiochem). ChIP from NPCs was performed as described (Dahl and Collas, 2008), using 2×10^5 (FLAG-Hes5) or 1×10^6 (pan-GRG) cells. For H3K9ac ChIP, supporting cells were FACS purified from the p27/GFP transgenic mouse line (~25,000 supporting cells per sample). A detailed ChIP protocol and primers are provided in the supplementary Materials and Methods and Table S2.

Reporter assay

Reporter assays were conducted using HEK 293 cells (2×10^5) seeded in each well of a 24-well culture dish and transfected 14-16 h later with 150 ng *Atoh1* reporter plasmid (GFP or mCherry, Fig. 2) and 600 ng pCS2-CMV-FLAG-Hes5 plasmid, or empty plasmid control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For the mutational analysis of the C-sites in the *Atoh1* promoter region, 150 ng pCS2-CMV-H2BmRFP plasmid was also cotransfected as an internal control for transfection efficiency. 48 h post-transfection, the number of positive cells was determined by flow cytometry using 1×10^5 cells and analyzed by BD FACSDiva software. Two-tailed Student's *t*-test was used to determine statistical significance. Further details of FACS analysis of reporter assays are provided in the supplementary Materials and Methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.A. and N.S. developed the concepts, analyzed the data and wrote the manuscript; Y.A. designed and performed the experiments; Z.S. performed histone ChIP, contributed intellectual advice and edited the manuscript.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.129320/-/DC1

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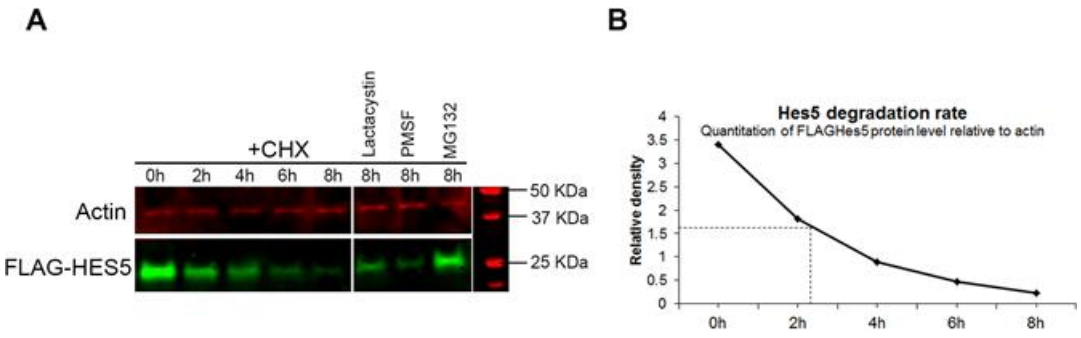

Fig. S1, related to Fig. 1. HES5 protein stability *in vitro*. 293 cells were transfected with CMV-FLAG-Hes5 plasmid. After 24h cells were treated with cycloheximide (CHX) alone, or cycloheximide and proteasome inhibitors, and collected at the indicated time points. Whole-cell extracts were made according to (Gallagher, 2007) and western blotted with anti-FLAG antibody (A). The intensity of the signals relative to actin was measured using Image J (NIH) (B). Values shown in B are average of two independent experiments. HES5 is degraded with a half-life of 2.5 hours. Proteasome pathway inhibitors MG132 or Lactacystin increased HES5 stability, while the protease inhibitor PMSF had no effect.

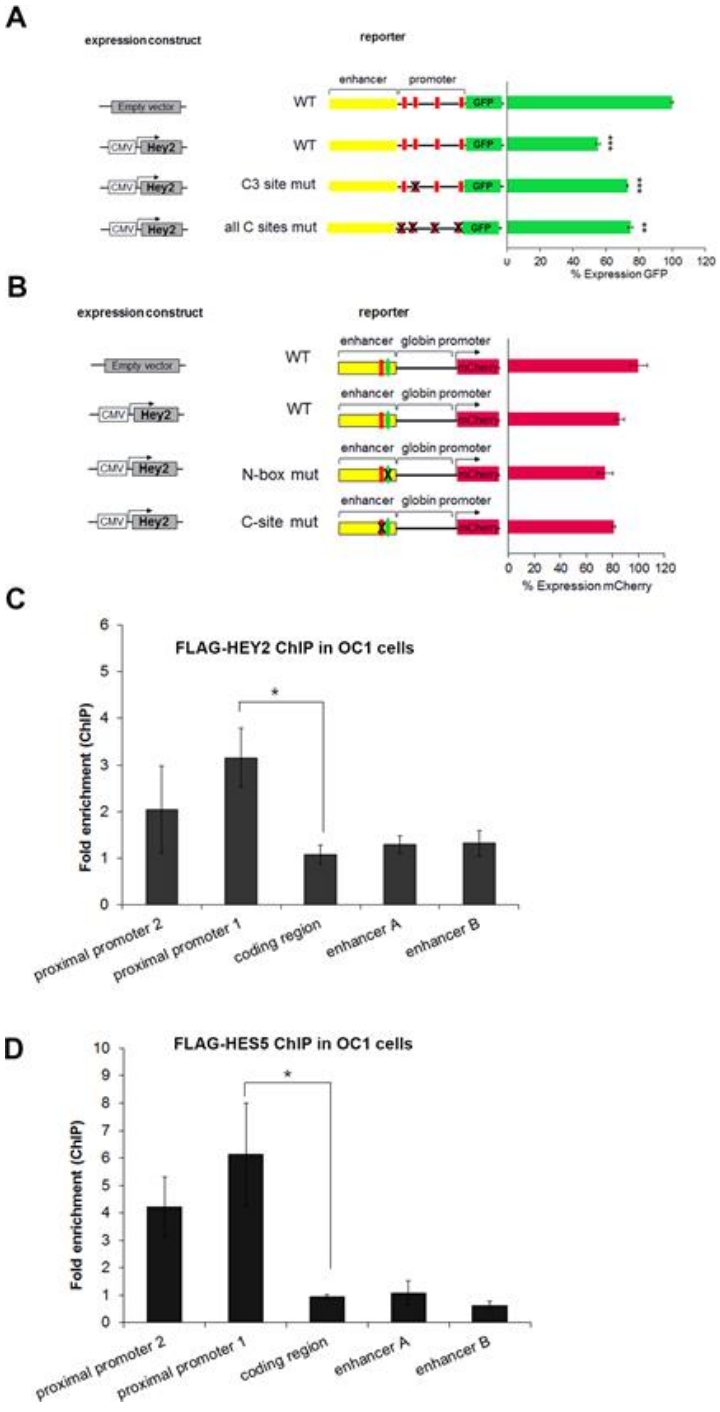

Fig. S2, related to Fig. 2. HEY2 is capable of repressing Atoh1 transcription through both the promoter and enhancer regions. Quantification of flow cytometry analysis in 293 cells transfected with the indicated plasmids. **(A)** The promoter C-sites were mutated as described in Fig. 2. Hey2 repression of Atoh1 promoter was dependent on the conserved C3 site. Mutation of additional C-sites

only slightly increased the percentage of GFP^+ cells, suggesting that part of HEY2-mediated repression of Atoh1 is likely independent of the C-sites in the promoter region. Values are mean ± SEM, n = 3; [***] P < 0.0005, [**] P < 0.005, Student's t-test. **(B)** The C-site and N-box in the Atoh1 3' enhancer sequence were mutated by site directed mutagenesis (Methods). Hey2's weak inhibitory effect (as seen in A) is not dependent on the N-box or C-site within the Atoh1 enhancer. Values are mean ± SEM, n = 3. **(C and D)** HEY2 binds to Atoh1 promoter *in vitro* similar to HES5. ChIP-qPCR result in OC1 (organ of Corti 1) cells that were transfected with CMV-FLAG-Hey2 (C), CMV-FLAG-Hes5 (D) plasmid or CMV-GFP plasmid (control) for 48 hours. Chromatin immunoprecipitation was performed with anti-FLAG antibody. The result is reported as fold enrichment (Hey2 or Hes5 transfected % input/GFP transfected % input). Shown are the values as mean ± SEM for three independent replicates. [*] $p < 0.05$, Student's *t*-test.

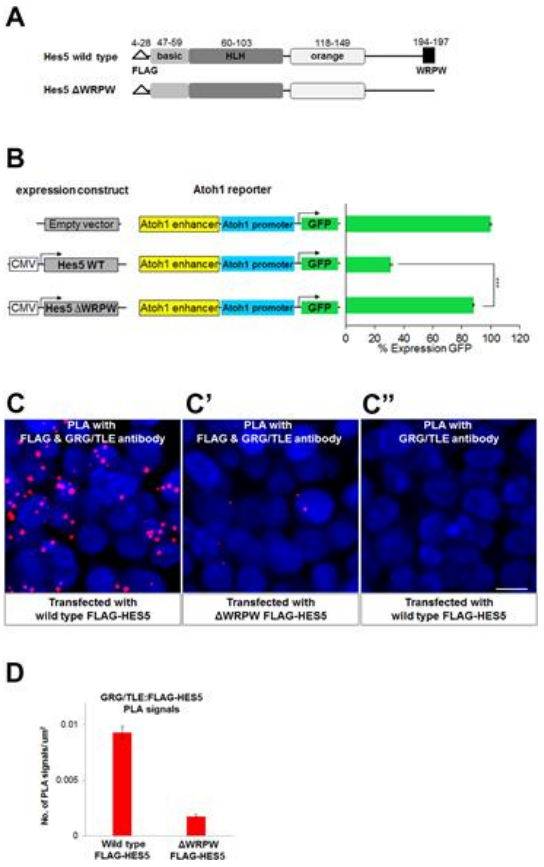

Fig. S3, related to Fig. 5. The HES5 WRPW motif is required for the repression of the *Atoh1* promoter and for interaction with GRG/TLE. (A) Schematic representation of mouse HES5 protein showing the amino acid sequence and different domains. Four amino acids of the C-terminal WRPW motif were deleted in Hes5 ΔWRPW**. (B)** Quantification of flow cytometry analysis of 293 cells transfected with the indicated plasmids reported as a percentage of cells expressing GFP relative to RFP control (not shown, empty vector control set to 100%). When Hes5 C-terminal motif WRPW was deleted, HES5 inhibitory effect on Atoh1 reporter was significantly reduced. Values are mean ± SEM, n = 3. [***] $p < 0.0005$. **(C)** Proximity ligation assay showing requirement for the WRPW motif for interaction with endogenous GRG/TLE. Confocal images of 293 cells transfected with FLAG-Hes5 wild type or WRPW-deleted expression plasmids (red dots indicate interaction). DAPI (blue). Scale bar = 20 µm. **(D)** Quantification of PLA signals shown in (C).

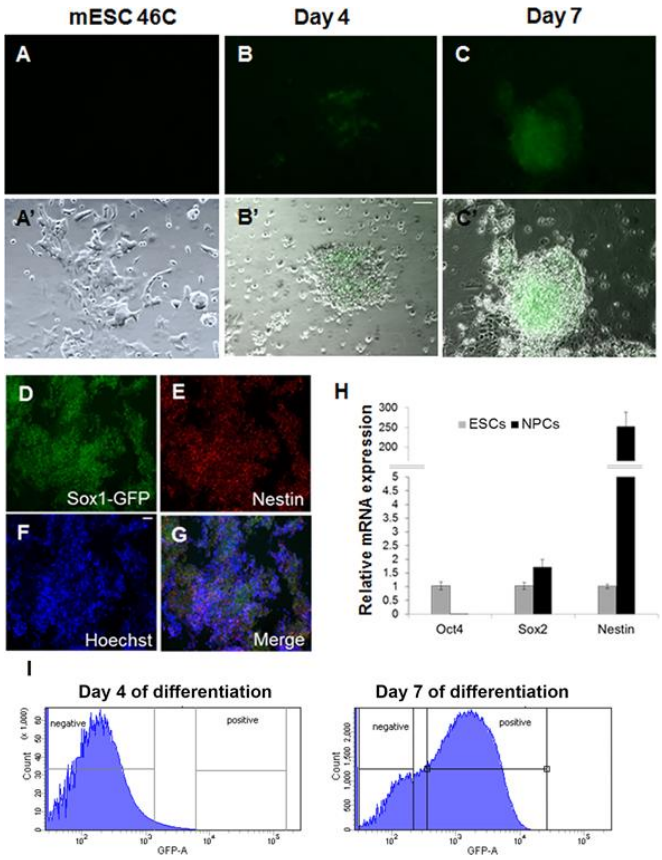

Fig. S4, related to Fig. 5. Sox1-GFP knock-in mouse embryonic stem cell (mESC) line 46C expresses GFP and Nestin upon neural differentiation. ESCs (line 46C) were cultured and differentiated to neural progenitor cells (NPCs) following a monolayer cell culture protocol (Ying and Smith, 2003; Ying et al., 2003). The expression of GFP allows monitoring of the differentiation procedure and also FACS-purification to obtain a highly purified population of NPCs. **(A-C)** Fluorescent and **(A'-C')** brightfield images of differentiation of mES cells. mESCs were cultured without (A and A') and with differentiation media for 4 days (B and B'), or 7 days (C and C'). (**D-H**) ESCs in differentiation media downregulate Oct4 and upregulate Nestin by day 7 as shown by immunostaining (D-G), and real time quantitative PCR (H). **(I)** The fluorescence activated cell sorting (FACS) analysis of 46C ES cells on day 4 and 7 of differentiation. As the cell differentiate, the percentage of Sox1-GFP poisitive cell increases. Scale bars = 50 µm.

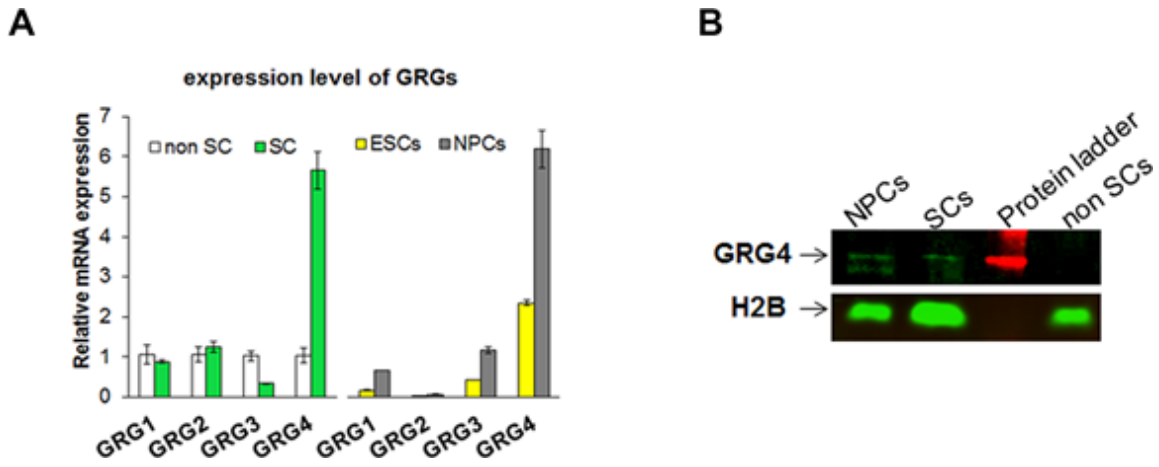

Fig. S5, related to Fig. 5. (A) GRG4 is the predominant family member expressed in supporting cells and neural progenitor cells (NPCs). qPCR analysis of FACS-purified supporting cells ($p27^+$ SC), non-supporting cells ($p27^-$ non SC), 46C ES cells and NPCs. n = 4. Values are mean ± SEM. **(B)** GRG4 is expressed in NPCs and supporting cells. Immunoblotting of GRG4 with 25,000 FACS-purified NPCs, purified $p27^+$ supporting cells (SCs) and $p27^-$ non-supporting cells (non SCs) at P1. Histone H2B was used as control.

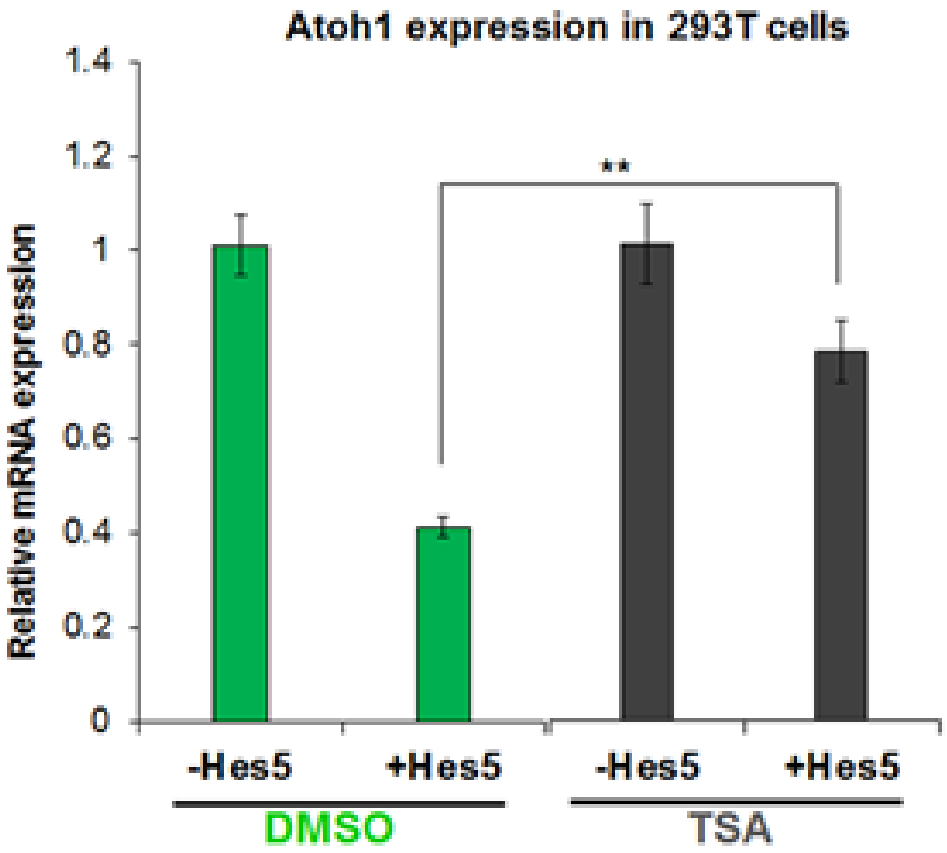

Fig. S6, related to Fig. 6. HES5 cannot repress Atoh1 expression in the absence of HDAC activity. Hes5 overexpression in 293 cells in the absence and presence of HDAC inhibitor trichostatin A (TSA) for 15h. Values are mean ± SEM, n = 3; [**] P< 0.001, Student's *t*-test.

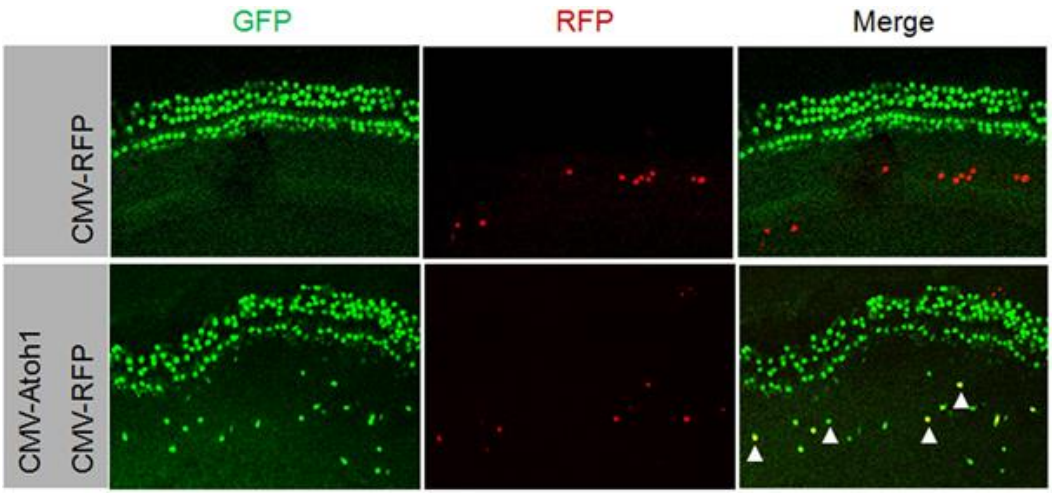

Fig. S7, related to Fig. 7. The Atoh1 enhancer acts as an autoregulatory element in response to ectopic expression of Atoh1. A CMV-RFP expression plasmid with or without an Atoh1 expression plasmid was electroporated into GER (greater epithelial ridge, epithelial cells lying medial to the organ of Corti) of Atoh1 enhancer/β-globin promoter/GFP transgenic mouse at P1. Overexpression of Atoh1 in this area, where Atoh1 is not normally expressed, induced the Atoh1 enhancer activity in the transgene and resulted in the appearance of GFP-positive cells in the GER. This confirms the autoregulatory activity of the Atoh1 enhancer (Helms et al., 2000), and suggests the presence of a positive feedback loop in the organ of Corti.

Supplementary Materials and Methods

Embryonic stem cell culture and differentiation to neural progenitor cells (NPCs)

46C ES cells were cultured on gelatin-coated tissue culture plates in GMEM (Invitrogen) supplemented with 15% Embryonic Stem (ES) Screened FBS (HyClone), 1 mM sodium pyruvate (Invitrogen), non-essential amino acid (Invitrogen), 0.1% 2-Mercaptoethanol (Invitrogen) and 10 ng/ml leukemia inhibitory factor (LIF) (Millipore). For neural differentiation, 46C cells were cultured on gelatin-coated 6-well plates at a density of $1\times10^4/cm^2$ (10^5 cells per well). Culture medium was DMEM/F12 combined 1:1 with Neurobasal medium (Invitrogen) supplemented with 1% B27 (Invitrogen), 0.5% N2 (Invitrogen), 25 µg/ml bovine serum albumin (Sigma). Medium was changed every other day for six days. On day six the medium was changed to DMEM/F12 combined 10:1 with Neurobasal medium supplemented with 0.1% B27, 0.5% N2 and 20 ng/ml FGF and EGF (R&D).

Plasmids and reporter constructs

Hes5 expression plasmids:

The pCS2-CMV-FLAG-Hes5 expression plasmid included 1.4 kb Hes5 coding region (generously provided by Verdon Taylor (Basak and Taylor, 2007), University of Basel, Switzerland) tagged by 5' 3X FLAG epitope inserted downstream of CMV enhancer/promoter in pCS2. Cloning details upon request.

Atoh1 reporter plasmids:

The Atoh1 enhancer/β-globin promoter/GFP plasmid, in which the mouse Atoh1 enhancer (GenBank: AF218258.1) and the human β-globin basal promoter drive the expression of GFP, was a generous gift from Jane Johnson, University of Texas (Lumpkin et al., 2003). The mCherry reporter (Fig. 2) was constructed by cloning a histone H2B-mCherry fusion into the Atoh1 enhancer/β-globin

promoter/GFP plasmid to replace the GFP coding region. Atoh1 enhancer/Atoh1 promoter/GFP reporter was constructed using a 226 bp fragment upstream of the *Atoh1* transcription start site, and 177 bp of *Atoh1* 5'-UTR to replace the β-globin promoter sequence in Atoh1 enhancer/β-globin promoter/GFP. For the Atoh1 enhancer/Atoh1 promoter/tdTomato reporter, tdTomato sequence was PCR amplified from FUtdTW (a gift from Connie Cepko (Rompani and Cepko, 2008), Addgene plasmid # 22478) and inserted in the Atoh1 enhancer/Atoh1 promoter/GFP reporter to replace the GFP sequence. Expression of the reporters carrying the Atoh1 autoregulatory enhancer element (Fig. S7) was observed in 293 cells, likely as a result of low level *Atoh1* expression as previously reported (Neves et al., 2012).

Hey2 expression plasmid (CMV-FLAG-Hey2) was generously provided by Manfred Gessler (University of Wuerzburg, Germany).

Flow cytometric analysis of reporter assays

48h post-transfection of 293 cells were suspended in DMEM/10% FBS, passed through a 40 µm cell strainer and analyzed using BD FACSAria II cytometer with lasers at 488- and 561-nm wavelengths and 100 µm nozzle. Cells were initially gated (P1) using forward scatter (FSC-A) and side scatter (SSC-A). Two sequential gates were used to exclude the cellular debris and clumps (P2: SSC-H vs. SSC-W, P3: FSC-H vs. FSC-W). Untransfected and single-transfected (GFP, mCherry or RFP) cells were used to determine the gates for the positive and negative populations, as well as to set up the compensation. For each sample about $1x10^5$ cells in gate P3 were analyzed. BD FACSDiva software was used to operate the system and analyze the cell populations. Two-tailed Student's t-test was used to determine the statistical significance.

Western blotting

Proteins extracted from transfected 293 cells were separated under reducing conditions on 12% NuPage Novex Bis-Tris gels (Invitrogen) and transferred to PVDF membranes (Millipore) using the XCell II Blot Module (life technologies). Membranes were blocked using Odyssey blocking buffer (LI-COR Biosciences) and probed with the following antibodies: anti-FLAG (1:1000, Sigma F7425); anti-actin (1:2000, Sigma A3853); anti-TLE4 (1:1000, Novus NB100-92363); anti-histone H2B (1:2000, Millipore 07-371); IRDye 800CW Goat anti-Rabbit IgG (1:20000); and IRDye 680RD Goat anti-Mouse IgG (1:10000) (LI-COR).

Proximity ligation assay

To visualize GRG/TLE:FLAG-HES5 interaction, proximity ligation assay (Soderberg et al., 2006) was performed using the Duolink kit (Sigma-Aldrich) in transfected 293 cells according to manufacturer's manual with the following antibodies: anti-FLAG (1:500, Sigma F7425); anti-pan GRG/TLE (1:100, Santa Cruz sc-13373 X).

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP experiments were done according to (Dahl and Collas, 2008). FACS-purified NPCs were fixed with 1% formaldehyde for 10 minutes and quenched by 125 mM Glycine for 5 minutes at room temperature. Cross-linked cells were centrifuged at 470g for 10 minutes at 4°C, washed twice with ice-cold PBS, lysed with 50 µl lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% (wt/vol) SDS, PMSF and protease inhibitor cocktail for 8 minutes on ice. RIPA buffer (10 mM Tris–HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 0.1% (wt/vol) Na-deoxycholate, protease inhibitor cocktail and PMSF) was added before sonication. Chromatin was sonicated to an average size of 200-300bp with High Intensity Ultrasonic Processor (50 Watt Model) on ice for 4 X 30s, with 30s intervals. After sonication, samples were centrifuged at 12000g for 10 minutes

at 4°C and 1% of the supernatant chromatin was set aside as input. The remaining chromatin was added to antibody-Dynabeads protein G complexes (4 µg FLAG antibody (Sigma F1804) or GRG/TLE antibody (Santa Cruz sc-13373 X) and 25 µl protein G beads (Invitrogen) were preincubated on a rotator for 2h at 4°C). The tubes containing the chromatin-antibody-protein G and the input tubes were placed on a rotor for 16h at 4°C. The beads were then captured in magnetic rack, washed with RIPA and TE. The washed beads were reverse cross-linked with elution buffer (20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl, 1% (wt/vol) SDS, 50 µg/ml proteinase K) at 1300 rpm for 4h at 68°C. ChIP and input DNA was purified by phenol–chloroform–isoamyalcohol extraction and ethanol precipitation and dissolved in TE. qPCR reactions were done in triplicate. H3K9ac ChIP with about 25,000 supporting cells (from p27/GFP mouse line) per experiment was done as above with minor differences. FACS-purified supporting cells were cross-linked for 8 minutes and sonicated for 8 X 30s, with 30s intervals. 2.4 µg H3k9ac antibody (Active Motif 39137) and 10 µl Dynabeads Protein A (Invitrogen) per sample were used. ChIP-qPCR primers are listed in table 2.

Table S1. Primers used in real time quantitative PCR (qPCR).

Gene	Forward (5'→3')	Reverse (5'→3')
Atoh1	GAGTGGGCTGAGGTAAAAGAGT	GGTCGGTGCTATCCAGGAG
Hes5	GCACCAGCCCAACTCCAA	GGCGAAGGCTTTGCTGTGT
GFP	CTGCTGCCCGACAACCA	TGTGATCGCGCTTCTCGTT
Gapdh	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA
Hey1	CACTGCAGGAGGGAAAGGTTAT	CCCCAAACTCCGATAGTCCAT
Oct4	ACATCGCCAATCAGCTTGG	AGAACCATACTCGAACCACATCC
Nestin	GCTGGAACAGAGATTGGAAGG	CCAGGATCTGAGCGATCTGAC
Sox2	CTGTTTTTTCATCCCAATTGCA	GGAGATCTGGCGGAGAATA
GRG1	GACAGCCTAAGAGGCACAGAT	GGTCCTCGTTAGACACATCCA
GRG2	TGAGGACCAACCGTCAGAG	GCTGGACTGTCTGTGAGGT
GRG3	TGGATGTCTCTAATGAGGACCC	TTCAGACCACGGGCTTTGTC
GRG4	ATTGCAGCTCGCTATGACAGT	GAGGAGTCGTGTCTTGTCCAG
hAtoh1	TTGTCCGAGCTGCTACAAACG	GAGAAGCGAGTCCGGCAAC
hGAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG

Table S2. Primers used in ChIP-qPCR. Numbers refer to the 5' end of the forward primer.

Primer	Forward (5'→3')	Reverse (5'→3')	Amplicon size (bp)
Atoh1-proximal promoter 2 (-354)	CCCTCACTCAGGTCGCCTG	CGTGCGAGGAGCCAATCA	205
Atoh1-proximal promoter 1 (-87)	GGGGAGCCGGGGGAGATACAC	ACCAGGTCGCGTGCAACGAAG	93
Atoh1-distal promoter (-1130)	ACAGAGCGGGACAGGTGGGT	CCTCGGGAGGCCCCGGTTTA	86
Atoh1-coding region (+1156)	ACATCTCCCAGATCCCACAG	GGGCATTTGGTTGTCTCAGT	119
Atoh1 enhancer-B (+5346)	AGAGCGGCTGACAATAGAGG	GTGCGCTCACCAGCTGAC	93
Atoh1 enhancer-A (+4264)	CACACCCCATTAACAAGCTG	GTCTGGCATATGGGGAATGA	112
Gapdh (+32)	GGGTTCCTATAAATACGGACTG	CTGGCACTGCACAAGAAGA	90

Table S3. Genotyping Primers.

Gene	Forward (5'→3')	Reverse (5'→3')
GFP	GTGAAGTTCGAGGGCGACAC	CGGACTGGGTGCTCAGGTAG
tdTomato	GTGACCGTGACCCAGGACTC	TGACGGCCATGTTGTTGTCCTC

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