HIC1 Represses Atoh1 Transcription and Hair Cell Differentiation in the Cochlea

Dunia Abdul-Aziz,1,2 Nicolai Hathiramani,2 Lauren Phung,2 Vittoria Sykopetrites,2,3 and Albert S.B. Edge1,2,4,*
1Department of Otolaryngology, Harvard Medical School, Boston, MA, USA
2Eaton Peabody Laboratory, Massachusetts Eye and Ear, Boston, MA, USA
3Università degli Studi di Milano, Milan, Italy
4Harvard Stem Cell Institute, Cambridge, MA, USA
*Correspondence: albert_edge@meei.harvard.edu
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SUMMARY

Across species, expression of the basic helix-loop-helix transcription factor ATOH1 promotes differentiation of cochlear supporting cells to sensory hair cells required for hearing. In mammals, this process is limited to development, whereas nonmammalian vertebrates can also regenerate hair cells after injury. The mechanistic basis for this difference is not fully understood. Hypermethylated in cancer 1 (HIC1) is a transcriptional repressor known to inhibit Atoh1 in the cerebellum. We therefore investigated its potential role in cochlear hair cell differentiation. We find that Hic1 is expressed throughout the postnatal murine cochlear sensory epithelium. In cochlear organoids, Hic1 knockdown induces Atoh1 expression and promotes hair cell differentiation, while Hic1 overexpression hinders differentiation. Wild-type Hic1, but not the DNA-binding mutant C521S, suppresses activity of the Atoh1 autoregulatory enhancer and blocks its responsiveness to β-catenin activation. Our findings reveal the importance of HIC1 repression of Atoh1 in the cochlea, which may be targeted to promote hair cell regeneration.

INTRODUCTION

Within the cochlea, sensory hair cells reside interdigitated with specialized supporting cells and mechanotransduce sound into electrical signal that they transmit to auditory neurons. In the mature mammalian cochlea, these sensory hair cells and supporting cells do not turn over, and as a consequence, damage induced by factors such as noise and aging results in irreversible hearing loss (Doetzhof et al., 2006; White et al., 2006). However, spontaneous regeneration occurs in non-mammalian cochlear sensory epithelia as well as the mammalian vestibular epithelia (Atkinson et al., 2015; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Warchol, 2011). Recent results have also demonstrated spontaneous regeneration of hair cells after damage in the newborn mammalian cochlea (Bramhall et al., 2014; Cox et al., 2014; Hu et al., 2016), where, upon damage and loss of Notch-mediated lateral inhibition, progenitor cells, which comprise a subset of supporting cells, express the basic helix-loop-helix transcription factor ATOH1 and differentiate into hair cells. These progenitor cells, which are marked by the stem cell marker LGR5, are Wnt responsive and re-enter the cell cycle to asymmetrically divide into supporting cell and hair cell lineages (Bramhall et al., 2014; Chai et al., 2012; Shi et al., 2013). But the regenerative potential of the newborn mammalian cochlea is lost in the first postnatal week, even when driven with exogenous stimuli (including Atoh1 activation) (Kelly et al., 2012; Samarajeewa et al., 2018; Shi et al., 2013). The quiescence of the cochlear progenitor cells in the maturing cochlea suggests a broadly repressive regulatory network, such as a nonpermissive chromatin signature. These observations have prompted us to study signals that modulate the epigenetic status of cochlear genes in the first postnatal week.

Hypermethylated in cancer 1 (HIC1) is an evolutionarily conserved transcriptional repressor that has been previously shown to directly bind to and suppress the regulatory regions around the Atoh1 gene during cerebellar development (Briggs et al., 2008). Atoh1 is highly expressed in cerebellar granule progenitor cells (CGPs), and its expression drops during their differentiation, concurrent with the rise of Hic1 expression (Ben-Arie et al., 2000; Briggs et al., 2008; Jensen et al., 2004; Lumpkin et al., 2003). Knockdown of Hic1 contributes to the formation of medulloblastoma, a cerebellar tumor characterized by reactivation of Atoh1 expression (Ayrault et al., 2010; Briggs et al., 2008; Flora et al., 2009; Grausam et al., 2017; Jensen et al., 2004). The human HIC1 is deleted in the contiguous gene disorder Miller-Dieker syndrome (Carter et al., 2000; Grimm et al., 1999), and Hic1 knockout mice are embryonic lethal with defective craniofacial, gastrointestinal tract, and kidney development. Hic1-deficient intestinal epithelium derived from conditional Hic1-loxp/loxP crossed with Villin-Cre mice demonstrated increased number of secretory Paneth cells, which are driven by the transcription factors ATOH1 and SOX9, suggesting that Hic1 deletion potentiates Atoh1 expression and intestinal Paneth cell differentiation (Janeckova et al., 2015). HIC1 represses Atoh1 by a mechanism involving recruitment of corepressor complexes, including C-terminal binding...
protein (CtBP) (Deltour et al., 2002), nucleosome remodeling and deacetylase (NuRD) (Van Rechem et al., 2010), and polycomb repressive complex 2 (PRC2) (Boulay et al., 2012), which mediate chromatin changes resulting in repression. In addition, HIC1 negatively regulates Wnt signaling through direct protein-protein interactions via recruitment of T cell factor (TCF) and β-catenin to nuclear “HIC1 bodies” (Valenta et al., 2006), thereby preventing β-catenin activation of Wnt-responsive genes, which include Atoh1 (Shi et al., 2010). The role of HIC1 in the regulation of Atoh1 and hair cell differentiation in the inner ear has not been examined.

We hypothesized that HIC1 could contribute to Atoh1 repression in the cochlea through transcriptional regulation and interaction with Wnt. Using qRT-PCR and in situ hybridization specific to Hic1, we show here that Hic1 is expressed ubiquitously in the postnatal murine sensory epithelium, including hair cells and supporting cells. We use a murine cochlear organoid system comprising expanded postnatal LGR5+ progenitor cells to study the effect of HIC1 transcriptional silencing on activation of the Atoh1 autoregulatory enhancer, expression of hair cell genes, and hair cell differentiation. We show that short hairpin (sh)RNA-mediated Hic1 knockdown in inner ear organoids activates the Atoh1 enhancer, increases expression of hair cell genes, and potentiates hair cell development. Conversely, forced overexpression of HIC1 inhibits hair cell differentiation. Using a luciferase reporter system to study the mechanism of HIC1-mediated repression of Atoh1, we demonstrate that wild-type HIC1, but not the zinc-finger DNA-binding mutant C521S, is able to inhibit Atoh1 and Wnt reporter activation. Together, our findings suggest an important role for HIC1 in mediating Atoh1 repression, at least in part through repression of TCF/β-catenin signaling, and that its targeted inhibition may potentiates hair cell regeneration in the mature mammalian cochlea.

RESULTS

Hic1 is Expressed in the Sensory Epithelium of Postnatal Mice Across Developmental Time Points

Given the potential regulatory function of HIC1 in the mammalian cochlear sensory epithelium, we first probed its expression in newborn versus adult murine cochleae (Figure 1). qRT-PCR of dissected cochlear sensory epithelia from postnatal day 1 (P1), P7, and P14 mice demonstrated progressively increased expression of Hic1, while corresponding expression of Atoh1 precipitously declined (Figure 1A). Immunohistochemistry using a commercially available HIC1 antibody showed nonspecific binding, as others have reported (Pospichalova et al., 2011). Therefore, to specify at the cellular level the relative expression of Hic1 in the sensory epithelium (schematized in Figure 1B), we used single-molecule in situ hybridization to provide a semiquantitative and highly specific Hic1 expression pattern in the cochlea (Figures 1C and S1) and control murine intestinal epithelia (Figure S1). Across ages, Hic1 is expressed throughout the sensory epithelium, including hair cells, supporting cells, and cells of the medial and lateral compartments.

Hic1 Knockdown in Cochlear Organoids Activates the Atoh1 Enhancer and Potentiates Hair Cell Differentiation

To investigate whether HIC1 activity is relevant to cochlear hair cell differentiation, we tested the effect of Hic1 silencing on Atoh1 expression and hair cell differentiation in cochlear organoids (Lenz et al., 2019; McLean et al., 2017). Inner ear organoids, similar to other tissue-specific organoid models (for example, the intestine, skin, or brain), recapitulate in vivo hair cell development (Clevers, 2016; Roccio and Edge, 2019). They are derived from newborn mouse cochlear sensory epithelia following an established protocol in which isolated LGR5+ progenitor/supporting cells are selectively expanded and then induced to differentiate to immature hair cells that contain stereocilia and express key hair cell markers, including Atoh1, Gfi1, Pou4f3, and Myo7a (schematized in Figure 2A) (Lenz et al., 2019; McLean et al., 2017).

We examined the relationship between Hic1 and Atoh1 expression levels following shRNA-mediated knockdown of Hic1. In these experiments, we generated cochlear organoids from Atoh1-nGFP transgenic mice, which provide a reliable fluorescent reporter of Atoh1 expression and hair cell differentiation by expression of GFP in hair cells under the control of the Atoh1 3’ enhancer (Abdolazimi et al., 2016; Lenz et al., 2019; Lumpkin et al., 2003; McLean et al., 2017). Organoids were analyzed at various time points of differentiation (D10, D15, D20), which reflect relative maturation of the hair cells (McLean et al., 2017). Dissociated cochlear progenitor cells were transduced with lentivirus delivering the pLKO-mScarlet vector, which results in constitutive expression of shRNA targeting Hic1 (shHic1) or control nontargeting shRNA (shControl) and mScarlet to mark transduced cells. Following the initial 10 day proliferation period, live cell imaging and flow cytometry showed a significantly greater percentage of mScarlet+/Atoh1-nGFP+ double-positive cells in shHic1-infected cells as opposed to shControl (Figures 2B and 2C), indicating that Hic1 knockdown alone (in the absence of any other differentiation-promoting factors) potentiates Atoh1 expression. The proportion of Atoh1-nGFP+ cells, indicative of Atoh1 enhancer activation, in both transduced and untransduced cells was...
highest at D10 and reflects the relative priming of progenitor cells that have an accessible/active Atoh1 enhancer during the end of proliferation/start of differentiation period in organoids. This observation is consistent with single-cell expression data that demonstrate Atoh1 expression levels to be highest at this early time point (Lenz et al., 2019). The ability of shHic1 alone to activate the Atoh1-nGFP reporter persisted over the 10 day window of differentiation studied, in marked contrast to untransduced and shControl-transduced cells in which Atoh1-nGFP activity decreased over time (D15; Figure 2C). These findings indicate that downregulation of HIC1 in supporting cells promotes Atoh1 expression which is essential for differentiation to hair cells.

To confirm induction of hair cell fate, we performed immunostaining for an additional hair-cell-specific marker, Myosin VIIA (MYO7A), an unconventional myosin isozyme essential for hair cell function. Organoids were fixed and analyzed by high-resolution immunohistochemistry and showed significantly increased co-expression of mScarlet and MYO7A in a greater proportion of shHic1-treated organoids in contrast to shControl (Figure 2D). qRT-PCR analysis of sorted, transduced cells at 10 days of differentiation revealed significantly increased
Figure 2. Hic1 Knockdown in Cochlear Organoids Potentiates Atoh1 Activation and Hair Cell Differentiation
(A) Schematic of the cochlear organoid protocol. Dissociated postnatal day 3 supporting cells isolated from wild-type or Atoh1-nGFP transgenic reporter mice are transduced at D0 with lentivirus and expanded in proliferation medium for 10 days, followed by induction of

(legend continued on next page)
levels of hair-cell-specific genes, *Myo7a* and *Pou4f3*, in shHic1-transduced cells (Figure 2E), which turn on after *Atoh1* induction during development (Cai and Groves, 2015).

Reminiscent of in vivo mammalian hair cell differentiation, expanded supporting cells in organoids do not spontaneously differentiate to *Atoh1*+ hair cells to a significant degree (1%–5%) but can be potentiating through known pathways, including Wnt activation (McLean et al., 2017; Samaraieeawa et al., 2018; Shi et al., 2010, 2013) and Notch inhibition (Abdolazimi et al., 2016; Brown and Groves, 2020; Jeon et al., 2011; Mizutari et al., 2013). Both Wnt and Notch pathways act through effector transcription factors, TCF/LEF and HES/HEY, respectively, at the *Atoh1* regulatory promoter and enhancer regions to regulate *Atoh1* at the transcriptional level. To assess the interplay of these pathways and their potential to promote hair cell differentiation, organoids were treated with drugs that promote differentiation, the GSK3β inhibitor CHIR99021 (CHIR), to potentiate Wnt signaling, in combination with the Notch inhibitor LY411575 (LY), compared with vehicle control (DMSO), and analyzed by flow cytometry. Consistent with results from earlier time points, Atoh1-nGFP organoids transduced with shHic1 generated a significantly greater proportion of double-positive (GFP/+mScarlet+) cells and potentiated the effect of CHIR in combination with LY (CHIRLY) relative to shControl (Figure 2F).

In addition to the downstream 3′ enhancer, the *Atoh1* proximal promoter has been shown to be important in the regulation of *Atoh1* expression, being the primary site of action of HES/HEY repression by binding to canonical E-box C-site motifs (Figure S2) (Abdolazimi et al., 2016). Query of the murine *Atoh1* genomic locus for a biochemically determined HIC1 binding motif (TGCC[A/C]) (Pint et al., 2004) reveals several putative binding sites (Figure S2), and publicly available chromatin immunoprecipitation sequencing (ChIP-seq) data of HIC1 binding in HEK293 cells provided through the ENCODE Project (dataset: ENCSR803GYT, www.encodeproject.org) (ENCODE

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**differentiation at D10.** Analysis by immunofluorescence, RT-PCR, and flow cytometry was performed at various time points—D10, D15, and D20—of differentiation that correlate with relative maturation of hair cells in the organoids.

(B) **Frequency of *Atoh1* activation (Atoh1-nGFP+) in cochlear organoids among cells transduced (mScarlet+) with shControl and shHic1 after initial 10 days of expansion (D10) as measured by flow cytometry (**n** = 4). At the onset of differentiation, when *Atoh1* levels are highest, significantly more supporting cells transduced with shHic1 activate the Atoh1-nGFP reporter relative to shControl (7.4% ± 0.4 [shControl] versus 31.7% ± 0.5 [shHic1]), as well as relative to untransduced mScarlet− populations in either group (4.7% ± 0.6 [untreated, shControl], 5.9% ± 0.7 [untreated, shHic1]).

(B') **Representative bright-field images showing greater proportion of double-positive (GFP+/mScarlet+) cells in shHic1-treated organoids relative to shControl. Scale bar, 150 μm.**

(B'') **Representative flow cytometry.**

(C) **Frequency of double-positive (GFP+/mScarlet+) cells among shHic1 and shControl transduced cells in D15 organoids as measured by flow cytometry, with representative plots (**n** = 5). With shControl, 1.7% ± 0.3 of the total transduced mScarlet− supporting cells demonstrate persistent Atoh1-nGFP expression, while with shHic1, 10.7% ± 2.5 of the total mScarlet− supporting cells activate Atoh1-nGFP.

(C') **Representative flow cytometry.**

(D) **Quantification (**n** ≥ 18 organoids) of the hair-cell-specific marker, MYO7A+, in shControl and shHic1 organoids. Representative immunohistochemistry for mScarlet, Atoh1-nGFP, and MYO7A in cochlear organoids.

(D') **Hair cell differentiation and expression of hair-cell-specific markers, Atoh1-nGFP and MYO7A, are induced in a subset of organoids transduced with shHic1 (D15 organoids). Scale bar, 20 μm.**

(E) **mRNA expression of hair cell genes in D20 FACS-sorted, mScarlet+ cells from shHic1 and shControl organoids. Knockdown of Hic1 potentiates expression of hair cell transcripts, Myo7a and Pou4f3, following 10 days of differentiation.** Results are presented as fold change in expression normalized to Gapdh relative to shControl. Student’s t test, *p < 0.05.

(F) **Frequency of *Atoh1* activation (Atoh1-nGFP+) in cochlear organoids among cells transduced (mScarlet+) with shControl and shHic1 alone and in combination with CHIRLY demonstrates that shHic1 augments CHIRLY to drive differentiation of supporting cells to hair cells, as measured by increased double-positive (GFP+/mScarlet+) cells in shHic1-treated cells, relative to shControl (D20 organoids, **n** = 5): shControl, 1.4% ± 0.3; shHic1, 11.1% ± 2.9; shControl + CHIRLY, 13.6% ± 2.0; shHic1 + CHIRLY, 28.0% ± 4.3.

(F') **Representative flow cytometry of different treatments.**

(G) **Schematic of the *Atoh1* enhancer-promoter-nanoLuc (Atoh1-Enh-Prom-NLuc) lentivirus construct.**

(H) **Results of dual nanoLuc/firefly luciferase reporter assay in cochlear organoids.** Cochlear progenitor cells were transduced with Atoh1-Enh-Prom-NLuc reporter, constitutively active firefly luciferase control reporter, and TET-pLKO-shHic1 and subsequently differentiated with specified treatments with and without Dox (1 μg/mL), and the nanoLuc/firefly luciferase ratio was quantified. Knockdown of Hic1 potentiates differentiation and activation of the *Atoh1* promoter and enhancer (**n** = 3): DMSO, 1.00 ± 0.09; DMSO + Dox, 2.28 ± 0.53; CHIR 2.44 ± 0.12; CHIR + Dox, 4.12 ± 0.34; CHIRLY, 4.78 ± 0.48; CHIRLY + Dox, 8.55 ± 0.20 (unless otherwise indicated, data reflect mean ± SD from **n** independent experiments; one-way ANOVA, *p < 0.05, **p < 0.001, ****p < 0.0001).
HIC1 Overexpression Suppresses Atoh1 Activation and Hair Cell Differentiation in Cochlear Organoids

To further test the hypothesis that HIC1 represses Atoh1 in the inner ear, we forced expression of HIC1 in organoids derived from Atoh1-nGFP mice using lentiviral delivery of HIC1 under a Dox-inducible system, with mScarlet to mark transduced cells. Dissociated cochlear cells were transduced with Dox-inducible HIC1 and, after 10 days of proliferation, organoids were forced to differentiate with CHIRLY, with (+) or without (−) Dox (Figure 3A). Relative to no-Dox control, mScarlet+ cells in which HIC1 was overexpressed (+Dox) showed a 50% reduction in differentiation and Atoh1-nGFP co-expression, indicating that HIC1 suppresses Atoh1 activation in these cells (Figures 3A and 3B). This suppression of GFP was not observed in transduced cells in which HIC1 overexpression was not induced (mScarlet−, −Dox), or in untransduced cells treated with Dox (mScarlet−, +Dox). There was no increased cell death with HIC1 overexpression, as assessed by live microscopy and DAPI viability gating across treatments. Together with the knockdown data, these findings indicate that HIC1 is a biologically relevant repressor of progenitor-to-hair cell differentiation in the ear.

HIC1 Represses the Atoh1 Regulatory Enhancer

Hair cell differentiation is driven by the transcription factor ATOH1 through regulation of its 1.7 kb downstream enhancer as well as promoter regions (Figure 4; Figure S2) (Galvez et al., 2017; Helms et al., 2000). Having observed that HIC1 knockdown and overexpression respectively increase and decrease hair cell differentiation in cochlear organoids, we proceeded to investigate whether HIC1 may act as a direct transcriptional regulator of Atoh1, as suggested by prior studies in the cerebellum where HIC1 was shown to directly bind to the two adjacent motifs in the Atoh1 enhancer (figure S2) (Briggs et al., 2008; Pintea et al., 2004). We therefore tested the effect of HIC1 on the Atoh1 3‘ enhancer in an orthologous system, HEK293 cells, which do not naturally express ATOH1 but do express HIC1 (Broad Institute Cancer Cell Line Encyclopedia: portals.broadinstitute.org/ccle). Co-transfection of a reporter containing the Atoh1 3‘ enhancer driving firefly luciferase with either a vector for constitutive HIC1 expression or a small interfering RNA (siRNA) targeting endogenous HIC1 showed the same pattern as observed in organoids, an inverse relation between HIC1 expression and Atoh1 enhancer activity, while empty vector or non-targeting siRNA had no effect (Figure 4A). ATOH1 is a potent activator of its own expression via its 3‘ enhancer (Klisch et al., 2011), and our results in cochlear organoids leave open the possibility that the effect of HIC1 may be indirect through ATOH1 (for example, regulating an upstream factor, which then turns on Atoh1 expression). Given the
absence of ATOH1 in HEK cells, these data indicate that HIC1 regulates Atoh1 by interacting with the 3' enhancer and is independent of the ATOH1 protein.

**HIC1 Inhibits TCF/β-Catenin Activation of Atoh1**
The activation of Atoh1 by Wnt and subsequent loss of Atoh1 responsiveness to Wnt are critical events regulating hair cell development (Samarajeewa et al., 2018; Shi et al., 2010). We therefore investigated whether HIC1 represses Atoh1 by inhibiting its activation by Wnt. Indeed, co-transfection of HEK293 cells with the Atoh1 enhancer reporter and β-catenin show activation of Atoh1 expression, as expected, and this activation is increased by knockdown of endogenous HIC1 and reversed by addition of exogenous HIC1 (Figure 4B) in a dose-dependent fashion (Figure 4C).

The Atoh1 gene locus contains several sites matching the HIC1 minimal DNA binding motif TGCC(A/C) (Figure S2), and ChIP-qPCR data indicate HIC1 occupancy at some of these sites (Briggs et al., 2008; Pinte et al., 2004). To test whether DNA binding is required for HIC1 regulation of Atoh1, we employed a previously characterized mutant of HIC1, which contains an amino acid substitution (C521S) in the third zinc-finger domain that abolishes DNA binding and repression activity (Figure 4B) (Pinte et al., 2004). We confirmed that wild-type HIC1 and not HIC1 C521S represses the HIC1 reporter (5·3 HiRE), as shown previously (Figure 4D) (Pinte et al., 2004). We found that HIC1 C521S is less effective at blocking Atoh1 enhancer activation by β-catenin (Figure 4A), supporting the view that HIC1 regulation of Atoh1 requires direct binding to the enhancer.

However, prior work has shown that HIC1 can inhibit β-catenin at the protein level by sequestering β-catenin/TCF in a nuclear complex with HIC1 (Valenta et al., 2006). Using an established TCF reporter, pTOPflash, which comprises evenly spaced repeat TCF binding motifs,
we studied the interaction between HIC1 and β-catenin on the Wnt reporter. Consistent with the observations of Valentia et al., we found that HIC1 can abolish β-catenin activation of the TCF reporter in a dose-dependent fashion (Figure 5A). HIC1 is not expected to bind the TCF reporter (Valenta et al., 2006), which implies that HIC1 inhibits Wnt and therefore represses Atoh1 independent of DNA binding. The previously proposed mechanism for this inhibition of Wnt is the formation of HIC1 nuclear bodies that sequester TCF and β-catenin (Valenta et al., 2006), which we observe as well, at least upon HIC1 overexpression (Figure 5B).
We found no inhibition of pTOPflash reporter activity by the HIC1 mutant C521S (Figure 5A), even though this mutant is still able to form the HIC1 nuclear bodies thought to sequester TCF (Figure 5B). These data suggest that HIC1 C521S may be defective not only in DNA binding but also in its interaction with TCF. In either case, our results indicate that HIC1 can block activation of \textit{Atoh1} by \textit{\beta}-catenin and that this interaction is disrupted by mutation of its third zinc-finger domain.

**DISCUSSION**

Our studies using Atoh1 and Wnt reporter systems demonstrate that HIC1 regulates \textit{Atoh1} expression and sensory hair cell differentiation in the inner ear. These findings suggest a more universal role for HIC1 in repressing \textit{Atoh1} and other Wnt target genes. We observed that silencing \textit{Hic1} alone was sufficient to activate the \textit{Atoh1} enhancer and increase \textit{Atoh1} expression during cochlear progenitor-to-hair cell differentiation, and that this effect was additive when combined with Wnt activation, which potentiates differentiation by upregulating \textit{\beta}-catenin. Conversely, HIC1 overexpression significantly inhibited differentiation of progenitor supporting cells to hair cells.

Our findings suggest that HIC1’s inhibition of TCF/\textit{\beta}-catenin may also rely on zinc-finger-mediated DNA binding. Intriguingly, although the C-terminal region of HIC1 was found to be essential for TCF interaction, Valenta et al. did not observe direct HIC1 binding to the TCF minimal

**Figure 5. HIC1, but not HIC1 C521S, Represses \textit{\beta}-Catenin Activation of TCF Reporter**

Results of dual luciferase reporter assays in heterologous HEK293 cells are shown. (A) TCF reporter pTOPflash assay in the absence (−) or presence (+) of \textit{\beta}-catenin, HIC1, and HIC1 C521S at increasing doses. \textit{\beta}-catenin potently activates the TCF reporter, which is suppressed by HIC1 in a dose-dependent manner. HIC1 C521S DNA-binding mutant fails to repress \textit{\beta}-catenin activation and leads to relative de-repression. Data reflect mean ± SD of normalized firefly/Renilla luciferase ratio relative to empty vector control (n = 3 independent experiments); one-way ANOVA, *p < 0.05, **p < 0.001, ***p < 0.0001.

(B) Representative confocal images of HEK293 cells transfected with HIC1-eGFP (C-terminal tag) and HIC1 C521S-eGFP and stained with anti-HIC1 antibody and DAPI nuclear stain demonstrate that HIC1 and HIC1 C521S are expressed and form “nuclear bodies.” Scale bar, 20 \textmu m.
binding motif (Valenta et al., 2006). The discrepancy between our findings and previous reports may relate to protein characteristics of the mutant in cells versus in vitro pull-down assays. We cannot exclude the possibility that the C521S mutation induced a significant structural change that affected the protein beyond DNA binding, though we confirmed that the mutant could be overexpressed and form protein aggregates much akin to wild-type HIC1 without resulting in untoward toxicity. Further work will focus on elucidating the mechanism of this HIC1 C-terminal region in mediating repression of TCF targets.

In the cerebellum, Hic1 expression is inversely correlated with Atoh1 expression (Briggs et al., 2008). A correlation between the timing of Hic1 upregulation and Atoh1 downregulation in the first few postnatal days supported a similar role for HIC1 in repression of Atoh1 in the cochlea. Hic1 expression comes up later and Atoh1 expression persists longer in the cerebellum (Briggs et al., 2008), and differences in the downstream targets of ATOH1 may explain contrasting roles in the cerebellum and cochlea (Cai and Groves, 2015; Klisch et al., 2011). We found evidence of expression in both hair cells and supporting cells in the postnatal cochlea, which raises the possibility that HIC1 may be contributing to the suppression of Atoh1 in supporting cells as well as in the maturing hair cells, where Atoh1 expression precipitously drops, as occurs in CGPs during maturation (Briggs et al., 2008). Recent work that takes advantage of the RiboTag system to enrich for cell-specific expression shows relative enrichment of Hic1 at least in outer hair cells (using PrestinCreERT2/+; RiboTagHA/+ mice) at various postnatal developmental stages (dataset source: umgear.org) (Hertzano et al., 2020; Matern et al., 2020). Future work will focus on characterizing the significance of changes in Hic1 expression in the cochlea.

We demonstrate here through shRNA-mediated knockdown of Hic1 that the expression level is physiologically relevant in suppressing Atoh1 and hair cell differentiation. Our data on the HIC1 C521S zinc-finger mutant suggest that the repressive effect on the Atoh1 and Wnt reporters more generally is dependent on DNA binding, possibly through recruitment of corepressor complexes. Indeed, HIC1 is known to recruit CtBP, NuRD, and PRC2 in different cellular contexts (Boulay et al., 2012; Deltour et al., 2002; Van Rechem et al., 2010). In turn, HIC1 itself is regulated by HDAC-dependent and independent mechanisms (Deltour et al., 2002), raising the possibility that HDAC inhibitors may be good candidates for (at least in part) reversing HIC1-mediated silencing of Atoh1 (Dehennaut et al., 2013; Van Rechem et al., 2010). Within the first postnatal week, we and others have shown that, while Wnt signaling is able to induce hair cell differentiation in the newborn, Wnt/β-catenin is no longer effective by as early as P5, despite still being active (Samarajeewa et al., 2018). These observations are consistent with the current pedagogy regarding cellular reprogramming: they imply that transcriptional reprogramming of precursor cells toward a hair cell lineage ultimately requires both forced activation of essential hair cell transcriptional programs and removal of key inhibitory signatures (Hochedlinger and Jaenisch, 2015; Takahashi, 2014; Takahashi et al., 2007). Targeting HIC1, either directly using genetic means (shRNA, CRISPR-Cas9) or indirectly by targeting components of the repressor complexes (e.g., HDAC inhibitors), may potentiate the transcriptional response to Wnt signaling and help drive hair cell differentiation during this period.

Future work will seek to characterize the cochlear-specific HIC1 targets at the Atoh1 locus and genome-wide and to define the epigenetic changes resulting from HIC1 knockdown and overexpression and their effects on hair cell differentiation. Additional work will focus on targeting HIC1 in vivo to test its role in regeneration in the adult cochlea following hair cell damage. This study supports a growing paradigm wherein repressor complexes recruit epigenetic modifiers that alter key signaling pathways (e.g., Wnt) on fate-determining target genes, like Atoh1. Moreover, the use of inner ear organoids to model progenitor-to-hair cell differentiation and targeted knockdown and overexpression, as demonstrated here with HIC1, provides a framework in which to test the effects of candidate factors on hair cell differentiation in a physiologically relevant model. Indeed, elucidating the full repertoire of repressors at the Atoh1 gene in the inner ear will help instruct efforts in targeted therapy aimed at relieving this repression and potentiating hair cell regeneration.

**EXPERIMENTAL PROCEDURES**

**Experimental Mice**

Mice were under the husbandry care of the Massachusetts Eye and Ear Animal Facility. P3 Atoh1-nGFP mice (Lumpkin et al., 2003) (provided by Dr. Jane Johnson) were used for organoid dissection to identify differentiated hair cells. Alternatively, for immunohistochemistry/in situ hybridization experiments, wild-type C57B6 mice were used at the stated ages. All mouse work was reviewed and approved by the Massachusetts Eye and Ear Animal Care Committee.

**Progenitor Cell Isolation and 3D Organoid Culture**

Progenitor cells were dissected from the cochlear sensory epithelium and allowed to expand as inner ear organoids using the previously established protocol, with some modifications (Lenz et al., 2019; McLean et al., 2017). Briefly, the sensory epithelium was mechanically dissected from the cochlea with the aid of a cell recovery solution (Corning) and ACCUMAX, and dissociated single cells were embedded in Matrigel (Corning) at a ratio of 1 cochlea per well, in a 24 well plate. Cells were expanded for 10 days in
proliferation medium consisting of valproic acid (1 mM), CHIR (3 μM), 2-phospho-L-ascorbic acid (280 μM), fibroblast growth factor, epidermal growth factor, and insulin growth factor (50 ng/mL each). Organoid proliferation conditions were withdrawn and cells were allowed to differentiate under different treatment conditions. Medium was changed every 2 days.

Plasmid Construction, Lentivirus Production, and Infection
Details of vectors, cloning strategies, and lentivirus production are given in the supplementary information.

In Situ Hybridization Followed by Immunohistochemistry
Single-molecule in situ hybridization was performed using the RNAscope 2.0 HD detection kit (Advanced Cell Diagnostics), with a commercially available mRNA probe to mHic1, according to the standard fixed protocol, until the final step, when immunostaining was performed prior to mounting (see supplemental experimental procedures).

Immunohistochemistry
Murine cochleae, organoids, and HEK cells were fixed, permeabilized, and blocked, followed by staining using established protocols. Details, including reagents, are provided in the supplementary information.

Organoid Live Cell Imaging
Live organoids were imaged on the Axio Observer Z1 imaging system (Carl Zeiss) and analyzed using Zen 2 software (Carl Zeiss). Laser intensity was kept constant and adjustments for brightness and contrast were kept uniform to allow comparisons of organoids across treatments.

Flow Cytometry
Organoids were dissociated by incubation first in cell recovery solution (Corning), followed by ACCUMAX cell detachment solution. Transduced mScarlet⁺ cells were isolated on a BD FACS Aria II SORP cell sorter into base medium or RLT buffer (QIAGEN). Dead cells were excluded from the analysis using DAPI staining. FACS plots were analyzed and generated using FlowJo software version 10.4 (Becton Dickinson).

Hair Cell Quantification
Fluorescence images of fixed cochlear organoids at 20× magnification were obtained with an SP8 confocal microscope and formatted with Fiji ImageJ (https://fiji.sc/). MYO7A⁺ cells were quantified using the Cell Counter plugin from cross-sectional images of mScarlet⁺ organoids in 5,000 μm² regions to account for the variability in organoid size and shape (only regions in the grid that fell completely within an organoid were analyzed, allowing sampling of approximately 80% of a given organoid). Counts were summed across regions and averaged for each organoid. At least 18 mScarlet⁺ organoids per condition were counted. Statistical analyses were conducted using GraphPad Prism 8.4 software (Graph-Pad). Two-tailed, unpaired Student’s t test was used to determine statistical significance. Data are shown as mean ± SD.

RNA Extraction and qRT-PCR
Fixed cochleae, whole organoids from a single well, and sorted cells were lysed into RLT buffer and total RNA was isolated using the RNeasy Micro Kit (QIAGEN) according to the manufacturer’s instructions. RNA was converted to cDNA with the qScript XLT cDNA kit (Quanta BioSciences). qRT-PCR was performed with diluted cDNA (1:4) in two wells for each primer and TaqMan probes and SYBR green master mix (Bio-Rad) on the Bio-Rad iCycler RT-PCR detection system.

Luciferase Assays
HEK293 cells were seeded into 24-well plates one day before transfection. Two hundred fifty nanograms of Atoh1 enhancer-luciferase reporter construct (Shi et al., 2010), or 250 ng of pTOPFlash (Molenaar et al., 1996; Shi et al., 2010), with 50 ng of Renilla luciferase construct and varying amounts of empty vector (eGFP), HIC1-GFP, HIC1 C521S-GFP, and β-catenin (Shi et al., 2010) expression constructs were transfected using Lipofectamine 2000 per the standard protocol. The total amount of DNA was kept constant. For siRNA experiments, negative control DsiRNA and HIC1 DsiRNA (hs.Ri.HIC1.13.1) were used at 50 nM final concentration (Integrated DNA Technologies). Cells were lysed after 36 h, and firefly/Renilla luciferase activity was measured using the Dual-Glo luciferase reporter assay system (Promega) in a TD-20/20 Luminometer (Turner Designs). Firefly luciferase signal was normalized to Renilla luciferase signal and calculated as fold change relative to control.

For the Atoh1 enhancer-promoter-nanoLuc reporter in inner ear organoids, dissociated cochlear cells were transduced with reporter constructs as well as Tet-pLKOmScarlet-Hic1 according to the above protocol and allowed to expand in Matrigel in a 24 well plate. At the time of differentiation, individual wells were treated with different conditions, with and without 1 μg/mL Dox. Following 10 days of differentiation conditions, the wells were washed with PBS for 1 h and lysed, and nanoLuc/firefly luciferase activity was measured using the Nano-Glo Dual-Luciferase kit (Promega).

Statistical Analysis of Data
The samples used for each experiment are detailed in the figure legends. All experiments were repeated on at least three separate occasions (independent experiments). For organoid assays, one to three wells per group were pooled and analyzed. All center values shown in graphs refer to the mean ± SD. For statistical significance of the differences between the means of two groups, we used two-tailed Student’s t tests. Statistical significance for the means of several groups was calculated by performing one-way ANOVA with Tukey’s multiple comparisons of the means for each group. A p < 0.05 was considered significant. Animals were randomly assigned to groups and contained a mix of male and female littermates.

Data and Code Availability
Source data for HIC1 ChIP-Seq in HEK293 (Figure S3) are available through the ENCODE Project (dataset: ENCSR803GYT, www.encodeproject.org).
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2021.02.022.

AUTHOR CONTRIBUTIONS

D.A. and A.E. developed the concepts, analyzed the data, and wrote the manuscript; D.A. designed, performed, and analyzed the experiments; N.H., L.P., and V.S. assisted with experiments and analysis.

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