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COUP-TFI controls Notch regulation of hair cell and support cell differentiation

Louisa S. Tang, Heather M. Alger* and Fred A. Pereira†

The orphan nuclear receptor COUP-TFI (Nr2f1) regulates many aspects of mammalian development, but little is known about its role in cochlear hair cell and Deiter's support cell development. The COUP-TFI knockout (*COUP-TFI*$^{-/-}$) has a significant increase in hair cell (HC) number in the mid-to-apical turns. The total number of hair cells is not increased over wild type, perhaps because of displaced hair cells and a shortened cochlear duct. This implicates a defect of convergent-extension in the COUP-TFI$^{-/-}$ duct. In addition, excess proliferation in the *COUP-TFI*$^{-/-}$ sensory epithelium indicates that the origin of the extra HCs in the apex is complex. Because loss-of-function studies of Notch signaling components have similar phenotypes, we investigated Notch regulation of hair cell differentiation in *COUP-TFI*$^{-/-}$ mice and confirmed misregulation of Notch signaling components, including *Jag1*, *Hes5* and *Lfng*, in a manner consistent with reduced Notch signaling, and correlated with increases in hair cell and support cell differentiation. The disruption of Notch signaling by a γ-secretase inhibitor in an in vitro organ culture system of wild-type cochleae resulted in a reduction in expression of the Notch target gene *Hes5* and an increase in hair cell differentiation. Importantly, inhibition of Notch activity resulted in a greater increase in hair cell differentiation in *COUP-TFI*$^{-/-}$ cochlear cultures than in wild-type cultures, suggesting a hypersensitivity to Notch inactivation in *COUP-TFI*$^{-/-}$ cochlea, particularly at the apical turn. Thus, we present evidence that reduced Notch signaling contributes to increases in hair cell and support cell differentiation in *COUP-TFI*$^{-/-}$ mice, and suggest that COUP-TFI is required for Notch regulation of hair cell and support cell differentiation.

KEY WORDS: COUP-TFI, Cochlea, Hair cell, Deiter's cell, Cell proliferation, Differentiation, Migration, Notch, *Jag1*, *Hes5*, *Lfng*, Organ culture, γ-secretase, DAPT, Myosin VIIa

INTRODUCTION

Orphan nuclear receptors (ONRs) are members of the nuclear receptor superfamily for which ligands and physiological functions have not been identified (Mangelsdorf et al., 1995; Pereira et al., 2000). COUP-TFI (chicken ovalbumin upstream promoter transcription factor I; also known as NR2F1) is an ONR proposed to function as a ligand-regulated transcription factor (Cooney et al., 1992; Wang et al., 1989). In the absence of a specific ligand or of binding to an antagonist, ONRs repress the expression of target genes through interaction with co-repressor proteins (Shibata et al., 1997). Upon binding to a ligand or agonist, ONRs undergo conformational changes that displace co-repressors with co-activators, leading to the expression of target genes (McKenna and O'Malley, 2002). COUP-TFI has been shown to play important roles in the central and peripheral nervous systems (Pereira et al., 2000). Mice deficient in COUP-TFI exhibit reduced axon arborization (Qiu et al., 1997), thalamocortical projections (Zhou et al., 1999), disrupted regionalization of the forebrain neocortex (Zhou et al., 2001), a delay in axon myelination (Yamaguchi et al., 2004), and defects in tangential cell migration in the basal forebrain (Tripodi et al., 2004). Most recently, the paralog COUP-TFII (Nr2f2) was implicated in epithelial-mesenchymal interactions for radial and anteroposterior patterning of the stomach (Takamoto et al., 2005), and in the specification of arteries through regulation of the Notch pathway (You et al., 2005).

Huffington Center on Aging, Department of Otolaryngology – Head and Neck Surgery, Department of Molecular and Cellular Biology, Program in Cell and Molecular Biology, Baylor College of Medicine, Houston, TX 77030, USA.

*Present address: Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA
†Author for correspondence (e-mail: fpereira@bcm.edu)

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The Notch gene family encodes transmembrane receptors and ligands that are crucial for cell fate decisions during development in metazoans (Heitzler and Simpson, 1991; Swiatek et al., 1994). Notch signaling governs the choice of cell fate by lateral inhibition, in which one cell inhibits a group of adjacent cells from taking a specific fate, and by lineage decisions, in which one daughter cell adopts a fate different from its sibling (Artavanis-Tsakonas et al., 1995). The restricted expression of Notch signaling components mediates the regulation of Notch activity (Robey, 1997). Notch receptors and their ligands are initially expressed in the same cells, and modulation of expression of a specific ligand affects the activity in the adjacent cell expressing the receptors (Heitzler and Simpson, 1991). Ligand binding induces a proteolytic cascade, culminating in the release and activation of the Notch intracellular domain (NICD). A presenilin-dependent γ-secretase complex mediates the activating cleavage of the NICD (De Strooper et al., 1999). The NICD then translocates to the nucleus and switches a DNA-bound factor associated with co-repressors into a complex associated with co-activators that stimulate the transcription of Notch target genes, such as the hairy/enhancer of split (Hes) genes (Barrick and Kopan, 2006; Jarriault et al., 1995; Kao et al., 1998; Kopan and Turner, 1996; Struhl and Adachi, 1998).

In humans, deregulated Notch signaling causes several developmental abnormalities and diseases. Mutation of *JAG1* causes Alagille syndrome, an autosomal dominant disorder characterized by deafness and developmental abnormalities of various organs (Li et al., 1997; Oda et al., 1997). Mutation in *NOTCH3* leads to CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), an inherited vascular dementia syndrome (Joutel et al., 1996; Kalaria et al., 2004) with characteristics that include sensorineural hearing loss (Baudrimont et al., 1993; Phillips et al., 2005). The formation of cellular diversity in the sensory epithelium is a highly regulated developmental event involving proliferation, cell fate decisions, pattern formation, and

differentiation and Notch signaling. Notch signaling cascade proteins, including Notch1, Dll1, Jag1, Jag2, Hes1 and Hes5 are expressed in hair cells, supporting Deiter's cells or their precursors (Lanford et al., 1999; Lanford et al., 2000; Morrison et al., 1999; Zheng et al., 2000; Zine et al., 2001). Mutations of Notch and its signaling components in mice result in extra rows of either inner or outer hair cells, *Notch1* (Zhang et al., 2000), *Jag1* (Kiernan et al., 2001), *Jag2* (Lanford et al., 1999; Zhang et al., 2000), *Hes1* (Zheng et al., 2000; Zine, 2003) and *Hes5* (Zine, 2003), consistent with the notion that lateral inhibitory mechanisms were disrupted. Additionally, antisense *Notch1* and *Jag1* oligonucleotides (Zine et al., 2000), or use of γ-secretase inhibitors (Yamamoto et al., 2006) that block Notch signaling, in cochlear organ cultures produce supernumerary hair cells. These and other studies have led to the conclusion that Notch signaling is essential for the differentiation of hair cells in vertebrates (Daudet and Lewis, 2005; Haddon et al., 1998; Kiernan et al., 2001; Kiernan et al., 2005; Kiernan et al., 2006; Lanford et al., 1999; Weir et al., 2000; Woods et al., 2004; Yamamoto et al., 2006; Zhang et al., 2000; Zheng et al., 2000; Zine et al., 2000).

We have recently described a detailed expression profile of COUP-TFI and COUP-TFII in the developing and mature mouse cochlea (Tang et al., 2005). We showed that COUP-TFI is expressed in the otocyst and developing sensory epithelium prior to and during hair cell and support cell differentiation. Here, we report for the first time that *COUP-TFI* deficiency causes altered sensory epithelial development, resulting in supernumerary hair cells and support cells in the apical turn: there are frequent inner hair cell (IHC) duplications, four rows of outer hair cells (OHC) in the middle turn and up to six to seven rows of OHCs at the apex, all with an equal number of underlying Deiter's support cells. Because the phenotype was strikingly similar to that caused by inhibition of Notch signaling (Zine et al., 2000; Kiernan et al., 2005; Kiernan et al., 2006; Brooker et al., 2006), we investigated the expression of Notch signaling genes in the developing sensory epithelium of *COUP-TFI*$^{-/-}$ mice by in situ hybridization and by quantitative RT-PCR (qRT-PCR) in an in vitro cochlea organ culture system. These studies showed qualitative and quantitative changes in several Notch signaling components, which indicated that Notch signaling was attenuated in *COUP-TFI*$^{-/-}$ cochleae. We show that a γ-secretase inhibitor (DAPT) suppressed Notch signaling in a dose-dependent fashion and induced hair cell differentiation in both wild type and in *COUP-TFI*$^{-/-}$ cochleae in organ cultures. DAPT suppressed Notch signaling to a greater extent in *COUP-TFI*$^{-/-}$ cochleae, suggesting a hypersensitivity to inhibition of Notch signaling in *COUP-TFI*$^{-/-}$, particularly at the apical turn. These results are consistent with a function for COUP-TFI in modulating Notch regulation of differentiation and patterning of hair cells and support cells.

MATERIALS AND METHODS

Animal husbandry and genotyping

Generation, genotyping and husbandry of COUP-TFI deficient *129SvEv* mice by gene targeting has been described (Qiu et al., 1997) and is in accordance with the Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act approved by the Institutional Animal Care and Use Committee of the Baylor College of Medicine. The onset of gestation was identified by the appearance of a vaginal plug the morning after mating and was designated as embryonic day (E) 0.5, with the day of birth set as P0.

Counts of phalloidin-stained hair cells

P10 cochleae were dissected to open the cochlear capsule and fixed in 2% paraformaldehyde and 0.1% Triton X-100 in PBS for 10 minutes. After PBS washes and incubation in Alexa 488-conjugated phalloidin (Molecule Probes, Eugene, OR; 1:200, diluted in PBS) for an hour at room temperature, the stria vascularis and tectorial membrane were removed to expose the organ of Corti. Cochleae were then flat-mounted with Vectashield DAPI medium (Vector Laboratories, Burlingame, CA) on glass slides and examined under epi-fluorescence (Carl Zeiss, Gottingen, Germany). Eight samples from each genotype were studied. Hair cell counts were estimated from three equal portions (basal, middle and apical) of the cochlear duct, and counted in at least four random microscopic fields (400×) from three cochleae of each genotype. Numbers are means±s.e.m., with statistical significance at $P<0.05$.

Paint-filling of cochlear ducts

Inner ears at E13, E15 and E17 were isolated from wild-type and null embryos, fixed in Bodian's fixative, dehydrated in an ethanol series, and cleared in methyl salicylate. Latex paint was injected to visualize the membranous labyrinths as previously described (Morsli et al., 1998). At least four pairs of inner ears were injected for each stage studied.

Histology

Embryonic heads of E14.5 to E16.5 mice, or isolated cochleae at P10, were fixed in neutral-buffered formalin, dehydrated and embedded in paraffin. Sets of 7-10 μm serial sections through the cochlear duct were collected and processed for Hematoxylin and eosin staining, immunofluorescence analyses or in situ hybridization.

Immunofluorescence and proliferating cell counts

Immunofluorescence was performed with anti-p27 (1:200, NeoMarkers, Fremont, CA), Ki67 (1:200, BD Biosciences, San Diego, CA), anti-BrdU (DAKO, Carpinteria, CA), anti-myosin VIIa (Tama Hasson, Department of Biological Sciences, University of California at San Diego, San Diego, CA; or Proteus Biosciences, 25-6790), *Griffonia simplicifolia* lectin I (Vector Laboratories, Burlingame, CA), anti-β-tubulin (Sigma, St Louis, MO) and anti-COUP-TFI (Tang et al., 2005) antibodies, as published previously (Zhou et al., 1999), except the secondary antibodies were conjugated to Alexa Fluors (594 red or 488 green, and used at 1:1000; Molecular Probes, Eugene, OR). Nuclei were counterstained with DAPI (Vector Laboratories, Burlingame, CA). For BrdU labeling, pregnant mice were injected with 100 μg/g BrdU (Amersham, Piscataway, NJ) 3 hours prior to euthanasia. At least six cochleae from each group were analyzed. The number of BrdU-positive and Ki67-positive cells in the outer sulcus region of wild-type and *COUP-TFI*$^{-/-}$ cochlea were counted from at least 12 sections from each of three cochleae/genotype.

In situ hybridization

In situ hybridization was performed on E14.5 to E16.5 sections essentially as published (Tang et al., 2005). Non-radioactive antisense digoxigenin (DIG)-11-UTP (Roche, Indianapolis, IN) riboprobes were synthesized from linearized plasmids, 1800 bp (mouse *Jag1*), 750 bp (mouse *Lfng* provided by Dr Sean Egan, University of Toronto, Canada) and 1400 bp (mouse *Hes5*) cDNA (kindly provided by Dr Tim Mitsiadis, Kings College, London, UK). At least eighht samples were analyzed at each stage.

Quantitative real-time PCR

Total RNA was purified using RNeasy spin columns (Qiagen, Valencia, CA) and treated with RNase-free DNase (Ambion, Austin, TX), before reverse transcription into cDNA with oligo(dT) using MMLV-reverse transcriptase (Ambion, Austin, TX), in accordance with manufacturers' protocols. A control reaction lacking reverse transcriptase ensured fidelity of the amplified products. Primers (Table 1) were designed using Primer Express (Applied Biosystems, Foster City, CA). SYBR-green quantitative PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Reactions contained 12.5 ng template DNA, 12.5 μl 2×SYBR-green PCR Master Mix (Sigma, St Louis, MO) and 50 nM primers, in a final volume of 25 μl. Cycling conditions were: 95°C for 10 minutes to activate the Taq and then 40 cycles of sequential denaturation (95°C for 15 seconds) and annealing/extension (72°C for 60 seconds). A housekeeping gene, Gapdh, was also analyzed to normalize and correct for variations in RNA and/or cDNA quality and quantity. Data analysis was performed using the ABI Prism 7000 SDS Software (Applied Biosystems,

Table 1. Primer pairs used for quantitative real-time PCR

Gene	GenBank Accession number	Primer	Primer sequence (5′→3′)	Amplicon size (bp)
Jag1	AF171092	Forward	CACTTATTGCTGCGGTTGCA	51
		Reverse	TTTTCAGAGGACGCCTCTGAAC	
Lfng	U94351	Forward	CCACCCTCTGATGCCCTATC	51
		Reverse	GCTCTGAACCCCACTTGGTGT	
Hes5	D32132	Forward	ATTTCAGCAAGTGACTTCTGCG	51
		Reverse	ATAGAACCCCCGGTGGTGAC	
Myosin VIIa	U81453	Forward	CTGCCACGAGGTCCAGACTC	51
		Reverse	GCAAACCCAGATGCCATAGGT	
COUP-TFI	U07625	Forward	CAGCCCAGCCGCTTTG	51
		Reverse	TGTGCGAAGAGAGGGCAATC	
COUP-TFII	NM_009697	Forward	AGAAGGAACTGTGGAATTTATTGGC	51
		Reverse	TTGTGTCTTGGACACATTCCTTG	
Gapdh	M32599	Forward	AACGACCCCTTCATTGAC	191
		Reverse	TCCACGACATACTCAGCAC	

Foster City, CA). Four cochleae from each group were pooled together as one biological sample. At least three biological replicates (each with three technical replicates) for each group were analyzed. The Ct value, which represents the cycle number at which a fluorescent signal rises statistically above background, was determined for each transcript. After normalization with Gapdh, the change (Δ) in Ct values was presented. In these analyses, the Ct value for each biological replicate represented the average of the three technical replicates. The data are presented as average Ct±s.d. Statistical significance (when $P<0.05$) was determined by two-tailed *t*-test, with respect to the corresponding dimethyl sulfoxide (DMSO group).

Organotypic cochlear culture and hair cell counts

Cochlear cultures were established as previously described (Montcouquiol and Kelley, 2003), with some modifications. Briefly, cochleae were dissected from E16.5 embryos in cold HBSS and cultured in DMEM, 10% fetal bovine serum and N-2 supplement with antibiotics (all from Invitrogen, Carlsbad, CA). To modulate Notch signaling, the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Calbiochem, San Diego, CA) at concentrations from 0.5 to 5 μM was used. DMSO solvent was used as control. Explants were cultured for 5 days with daily media changes. The numbers of outer and inner hair cell rows in the explants were counted (at 400× magnification) in every fourth 7-μm thick section previously labeled with myosin VIIa antibodies. An average of 12 sections from each explant and at least six explants from each group were counted. Data are presented as mean±s.e.m. and statistical significance ($P<0.05$) was determined by a paired *t*-test.

Western analysis

Myosin VIIa (Myo7a) expression in cochlear explants were determined by western blot analyses. Cochleae lysates (30 μg) were fractionated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tang et al., 2005). γ-Tubulin served as an internal control for protein loading. Four cochleae from each group were pooled together as one biological sample. At least three biological replicates for each group were analyzed.

RESULTS

COUP-TFI$^{-/-}$ cochleae exhibit supernumerary hair cells and supporting cells

Hair cells are arranged stereotypically within the sensory epithelium in a spatially organized and invariant manner. The maturing wild-type organ of Corti at P10 contains a typical pattern of four mechanosensory hair cell rows: a single row of inner hair cells (IHC) and three rows of outer hair cells (OHC) along the entire length of the cochlear duct (Fig. 1A, parts a,c,e,g,i). Occasionally, the apical turn exhibits four rows of OHCs in wild-type mouse ears (Fig. 1A, part e). In the *COUP-TFI*$^{-/-}$ cochlear duct, extra inner hair cells were seen routinely in the organ of Corti at the basal and apical region but less frequently in the middle turn (Fig. 1A, parts b,d,f,h,j, arrowheads). In the middle-to-apical regions, the mutant organ of Corti exhibited extra rows of OHCs, ranging from one extra row in the middle turn (Fig. 1A, part d), to three or four extra rows in the apical-most regions (Fig. 1A, parts f,h,j). Notably, the *COUP-TFI*$^{-/-}$ organ also exhibits supernumerary Deiter's supporting cells beneath each hair cell (Fig. 1A, part j). In addition, there were occasional IHCs (Fig. 1A, part b) and OHCs (Fig. 1A, parts f,h, arrows) with stereociliary bundles displaced from the normal orientation in the *COUP-TFI*$^{-/-}$ organ of Corti, which suggests a defect in planar polarity or simple displacement of hair cells as a result of packing limitations within the epithelium.

Paint-filling of the membranous labyrinths demonstrated that *COUP-TFI*$^{-/-}$ have a shorter cochlear duct (arrows in Fig. 1B, parts b,d,f) than wild-type controls (Fig. 1B, parts a,c,e). The cochlear duct lengths were similar at E13; however, the wild-type duct was longer than that of *COUP-TFI*$^{-/-}$ embryos by E15 and E17. We next determined the number of hair cells (IHC and OHC) at different regions of the flat-mounted, phalloidin-stained cochlear ducts. We found more hair cells per unit area in *COUP-TFI*$^{-/-}$ than in the wild type at the middle and apical regions, but because of the shorter duct, there was a reduction in total hair cells in the base and middle turns of *COUP-TFI*$^{-/-}$ cochlear ducts. In addition, despite having a shorter duct, the total number of hair cells in *COUP-TFI*$^{-/-}$ was similar to in wild type (Fig. 1C).

The COUP-TFI$^{-/-}$ organ of Corti exhibits continued proliferation and ectopic hair cell differentiation

Because there were extra hair cells per unit area in the middle and apical turns of *COUP-TFI*$^{-/-}$ organ of Corti, we analyzed the expression domain of the cyclin-dependent kinase inhibitor p27, which demarcates a zone of non-proliferation (ZNP) in the developing organ of Corti where hair cells and support cells differentiate (Chen et al., 2002). The ZNP was similar at the basal and middle turns, and hair cells differentiated appropriately near the junction between the ZNP and the inner sulcus region of proliferating cells in the wild-type and *COUP-TFI*$^{-/-}$ sensory epithelia (arrowheads in Fig. 2A-D, see also Fig. 2I-L). However, at the apical turn, the ZNP domain was consistently widened and encompassed the extra hair cells in *COUP-TFI*$^{-/-}$ mice (Fig. 2, compare double arrows in 2F,H with 2E,G).

We next determined whether there were changes in proliferation. No differences were found in BrdU labeling at time-points before E16.5 (data not shown), after which there were extra BrdU-positive cells in the outer sulcus region at the apical turns in the *COUP-TFI*$^{-/-}$ organ of Corti (Fig. 2J, arrows). Compared with the ZNP region in adjacent serial sections, the extra BrdU-positive proliferating cells were found outside the ZNP in the lateral region where Hensen's cells would differentiate in *COUP-TFI*$^{-/-}$ epithelia (compare Fig. 2J

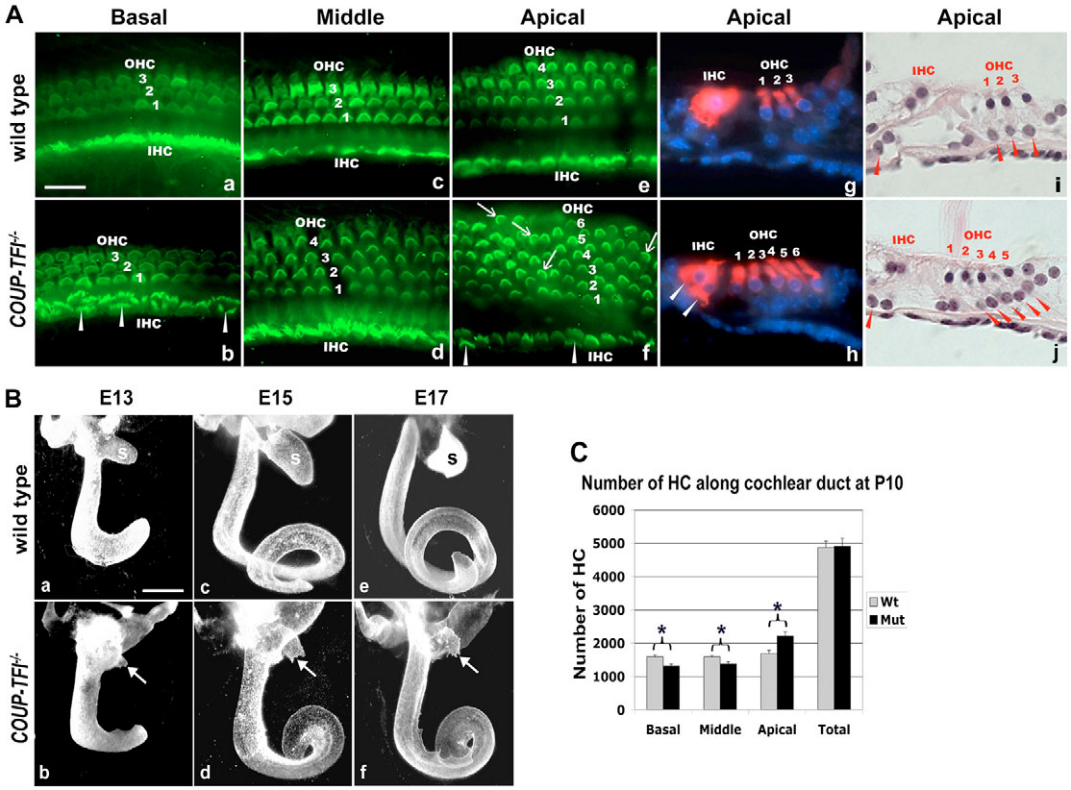

Fig. 1. *COUP-TFI*$^{-/-}$ cochlea exhibit supernumerary hair cells and Deiter's supporting cells. (**A**) Flat-mounts (a-f) and cross-sections (g-j) of wild-type (a,c,e,g,i) and *COUP-TFI*$^{-/-}$ (b,d,f,h,j) cochlear duct at P10, stained with Alexa 488-conjugated phalloidin (a-f), anti-myosin VIIa (g,h), and Hematoxylin and Eosin (H&E, i,j). Extra inner hair cells (IHC) were seen in the basal (compare b with a) and apical (compare f with e) regions, and extra outer hair cells (OHC) were seen in the middle (compare numbered rows in d with c) and apical (compare rows in f with e) regions of *COUP-TFI*$^{-/-}$ cochlea. OHC stereociliary bundles were often misoriented in *COUP-TFI*$^{-/-}$ cochlea (direction of arrows is perpendicular to the kinocilium in f). (g,h) Cross-sections through the apical region of the cochlear duct show the typical pattern of one row of IHC and three rows of OHC expressing myosin VIIa (red) in the wild type, but the *COUP-TFI*$^{-/-}$ mutant has supernumerary hair and support cells (arrowheads). Nuclei were counterstained with DAPI (blue). (i,j) H&E-stained sections of apical regions show individual supporting cells underlying every hair cell (arrowheads, compare j with i). Scale bar: 15 μm. (**B**) Paint-filling of wild-type (a,c,e) and *COUP-TFI*$^{-/-}$ (b,d,f) cochlear ducts at E13, E15 and E17 show that *COUP-TFI*$^{-/-}$ mutants have a shorter cochlear duct. Scale bar: 100 μm. (**C**) Graph of hair cell counts (IHC and OHC) from different regions of the cochlear duct at P10, showing more hair cells in the apical region of the *COUP-TFI*$^{-/-}$ duct than in the wild type (*P<0.05).

with 2L). The presence of ectopic proliferating cells was corroborated by Ki67 staining, another cell proliferation marker (Fig. 2M,N), and quantification was performed at this stage (Fig. 2Q). At older stages (P0), when the wild-type organ of Corti was postmitotic (Ruben, 1967), some Ki67-positive cells were still found at the apical turn in the *COUP-TFI*$^{-/-}$ mutants (Fig. 2O,P), suggesting that proliferation in the *COUP-TFI*$^{-/-}$ sensory epithelium was deregulated. Routine myosin VIIa immunolabeling to mark hair cells unexpectedly revealed its ectopic expression in the supporting cell region in both the basal and apical turns of *COUP-TFI*$^{-/-}$ epithelia (asterisk in Fig. 2R-T). The cells may represent displaced hair cells, as has been found in the pRB mutants (Mantela et al., 2005).

Jag1, *Hes5* and *Lfng* transcripts are deregulated in *COUP-TFI*$^{-/-}$ cochleae

Because similar supernumerary hair cell phenotypes were observed in Notch1 and Jag1 loss-of-function rat cochlea cultures (Zine et al., 2000), and loss-of-function mouse mutants of the Notch signaling pathway have alterations in the number and rows of hair cells (Brooker et al., 2006; Kiernan et al., 2001; Lanford et al., 1999; Zhang et al., 2000; Zheng et al., 2000; Zine, 2003), we analyzed the expression of crucial components in the Notch signaling pathway. We used in situ hybridization to examine the expression of the Notch receptors: *Notch1*, *Notch 2* and *Notch3*; Notch ligands, *Jag1*, *Jag2*, *Delta-like1* and *Delta3*; Notch downstream target genes, *Hes1* and *Hes5*; and a modulator of Notch intracellular domain cleavage, *Lfng*. Among these transcripts, *Jag1*, *Hes5* and *Lfng* showed altered expression in *COUP-TFI*$^{-/-}$ cochleae (Fig. 3), and no significant changes in the expression of other genes were seen (data not shown).

Jag1 was expressed in a broad domain in the sensory epithelium encompassing the presumptive hair and support cell precursor region (Fig. 3B) from the apex to the base in the wild-type E15.5 cochlea. As the sensory epithelium progressed in development in the apical to basal direction (Fig. 3B-D), the signal intensity within the center of this domain decreased and hair cells are seen to differentiate (Fig. 3B, arrow points to hair cells with large nuclei devoid of in situ signal). By contrast, there was a consistent reduction of *Jag1* expression levels throughout the *COUP-TFI*$^{-/-}$ cochlear duct, and no significant changes in signal intensity were seen from apex to base (Fig. 3F-H).

At E16.5, the expression of *Hes5* in the wild-type organ of Corti was restricted to the hair cells and support cells, with the highest level in the Deiter's supporting cells below OHCs (Fig. 3I-L). In the *COUP-TFI*$^{-/-}$ organ of Corti, *Hes5* expression was seen in a much

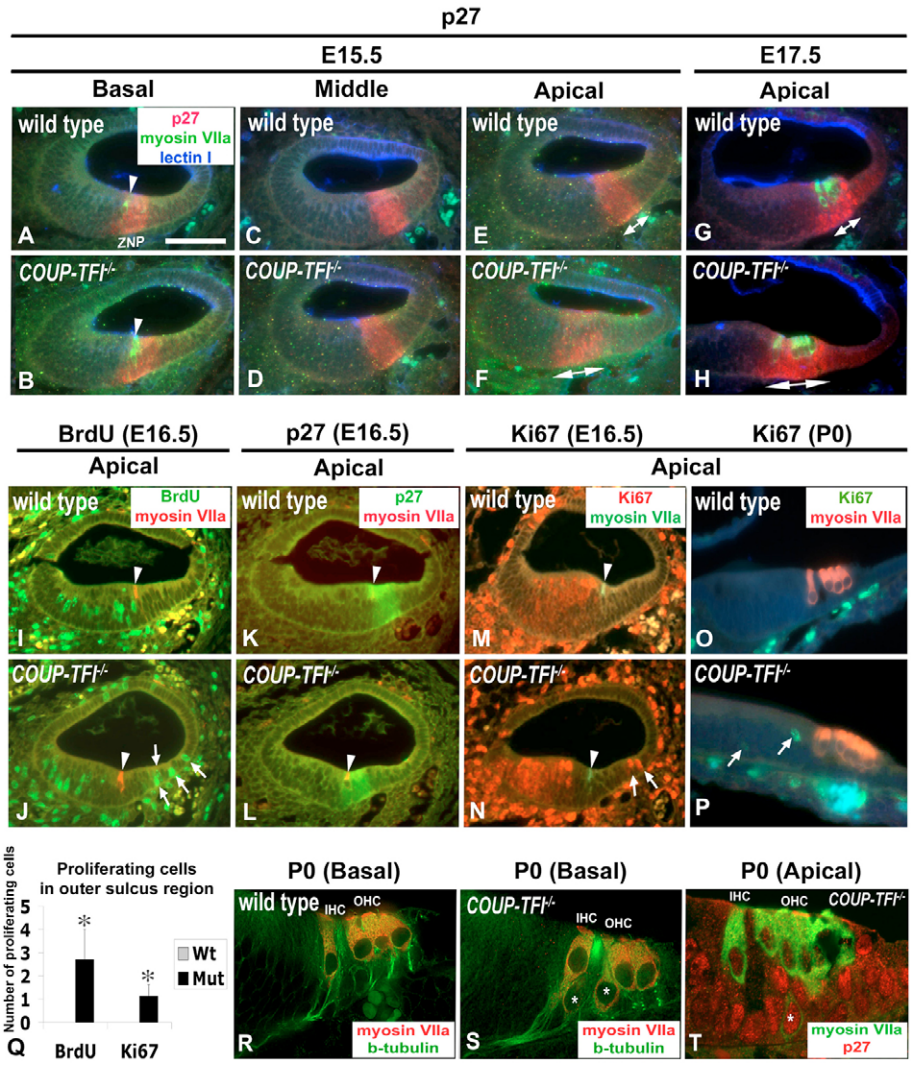

Fig. 2. *COUP-TFI*$^{-/-}$ sensory epithelium exhibited extra proliferating cells and ectopic hair cell differentiation in the supporting cell region.
(**A-H**) Immunofluorescence detection of p27 (red) to mark the zone of non-proliferation (ZNP) in the sensory epithelia in wild-type (A,C,E,G) and *COUP-TFI*$^{-/-}$ (B,D,F,H) cochlea at E15.5 (A-F) and E17.5 (G,H). The ZNP was similar at the basal and middle cochlear turns (A-D). However, the ZNP domain at the apex was widened (compare double arrows in E with F) and encompassed extra hair cells (compare G with H) in the *COUP-TFI*$^{-/-}$ mutants (myosin VIIa, green; GFI lectin I, blue). The onset of hair cell differentiation was unchanged in the *COUP-TFI*$^{-/-}$ basal (A,B) and apical (E,F) regions (arrowheads point to myosin VIIa-positive cells). (**I**,**J**) BrdU labeling (green) in wild-type (I) and *COUP-TFI*$^{-/-}$ (J) epithelia at E16.5 shows extra positive cells in the outer sulcus region of the apical turn in the *COUP-TFI*$^{-/-}$ mutants (arrows). Sections were co-localized with myosin VIIa (red, arrowheads). (**K**,**L**) p27 (green) expression in adjacent sections labeled with BrdU, confirmed the BrdU-positive cells in outer sulcus region were outside the ZNP. (**M-P**) Immunolocalization of Ki67 in wild-type (M,O) and *COUP-TFI*$^{-/-}$ (N,P) cochlea at E16.5 (M,N) and P0 (O,P). Proliferating cells were found in the outer sulcus region at E16.5 (red) and inner sulcus region at P0 (green) in the apical turn of *COUP-TFI*$^{-/-}$ mutants (arrows). (**Q**) Quantification of BrdU-positive and Ki67-positive cells at E16.5. (**R-T**) Deconvolution microscopy images show ectopic expression of myosin VIIa (asterisks in S and T) in the supporting cell compartment of the *COUP-TFI*$^{-/-}$ duct at P0. Scale bars: in A-P, 40 μm; in R-T, 100 μm.

more restricted domain, and consistently at a lower intensity in the middle turn at E16.5 (Fig. 3M-P). By contrast, the expression domain of *Lfng* at E14.5 was confined to a narrow region of the sensory epithelium in the wild-type cochlear duct (Fig. 3R, circle), whereas in *COUP-TFI*$^{-/-}$ ducts, the expression domain was significantly expanded (Fig. 3U, circle). The COUP-TFI expression domain in wild type (Fig. 3S) encompassed the expanded *Lfng* expression domain within the sensory epithelium (compare arrowheads in Fig. 3R,U with 3S). By E15.5, *Lfng* expression was primarily confined to the morphologically defined greater epithelial ridge (GER) (Sher, 1971) in the wild-type organ (Fig. 3W-Y, arrows), whereas, in the *COUP-TFI*$^{-/-}$ duct, *Lfng* exhibited a reduced level and was detected in a widened domain encompassing the GER and lesser epithelial ridges (LER) along the length of the duct (Fig. 3A′-C′, arrows; compare expression in the middle to apical turns in 3B′ to 3X, and 3C′ to 3Y). These changes in expression levels were confirmed by quantitative real-time RT-PCR (data not shown).

Dose-dependent inhibition of γ-secretase induces myosin VIIa expression in a cochlea organ culture

We used an in vitro organ culture system (Montcouquiol and Kelley, 2003) to determine the role of Notch signaling in regulating hair cell differentiation in *COUP-TFI*$^{-/-}$ cochlea. We used an inhibitor of γ-secretase, DAPT, to inhibit Notch signaling (Dovey et al., 2001; Geling et al., 2002), as presenilin-dependent γ-secretase mediates proteolytic cleavage and activation of the Notch intracellular domain (De Strooper et al., 1999). We used qRT-PCR and western blotting to analyze changes in expression of myosin VIIa levels as a marker of hair cell differentiation (Hasson et al., 1995). Myosin VIIa transcripts (Fig. 4A) and protein (Fig. 4B) were increased in a dose-dependent manner upon addition of DAPT (0.5 to 5 μM) to organ cultures, with a statistically significant increase at 5 μM DAPT with respect to the DMSO control group.

Expression of *COUP-TFI* and *COUP-TFII* are unchanged by DAPT treatment

We next determined whether DAPT treatment affected COUP-TFI and its homolog *COUP-TFII* in the in vitro organ culture model (Fig. 4C). Addition of DAPT in the cochlea cultures did not alter *COUP-TFI* and *COUP-TFII* expression, even at the highest DAPT concentration that induced hair cell differentiation (5 μM, Fig. 4A). The expression of *COUP-TFI* and *COUP-TFII* transcripts was unchanged by DAPT treatment, even after normalizing to the values for myosin VIIa to exclude the possibility of changes in *COUP-TFI* and *COUP-TFII* expression as a consequence of hair cell number changes induced by Notch inactivation.

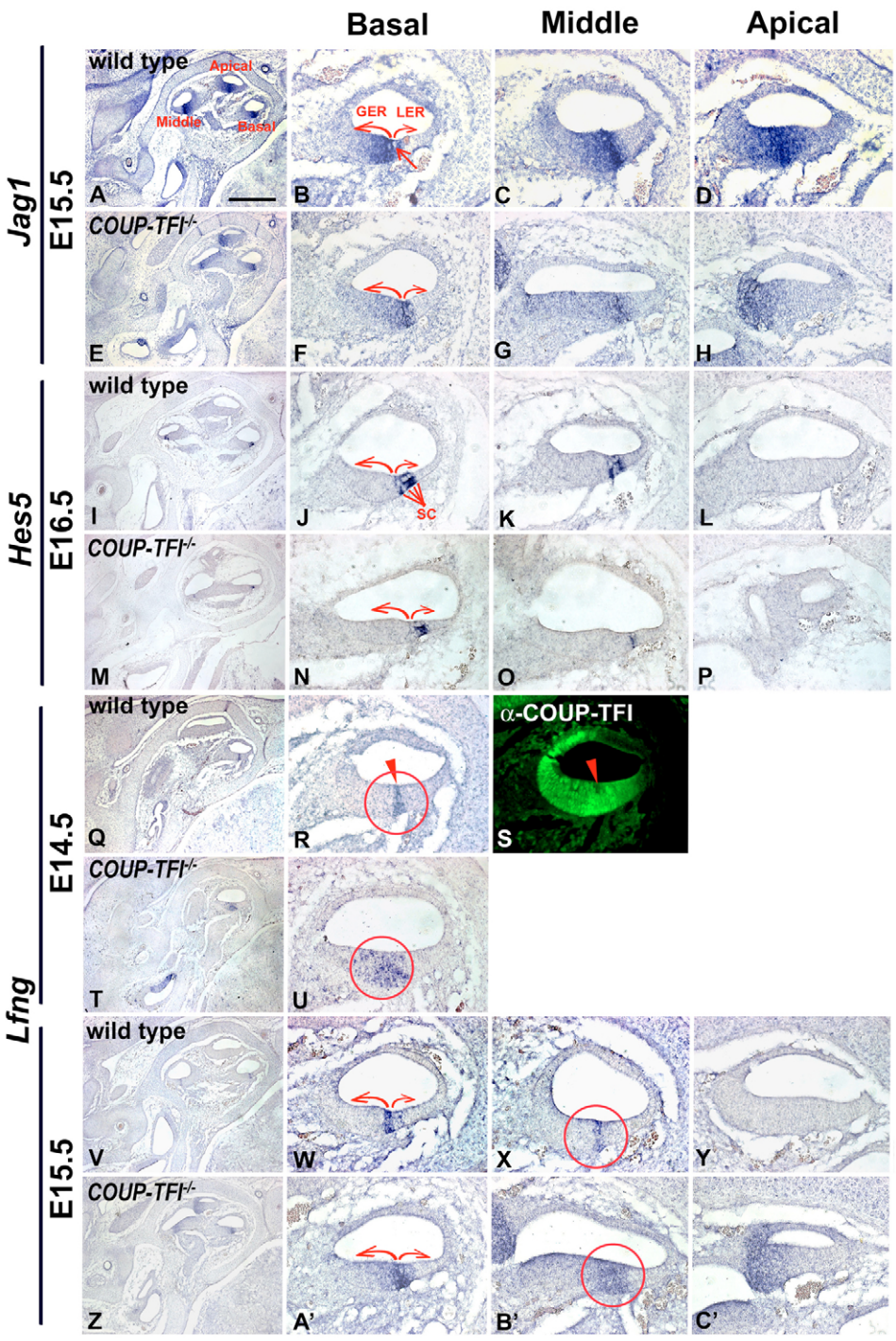

Fig. 3. Deregulation of Notch signaling molecules in *COUP-TFI*$^{-/-}$ cochlea. (**A-C'**) In situ hybridization on sections of wild-type (A-D,I-L,Q-R,V-Y) and *COUP-TFI*$^{-/-}$ (E-H,M-P,T,U,Z-C') cochlea for *Jag1* (A-H), *Hes5* (I-P), and *Lfng* (Q,R,T-C'). (A-D) At E15.5, *Jag1* in wild type was predominately expressed in the region where the non-sensory cells (arrow) are differentiating. (E-H) In *COUP-TFI*$^{-/-}$ cochlea, there was a lack of upregulation of *Jag1* in the supporting cells region. (I-P) *Hes5* expression in wild type (I-L) was restricted to the Deiter's supporting cells (SC) of the sensory epithelium, whereas in *COUP-TFI*$^{-/-}$ mutants (M-P), *Hes5* expression was downregulated. (Q,R,T-C') At E14.5, there was a dramatic expansion in the *Lfng* expression domain (compare R and U, circles); at E15.5, expansion to the greater epithelial ridge (GER) was observed only in the wild type (W-Y), whereas expansion to the lesser epithelial ridge (LER) was also observed in *COUP-TFI*$^{-/-}$ mutants (A'-C'). (S) Immunofluorescence staining of COUP-TFI (α-COUP-TFI) in wild-type sensory epithelium at E14.5 (arrowhead marks a hair cell). Scale bar: in A,E,I,M,Q,T,V,Z, 200 μm; in B-D,F-H,J-L,N-P,R,S,U,W-Y,A'-C', 50 μm.

Dose-dependent inactivation of Notch signaling induces hair cell differentiation in cochlea organ cultures

In order to confirm that DAPT treatment of the cochlea cultures resulted in inhibition of Notch signaling, we studied the expression of *Hes5*, a direct downstream target of Notch in hair cell differentiation (Lanford et al., 2000; Zine et al., 2001). *Hes5* expression was reduced with increasing concentrations of DAPT, reaching statistical significance at 2.5 and 5 μM (Fig. 4D). This reduction was detected in both wild-type and *COUP-TFI*$^{-/-}$ cochleae. Interestingly, there was also a dramatic reduction in *Hes5* expression in *COUP-TFI*$^{-/-}$ cochleae when compared with the wild-type culture with addition of 5 μM DAPT, and when compared with the levels of both wild-type and *COUP-TFI*$^{-/-}$ cochleae at 2.5 μM DAPT. We expected to see changes in *Hes1*, as *Hes1*$^{-/-}$ cochleae had an increase of IHCs (Zine et al., 2001); however, the changes we detected by RT-PCR were not statistically significant. This suggests that *COUP-TFI*$^{-/-}$ cochleae were more sensitive to Notch inactivation.

Analysis of myosin VIIa expression in DAPT-treated cultures confirmed that Notch inactivation resulted in a dose-dependent increase in myosin VIIa expression in wild-type and *COUP-TFI*$^{-/-}$ cochleae (Fig. 5A). Again, statistical significance was reached at 2.5 and 5 μM DAPT, with respect to the corresponding DMSO control

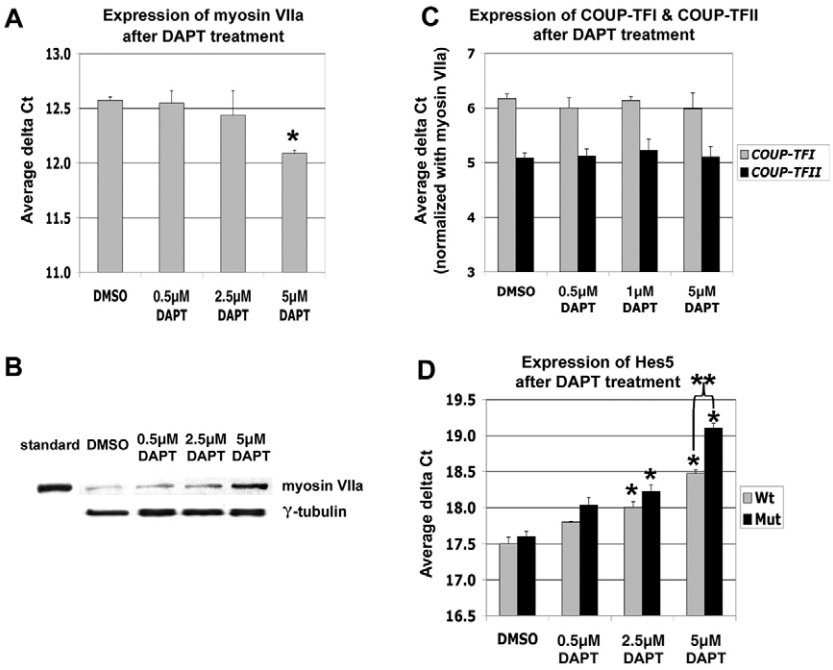

Fig. 4. DAPT treatment of cochleae in culture induces hair cell differentiation. (**A**) Real-time PCR analysis of myosin VIIa transcripts is presented in terms of average ΔCt (see Materials and methods). A greater Ct value represents a lower transcript expression level. Histogram and bars represent average ΔCt±s.d. (*P<0.05). High DAPT doses increase myosin VIIa expression, with significant induction at 5 μM DAPT. (**B**) Western blot confirmation of myosin VIIa protein expression changes with DAPT dose. The myosin band of Kaleidoscope prestained standard is used as control. (**C**) Expression of *COUP-TFI* and *COUP-TFII* transcripts in cochleae treated with DAPT. *COUP-TFI* and *COUP-TFII* expression was not changed with DAPT, even at the highest concentration of 5 μM. Because DAPT can induce hair cell differentiation (in terms of myosin VIIa expression as shown in Fig. 4A), the value is presented here after further normalization with the myosin VIIa values. (**D**) Expression of *Hes5* in cochleae after culture with DAPT. *Hes5* expression is reduced with increasing concentrations of DAPT, with statistically significant differences at 2.5 and 5 μM (*P<0.05). A statistically significant reduction was also found between *COUP-TFI*$^{-/-}$ and wild type at 5 μM (**P<0.05).

groups. Most importantly, at the 5 μM dosage, the elevation of myosin VIIa expression in the *COUP-TFI*$^{-/-}$ cochleae was statistically significant when compared with the wild type. The increase in myosin VIIa transcripts was accompanied by a concomitant increase in protein (Fig. 5B) that was restricted to hair cells (refer to Fig. 5D). These data confirm that *COUP-TFI*$^{-/-}$ cochleae have a greater sensitivity for hair cell differentiation than wild-type cochleae in culture.

Apical hair cell differentiation is sensitized to Notch inactivation

To quantify the induction of hair cell differentiation along the length of the cochlear duct, we counted the number of inner and outer hair cell rows from serial sections of cochleae (n=6 per group) after culture (Fig. 5C). Wild-type cochleae treated with DMSO consistently displayed four hair cell rows along the entire length of the duct (Fig. 5C), whereas DMSO-treated *COUP-TFI*$^{-/-}$ cochleae exhibited an elevated number of hair cell rows with an increase in the mid-basal to apical direction, having six rows at the apical turn (Fig. 5C). Addition of 5 μM DAPT induced an increase in the number of hair cell rows along the length of duct in both wild-type and *COUP-TFI*$^{-/-}$ cochleae (Fig. 5C), with a greater induction in *COUP-TFI*$^{-/-}$, as suggested by the myosin VIIa expression data (Fig. 4). More importantly, there was a significant elevation in hair cell rows at the apical turn in both wild-type (seven rows) and *COUP-TFI*$^{-/-}$ (nine rows) cochleae. Notably, there was also a significant difference in the induction of hair cells in wild-type and *COUP-TFI*$^{-/-}$ cochlea at the apical turn. Representative images from wild-type and *COUP-TFI*$^{-/-}$ cochlea with the treatment of DMSO and DAPT at apical turn are shown in Fig. 5D.

DISCUSSION

The *COUP-TFI*$^{-/-}$ organ of Corti exhibits an increase in hair cell rows, particularly OHCs in the apical turn. The increase in OHCs in *COUP-TFI*$^{-/-}$ mice superficially resembles Notch signaling loss of function. Expression of the Notch signaling pathway molecules *Jag1* and *Hes5* was reduced, whereas *Lfng* expression was enhanced in a broadened domain in *COUP-TFI*$^{-/-}$ sensory epithelium, consistent with a suppression of Notch signaling. Suppressing Notch activation in cochleae organ cultures increases hair cell number without altering *COUP-TFI* and *COUP-TFII* levels in the wild type. This treatment still exacerbates the in vivo cochlear phenotype in *COUP-TFI*$^{-/-}$ and yields further increases in hair cell numbers compared with the wild-type cochleae cultures. On the basis of these results, we conclude that the misregulation of the Notch signaling pathway is a primary factor in the development of the supernumerary hair cells and support cells in *COUP-TFI*$^{-/-}$ cochlea.

Suppression of Notch signaling induces hair cell and support cell differentiation

Notch signaling is important in cell fate determination, and for hair cell and support cell differentiation (Brooker et al., 2006; Daudet and Lewis, 2005; Heitzler and Simpson, 1991; Kiernan et al., 2001; Lanford et al., 1999; Zhang et al., 2000; Zine et al., 2000). Notch signaling plays at least two roles during inner ear development. The Notch ligand Jag1 regulates neuroepithelial patterning (Kiernan et al., 2001; Tsai et al., 2001) and lateral induction of sensory progenitors (Brooker et al., 2006; Daudet and Lewis, 2005; Kiernan et al., 2006; Murata et al., 2006). Within each patch of sensory progenitors, the ligands Dll1 and Jag2 act synergistically through Notch1 to mediate lateral inhibition, restricting the proportion of cells that differentiate as hair cells or supporting cells (Brooker et al., 2006; Kiernan et al., 2005; Murata et al., 2006).

In *COUP-TFI*$^{-/-}$ cochleae, deregulated Notch signaling included expansion of the *Lfng* expression domain to encompass the LER and GER in the early sensory epithelium before hair cells and supporting cells differentiated. *Lfng* is expressed in support cells, and *Lfng*$^{-/-}$ mice have no overt hair cell phenotype but mutation of *Lfng* acts epistatically with *Jag2* to suppress the production of extra IHCs (Zhang et al., 2000). The fringe genes modulate Notch activation (Bruckner et al., 2000; Moloney et al., 2000) and, thus, the expansion of the *Lfng* expression domain in *COUP-TFI*$^{-/-}$ sensory epithelium should have resulted in reduced Notch activation. Reduced Notch signaling was detected as a decrease in the Notch

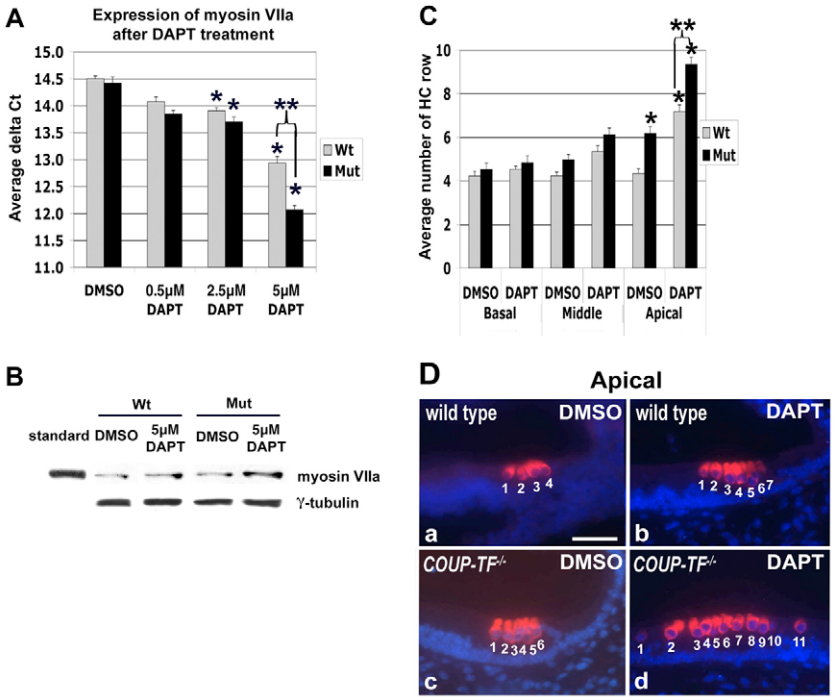

Fig. 5. Quantification of hair cell differentiation and rows in wild-type and *COUP-TFI*$^{-/-}$ cochlea after DAPT treatment. (**A**) Real-time PCR analysis of myosin VIIa transcripts shows hair cell differentiation is dependent on DAPT dose. There is a significant difference in the *COUP-TFI*$^{-/-}$ compared with wild type at the concentration of 5 μM (**P<0.05). (**B**) Western blot confirmation of myosin VIIa protein expression after treatment with 5 μM DAPT shows an increase with DAPT treatment. The myosin band of Kaleidoscope prestained standard is used as control. (**C**) Hair cell row counts (myosin VIIa-positive inner and outer hair cells) along the length of the cochlear duct after culture with 5 μM DAPT. Both wild-type and *COUP-TFI*$^{-/-}$ cochleae showed significant increases in hair cell rows after DAPT treatment (*P<0.05), and *COUP-TFI*$^{-/-}$ mutants had a significantly greater number than wild type in the apex (**P<0.05). (**D**) Immunostained sections of DAPT-treated cochleae at the apical region show an increase in hair cell rows in the *COUP-TFI*$^{-/-}$ mutants (c,d) compared with wild type (a,b; note, image in D part d is an extreme case of extra hair cells). Sections show hair cells expressing myosin VIIa (red) and nuclei counterstained with DAPI (blue). Scale bar: 20 μm.

target gene *Hes5*, which would have relieved the inhibitory action of Notch on Math1 (Chen et al., 2002; Kawamoto et al., 2003; Woods et al., 2004; Zheng and Gao, 2000). This resulted in hair cell differentiation in a widened ZNP in the *COUP-TFI*$^{-/-}$ cochleae (see Fig. 6). More studies will be needed to understand the full potential of elevated LFNG in modulating hair cell and support cell differentiation.

Suppressing the activating cleavage of Notch with a γ-secretase inhibitor modulates Notch signaling and induces hair cell differentiation. DAPT has been shown to regulate γ-secretase in a dose-dependent manner, and hence Notch signaling in mouse kidney (Cheng et al., 2003) and thymus cultures (Doerfler et al., 2001; Hadland et al., 2001), and in whole animals (Dovey et al., 2001; Geling et al., 2002). Although γ-secretase modulates the activation of several proteins (Kimberly and Wolfe, 2003), inhibition of γ-secretase produces phenotypes similar to those of Notch mutations in zebrafish (Geling et al., 2002) and *Drosophila* (Micchelli et al., 2003), strongly suggesting that the disruption of Notch signaling is the dominant effect of DAPT treatment. Using increasing concentrations of DAPT in cochleae explants, we have discovered that Notch regulates hair cell differentiation in a dose-dependent manner in the mouse cochlea. The concentrations of DAPT used were not toxic and did not seem to modify levels of genes non-specifically, as the expression levels of γ-tubulin, COUP-TFI and COUP-TFII were unaltered. Pharmacological suppression of Notch activation with a different γ-secretase inhibitor demonstrated a Notch-dependent regulation of excess hair cell development, primarily in the middle to basal turns (Yamamoto et al., 2006). It is unclear whether this difference of where in the duct excess hair cells differentiate is due to the different γ-secretase inhibitor used, the stage of culture, or both. Importantly, high doses of DAPT further attenuated Notch signaling to produce a greater number of hair cells in the apex in *COUP-TFI*$^{-/-}$ than in wild-type cochleae. Consistent with our results, genetic redundancy and gene-dose effects of Notch signaling components have been documented for several Notch signaling mutants that produce excess hair cells in the organ of Corti (Kiernan et al., 2005; Zhang et al., 2000; Zine et al., 2000), further indicating that Notch signaling was not completely suppressed in *COUP-TFI*$^{-/-}$ mice in vivo.

Conditional deletion of Jag1 results in loss of hair cells, particularly OHCs (Brooker et al., 2006; Kiernan et al., 2006), thus its reduction does not explain the excess hair cells in the *COUP-TFI*$^{-/-}$ cochlea. A comparison with the Dll1 conditional-deletion cochlea reveals phenotypical similarities to *COUP-TFI*$^{-/-}$ that include excess OHCs and an occasional duplication of IHCs, with expansion of the ZNP/prosensory region from the middle turn to apex and a shortened cochlear duct. In addition, deletion of a Notch downstream effector, *Foxg1*, produced a large increase in hair cells and supporting cells in the mid-apical turn, but it was unclear whether there was an equivalent number of hair cells and support cells because the organ of Corti was significantly disrupted (Pauley et al., 2006). These similarities further support our contention that COUP-TFI modulates hair cell and support cell development by regulating Notch signaling.

It has been suggested that signals required for hair cell differentiation are conveyed within the plane of the epithelium along the basal-to-apical axis of the developing cochlea (Montcouquiol and Kelley, 2003). Our data suggest that the potential for hair cells and support cells to differentiate depends on their position in the cochlear duct, and that those in the apical region have the greatest susceptibility to Notch signaling. This may explain why it is not uncommon to find more than the typical three rows of outer hair cells in the apical region of the wild-type cochlear duct in vivo. COUP-TFI protein and mRNA expression are highest at the apical turn during hair cell differentiation (Tang et al., 2005), further supporting the hypothesis that COUP-TFI function is required to regulate hair cell and support cell differentiation in a Notch-dependent and position-dependent manner.

Relationship of the shortened cochlear duct and excess hair cells

Radial and mediolateral intercalation of cells is the basis for the movements of convergence and extension that function in gastrulation, neurulation and formation of the vertebrate body axis

(Keller et al., 2000; Zajac et al., 2000). The process of convergent-extension is suggested to promote outgrowth and morphogenesis of the avian basilar papilla (Goodyear and Richardson, 1997) and the mammalian cochlear duct (Chen et al., 2002). The lack of longitudinal extension of the *COUP-TFI*$^{-/-}$ duct may have resulted in a short cochlear duct, and displacement and accumulation of precursor cells that differentiated at an inappropriate position but still resulted in a similar total number of the hair cells as in the wild type. Alternatively, the ectopic differentiation of support cells into hair cells in *COUP-TFI*$^{-/-}$ apex, which may have occurred at the expense of the support cells, was sufficiently compensated by continued proliferation in the sensory epithelium and resulted in a relatively well-patterned organ of Corti with corresponding numbers of hair cells and support cells. This hypothesis is not without precedent: hair cells continued to be produced postnatally because of an uncontrolled cell cycle and continued proliferation in cyclin kinase inhibitor *p27*$^{-/-}$ mutants (Chen and Segil, 1999), and *Dll1/Jag2* double mutants have supernumerary hair cells that arose through a switch in cell fate but also have prolonged cell proliferation (Kiernan et al., 2005). Thus, excess hair cell differentiation may hinder the process of convergent-extension, or defects in convergent-extension may promote excess hair cell differentiation at the middle to apex. Nevertheless, inhibiting lateral inhibition has similar effects, causing the production of excess hair cells and support cells and a shortened cochlear duct; subtle differences may occur depending on the method/target of mutation.

Model of COUP-TFI regulation of Notch-dependent differentiation of hair cells and support cells

Studies of Math1 (Atoh1) mutation (Bermingham et al., 1999), over- or ectopic expression (Chen et al., 2002; Kawamoto et al., 2003; Woods et al., 2004; Zheng and Gao, 2000), neurogenin1 (Ma et al., 2000; Matei et al., 2005) and *Foxg1* (Pauley et al., 2006) mutations, Notch overexpression (Daudet and Lewis, 2005) and underexpression (Brooker et al., 2006; Kiernan et al., 2005; Kiernan et al., 2006; Yamamoto et al., 2006; Zine et al., 2001), localization of cell-specific and timing of Notch activation (Murata et al., 2006), retinoid agonist and antagonist treatments in culture (Kelley et al., 1993; Raz and Kelley, 1997), cell ablation studies (Kelley et al., 1995), and other studies, have suggested ways by which supernumerary hair cells and/or supporting cells may arise. Extra cells may arise by: (1) expansion of the precursor cell pool (Daudet and Lewis, 2005); (2) secondary division of precursor cells (Brooker et al., 2006; Chen et al., 2002; Kiernan et al., 2005; Kiernan et al., 2006); (3) proliferation of supporting cells that then transdifferentiate into hair cells (Malgrange et al., 1998; Zheng and Gao, 2000); and (4) the recruitment and differentiation of adjacent non-sensory cells, including cells from the GER and outer sulcus (Brooker et al., 2006; Goodyear and Richardson, 1997; Woods et al., 2004; Yamamoto et al., 2006; Zheng and Gao, 2000).

A functional hierarchy of Notch and COUP-TFI regulation of hair cell and support cell differentiation is presented in Fig. 6. Our data suggest that the expansion of *Lfng* expressing cells and reduced Notch signaling in the precursor cell pool contributed to the increase in hair cell and support cell numbers in *COUP-TFI*$^{-/-}$ cochlea. The fact that *COUP-TFI* transcripts were unaffected by DAPT inhibition of Notch signaling suggests that COUP-TFI acts in parallel or upstream of Notch to regulate hair cell and support cell differentiation. COUP-TFI can directly regulate cell proliferation, differentiation and migration in different tissues. For example, the *Drosophila* homolog, *seven-up*, controls cell proliferation in the Malpighian tubules (Kerber et al., 1998); overexpression of COUP-TFI in neurons blocks morphological differentiation (Neuman et al., 1995); and COUP-TFI regulates tangential cell migration in the developing forebrain (Tripodi et al., 2004). As *COUP-TFI* transcripts and protein co-localize with *Lfng* and *Jag1* in the sensory epithelium (Tang et al., 2005), COUP-TFI might directly modulate the transcription of these Notch component genes and, thus, may constitute a regulatory mechanism to control hair cell and support cell numbers.

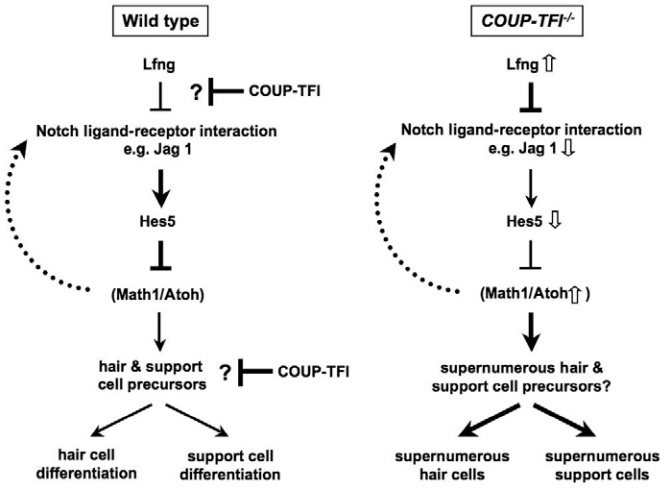

Fig. 6. A model of the functional hierarchy of COUP-TFI modulation of Notch signaling in hair cell and support cell differentiation. In the wild type, *Lfng* modulates Notch activities by limiting Notch ligand-receptor interactions. This process is regulated by the restricted expression of *Lfng* that allows strong binding of Notch with ligands such as *Jag1*, which promotes Notch signaling and the expression of downstream target genes, such as *Hes5*. *Hes5* inhibits the expression of *Math1*, leading to a normal limit of hair cell and support cell differentiation. COUP-TFI may directly modulate these Notch components in regulating hair cell and support cell differentiation. The absence of COUP-TFI results in expansion of the *Lfng* expression domain and an increasing inhibition of Notch ligand-receptor interactions, and results in a reduced expression of *Hes5*. Reduced Hes5 levels have a less inhibitory effect on *Math1* transcription, which allows Math1 to induce extra hair cell differentiation.

In summary, the orphan nuclear receptor COUP-TFI plays a crucial role in regulating cochlear hair and support cell differentiation. Inactivation of the *COUP-TFI* gene results in supernumerary hair cells and support cells, especially at the apical turn, which is also the region most susceptible to Notch inactivation. We conclude that COUP-TFI plays an important role in modulating Notch-mediated hair cell differentiation.

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