β-Catenin Up-regulates Atoh1 Expression in Neural Progenitor Cells by Interaction with an Atoh1 3′ Enhancer*§

Received for publication, August 25, 2009, and in revised form, October 27, 2009 Published, JBC Papers in Press, October 28, 2009, DOI 10.1074/jbc.M109.059055

Fuxin Shi1,9, Yen-fu Cheng9,*, Xiaohui L. Wang†, and Albert S. B. Edge15,2

From the 1Department of Otology and Laryngology, Harvard Medical School, Boston, Massachusetts 02115, the 2Eaton-Peabody Laboratory, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts 02114, the 3Program in Speech and Hearing Bioscience and Technology, Division of Health Science and Technology, Harvard and MIT, Cambridge, Massachusetts 02139, and the 4Department of Internal Medicine, Boston University School of Medicine, Boston, Massachusetts 02118

Atoh1, a basic helix-loop-helix transcription factor, plays a critical role in the differentiation of several epithelial and neural cell types. We found that β-catenin, the key mediator of the canonical Wnt pathway, increased expression of Atoh1 in mouse neuroblastoma cells and neural progenitor cells, and baseline Atoh1 expression was decreased by siRNA directed at β-catenin. The up-regulation of Atoh1 was caused by an interaction of β-catenin with the Atoh1 enhancer that could be demonstrated by chromatin immunoprecipitation.

We found that two putative Tcf-Lef sites in the 3′ enhancer of the Atoh1 gene displayed an affinity for β-catenin and were critical for the activation of Atoh1 transcription because mutation of either site decreased expression of a reporter gene downstream of the enhancer. Tcf-Lef co-activators were found in the complex that bound to these sites in the DNA together with β-catenin. Inhibition of Notch signaling, which has previously been shown to induce bHLH transcription factor expression, increased β-catenin expression in progenitor cells of the nervous system. Because this could be a mechanism for up-regulation of Atoh1 after inhibition of Notch, we tested whether siRNA to β-catenin prevented the increase in Atoh1 and found that β-catenin expression was required for increased expression of Atoh1 after Notch inhibition.

Progenitor cells in several tissues require the basic helix-loop-helix (bHLH) transcription factor, Atoh1, for their development into mature neurons or epithelial cells (1, 2). Upstream regulators of Atoh1 are likely to have an important role in the regulation of development in the central and peripheral nervous systems and in the intestinal epithelium, all of which rely on Atoh1 for differentiation. This finding was clear from the analysis of an Atoh1-null mouse, which lacks many of the cell types of the intestinal epithelium, and has incomplete development of cerebellar and spinal neurons and a complete lack of inner ear hair cells (1). The expression of bHLH transcription factors is partly regulated by components of the Notch pathway (3–5), but these may be only a part of the complex regulatory circuits governing the timing and amount of bHLH transcription factor expression as well as the tissue specificity of expression.

In a search for genes that affected Atoh1 expression, a number of genes were tested for their effect on Atoh1 expression by screening of an adenoviral library that allowed us to express the genes in various cell types. One such gene was β-catenin, the intracellular mediator of the canonical Wnt pathway. Its overexpression in neural progenitor cells increased activity of a reporter construct containing GFP under the control of one of the Atoh1 enhancers (12). Atoh1 has a 1.7-kb enhancer 3′ of its coding region, which is sufficient to direct expression of a heterologous reporter gene in several Atoh1 expression domains in transgenic mice (13). A region with high homology is present in the human gene (13).

Previous studies had shown that Atoh1 suppression was controlled by Notch signaling4 but did not identify the factors that increased Atoh1 after Notch inhibition. We found that β-catenin expression was increased after inhibition of Notch signaling and that this increase accounted for the effect of Notch inhibitors on Atoh1 expression. This indicated that expression of β-catenin was normally prevented by active Notch signaling and that β-catenin occupied a position upstream of Atoh1 in these cells. We found that β-catenin bound to the Atoh1 enhancer along with Tcf-Lef transcriptional co-activators, indicating that it directly affected Atoh1 expression.

**MATERIALS AND METHODS**

**Cell Culture**—Neuro2a cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum, 2 mM Glutamax, and penicillin (100 units/ml)/streptomycin (100 μg/ml).

---

*This work was supported, in whole or in part, by National Institutes of Health Grants R01 DC007174 and P30 DC05209 from the NIDCD and by the Hamilton H. Kellogg and Mildred H. Kellogg Charitable Trust.*

†Supported by scholarships from the Yen Tjing Ling Medical Foundation and Foundation for Poison Control, Taiwan.

‡To whom correspondence should be addressed: Eaton-Peabody Laboratory, Massachusetts Eye and Ear Infirmary, 243 Charles St., Boston, MA 02114. Tel.: 617-573-4452; Fax: 617-720-4408; E-mail: albert_edge@meei.harvard.edu.

§The abbreviations used are: bHLH, basic helix-loop-helix; GFP, green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; MSC, mesenchymal stem cells; ChIP, chromatin immunoprecipitation; DAPT, N-(N-(3,5-difluorophenacetyl-L-alanyl))S-phenylglycine t-buty ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
ROSA26 mouse embryonic stem cells (15) and Pofut1\(^{-/-}\) and Pofut1\(^{+/+}\) embryonic stem cells (16) were grown in KO-DMEM (Invitrogen) supplemented with 15% fetal calf serum, 100 \(\mu\)M non-essential amino acids, 55 \(\mu\)M \(\beta\)-mercaptoethanol, 2 mM Glutamax, 12 ng/ml LIF (Chemicon), penicillin (100 units/ml), and streptomycin (100 \(\mu\)g/ml). The cells were dissociated and cultured in suspension in DMEM supplemented with N2 (Invitrogen) to generate nestin-positive neural progenitors (17). Mesenchymal stem cells (MSC) were isolated from human bone marrow cells as described (18). The cells were expanded once before use and cultured in MEM-\(\alpha\) supplemented with 9% horse serum, 9% fetal calf serum, and penicillin (100 units/ml) and streptomycin (100 \(\mu\)g/ml).

Cells were treated with a \(\gamma\)-secretase inhibitor (DAPT, N-[N-(3,5-difluorophenacetyl-l-alanyl)]-S-phenylglycine \(\beta\)-butyly ester, Calbiochem-EMD Bioscience) or a GS3K\(\beta\) inhibitor, (SB415286, Sigma). The compounds were stored as 40 mM stock in DMSO at \(-20^\circ\)C and added at the concentrations given in the text. Wnt3a-conditioned medium was harvested from L-Wnt3a cells (from ATCC, CRL-2647), which were stably transfected with a Wnt3a expression vector and secrete biologically active Wnt3a protein. Control conditioned medium harvested from the parental cell line L (from ATCC, CRL-2648) was used in experiments involving the Wnt3a-conditioned medium. L cells and L-Wnt3a cells were culture in DMEM with 10% fetal calf serum and supplement of 0.4 mg/ml G-418 for L-Wnt3a cells. The conditioned medium was harvested according to the ATCC protocol, sterile-filtered, and stored at \(-20^\circ\)C until use.

**Plasmid Constructs and Site-directed Mutagenesis**—Atoh1-Luc with the Atoh1 3’ enhancer controlling expression of firefly luciferase (Luc) was described previously.\(^{5}\) Site-directed mutagenesis was performed using the QuikChange\textsuperscript{TM} II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Atoh1-Luc was denatured and annealed to the oligonucleotide primers, TAT CAC CCA AAC AAA tcc gGA GTC AGC ACT TCT T (965–1012)/CAG AGG CAA GGA GTC ACC CCC ggc acg TCT GGC TCC TAA CTG AAA AAG (945–992), with the mutations in Tcf-Lef binding sites (lowercase) underlined in the primers. Following temperature cycling, circular DNA was generated from the template vector containing the incorporated mutated Tcf-Lef binding sites using PfuTurbo DNA polymerase, and methylated, parenteral DNA was digested with DpnI endonuclease. Finally the circular, nicked dsDNA was transformed into competent cells for repair.

**Gene Silencing and Transfection**—siRNAs for silencing of Atoh1 (NM_007500, NM-005172, gca acg uaa ucc cgu ccu u UAA CAG CGA UGA UGG CAC A) and \(\beta\)-catenin (NM_007614, NM_001904, GCG CUU GGC UGA ACC AUC AUU, GUG AAA UUC UUG GCU AUU AUU) were obtained from Dharmacon. The more efficient of two siRNA sequences for gene silencing based on quantitative PCR was chosen for each gene. The siRNA (200 nM) was combined with siRNA transfection reagent GeneSilencer\textsuperscript{TM} (5 \(\mu\l/ml; Gene Therapy Systems) and incubated with the cells for 16 h. Cells were harvested at 48 h. Non-targeting siRNA was transfected in parallel as a control. Transfection efficiency was determined with the fluorescent-labeled non-targeting siRNA. Cells were counted on an epifluorescent microscope (Axioskop 2 Mot Axiocam; Zeiss) and analyzed with a Metamorph Imaging System. Real-time RT-PCR was performed after exposure to the targeting siRNA and the non-targeting siRNA to confirm gene silencing.

Cells to be used for the measurement of gene expression were seeded onto 10-cm dishes and transfected with Atoh1 (18), GFP, control pcDNA3 vector, Notch intracellular domain (NICD) (20), \(\beta\)-catenin (21), or dominant-negative Tcf4 expression vector (22) using 5 \(\mu\)g of DNA per 15 \(\mu\l\) of Lipofectamine 2000 (Invitrogen) in 5 ml of opti-MEM for 10\(^6\) cells seeded. Transfection was carried out for 4 h, and after washing, followed by incubation with medium. Cells were harvested at 24 h.

**RT-PCR**—Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen), and 1 \(\mu\g\) of RNA was subjected to RT-PCR with SuperTranscript\textsuperscript{TM} III and TaqDNA polymerase (New England Biolabs). The primer sequences were as follows: Atoh1: forward, AGA TCT ACA TCA ACG CTC TGT C; reverse, ACT GGC CTC ATC AGA GTC ACT G (449bp); GAPDH, forward, AAC GGG AAG CCC ATC ACC; reverse, CAG CCT TGG CAG CAC CAG (442bp); \(\beta\)-catenin, forward, ATG CGC TCC CCT CAG ATG GTG TC; reverse, TCG CGG TGG TGA GAA AGG TGG TGC (113bp). Annealing temper-ature and cycles were optimized for each primer. PCR primers for real-time PCR of Atoh1 and S18 were ordered from Applied Biosystems, and PCR was performed in a Perkin Elmer ABI PRISM\textsuperscript{TM} 7700 Sequence Detector (PE Applied Biosystems).

**Chromatin Immunoprecipitation**—In brief, 5 \(\times\) 10\(^5\) Neuro2a cells (175-cm\(^2\) culture flasks) were cross-linked with 1% formaldehyde in DMEM for 10 min, followed by 5 min at 37 °C in formaldehyde saturated with glycine. The cells were washed and in ice-cold phosphate-buffered saline and pelleted for 10 min at 720 \(\times\) g at 4 °C. The nuclei were released in a Dounce homogenizer in PBS containing protease inhibitors and collected at 4 °C by centrifugation at 2,400 \(\times\) g. Sheared chromatin was collected in the supernatant by centrifugation (8,000 \(\times\) g at 4 °C for 10 min) after treatment of the nuclei with the enzymatic mixture from a ChIP-IT\textsuperscript{TM} Express kit (Active Motif) for 10 min at 37 °C. An aliquot (10 \(\mu\l\) of the sheared DNA was saved as the input sample for PCR, and the rest (3 \(\times\) 80 \(\mu\l\)) was split for immunoprecipitation using 1 \(\mu\g\) of mouse anti-\(\beta\)-catenin antibody (Upstate, 05-601, 1:100), mouse anti-LEF-1 antibody (Sigma L7901), or nonimmune mouse serum (Sigma). The precipitated chromatin was recovered after reversing cross-links, and the proteins were digested with protease K. Target Atoh1 regulatory DNA (AF218258) was amplified by PCR using primers: forward, ACG TGT GGC AGC TCC CTC TC; reverse, ATA GTT GAT GCC TTT GTT AGT A (33-72); forward, ATT CCC CAT ATG CCA GAC CAC; reverse, GGC AAA GAC AGA ATA TAA AAC AAG (148-434); forward, AAT CGG GGT AGT TCT TTG; reverse, ACT CCC CCT CCT TTT CTT GTA (349-609); forward, CAC GGG GAG CTG AAG GAA G; reverse, TTT TAA GTT AGC AGA GGA GAT GTA (501-742); forward, CTG AGG CCC AAA GTT GTA ATG TT; reverse, TGG GGT GCA GAG AAG

---

\(^{5}\) S. J. Jeon, M. Fujioka, K. I. Seyb, E. R. Schuman, M. L. Michaelis, M. Glicksman, and A. S. B. Edge, in preparation.
Activation of the Atoh1 Enhancer by β-Catenin Binding

ACT AAA (675–939); forward, ACC CCA GGC CTA GTG TCT CC; reverse, TGC CAG CCC CTC TAT TGT CAG (296–1161); forward, GTG GGG GTA GTT TGC CGT AAT GTG; reverse, GGC TCT GGC TTC TGT AAA CTC TGC (1094–1367).

DNA Pull-down Assay—Nuclei were isolated from 10^6 Neuro2a cells following mechanical disruption with a 20-gauge needle. Proteins were extracted from nuclei in 200 μl of radio-immune precipitation assay buffer (RIPA) (Sigma) with fresh proteinase inhibitors (2 mM phenylmethylsulfonyl fluoride; protease inhibitor mixture, Sigma) at 4 °C for 60 min. Chromatin DNA was pelleted at 14,000 × g for 15 min at 4 °C, and the nuclear lysate in the supernatant was collected. Biotin-labeled DNA probes (0.3 μg) with or without 10 μg of unlabeled DNA probe were incubated with 40 μl of nuclear lysate with proteinase inhibitors for 30 min at room temperature in binding buffer (10 mM Tris, 50 mM KCl, 1 mM dithiothreitol, 5% glycerol, pH 7.5, 40 mM 20 mer poly A and poly C). Probes used were (mutations shown in lowercase): ATC ACC ACA AAG AAA GAG TCA GCA CTT (297–326); ATC ACC ACA AAG ACG aAG TCA GCA CTT (297–326); GTT AGG AGC GAG TCA GCA CTT (297–326); ATC ACC CAA ACA AAC AAA ATC ACC CAA ACA AAC AAA ATC ACC CAA ACA AAC AAA.

RESULTS

Overexpression of β-Catenin Up-regulates Atoh1 Expression—The effect of β-catenin overexpression and silencing on Atoh1 levels was measured in two neural cell types, a neuroblastoma cell line and neural progenitors derived from embryonic stem cells.

Overexpression of β-catenin in Neuro2a cells increased expression of Atoh1 mRNA based on RT-PCR (β-cat, Fig. 1A). Expression of Atoh1 was also up-regulated in neural progenitors after overexpression of β-catenin (β-cat, Fig. 1A). The increase was significant for both Neuro2a cells (871.3 ± 141.3) and neural progenitors (741.2 ± 218.2) as determined by quantitative RT-PCR compared with cells transfected with a GFP vector without β-catenin (1.1 ± 0.1) or untreated cells (1 ± 0.2) (p < 0.01, Fig. 1B). Atoh1 expression was also increased after transfection with Atoh1 cDNA as a control (Atoh1, Fig. 1, A and B).

Atoh1 expression was decreased by Atoh1 siRNA (siRNA-Atoh1, Fig. 1C), used as a positive control, but not by non-targeting siRNA (siRNA-non-targ, Fig. 1C) or no siRNA (Ctl, Fig. 1C). The extent of the decrease in Atoh1 mRNA after silencing with siRNA directed to Atoh1 was 57 ± 1.4% (Fig. 1D). β-Catenin siRNA decreased β-catenin expression by 62 ± 6% as determined by quantitative RT-PCR. Silencing β-catenin expression with siRNA directed to β-catenin decreased Atoh1 mRNA levels in Neuro2a cells and neural progenitors based on RT-PCR (Fig. 1C). Down-regulation of β-catenin decreased Atoh1 expression by 37.2 ± 1.0% in Neuro2a cells and 21.8 ± 0.5% in neural progenitors. The decrease in Atoh1 expression determined by quantitative RT-PCR after treatment with β-catenin siRNA was significant (p < 0.01, Fig. 1D).

The level of β-catenin activity as measured by the TOPFlash reporter, which contains multiple binding sites for β-catenin complexed to Tcf-Lef co-activators, was proportional to the concentration of β-catenin cDNA (Fig. 1E), and the increase in TOPFlash signal correlated with an increased expression of Atoh1. Increased amounts of β-catenin cDNA also raised the level of the active fraction of nuclear β-catenin, which is recognized by an antibody (24) to the unphosphorylated form (β-catenin*) in correlation with Atoh1 (Fig. 1F). Wnt3a-conditioned medium (Wnt3a) also resulted in parallel increases in the level of both nuclear unphosphorylated β-catenin and Atoh1, whereas overexpression of dominant-negative Tcf4 (dnTcf4), which lacks the β-catenin binding site, decreased the level of Atoh1. These experiments demonstrated a correlation between the level of expression of β-catenin and Atoh1.

β-Catenin Binds to Tcf-Lef Sites within the Atoh1 Enhancer and Binding Leads toActivation of the Enhancer—To define the binding sites on the mouse Atoh1 enhancer, we searched the 3' enhancer sequence. The software indicated two potential binding sites for β-catenin in combination with Tcf-Lef transcriptional co-activators at 309–315 and 966–972 (AF218258). To investigate whether β-catenin in combination with Tcf-Lef factors has a direct interaction with regulatory regions of the Atoh1 gene, we analyzed the DNA binding to β-catenin and Tcf-Lef by chromatin immunoprecipitation. Sheared and cross-linked chromatin was immunoprecipitated with β-cate-
Activation of the Atoh1 Enhancer by β-Catenin Binding

FIGURE 1. β-Catenin overexpression increased Atoh1 mRNA levels, and β-catenin silencing decreased Atoh1 mRNA levels. A, analysis of Atoh1 mRNA expression by RT-PCR showed that transfection of β-catenin (β-cat) into both Neuro2a cells and neural progenitors increased Atoh1 mRNA compared with untransfected cells (Ctl), whereas GFP transfection did not increase Atoh1 mRNA. Atoh1 transfection (Atoh1) was used as a positive control, and GAPDH was used as an internal control. B, increase in Atoh1 expression was quantified by real-time PCR in Neuro2a cells and neural progenitors from two independent experiments (each experiment in triplicate). The cells were transfected with either β-catenin (β-cat) or Atoh1 (Atoh1) as positive controls or GFP (GFP) as a negative control. Atoh1 levels are expressed relative to untreated control cells (Ctl) and normalized to S18, a housekeeping gene. The increase in Atoh1 expression relative to the control was significant for both cell types transfected with β-catenin or Atoh1 (marked by asterisk). C, Atoh1 mRNA expression was analyzed by RT-PCR in Neuro2a cells and neural progenitors treated with siRNA. Atoh1 expression was increased in the cells treated with β-catenin siRNA (siRNA-β-cat) compared with non-targeting siRNA (siRNA-non-targ) or no siRNA (Ctl). Cells treated with Atoh1 siRNA (siRNA-Atoh1) were used as a positive control. D, decrease in Atoh1 expression was quantified by real-time PCR from two independent experiments (each experiment in triplicate). The cells were transfected with β-catenin siRNA (siRNA-β-cat) or non-targeting siRNA (siRNA-non-targ). Atoh1 levels are expressed relative to untreated control cells (Ctl) and normalized to S18. Significant decreases in expression of Atoh1 are indicated by asterisks. E, activation of Tcf-Lef-mediated transcription was measured by TOPFlash luciferase reporter, and increased Atoh1 expression was quantified by real-time RT-PCR. Transfection of β-catenin (1 μg/ml or 5 μg/ml) increased TOPFlash activity (TOPFlash, dashed line) and Atoh1 mRNA expression in neural progenitor cells. FOPFlash (FOPFlash, dotted line), which contains mutant Tcf/Lef-binding sites remained unchanged in the cells transfected with β-catenin. F, nuclear fraction of unphosphorylated β-catenin and Atoh1 was examined by Western blotting in neural progenitors transfected with β-catenin, treated with Wnt3a, or transfected with dominant-negative Tcf4 (dnTcf). Overexpression of β-catenin increased the level of activated nuclear β-catenin (β-catenin*) and Atoh1. Wnt3a-conditioned medium compared with control conditioned medium (Ctl) also increased the level of active nuclear β-catenin and Atoh1. Conversely, overexpression of dominant-negative Tcf4 decreased the level of Atoh1.

β-Catenin and Tcf-Lef antibody immunoprecipitated DNA at the 5′- and 3′-ends of the 1.3-kb sequence indicating that DNA in the regions had an affinity for both of the proteins (Fig. 2, β-catenin and Tcf/Lef). The intermediate sequences did not display this affinity suggesting that this sequence contained binding sites at its ends as predicted. These sequences were not amplified by PCR from chromatin immunoprecipitated with nonimmune IgG (Fig. 2, serum), whereas the same DNA fragments were co-precipitated by β-catenin and Tcf-Lef antibodies. This shows that these proteins bound to the same sequences of DNA. The precise localization of the binding sites could not be ascertainment from the PCR data because the shearing of the DNA was variable, and, despite repeated attempts, the variable lengths of sheared DNA led to different patterns of bands from the overlapping primers.

To determine the precise sequences that had an affinity for β-catenin and Tcf-Lef we performed DNA pull-down assays with two biotin-labeled oligonucleotides probes, covering bases 297–326 and 956–986 of the Atoh1 sequence, which contained the predicted Tcf-Lef-binding sites and surrounding nucleotides. Incubation of the probes with nuclear lysate from Neuro2a cells was followed by precipitation with streptavidin beads. By Western blotting of the proteins interacting with the probes, both β-catenin and Tcf-Lef could be detected (Fig. 3A, probe 309 and probe 966). The binding of β-catenin and Tcf-Lef to the probes was reduced by competition with unlabeled probes (Fig. 3A, comp 309 and comp 966), and mutation of the predicted binding sites reduced binding in both cases (Fig. 3A, mutant 309 and mutant 966). The correlation between β-catenin binding and Tcf-Lef binding suggested that the proteins were involved in a complex, and this agreed with the ChIP data showing that both proteins could be precipitated from the binding sites in the native DNA. This experiment also suggested that both of the potential binding sites on the Atoh1 enhancer bound to the complex of Tcf-Lef with β-catenin.
The expression of Atoh1 in Neuro2a cells transfected with β-catenin (1 μg/ml) or co-transfected with dominant-negative Tcf4 was analyzed by real-time PCR from two independent experiments (each experiment in triplicate). The increase in Atoh1 expression after overexpression of β-catenin was inhibited in reverse proportion to the concentration of dominant-negative Tcf4 (p < 0.01, Fig. 3B), and the extent of inhibition was nearly complete at the higher level, indicating that a complex with Tcf-Lef was required for activation of Atoh1 by β-catenin.

To determine whether the two β-catenin binding sites on the Atoh1 enhancer increased the functional activity of the Atoh1 enhancer, we constructed Atoh1 enhancer-reporter genes with an intact or mutated Atoh1 3′ enhancer. Each of the β-catenin binding sites in the Atoh1 enhancer were mutated, alone or in combination, in a luciferase reporter construct (Fig. 4). We found that overexpression of β-catenin had no effect on a luciferase construct without the Atoh1 enhancer (Fig. 4, Luc), but transfection of β-catenin at the same time as the reporter containing the native Atoh1 enhancer increased reporter gene expression (Fig. 4, Atoh1-Luc). Up-regulation of Atoh1 enhancer activity was reduced when the first β-catenin binding site was mutated (Fig. 4, Atoh1-Luc, mutant 309). β-Catenin-mediated up-regulation of the Atoh1 reporter was also reduced when the second β-catenin binding site in the Atoh1 enhancer was mutated (Fig. 4, Atoh1-Luc, mutant 966). Double mutation of the binding sites completely abolished β-catenin-mediated up-regulation (Fig. 4, Atoh1-Luc, 2X mutant) (p < 0.01). This indicated that β-catenin binding to the Atoh1 enhancer at the binding sites at 309–315 and 966–972 increased activity of the enhancer. The absolute level of the increase likely results from this activity combined with subsequent auto-activation of the 3′ enhancer caused by binding of Atoh1 to its own enhancer (25). To determine the contribution of this auto-feedback loop, we directly transfected the cells with Atoh1 in combination with the Atoh1-luciferase reporter. Atoh1 overexpression up-regulated both unaltered and double mutant Atoh1 reporter about 1.5-fold, indicating that auto-feedback did not account for the level of activation found with β-catenin, but could add to the reporter activity by binding independently to the Atoh1 enhancer.

Increased Expression of Atoh1 after Notch Inhibition Is Partly Caused by β-Catenin Expression—Because Notch also regulates Atoh1 expression, we sought to determine whether increased expression of Atoh1 after treatment with a γ-secretase inhibitor to inhibit Notch signaling was affected by β-catenin.

To observe the effect of a γ-secretase inhibitor, we assessed expression of β-catenin in bone marrow-derived MSCs, cells that were previously shown to have increased Atoh1 expression after Notch inhibition.3 Inhibition of Notch signaling by the γ-secretase inhibitor and up-regulation by transfection of Notch intracellular domain was confirmed in these cells with a CBF-1 luciferase reporter (data not shown). The expression of β-catenin was increased in MSCs treated with a γ-secretase inhibitor (Fig. 5A, DAPT; quantification of these results is shown in the supplemental data). Atoh1 expression was increased by the inhibition of Notch signaling in the cells that had increased β-catenin (Fig. 5A). A GSK3β inhibitor (GSKi) also increased Atoh1, consistent with its ability to increase β-catenin in these cells (Fig. 5A).

To determine whether β-catenin expression was related to increased expression of Atoh1 seen after treatment of these cells with a γ-secretase inhibitor, we blocked β-catenin expression with siRNA and measured the influence on Atoh1. Incubation of MSCs with siRNA to β-catenin decreased β-catenin expression as much as 70% based on quantitative RT-PCR (Fig. 5B). The siRNA prevented the increase in β-catenin that could be seen after treatment with a γ-secretase inhibitor (Fig. 5C) and decreased the level of Atoh1 expression (Fig. 5C).

We tested the effect of disrupting the Notch pathway by a method other than a γ-secretase inhibitor. Comparison of Atoh1 in wild-type neural progenitor cells and Pofut1−/− cells that have a mutation that prevents Notch signaling showed that Atoh1 expression was higher in the cells that lacked Notch signaling (Fig. 5D), and a γ-secretase inhibitor, which increased Atoh1 expression in control cells, did not further increase Atoh1 expression in Pofut−/− cells, as determined by quantitative RT-PCR (Fig. 5D). To see if increased Atoh1 was caused by the higher expression level of β-catenin, we blocked β-catenin expression with siRNA. The deceased level of β-catenin resulted in a decrease in expression of Atoh1 (Fig. 5D), confirming that β-catenin signaling was partly responsible for the increased level of Atoh1 found under conditions of Notch blockage.
Activation of the Atoh1 Enhancer by β-Catenin Binding

Disruption of β-catenin-mediated transcription by overexpression of dominant-negative Tcf (dnTcf) reversed the increase of Atoh1 expression in cells treated with the Notch inhibitor (Fig. 5E). Conversely, whereas β-catenin and Atoh1 expression were diminished in cells after elevation of Notch activity, activation of β-catenin-mediated transcription by Wnt3a (Wnt3a) returned the expression of Atoh1 to control levels.

Notch could influence the level of β-catenin by regulation of GSK3β activity. Inhibition of Notch decreased the level of phosphorylated GSK3β (GSK3β*/Y216) and increased the nuclear fraction of unphosphorylated β-catenin (Fig. 5F, β-catenin*), resulting in increased Atoh1 and suggesting that Notch could down-regulate β-catenin by increasing its degradation via increased GSK3β activity. Activation of Notch increased phosphorylated GSK3β but left total GSK3β unchanged, and this appeared to correlate with a decrease in Atoh1 3′ enhancer (13). Thus, whatever factor first activates Atoh1 transcription has a powerful role. Upstream regulators of Atoh1 have been postulated as initiators of the self-perpetuating process and the maintenance of the cochlea does not occur until after this time (1). Hence, Atoh1 expression, β-Catenin expression was required for the increased expression of Atoh1 caused by Notch inhibition because siRNA to β-catenin prevented the increase in Atoh1.

Finding upstream regulators of Atoh1 is a key to understanding its role in cell specification because, once it is activated, Atoh1 transcription is self-perpetuating. This is due to binding of Atoh1 protein to the unphosphorylated β-catenin and a reduction in Atoh1 expression (Fig. 5F). Thus, Notch regulated GSK3β activity and thereby controlled the level of β-catenin and Atoh1.

**DISCUSSION**

Multiple influences control the timing of Atoh1 expression, a key factor in development of the nervous system and the maintenance of intestinal epithelial cell. We show here that β-catenin signaling triggers Atoh1 expression by interacting with sites within the 3′ enhancer of the Atoh1 gene. Binding of β-catenin to the enhancer occurred in conjunction with Tcf-Lef co-activators. Putative Tcf-Lef sites found in this regulatory region both displayed an affinity for Tcf-Lef and β-catenin. We found that both sites were critical for full activation of the Atoh1 gene, and this led to increased transcription of Atoh1 from the endogenous gene. Inhibition of Notch signaling, which has previously been shown to induce Atoh1 expression, was found to increase β-catenin expression in progenitor cells of the nervous system. β-Catenin expression was required for the increased expression of Atoh1 caused by Notch inhibition because siRNA to β-catenin prevented the increase in Atoh1.

Finding upstream regulators of Atoh1 is a key to understanding its role in cell specification because, once it is activated, Atoh1 transcription is self-perpetuating. This is due to binding of Atoh1 protein to the unphosphorylated β-catenin and a reduction in Atoh1 expression (Fig. 5F). Thus, Notch regulated GSK3β activity and thereby controlled the level of β-catenin and Atoh1.

**DISCUSSION**

Multiple influences control the timing of Atoh1 expression, a key factor in development of the nervous system and the maintenance of intestinal epithelial cell. We show here that β-catenin signaling triggers Atoh1 expression by interacting with sites within the 3′ enhancer of the Atoh1 gene. Binding of β-catenin to the enhancer occurred in conjunction with Tcf-Lef co-activators. Putative Tcf-Lef sites found in this regulatory region both displayed an affinity for Tcf-Lef and β-catenin. We found that both sites were critical for full activation of the Atoh1 gene, and this led to increased transcription of Atoh1 from the endogenous gene. Inhibition of Notch signaling, which has previously been shown to induce Atoh1 expression, was found to increase β-catenin expression in progenitor cells of the nervous system. β-Catenin expression was required for the increased expression of Atoh1 caused by Notch inhibition because siRNA to β-catenin prevented the increase in Atoh1.

Finding upstream regulators of Atoh1 is a key to understanding its role in cell specification because, once it is activated, Atoh1 transcription is self-perpetuating. This is due to binding of Atoh1 protein to the unphosphorylated β-catenin and a reduction in Atoh1 expression (Fig. 5F). Thus, Notch regulated GSK3β activity and thereby controlled the level of β-catenin and Atoh1.

---

**FIGURE 3. β-Catenin interacts with the Atoh1 3′ enhancer in a complex with Tcf-Lef.** A, nuclear lysate from Neuro2a cells was incubated with biotin-labeled DNA probes corresponding to enhancer sequence 297–326 (probe 309) and 956–985 (probe 966), which contained two predicted β-catenin/Tcf-Lef binding sequences, and Atoh1 enhancer-binding proteins were collected with magnetic beads coupled to streptavidin. Proteins were analyzed by Western blotting using antibodies specific for β-catenin and Tcf-Lef. β-Catenin (β-catenin) was found in the bead-eluted proteins (probe 309 and probe 966), indicating an interaction with the probes; binding was inhibited by competition with excess unlabeled probes (comp 309 and comp 966). Binding was inhibited when predicted β-catenin/Tcf-Lef binding sequences were mutated in the probes (mutant 309 and mutant 966). Tcf-Lef was also found in this fraction (Tcf-Lef) indicating formation of a complex involving Tcf-Lef factors and β-catenin. B, expression of Atoh1 in untransfected Neuro2a cells and neural progenitors (Ctl) was increased in the same cells transfected with 1 μg/ml β-catenin (β-cat). The level of Atoh1 was decreased in a dose-dependent manner when cells were co-transfected with dominant-negative Tcf4 (β-cat + dn Tcf). The Atoh1 levels were analyzed by real-time PCR in two independent experiments (each experiment in triplicate). The asterisks mark significant differences in Atoh1 expression compared with control.
inner ear is consistent with the possibility that β-catenin stimulates Atoh1 expression in the early embryo.

Because Notch is another key regulator of Atoh1 expression, we investigated whether disruption of Notch signaling could lead to alterations in β-catenin that would account for the increased expression of Atoh1. We found that, when released from Notch regulation, β-catenin expression was increased in neural progenitor cells and MSCs. Increased expression of β-catenin was demonstrated after preventing Notch activity with a γ-secretase inhibitor. Notch exerted its effect by a mechanism involving phosphorylation at Tyr-216 of GSK3β, a key enzyme in the regulation of β-catenin activity. The increase in β-catenin resulted in an up-regulation of Atoh1 in these cells. A similar increase was found in cells that carry a mutation that prevents Notch signaling. When the mutant cells were treated with siRNA to β-catenin and siRNA to β-catenin, indicating that β-catenin was necessary as a mediator of increased Atoh1 expression after disruption of Notch signaling.

Notch is a negative regulator of Atoh1 expression, but it has not been clear how Atoh1 expression could be initiated. Notch signaling is thought to prevent proneural bHLH transcription factor activity through the inhibitory actions of Hes family members (5). Previous studies have shown that Notch signaling regulates activity of β-catenin and can prevent nuclear localization and up-regulation of β-catenin genes, although other studies have shown contradictory effects of Notch on Wnt signaling. Models in which Notch signaling has been manipulated showed that β-catenin activity was inhibited by Notch in stromal cells from bone marrow and in muscle stem cells (28). These studies concluded that Notch signaling inhibited Wnt signaling by direct action of Hes1 or by activation of GSK3β (28). Activation of GSK3β was the apparent mechanism of Wnt inhibition in this study. Our study agrees with the conclusion that Notch inhibits Wnt signaling in neural progenitors and MSCs and could explain how inhibition of
to that of the Atoh1 knock-out mouse (2). Inhibition of Notch led to increased goblet cell differentiation, which is dependent on β-catenin signaling and Atoh1 expression (11). Overexpression of β-catenin stimulated Atoh1 in lung epithelium where it is not normally expressed (31). Our study for the first time shows that β-catenin directly regulates Atoh1 and provides a mechanism for the previous observation of an effect of β-catenin on expression of the Atoh1 gene.

Other bHLH transcription factors contain regulatory regions for β-catenin binding and have been shown to respond to β-catenin binding by increased expression. Cortical neural progenitor cells exposed to Wnt or transfected with activated β-catenin increased expression of Ngn1 in conjunction with their increased differentiation to neurons (32), and this was shown to be due to direct binding and activation of the Ngn1 promoter by β-catenin. A similar binding and increased activity of the Ngn1 promoter and the Ngn2 enhancer was reported in P19 embryonal carcinoma cells (33). Wnt plays a dual role in progenitor cells that can lead to their proliferation or differentiation. Wnt signaling is required for the expansion of progenitors in the CNS (8, 34). However, Wnt signaling can provide a signal that leads to terminal differentiation of neurons (32, 33, 35, 36) consistent with the activation of bHLH transcription factors as we have demonstrated in neural progenitors.

Unlike the activation of Atoh1 by β-catenin in neural progenitors, Wnt signaling is aberrant in tumor cells and the dependence of Atoh1 expression on Wnt signaling does not follow the normal pattern (2, 8, 19). Expression of Atoh1 protein was decreased in colon tumors despite increased nuclear β-catenin expression, and inhibition of β-catenin signaling increased Atoh1 expression (14). We have found an increase in Atoh1 mRNA expression but not protein expression in human colon cancer cells after overexpression of β-catenin, whereas both Atoh1 mRNA and protein are increased in HEK cells (data not shown). An explanation for this
Activation of the Atoh1 Enhancer by β-Catenin Binding

FIGURE 6. Schematic diagram illustrates regulation of Atoh1 by β-catenin. The positive sign and arrow represent the increased transcription of Atoh1 upon binding of β-catenin-Tcf/Lef to the Atoh1 3′-enhancer. The resulting Atoh1 acts to up-regulate its own expression by binding to the same enhancer. Stimulation by β-catenin accounts for the up-regulation of Atoh1 levels after Notch inhibition. Atoh1 levels are determined by negative regulation by transcription factors such as Hes1 and S when Notch signaling is active.

Atoh1 enhancer to activate expression of this key transcription factor.

Acknowledgments—We thank the following for generous gifts of reagents and cells: Oksana Berezovska for the NICD expression vector, Eric Fearon for the dominant-negative Tcf4 plasmid, Walter Birchmeier for the NICD expression vector, Diane Hayward for the CBF1-luciferase vector, and Pamela Stanley for the Pofut1−/− stem cells. We thank Kevin Jiang for technical assistance.

REFERENCES
1. Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eaton, R. A., Bellin, H. J., Lysakowski, A., and Zoghbi, H. Y. (1999) Science 284, 1837–1841
2. Yang, Q., Bermingham, N. A., Finegold, M. J., and Zoghbi, H. Y. (2001) Science 294, 2155–2158
3. Bertrand, N., Castro, D. S., and Guillemot, F. (2002) Nat. Rev. Neurosci. 3, 517–530
4. Hirata, H., Tomita, K., Bessho, Y., and Kageyama, R. (2001) EMBO J. 20, 4454–4466
5. Ross, S. E., Greenberg, M. E., and Stiles, C. D. (2003) Neuron 39, 13–25
6. Clevers, H. (2006) Cell 127, 469–480
7. Ohyama, T., Mohamed, O. A., Taketo, M. M., Dufort, D., and Groves, A. K. (2006) Development 133, 865–875
8. Pinto, D., and Clevers, H. (2005) Exp. Cell Res. 306, 357–363
9. Stevens, C. B., Davies, A. L., Battista, S., Lewis, J. H., and Fekete, D. M. (2003) Dev. Biol. 261, 149–164
10. van Es, J. H., Jay, P., Gregoireff, A., van Gijn, M. E., Jonkheer, S., Hatzis, P., Thiele, A., van den Born, M., Begthel, H., Brabletz, T., Taketo, M. M., and Clevers, H. (2005) Nat. Cell Biol. 7, 381–386
11. van Es, J. H., van Gijn, M. E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D. J., Radtke, F., and Clevers, H. (2005) Nature 435, 959–963
12. Lumpkin, E. A., Colisson, T., Parah, P., Omer-Abdalla, A., Haeberle, H., Chen, P., Doettloher, A., White, P., Groves, A., Segil, N., and Johnson, J. E. (2003) Gene Expr. Patterns 3, 389–395
13. Helms, A. W., Abney, A. L., Ben-Arie, N., Zoghbi, H. Y., and Johnson, J. E. (2000) Development 127, 1185–1196
14. Leow, C. C., Romero, M. S., Ross, S., Polakis, P., and Gao, W. Q. (2004) Cancer Res. 64, 6050–6057
15. Zambrowicz, B. P., Imamoto, A., Fiering, S., Herzenberg, L. A., Kerr, W. G., and Soriano, P. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 3789–3794
16. Stahl, M., Uemura, K., Ge, C., Shi, S., Tashima, Y., and Stanley, P. (2008) J. Biol. Chem. 283, 13638–13651
17. Corrales, C. E., Pan, L., Li, H., Liberman, M. C., Heller, S., and Edge, A. S. (2006) J. Neurobiol. 66, 1489–1500
18. Jeon, S. J., Oshima, K., Jho, E. H., and Joo, C. K. (2003) Cancer Res. 63, 517–530
19. Tsuchiya, K., Nakamura, T., Okamoto, R., Kanai, T., and Watanabe, M. (2007) Gastroenterology 132, 208–220
20. Berezovska, O., Jack, C., McLean, P., Aster, J. C., Hicks, C., Xia, W., Wolfe, M. S., Kimberly, W. T., Weinmaster, G., Selkoe, D. J., and Hyman, B. T. (2000) J. Neurochem. 75, 583–593
21. Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G., and Birchmeier, W. (2001) Cell 105, 533–545
22. Kolligs, F. T., Hu, G., Dang, C. V., and Fearon, E. R. (1999) Mol. Cell Biol. 19, 5696–5706
23. Hsieh, J. J., Henkel, T., Salmon, P., Robey, E., Peterson, M. G., and Hayward, S. D. (1996) Mol. Cell Biol. 16, 952–959
24. van Noort, M., Weerkamp, F., Clevers, H. C., and Staal, F. J. (2007) Blood 110, 2778–2779
25. Helms, A. W., Gowar, K., Abney, A., Savage, T., and Johnson, J. E. (2001) Mol. Cell Neurosci. 17, 671–682
26. Fritzsch, B., Pauley, S., Matei, V., Katz, D. M., Xiang, M., and Tessarollo, L. (2005) Hear Res. 206, 52–63
27. Riccomagno, M. M., Takada, S., and Epstein, D. J. (2005) Genes Dev. 19, 1612–1623
28. Brack, A. S., Conboy, I. M., Conboy, M. J., Shen, J., and Rando, T. A. (2008) Cell Stem Cell 2, 50–59
29. Proweller, A., Tu, L., Lepore, J. J., Cheng, L., Lu, M. M., Seykora, J., Millar, S. E., Pear, W. S., and Parmacek, M. S. (2006) Cancer Res. 66, 7438–7444
30. Deregowski, V., Gazzetello, E., Priest, L., Rydziel, S., and Canalis, E. (2006) J. Biol. Chem. 281, 6203–6210
31. Okubo, T., and Hogan, B. L. (2004) J. Biol. 3, 11
32. Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N., and Gotoh, Y. (2004) Development 131, 2791–2801
33. Israsena, N., Hu, M., Fu, W., Kan, L., and Kessler, J. A. (2004) Dev. Biol. 268, 220–231
34. Chenn, A., and Walsh, C. A. (2002) Science 297, 365–369
35. Lyu, J., Costantini, F., Jho, E. H., and Joo, C. K. (2003) J. Biol. Chem. 278, 13487–13495
36. Maretto, S., Cordenonsi, M., Dupont, S., Braggheita, P., Broccoli, V., Hassan, A. B., Volpin, D., Bressan, G. M., and Piccolo, S. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 3299–3304

400 JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 285 • NUMBER 1 • JANUARY 1, 2010