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Chibby Promotes Adipocyte Differentiation through Inhibition of β-Catenin Signaling▽

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The canonical Wnt/β-catenin signaling pathway plays diverse roles in embryonic development and disease. Activation of this pathway, likely by Wnt-10b, has been shown to inhibit adipogenesis in cultured 3T3-L1 preadipocytes and in mice. Here, we report that the β-catenin antagonist Chibby (Cby) is required for adipocyte differentiation. Cby is expressed in adipose tissue in mice, and Cby protein levels increase during adipogenic differentiation of 3T3-L1 cells. Ectopic expression of Cby induces spontaneous differentiation of these cells into mature adipocytes to an extent similar to that of dominant-negative Tcf-4. In contrast, depletion of Cby by RNA interference potently blocks adipogenesis of 3T3-L1 and mouse embryonic stem cells. In support of this, embryonic fibroblasts obtained from Cby-deficient embryos display attenuated differentiation to the adipogenic lineage. Mechanistically, Cby promotes adipocyte differentiation, in part by inhibiting β-catenin, since gain or loss of function of Cby influences β-catenin signaling in 3T3-L1 cells. Our results therefore establish Cby as a novel proadipogenic factor required for adipocyte differentiation.

Adipose tissue plays critical roles in the regulation of energy homeostasis by storing and releasing fuel as a reservoir and by secreting a number of hormones and cytokines as an endocrine organ (37). Excess body fat, or obesity, is a major public health problem, particularly in industrialized countries, increasing the risk of diabetes, cardiovascular diseases and several types of cancers (28, 37). Conversely, lipoatrophy, the lack of adipose tissue, is also associated with diabetes and a number of other metabolic abnormalities (32). Hence, understanding the signaling pathways that govern adipocyte differentiation is necessary to develop comprehensive therapeutic strategies for the prevention and treatment of these disorders.

Adipogenesis involves the formation of preadipocytes from mesenchymal progenitor cells and their differentiation into adipocytes (29, 33, 34). The cellular and molecular mechanisms of adipocyte differentiation have been extensively studied using preadipocyte culture systems, such as 3T3-L1 and 3T3-F442A cell lines (10, 31, 33, 34). In response to hormonal stimuli of adipogenesis, two transcription factors, CCAAT/enhancer-binding protein β (C/EBPβ) and C/EBPδ, are rapidly and transiently induced. These proteins then stimulate expression of the key adipogenic transcription factors, C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ), which act synergistically to induce expression of various adipocyte-specific genes.

Intracellular signaling by the Wnt family of secreted glycoproteins regulates cell proliferation, differentiation, and polarity throughout vertebrate embryonic development (27, 44, 46). β-Catenin plays a pivotal role as a transcriptional coactivator in the canonical Wnt pathway (39). In the absence of Wnt signaling, cytoplasmic β-catenin becomes phosphorylated by glycogen synthase kinase-3β (GSK-3β) in a complex containing Axin and the tumor suppressor Adenomatous polyposis coli and is targeted for ubiquitin-mediated proteasomal degradation (23, 30). Wnt binding to the seven transmembrane Frizzled receptors and the low-density lipoprotein receptor-related protein (LRP) coreceptors, LRP5 and LRP6, leads to inhibition of GSK-3β activity, resulting in stabilization of cytoplasmic β-catenin. Subsequently, stabilized β-catenin translocates into the nucleus and binds to the T-cell factor/lymphoid enhancer factor (Tcf/Lef) family of transcription factors, leading to activation of target genes (1, 2, 6, 26, 43).

The Wnt/β-catenin pathway has been shown to inhibit adipogenesis (3, 5, 7, 36). Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of C/EBPα and PPARγ. Wnt-10b is a good candidate for the endogenous signal because it is expressed in dividing and confluent preadipocytes, and its expression decreases during differentiation into mature adipocytes (5, 36). Forced expression of Wnt-10b or Wnt-1 in 3T3-L1 cells stabilizes free cytosolic β-catenin and blocks adipogenesis. In contrast, overexpression of negative regulators of the pathway, such as Axin or dominant-negative Tcf-4 (dnTcf-4), which binds to Tcf/Lef consensus binding sites but lacks the N-terminal β-catenin-binding domain, results in spontaneous adipogenesis. Moreover, transgenic mice overexpressing Wnt-10b in adipose tissues show a 50% reduction in total body fat and resist diet-induced obesity (22). Induction of PPARγ appears to further suppress canonical Wnt signaling by stimulating degradation of β-catenin by the proteasome (20, 21, 25).

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We previously reported a β-catenin-associated antagonist, Chibby (Cby) (11, 38). Cby is a 14.5-kDa nuclear protein evolutionarily conserved from fly to human. Cby physically interacts with the C-terminal activation domain of β-catenin and represses β-catenin-mediated transcriptional activation. Mechanistically, Cby competes with Tcf/Lef transcription factors to bind to β-catenin. Depletion of *Drosophila* Cby by RNA interference (RNAi) results in segment polarity defects that mimic the gain-of-function phenotype of a *Drosophila* Wnt homolog, *wingless* (*wg*), highlighting the biological importance of Cby's function (11, 38, 42).

In this report, we demonstrate that Cby is required for adipocyte differentiation. Cby protein levels increase during differentiation of 3T3-L1 preadipocyte cells. Intriguingly, ectopic expression of Cby in 3T3-L1 cells causes differentiation into mature adipocytes even without hormonal stimulation. In contrast, depletion of Cby by RNAi profoundly suppresses adipogenesis of 3T3-L1 and mouse embryonic stem (ES) cells. Mouse embryonic fibroblasts (MEFs) lacking Cby display a reduced ability to differentiate into the adipogenic lineage. Additionally, in 3T3-L1 cells, loss of Cby results in upregulation of β-catenin transcriptional activity, implying that Cby promotes adipogenesis, at least in part, by inhibiting β-catenin signaling. In support of this mechanism, overexpression of stabilized β-catenin partially suppresses Cby-induced adipogenesis, and furthermore, forced expression of dnTcf-4 rescues adipocyte differentiation of Cby-RNAi cells. Our findings suggest that Cby is a novel proadipogenic factor required for adipocyte differentiation.

MATERIALS AND METHODS

Cell culture and adipocyte differentiation. 293T cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% fetal bovine serum (FBS) and 100 units/ml penicillin-streptomycin.

Mouse 3T3-L1 preadipocytes (a kind gift from Jeffrey Pessin) were maintained in growth media containing DMEM supplemented with 10% calf serum (HyClone), 1 mM L-glutamine, and 100 units/ml penicillin-streptomycin at 37°C in 8% CO_2. 3T3-L1 cells were induced to differentiate into mature adipocytes as described previously (36). Briefly, 2 days after reaching confluence (day zero), cells were placed in differentiation media consisting of DMEM, 10% FBS, and the differentiation cocktail of 0.5 mM methylisobutylxanthine, 0.25 μM dexamethasone, and 1 μg/ml of insulin (MDI). Thereafter, the medium was replenished every 3 days.

Mouse ES cells were maintained in an undifferentiated state on gelatin-coated culture plates in Knock-out DMEM (Invitrogen) containing 10% Knockout-Serum Replacement (Invitrogen), 1% FBS (Atlanta Biologicals), 2 mM L-glutamine, 100 units/ml penicillin-streptomycin, 25 mM HEPES (Invitrogen), 300 μM monothioglycerol (Sigma), and 1,000 units/ml recombinant mouse leukemia inhibitory factor (Chemicon). The Cby knockdown ES and control cell lines were established by transfecting pSuppressor Retro-Cby short hairpin RNA (shRNA) or empty vector into R1 ES cells and selecting for 14 days with G418. Their differentiation into adipocytes was induced by the hanging-drop method essentially as described previously (9). Embryoid bodies were treated with retinoic acid prior to switching them to differentiation media.

To study adipogenesis of MEFs, wild-type and $Cby^{-/-}$ MEFs were prepared from embryonic day 12.5 (E12.5) embryos of $Cby^{+/-}$ crosses as described previously (24, 41). The generation and characterization of Cby knockout mice will be described elsewhere. MEFs were maintained in DMEM with 10% FBS and 100 units/ml penicillin-streptomycin and induced to undergo adipogenic differentiation 1 day postconfluence with MDI as described above for 3T3-L1 cells.

Retroviral constructs and infection. For ectopic expression, human Cby and dnTcf-4 cDNAs were amplified by PCR and subcloned into the retroviral vector pLXIN (neomycin resistant; Clontech). A stabilized form of human β-catenin (S33Y) and dnTcf-4 were subcloned into the retroviral vector pQCXIP (puromycin resistant; Clontech). A detailed description of the constructs is available upon request. The retroviral packaging cell line RetroPack PT67 (Clontech) was transfected with retroviral expression vectors using Lipofectamine 2000 (Invitrogen). After 48 h of transfection, the medium containing retroviruses was collected, filtered, treated with Polybrene, and used to infect 3T3-L1 fibroblasts. The infected cells were selected with either 500 μg/ml G418 (Invitrogen) alone or in combination with 2.5 μg/ml puromycin (Invitrogen) and maintained in growth media.

RNAi. For knockdown of Cby expression in 3T3-L1 and mouse ES cells, two complementary oligonucleotides for mouse Cby shRNA or Cby shRNA-ΔC harboring a single-base deletion in the targeted sequence were annealed and subcloned into the SalI and XbaI sites of the pSuppressor Retro vector (Imgenex) containing the U6 promoter and a neomycin resistance cassette. The target site corresponds to nucleotides 218 to 238 of the mouse Cby gene, with the A of the ATG start codon assigned as position 1. The sequences of the oligonucleotides were as follows: Cby shRNA-top, 5′-TCGAGTGGCAGACTCCGTGATTAGTTTCAAGAGAACTAATCACGGAGTCTGCCACTTTTT-3′; Cby shRNAi bottom, 5′-CTAGAAAAAGTGGCAGACTCCGTGATTAGTTCTCTTGAAACTAATCACGGAGTCTGCCAC-3′ (the underlined G is the deleted single nucleotide within the reverse primer). The C deleted in Cby shRNA-ΔC is underlined. All plasmids were verified by sequencing them. In order to examine the efficiency of RNAi, these constructs were transiently transfected into 293T cells in combination with a mouse Cby expression construct (38), and 24 h later, the cells were lysed and subjected to Western blotting for Cby as described below.

Western blot analyses. Monolayer cells or mouse epididymal fat pads were rinsed with phosphate-buffered saline (PBS), harvested in ice-cold lysis buffer (20 mM Tris-HCl [pH 8.0], 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, and 10% glycerol), and incubated for 20 min on ice with intermittent vortexing. After centrifugation for 30 min at 14,000 × *g* at 4°C, the supernatants were collected, and the protein concentrations of the extracts were determined by the Bradford assay (Bio-Rad). Equal amounts of protein were resolved by 4 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Invitrogen), transferred onto a nitrocellulose membrane (Bio-Rad), and immunoblotted with the following antibodies. Rabbit polyclonal anti-Cby antibodies were previously described (38). Anti-C/EBPα and anti-PPARγ antibodies were purchased from Santa Cruz. Anti-β-actin and anti-aP2 antibodies were from Abcam and ProSci, respectively. Anti-β-catenin was purchased from BD Transduction Laboratories, and anti-green fluorescent protein antibody was from Invitrogen. dnTcf-4 was tagged with the Flag epitope at the N terminus and detected with an anti-Flag M2 antibody (Sigma). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories. Membranes were visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce).

Tcf/Lef reporter assays. 3T3-L1 preadipocytes were transiently transfected using Lipofectamine Plus (Invitrogen). Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) as described previously (38, 40). Lucifearse activities were measured 48 h after transfection and normalized for transfection efficiency using the internal *Renilla* luciferase control.

Oil Red-O staining. Detection of neutral lipids with Oil Red-O was performed as described previously (36). Briefly, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 30 min, and then stained for 2 h at room temperature with Oil Red-O solution (0.5% Oil Red-O from Sigma in isopropanol). The cells were washed twice with PBS and visualized with an inverted microscope.

RNA extraction and RT-PCR. Total RNA was purified from epidydimal white adipose tissue (WAT), interscapular brown adipose tissue (BAT), and liver of 2-month-old C57BL/6J mice with RNeasy spin columns (QIAGEN), and synthesis of cDNA was performed with oligo(dT) primers using the ThermoScript reverse transcription (RT)-PCR System (Invitrogen) according to the manufacturer's instructions. The primer sequences were as follows: Cby (forward, 5′-CGTTTCCTCACTGAGTTAGG-3′, and reverse 5′-TAGTCTGCTAATCTGACGGG-3′); GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (forward, 5′-ACCACAGTCCATGCCATCAC-3′, and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′).

Total RNA from ES cells was extracted using the RNA aqueous kit (Ambion), and cDNA was synthesized as described above. Amplifications were performed using primers for C/EBPα (forward, 5′-GGACAAGAACAGCAACGAGTAC C-3′, and reverse, 5′-GGCGGTCATTGTCACTGGTC-3′), PPARγ (forward, 5′-CTCCTGTTGACCCAGAGCAT-3′, and reverse, 5′-ACCCTTGCATCCTTCACAAG-3′), aP2 (forward, 5′-GATGCCTTTGTGGGAACCTGG-3′, and reverse, 5′-TTCATCGAATTCCACGCCCAG-3′), and β-actin (forward, 5′-ATGGATGACGATATCGCTG-3′, and reverse, 5′-ATGAGGTAGTCTGTCAGGT-3′).

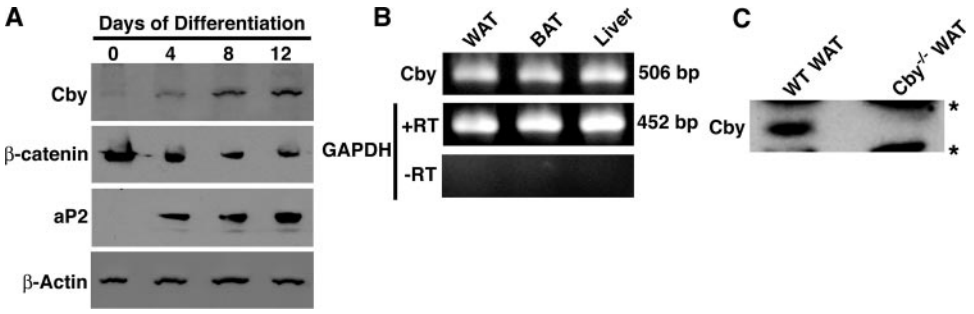

FIG. 1. Cby expression in cultured 3T3-L1 cells and murine adipose tissue. (A) Cby protein levels increase during differentiation of 3T3-L1 preadipocytes. Whole-cell lysates were extracted at the indicated days of differentiation and subjected to immunoblot analyses with antibodies against Cby, β-catenin, and the adipocyte differentiation marker aP2. β-Actin was used as a loading control. (B) Cby is expressed in WAT and BAT. Total RNA was prepared from epididymal WAT, interscapular BAT, and liver of 2-month-old C57BL/6J mice and used to synthesize cDNAs for RT-PCR for Cby. By Northern blot analysis of adult mouse tissues, liver was found to be one of the tissues in which Cby is highly expressed (data not shown), and it served as a positive control. GAPDH was used as a loading control. (C) Cby protein expression in WAT. Western blot analysis was performed on whole-cell lysates prepared from wild-type (WT) or Cby-deficient ($Cby^{-/-}$) epididymal WAT using anti-Cby antibodies. The stars denote nonspecific bands detected in both wild-type and $Cby^{-/-}$ samples.

RESULTS

Expression of Cby in differentiating 3T3-L1 cells and murine adipose tissue. Mouse 3T3-L1 preadipocytes are a well-characterized in vitro model of adipocyte differentiation that can reliably differentiate into mature fat cells upon exposure to MDI in the presence of FBS (12, 13). In order to investigate a potential role of Cby in adipogenesis, we first analyzed Cby expression levels during differentiation of 3T3-L1 preadipocytes into mature adipocytes. Interestingly, Western blot analysis demonstrated that Cby protein levels increased in a fashion similar to that of the adipocyte differentiation marker aP2 (adipocyte fatty acid-binding protein) and peaked at day 8 following hormonal stimulation (Fig. 1A). It is worthy of note that Cby protein levels inversely correlate with those of cytosolic β-catenin, and this is consistent with the previous notion that Wnt signaling needs to be suppressed for adipogeneis to occur (5, 36). In adult mice, relatively high levels of Cby mRNA expression were seen in both WAT and BAT (Fig. 1B). In agreement with this finding, Cby protein was detected in epididymal fat tissue from wild-type, but not Cby-null, animals (Fig. 1C).

Ectopic expression of Cby induces spontaneous differentiation of 3T3-L1 preadipocyte cells. The above-mentioned expression results suggested that Cby might be involved in adipocyte differentiation. It has been demonstrated that forced expression of negative regulators of the Wnt/β-catenin pathway, such as Axin or dnTcf-4 in 3T3-L1 cells, leads to spontaneous adipocyte differentiation in the absence of hormonal stimulation (36). Since Cby represses β-catenin-mediated transcriptional activation, overexpression of Cby in preadipocytes might similarly result in spontaneous adipogenesis.

To test this hypothesis, 3T3-L1 cells were infected with a retrovirus carrying human Cby or dnTcf-4 as a positive control, or a control retrovirus, and maintained without MDI treatment. As shown in Fig. 2A, the efficient expression of Cby in preadipocytes was confirmed by immunoblotting using anti-Cby antibodies. Two weeks postconfluence, control cells failed to differentiate and maintained fibroblast morphology (Fig. 2B). In accordance with the previous finding (36), ectopic expression of dnTcf-4 induced spontaneous differentiation of these cells as assessed by Oil Red-O staining. Cby-expressing

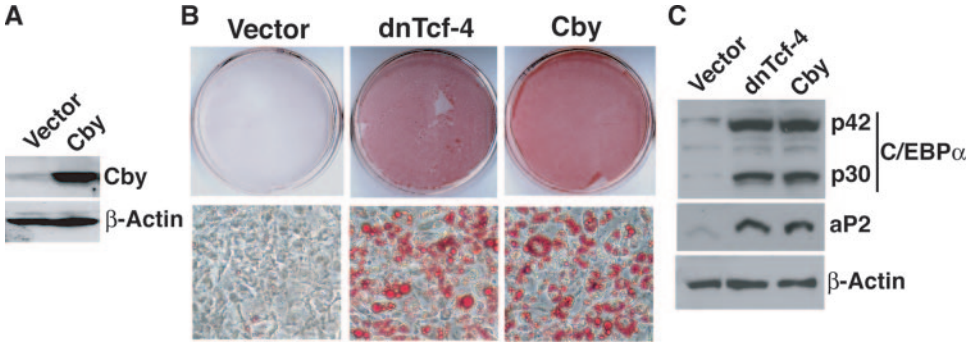

FIG. 2. Forced expression of Cby induces spontaneous differentiation of 3T3-L1 cells without hormonal stimulation. Preadipocytes were infected with a retrovirus vector alone (pLXIN) or retroviruses carrying dnTcf-4 or Cby, selected with G418, and maintained in growth media without the standard differentiation cocktail, MDI. (A) Total cell lysates were prepared from confluent cells for Western blot analyses using antibodies for Cby and β-actin. (B) Cells were stained with Oil Red-O to visualize lipid droplets 14 days postconfluence. (C) Cby-induced adipogenesis is accompanied by expression of the adipogenic markers C/EBPα and aP2. Total cell lysates were prepared 14 days postconfluence for Western blot analyses using the indicated antibodies.

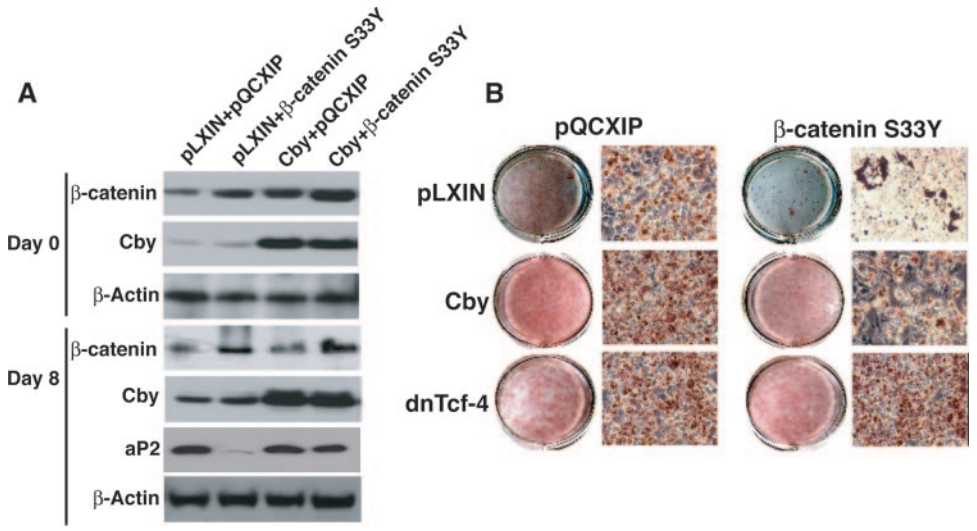

FIG. 3. Overexpression of β-catenin S33Y partially suppresses adipocyte differentiation of Cby-expressing 3T3-L1 cells. Control (pLXIN), Cby- or dnTcf-4-expressing cells were reinfected with a retrovirus vector alone (pQCXIP) or retroviruses carrying stabilized β-catenin S33Y and were coselected with G418 and puromycin. Two days postconfluence (day zero), the cells were induced to differentiate into mature adipocytes with MDI. (A) Whole-cell lysates were prepared at day 0 or 8 for Western blot analyses for β-catenin, Cby, aP2, and β-actin. (B) Cells were also stained with Oil Red-O to visualize lipid droplets 8 days postinduction.

cells also underwent spontaneous adipogenesis to an extent similar to that of dnTcf-4 (Fig. 2B).

To gain insights into the molecular mechanisms underlying these effects, we performed Western blot analyses for adipogenic markers (Fig. 2C). As expected, overexpression of Cby induced expression of C/EBPα and aP2. These data argue that forced expression of Cby in 3T3-L1 cells induces spontaneous differentiation, most likely by inhibiting endogenous β-catenin.

Cby antagonizes β-catenin signaling in 3T3-L1 cells. If our hypothesis were correct, providing excess β-catenin might overcome the effect of Cby overexpression, leading to a blockade of adipogenesis. In an attempt to examine this, control or Cby-overexpressing 3T3-L1 cells were reinfected with a control retrovirus or a retrovirus expressing β-catenin stabilized by a missense mutation of tyrosine for serine at codon 33 in the N-terminal GSK-3β phosphorylation site (β-catenin S33Y) (18). Cells were then induced to differentiate in the presence of MDI. Overexpression of β-catenin and Cby was verified by Western blot analyses (Fig. 3A). Consistent with a prior report (36), ectopic expression of β-catenin S33Y potently inhibited differentiation (Fig. 3B). Interestingly, Cby overexpression further facilitated adipocyte differentiation of these cells compared to the control retrovirus-infected cells. Under these conditions, β-catenin S33Y partially suppressed Cby-induced adipocyte differentiation, whereas it exhibited no effect on that of dnTcf-4-expressing cells. The extent of differentiation was further confirmed by Western blot analysis for aP2 at day 8 (Fig. 3A). These data suggest that Cby promotes adipocyte differentiation, at least in part by inhibiting β-catenin signaling.

To directly assess whether Cby negatively regulates β-catenin signaling in 3T3-L1 preadipocytes, we performed Tcf/Lef luciferase reporter assays (19). Expression of stabilized β-catenin S33Y stimulated the activity of TOPFLASH containing three optimal Tcf/Lef binding sites (Fig. 4B). Coexpression of Cby repressed β-catenin-mediated transcriptional activation. In order to test if silencing of endogenous Cby influences TOPFLASH activity in 3T3-L1 cells, we constructed an RNAi vector expressing shRNAs for mouse Cby. We first examined the efficiency of Cby RNAi by transient transfection into 293T cells. These cells were transfected with Cby shRNA or Cby shRNA-ΔC harboring a single-base deletion near the center of the Cby shRNA, together with a small amount of mouse Cby expression plasmid, followed by immunoblotting for Cby.

As shown in Fig. 4A, Cby shRNA markedly reduced Cby protein levels in a dose-dependent fashion. Importantly, Cby shRNA-ΔC had no major effect on Cby expression. In 3T3-L1 cells, expression of Cby shRNA resulted in a modest but consistent increase in TOPFLASH activity, whereas Cby shRNA-ΔC showed only a minor effect (Fig. 4B). As a control, cotransfection of Cby shRNA with a human Cby expression construct lacking the mouse Cby shRNA target sequence rescued TOPFLASH activity. These results suggest that Cby represses β-catenin-dependent transcription in 3T3-L1 cells.

Depletion of Cby attenuates adipocyte differentiation of 3T3-L1 and mouse ES cells. The above-mentioned data indicate that Cby positively regulates adipocyte differentiation. If so, one might hypothesize that knockdown of endogenous Cby would impair the differentiation process. To test this hypothesis, we infected 3T3-L1 preadipocytes with a retrovirus expressing Cby shRNA, Cby shRNA-ΔC, or a control virus, and the infected cells were selected with G418 and allowed to proliferate to confluence. We observed no major differences in morphology, viability, or growth rate between Cby shRNA and control retrovirus-infected preadipocyte cells. Two days later (day zero), these stable cells were induced to differentiate into mature adipocytes in the presence of MDI. Six days postinduction, total cell extracts were prepared and subjected to Western blot analysis for Cby. As shown in Fig. 5B, endogenous Cby protein was substantially decreased by Cby RNAi. In parallel, fat accumulation was visualized by staining lipids with Oil

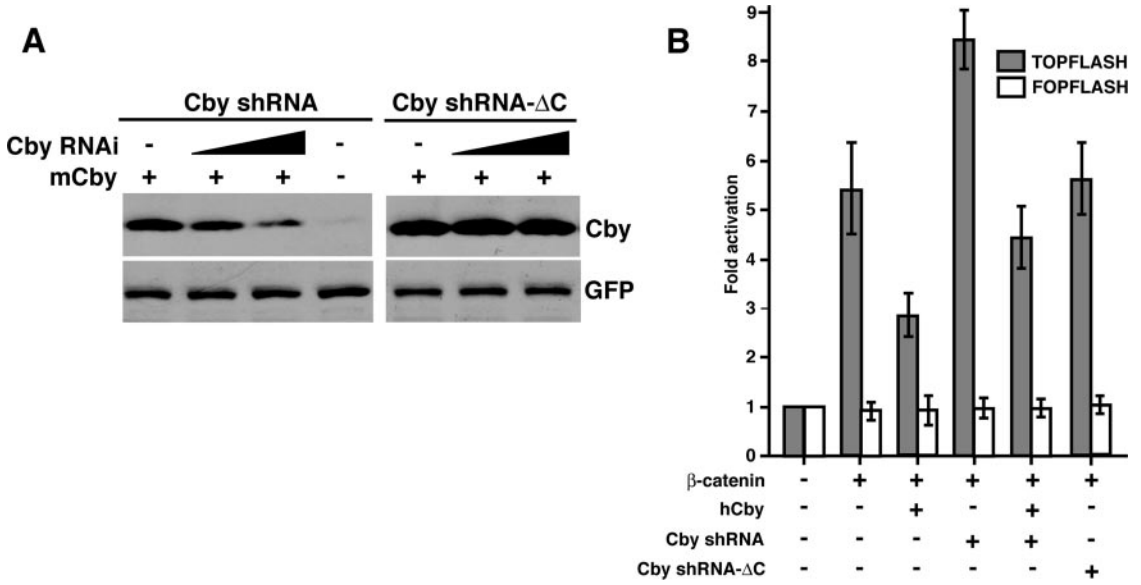

FIG. 4. Cby antagonizes β-catenin signaling in 3T3-L1 cells. (A) The efficiency of Cby RNAi was tested in 293T cells by transient transfection. 293T cells were transfected with 0.1 μg of a mouse Cby plasmid (mCby) (38) and increasing amounts (0.25, 0.5, or 1 μg) of an expression vector for Cby shRNA or Cby shRNA-ΔC, harboring a single-base deletion near the center of the targeted sequence, as indicated. Whole-cell lysates were analyzed by immunoblotting for Cby. A green fluorescent protein expression plasmid (0.1 μg) was cotransfectd as a control. (B) Tcf/Lef luciferase reporter assays in 3T3-L1 cells. 3T3-L1 preadipocytes were transfectd with 0.25 μg of TOPFLASH or the mutant FOPFLASH luciferase reporter and expression vectors for stabilized β-catenin S33Y (0.125 μg), human Cby (hCby) (2.5 μg) (38), Cby shRNA (0.5 μg), and Cby shRNA-ΔC (0.5 μg) in combinations as indicated, and relative luciferase activities were measured.

Red-O (Fig. 5A). 3T3-L1 preadipocytes infected with a control retrovirus underwent efficient differentiation, as expected. In marked contrast, Cby RNAi almost completely blocked the accumulation of lipid droplets, concomitant with reduced expression levels of adipogenic markers (Fig. 5B). A single-nucleotide deletion in the Cby shRNA sequence totally abolished these effects, confirming the specificity of Cby RNAi (Fig. 5A and B).

If loss of Cby by RNAi blocks adipogenesis via elevation of β-catenin activity, expression of dnTcf-4 could rescue adipogenic differentiation of Cby-RNAi cells. In an effort to test this possibility, the above-mentioned Cby shRNA-expressing 3T3-L1 cells were reinfected with a control retrovirus or a retrovirus expressing dnTcf-4 and maintained in the absence of MDI. As shown in Fig. 5C, expression of dnTcf-4 indeed rescued adipocyte differentiation of Cby-RNAi cells, with a concomitant induction of aP2 (Fig. 5D). These observations further support our model, in which Cby facilitates adipogenesis by suppressing Wnt/β-catenin signaling.

ES cells are known to differentiate into a variety of cell types in culture, including adipocytes (9, 17). To determine if Cby is involved in adipogenesis in pluripotent ES cells, Cby shRNA was stably expressed to suppress endogenous Cby expression in mouse R1 ES cells. Cby was expressed both in undifferentiated ES cells and during their in vitro differentiation, and Cby RNAi efficiently reduced Cby protein levels similar to those observed in 3T3-L1 cells expressing Cby shRNA (data not shown). Reduction of Cby did not exhibit any adverse effects on maintenance and early differentiation of ES cells (data not shown).

These stable lines were then differentiated by the standard hanging-drop method, and embryoid bodies were treated with retinoic acid and allowed to terminally differentiate into the adipogenic lineage in the presence of adipogenic hormones (9). Silencing of Cby expression significantly impaired adipocyte differentiation, while vector-transfected control cells efficiently differentiated into mature adipocytes under these conditions (Fig. 6A). As expected, expression of adipogenic markers was severely reduced in Cby knockdown cells, but not in control cells (Fig. 6B). Taken together, these results clearly demonstrate that Cby is required for adipocyte differentiation.

$Cby^{-/-}$ embryonic fibroblasts display impaired adipocyte differentiation. To further investigate the importance of Cby in adipogenesis, primary mouse embryonic fibroblasts (MEFs) were prepared from wild-type and $Cby^{-/-}$ embryos at E12.5, cultured to confluence, and then treated with MDI to induce adipocyte differentiation. As shown in Fig. 7A, Cby protein is not present in $Cby^{-/-}$ MEFs. Under these conditions, wild-type MEFs differentiated into adipocytes and accumulated lipid droplets (Fig. 7B). In contrast, $Cby^{-/-}$ MEFs exhibited reduced adipocyte differentiation, as verified by a decrease in aP2 levels (Fig. 7A). These observations strongly support the notion that Cby plays a proadipogenic role during adipocyte differentiation.

DISCUSSION

Recent studies established that Wnt/β-catenin signals inhibit adipogenesis (3, 5, 7, 25, 35). Wnt-10b is likely to mediate this effect, since it is highly expressed in preadipocytes, but its expression declines upon adipogenic differentiation (5, 36). Wnt signaling appears to exert its effect through regulation of mitotic clonal expansion (35). However, the precise mecha-

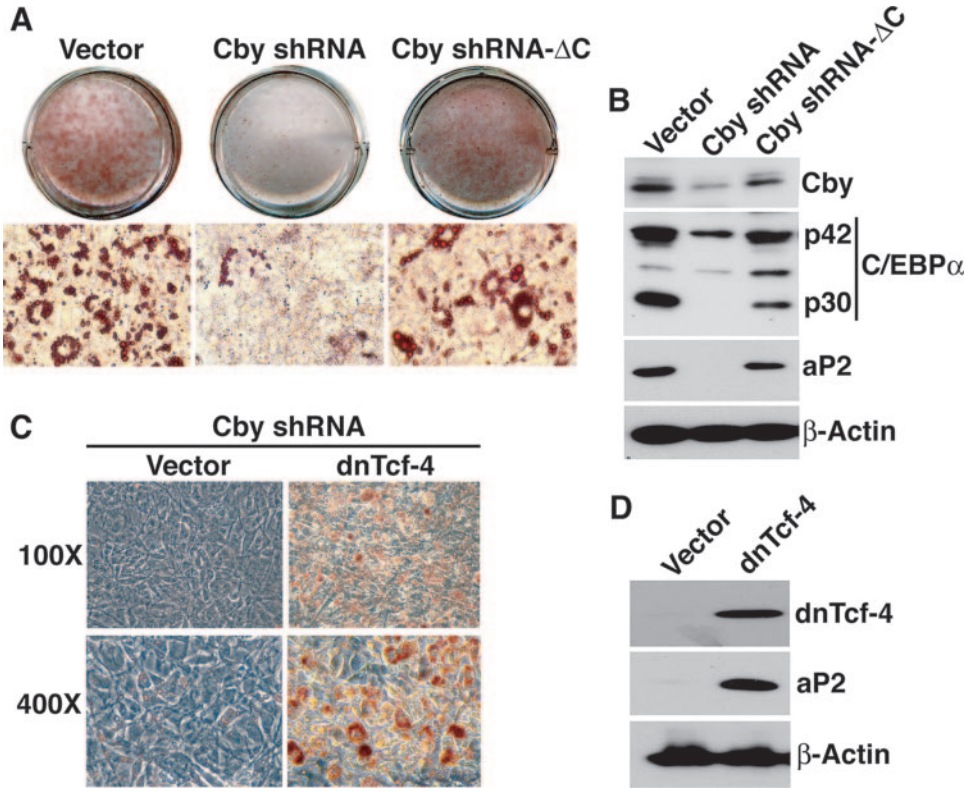

FIG. 5. Cby RNAi blocks the adipogenic conversion of 3T3-L1 cells, and dnTcf-4 rescues adipogenesis of Cby-RNAi cells. (A and B) 3T3-L1 cells were infected with a retrovirus vector alone (pSuppressor Retro) or a retrovirus expressing Cby shRNA or Cby shRNA-ΔC, selected with G418, and induced to differentiate using MDI. Six days later, the cells were stained with Oil Red-O to visualize the degree of lipid accumulation (A). Whole-cell extracts were subjected to immunoblot analyses with antibodies against the indicated proteins (B). (C and D) Cby shRNA-expressing cells were reinfected with a retrovirus vector alone (pQCXIP) or retroviruses carrying dnTcf-4, coselected with G418 and puromycin, and maintained in growth media without MDI for 14 days after confluence. The cells were then stained with Oil Red-O and photographed at two different magnifications (C). Total cell lysates were prepared for Western blot analyses using the indicated antibodies (D).

nisms through which Wnts block adipogenesis remain largely unknown.

Cby is an evolutionarily conserved nuclear protein that antagonizes β-catenin signaling (38). We have previously shown that Cby binds to the C-terminal portion of β-catenin and competes with Tcf/Lef transcription factors to repress gene transcription. In this study, we have uncovered a novel role of Cby in adipocyte differentiation. Cby protein levels increase during differentiation of 3T3-L1 preadipocytes. Overexpression of Cby facilitates adipogenesis of these cells, whereas depletion of Cby by RNAi inhibits adipocyte differentiation of 3T3-L1 and mouse ES cells. Furthermore, MEFs lacking Cby show a diminished ability to differentiate into the adipogenic lineage. Our gain- and loss-of-function studies clearly indicate that Cby is a novel proadipogenic factor required for adipocyte differentiation.

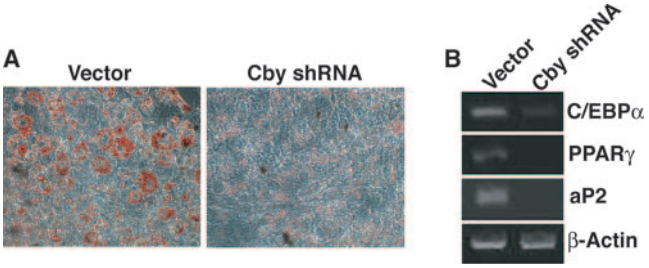

FIG. 6. Cby RNAi attenuates adipocyte differentiation of mouse ES cells. Mouse ES cells were stably transfected with an empty vector or Cby shRNA, selected by G418, and induced to differentiate into the adipogenic lineage for 20 days according to standard procedures. (A) Cells were stained with Oil Red-O. (B) RNA was purified and used to synthesize cDNAs for RT-PCR for adipogenic markers. β-Actin was used as a loading control.

It is worth noting that silencing of Cby by RNAi in 3T3-L1 or ES cells does not affect cell morphology or the rate of proliferation. This implies that Cby is not essential for normal growth. Rather, Cby is crucial for the adipocyte differentiation process per se. In addition, Cby RNAi profoundly decreases induction of C/EBPα and PPARγ (Fig. 5B and 6B), demonstrating that Cby functions upstream of these key adipogenic transcription factors. This is in agreement with the prior report that activation of Wnt signaling abrogates expression of C/EBPα and PPARγ (36).

In 3T3-L1 cells, ectopic expression of Cby represses β-catenin-mediated transcriptional activation, while loss of Cby by RNAi leads to elevation of β-catenin activity (Fig. 4). In support of this, forced expression of stabilized β-catenin partially suppresses Cby-induced adipocyte differentiation (Fig. 3), and moreover, expression of dnTcf-4 rescues adipogenesis of Cby shRNA-expressing cells (Fig. 5C and D), suggesting that Cby

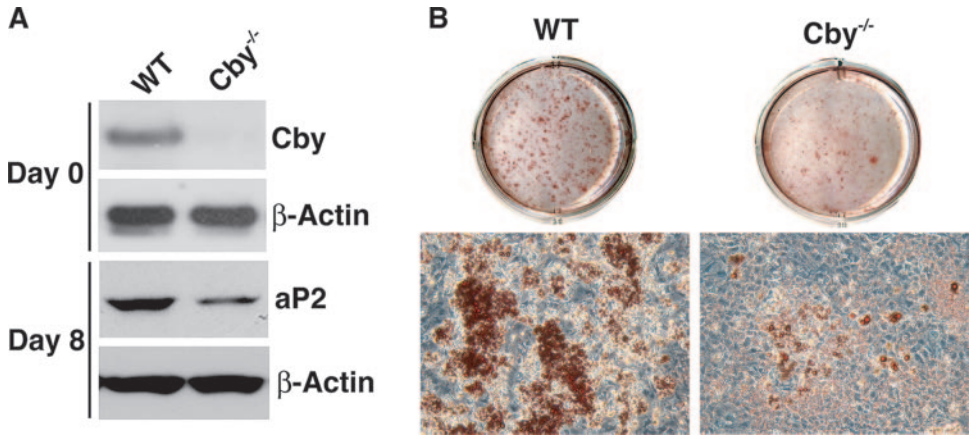

FIG. 7. Cby$^{-/-}$ MEFs differentiate poorly into the adipogenic lineage. MEFs were prepared from wild-type (WT) or Cby-deficient (Cby$^{-/-}$) E12.5 embryos. (A) Whole-cell lysates were extracted from confluent cells and subjected to Western blotting with the indicated antibodies. (B) MEFs were induced to differentiate into adipocytes for 10 days with MDI, followed by Oil Red-O staining to visualize lipid droplets.

promotes adipocyte differentiation in part through inhibiting β-catenin. This is consistent with the previous finding that the differentiation of preadipocytes into adipocytes requires suppression of the Wnt/β-catenin pathway (3, 25, 36). In this regard, Wnt-5b has been recently reported to promote adipogenesis by antagonizing the Wnt/β-catenin pathway (15, 16).

During differentiation of 3T3-L1 preadipocytes, nuclear β-catenin levels decrease; however, it remains present at basal levels in mature adipocytes (25). Cby protein levels inversely correlate with β-catenin levels (Fig. 1A). Thus, it is plausible that Cby binds to nuclear β-catenin and represses its activity in adipocytes. This may ensure terminal adipocyte differentiation by preventing ectopic transcriptional activation by β-catenin. This repressive activity of Cby is critical during *Drosophila* embryogenesis, since depletion of Cby by RNAi leads to ectopic activation of Wingless (Wg) target genes, even in a *wg*-null background, leading to embryonic lethality (38).

Little is known about the in vivo functions of the Wnt/β-catenin pathway in adipose tissue development. In contrast to in vitro observations and gain-of-function studies in mice, Wnt-10b knockout mice have no obvious defects in adipose tissue development (4, 45). However, it is possible that other Wnt family members compensate for the loss of Wnt-10b in the in vivo setting. Therefore, loss-of-function experiments are needed to rigorously document the involvement of β-catenin signaling in adipogenesis in vivo. In mice, Cby is expressed throughout embryonic development and in multiple adult tissues. Cby-null mice display severe digestive/absorption problems, and the vast majority die by weaning age, most likely due to starvation (K.-I. Takemaru, V. Voronina, and R. T. Moon, unpublished data). Although they manifest reduced adiposity in early postnatal life, it is difficult to assess whether it is a primary or secondary effect due to the nutritional problems. Hence, further studies will be needed to define potential deficits in adipocyte development in Cby-null animals.

In summary, our study provides compelling evidence that Cby is a novel proadipogenic factor required for adipocyte differentiation. More recently, mutations in the Wnt-10b gene have been described in human obesity (8), and LRP5 polymorphisms have been shown to be significantly associated with obesity phenotypes (14). These observations raise the possibility that manipulating Wnt signaling, such as blocking Cby's activity, may eventually provide means to develop new therapies for obesity and its associated disorders, such as diabetes.

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