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p300/β-Catenin Interactions Regulate Adult Progenitor Cell Differentiation Downstream of WNT5a/Protein Kinase C (PKC)*

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Maintenance of stem/progenitor cell-progeny relationships is required for tissue homeostasis during normal turnover and repair. Wnt signaling is implicated in both maintenance and differentiation of adult stem/progenitor cells, yet how this pathway serves these dichotomous roles remains enigmatic. We previously proposed a model suggesting that specific interaction of β-catenin with either of the homologous Kat3 co-activators, p300 or CREB-binding protein, differentially regulates maintenance *versus* differentiation of embryonic stem cells. Limited knowledge of endogenous mechanisms driving differential β-catenin/co-activator interactions and their role in adult somatic stem/progenitor cell maintenance *versus* differentiation led us to explore this process in defined models of adult progenitor cell differentiation. We focused primarily on alveolar epithelial type II (AT2) cells, progenitors of distal lung epithelium, and identified a novel axis whereby WNT5a/protein kinase C (PKC) signaling regulates specific β-catenin/co-activator interactions to promote adult progenitor cell differentiation. p300/β-catenin but not CBP/β-catenin interaction increases as AT2 cells differentiate to a type I (AT1) cell-like phenotype. Additionally, p300 transcriptionally activates AT1 cell-specific gene *Aqp-5*. IQ-1, a specific inhibitor of p300/β-catenin interaction, prevents differentiation of not only primary AT2 cells, but also tracheal epithelial cells, and C2C12 myoblasts. p300 phosphorylation at Ser-89 enhances p300/β-catenin interaction, concurrent with alveolar epithelial cell differentiation. WNT5a, a traditionally non-canonical WNT ligand regulates Ser-89 phosphorylation and p300/β-catenin interactions in a PKC-dependent manner, likely involving PKCζ. These studies identify a novel intersection of canonical and non-canonical Wnt signaling in adult progenitor cell differentiation that has important implications for targeting β-catenin to modulate adult progenitor cell behavior in disease.

Activation of endogenous somatic stem/progenitor cells is required for both normal homeostasis and repair of damaged tissues and organs. This fundamental process requires tight regulation of stem/progenitor cells to maintain the equilibrium between decisions for self-renewal and differentiation to produce daughter cells of a committed fate. Numerous critical signal transduction pathways are implicated in regulation of stem/progenitor cell fate during development and in the adult, including Wnt signaling. Stem/progenitor cells must integrate a complex array of environmental cues to arrive at a final decision regarding preservation of quiescence or cell cycle entry, and subsequent maintenance of progenitor status or differentiation. Aberrant regulation of cell fate decisions is associated with malignancy, fibrosis, and other disorders (1). Elucidation of pathways that regulate normal adult progenitor cell differentiation is key to understanding the contribution of abnormal differentiation to disease pathogenesis.

Wnt signaling is a developmental pathway that participates in maintenance and repair programs in the adult (2). Traditionally, Wnt signaling has been categorized as either "canonical" (β-catenin-dependent) or "non-canonical" (β-catenin independent). Canonical Wnt/β-catenin signaling leads to nuclear localization of β-catenin and activation of Wnt target genes (2, 3), whereas non-canonical Wnt signaling is mediated by alternative signal transduction pathways, including PKC (4, 5). Although it is clear that β-catenin-dependent Wnt signaling is important in regulation of stem/progenitor cell behavior, how this pathway serves dichotomous roles in stem/progenitor cell self-renewal/maintenance of potency *versus* differentiation remains somewhat elusive.

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β-Catenin recruits transcriptional co-activators cAMP response element-binding protein (CREB)[6]-binding protein (CBP) or the highly homologous co-activator p300 (E1A-binding protein, 300 kDa), as well as other components of the basal transcription machinery, to generate a transcriptionally active complex (2, 6). Although p300 and CBP are often viewed as functionally redundant (as reviewed by Kalkhoven (7)), increasing evidence suggests that usage of either of these co-activators, within the context of β-catenin-dependent signaling, differentially regulates target gene expression leading to different functional outcomes (1, 8–10). Based on studies in embryonic stem cells, we developed a model that highlights the distinct roles of co-activators CBP and p300 and provides a mechanistic basis to account for dichotomous behavior of Wnt/β-catenin-dependent signaling in controlling stem/progenitor cell function (8, 9). The critical feature of this model is that differential interactions between β-catenin and CBP or p300 activate transcriptional programs that promote either self-renewal or differentiation, respectively. How differential co-activator usage is controlled endogenously in adult stem/progenitor cell populations for normal tissue homeostasis and repair is unknown.

The goal of this study was to investigate mechanisms regulating shifts in endogenous β-catenin interactions with CBP or p300 in coordination of adult epithelial progenitor/progeny relationships. To this end, we utilized *in vitro* models of distal and proximal adult lung epithelial progenitor cell differentiation: alveolar epithelial cells (AEC) and tracheal epithelial cells in primary culture, respectively. Distal lung AEC are comprised of type 2 (AT2) cells, cuboidal surfactant-producing cells, and type 1 (AT1) cells, large flat cells that provide the surface area for gas exchange (11). AT2 cells are known progenitors of AT1 cells *in vivo* (12–14). Primary AT2 cells cultured *in vitro* transdifferentiate without an intervening cell division to AT1-like cells over 4–8 days, depending on the species (15–17), recapitulating AT2 to AT1 cell differentiation *in vivo*. Transdifferentiation of AEC grown on polycarbonate inserts is monitored by development of transepithelial electrical resistance due to formation of tight junctions, concurrent with loss of AT2 (*e.g.* prosurfactants A, B, and C (pro-SFTPA, -SFTPB, and -SFTPC)), and gain of AT1 (*e.g.* aquaporin-5 (AQP5), podoplanin (T1α), receptor for advanced glycation end products (RAGE), and caveolin-1 (CAV1)) cell phenotypic markers. In the proximal airways, including the trachea and bronchi, epithelial basal cells marked by expression of transformation-related protein 63 (TRP-63 or p63) serve as progenitor cells (18). $p63^+$ cell progeny differentiate to yield three major cell types, identified as secretory (club, $SCGB1A1^+$), mucous ($MUC5AC^+$), and ciliated cells (bearing multiple cilia identified by acetylated α-tubulin). Isolated tracheobronchial epithelial cells (hTEC) comprise a proliferative population of basal cells (19) that undergo differentiation in culture at air-liquid interface and give rise to a fully differentiated epithelium over 3 weeks that models the *in vivo* airway (20). Primary cells from proximal and distal regions of the lung constitute ideal adult progenitor cell differentiation model systems, because temporally regulated differentiation is well documented and can be closely monitored by changes in expression of phenotypic markers.

We describe a novel mechanism downstream of WNT5a/PKC that regulates the critical role of p300/β-catenin interaction in differentiation of both these adult epithelial progenitor cells. Studies in C2C12 myoblast to myocyte differentiation suggest that this paradigm can also be extended to differentiation of non-epithelial progenitors. Using AEC *in vitro* and $Wnt5a^{-/-}$ mice (21), we demonstrate that specific β-catenin co-activator interactions are the result of posttranslational modification of p300 regulated by WNT5a/PKC, providing novel insight into the coordination of canonical and non-canonical Wnt signaling in regulation of stem/progenitor cell maintenance *versus* differentiation.

Experimental Procedures

Cell Isolation, Culture, and Treatments—Rat AT2 (rAT2) cells were isolated as previously described (22) under a protocol approved by the Institutional Animal Care and Use Committee of the University of Southern California. Cells were grown in serum-free medium (23) on polycarbonate filters (Corning, Tewksbury, MA) for extraction of protein and RNA at various times between days 0 and 6 in culture, or fixation for immunofluorescence on day 4. IQ-1, a specific small molecule inhibitor of p300/β-catenin interaction, and ICG-001, a specific inhibitor of β-catenin/CBP interaction, have been previously described (8, 9). IQ-1 (5–20 μM) or DMSO (vehicle control) was added to media from the time of plating through completion of the experiment. Media were changed on day 3 and subsequently every other day. PKC inhibitor Gö6983 (5 μM, EMD, San Diego, CA) or DMSO (vehicle control), or PKCζ pseudo substrate (50 μM, Thermo Fisher Scientific, Carlsbad, CA) or H_2O (vehicle control) were added to media in the same manner as IQ-1, except media were changed on days 2 and 4. Dose responses to inhibitors were determined to assess efficacy and toxicity. Recombinant human WNT5a (100 ng/ml, STEMRD, Burlingame, CA) was added to freshly isolated rAT2 cells at the time of plating. Cell extracts were collected 24 h post-plating. Mouse lung epithelial (MLE-15) cells, an AT2-like cell line (J. Whitsett, University of Cincinnati), were cultivated in HITES medium (24). Mouse E10 cells, an AT1-like cell line, were cultured as previously described (25) with addition of gentamicin (40 μg/ml). hTEC were isolated from excess tracheal and bronchial tissue of normal lungs donated for transplantation under a protocol designated as non-human research by the human studies committee at Washington University. hTEC were expanded in growth factor-rich medium, followed by differentiation in media supplemented with 2% Nuserum (BD Biosciences) using air-liquid interface conditions as previously described (20). DMSO (vehicle control) or IQ-1 (10–20 μM) treatment was initiated ~5 days after plating, concurrent with removal of apical fluid to establish an air-liquid interface, and replenished three times per week. hTEC were harvested for RNA isolation or immunofluorescence analysis on days 8 (early ciliated cell differentiation) and 24 (completed differentiation).

[6] The abbreviations used are: CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; RAGE, receptor for advanced glycation end products; hTEC, human tracheobronchial epithelial cell; NS, non-silencing; MLE, mouse lung epithelial; DM, differentiation medium; DMSO, dimethyl sulfoxide; AEC, alveolar epithelial cell; qRT, quantitative RT; Rt, transepithelial electrical resistance.

C2C12 mouse myoblast cells (ATCC: CRL-1772) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were maintained at ≤70–80% confluence to minimize spontaneous differentiation. To differentiate C2C12 cells into myotubes, cells were seeded into 10-cm or 6-well plates at 50% confluence in normal growth medium and incubated at 37 °C with 5% CO_2 overnight. Subsequently, normal growth medium was aspirated and cells were washed with phosphate-buffered saline (PBS; pH 7.2) and incubated in differentiation medium (DMEM, 2% horse serum, and penicillin/streptomycin) for 5 days to induce myotube formation. IQ-1 (5 μM) or ICG-001 (10 μM) was added to appropriate media, which were changed every other day.

Isolation of Mouse Embryonic Lungs—Animals were maintained according to an approved IACUC protocol. Timed matings were set up between *Wnt5a*-heterozygous mice to generate homozygotes (21). Embryos were harvested at embryonic day (E) 18.5. Genotyping was performed as previously described (21). Distal lung tissues were isolated by removing the trachea and large airways followed by flash freezing in liquid N_2.

Transient Transfection Assays—MLE-15 cells were seeded at 4×10^4 cells/well in 24-well tissue culture plates. After 1.5 days, cells were transfected using Superfect (Qiagen) with a 4.3-kb rat *Aqp5*-luciferase reporter (26) together with p300 and constitutively active β-catenin S33Y (27) individually or in combination, with or without subsequent treatment with IQ-1 (5 μM). Cells were harvested 24 h after transfection for measurement of firefly luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Values were normalized to *Renilla* luciferase activity for transfection efficiency and empty vector or no vector controls. pCDNA3.1 and pCDNA3.1-p300 wild-type vectors were provided by D. Livingston (Dana-Farber Cancer Institute, Harvard Medical School), and pCDNA3.1-p300 S89A was generated using the QuikChange II Site-directed Mutagenesis Kit (Agilent, Cedar Creek, TX).

Lentivirus Production and shRNA Knockdown—The lentivirus backbone vectors pGIPZ, engineered to contain *p300* or non-silencing (NS) shRNA, or PLKO.1, engineered to contain *Wnt5a*, *Cbp*, or non-silencing (NS) shRNA (Thermo Scientific, Lafayette, CO), and packaging plasmids pCMVΔ8.91 and pMD2.G, were co-transfected into HEK293T cells. Virus was concentrated with PEG-it™ virus precipitation solution (System Biosciences, Mountain View, CA) and titered with HIV p24 ELISA (Cell Biolabs, San Diego, CA). Viral titers were on the order of 10^8 transducing units/ml. Freshly isolated rAT2 cells were plated on 24-well BD Primaria (BD Biosciences) plates at a density of 5×10^5 cells/well. On day 2, AEC were transduced with control pGIPZ or PLKO.1 non-silencing control, *p300*, or *CBP* shRNA-expressing lentivirus at a multiplicity of infection of 60 to obtain transduction efficiencies of ~70–80%. E10 cells were plated at a density of 6×10^4 cells/well on 12-well plates (or 10^6 cells/100-mm dish) 1 day prior to transduction with pLKO.1 non-silencing control or pLKO.1 *Wnt5a* shRNA lentivirus at a multiplicity of infection of 5. RNA or nuclear protein extracts were harvested 3 days post-transduction after a total of 5 days in culture.

Western Analysis—Protein was extracted from cells in 2% SDS lysis buffer containing protease inhibitor mixture III (Calbiochem, Billerica, MA) and phosphatase inhibitors (Sigma) for studies of protein phosphorylation. Protein concentrations were measured using DC Protein Assay (Bio-Rad) and all total protein amounts were equally loaded prior to SDS-PAGE gel separation. Proteins were resolved by SDS-PAGE and electrophoretically transferred onto ImmunoBlot PVDF membranes (Bio-Rad), followed by blocking in 5% nonfat milk or 5% bovine serum albumin (BSA) for phosphoantibodies. Primary antibodies (Abs) included rabbit anti-AQP5 (Alomone Labs, Jerusalem, Israel) -p300, -CBP, -Ser(P)-89, -Lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA), -RAGE, -CAV-1 (Abcam, Cambridge, MA), -pan phospho-PKC (Cell Signaling, Danvers, MA), -T1α (Affinity BioReagents, Golden, CO), -AQP5, pro-SFTPC (Millipore, Temecula, CA), and mouse anti-p300/CBP (NM11) (Santa Cruz Biotechnology), -GAPDH (Life Technologies), and -β-catenin (Sigma). Blots were incubated with horseradish peroxidase-linked anti-IgG conjugates (Promega) for 1 h at room temperature. Complexes were visualized by West Femto Super Sensitivity Kit (Thermo Scientific) with a FluorChem 8900 Imaging System (Alpha Innotech, San Leandro, CA). Precision Plus Protein™ Dual Color Standards (Bio-Rad) were used to assess molecular weight of bands. Location of standards are noted on Western blot figures.

Immunofluorescence—Rat AT2 cells or hTEC grown on polycarbonate filters were fixed in 4% paraformaldehyde and washed with PBS (pH 7.2). E18.5 mouse lungs were fixed in 4% paraformaldehyde and embedded in paraffin. Filters and deparaffinized slides were subjected to antigen retrieval using citric acid-based Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA), blocked in CAS block (Life Technologies), and incubated with rabbit anti-AQP5 (Millipore or Alomone Labs) or anti-pro-SFTPC (Seven Hills, Cincinnati, OH), mouse anti-acetylated α-tubulin (Clone 6-11-B1, Sigma) and anti-SCGB1A1 (B. Stripp, Cedars Sinai Medical Center) primary Abs overnight at 4 °C. After washing, filters were incubated with biotin-labeled secondary Abs, followed by incubation with Avidin DCS FITC (Vector Laboratories) or with Alexa Fluor® Dye-labeled secondary Abs (Life Technologies, Grand Island, NY). C2C12 cells were grown on glass coverslips in 6-well dishes. After treatment, cells were fixed in ice-cold methanol, blocked in 5% goat serum in PBS containing 2% Triton X-100, incubated with anti-myosin heavy chain (MHC) (K-16) Ab (Santa Cruz) overnight at 4 °C, and incubated with donkey anti-goat IgG H&L Alexa Fluor® 568 (Life Technologies) at room temperature for 1 h. Filters and coverslips were mounted on slides with media containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories) to counterstain DNA. Images were captured on a Nikon Eclipse Ti inverted microscope equipped with a DS-Qi1Mc camera (Melville, NY) (for AEC), a Leica DM5000 microscope (Wetzlar, Germany) with a Retiga 200R charge-coupled device camera (Q-Imaging, Surrey, Canada) interfaced with Q-Capture Pro software (Q-Imaging) (for hTEC) or a Leica DM6000 fluorescent microscope with a Leica DFC360 FX Monochrome Digital Camera (for C2C12 cells). Images were uniformly adjusted for bright-

ness and contrast using Photoshop (Adobe Systems, San Jose, CA).

qRT-PCR—RNA was extracted using TRIzol (Life Technologies) and DNase treated for 30 min using Turbo-DNase-free (Ambion, Austin, TX). cDNA was synthesized using the First Strand Synthesis Kit III (Life Technologies) or qScript™ cDNA Synthesis Kit (Quanta Bioscience, Gaithersburg, MD). qPCR was performed with the ABI 7900-HT Fast Real-time PCR System or the Bio-Rad MyiQ Thermal Cycler using SYBR Green (Applied Biosystems or Quanta Bioscience, Gaithersburg, MD, respectively). Primer sequences are available upon request. Data were analyzed using the $\Delta\Delta C_t$ method.

Co-immunoprecipitation of p300 and CBP—Rat AT2 cells were grown on Primaria 6-well plates (BD Biosciences) and nuclear extracts were collected at days 2 and 6 using the ProteoExtract Subcellular Proteome Extraction Kit (EMD) or NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific). 150–200 μg of nuclear extract was diluted in PBB (20 mM HEPES, 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.1% Nonidet P-40) or co-IP buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA), respectively, both containing protease inhibitor mixture III (Calbiochem). Diluted lysate was pre-cleared for 2 h and incubated overnight with 2 μg of rabbit IgG, rabbit anti-p300 or -CBP antibodies (Santa Cruz), or 5 μg of rabbit anti-Ser(P)-89 p300 Ab (Santa Cruz). Proteins were immunoprecipitated using protein A/G-Sepharose beads (Santa Cruz), washed 5× in PBB buffer, and collected by boiling beads in 2× SDS sample buffer for 5 min. Samples were then subjected to Western analysis.

Statistical Analysis—Statistical analysis was performed utilizing Prism Statistical software (GraphPad Software, La Jolla, CA). Data are presented as mean ± S.E. with *n* being the number of biological replicates used in statistical analysis. *p* values were calculated using one-way analysis of variance or unpaired Student's *t* tests compared with a hypothetical value.

Results

β-Catenin Interactions with Co-activators p300 and CBP Change Dynamically during AEC Differentiation—To investigate the dynamics of interactions between endogenous β-catenin and co-activators p300 and CBP during transdifferentiation from an AT2 to AT1 cell-like phenotype, we performed co-immunoprecipitation studies using nuclear extracts from AEC harvested early (day 2; AT2 cell-like) and late (day 6; AT1 cell-like) in primary culture (22). We observed stronger CBP interaction with β-catenin at day 2, which decreased at day 6 (Fig. 1*A*), whereas p300/β-catenin interaction increased over the same time frame. Immunoprecipitated p300 and CBP remained comparable over this period of time (Fig. 1*A*). These results establish that β-catenin co-activator interactions change dynamically during AEC transdifferentiation.

p300 Regulates Promoter of AT1 Cell-specific Gene Aqp5—Increased p300/β-catenin interaction during transdifferentiation led us to examine effects of p300 on transcriptional activation of *Aqp5*, a prototypic marker of AT1 cell differentiation (23, 28). We utilized a construct containing a 4.3-kb segment of the rat *Aqp5* promoter previously cloned 5′ to the luciferase

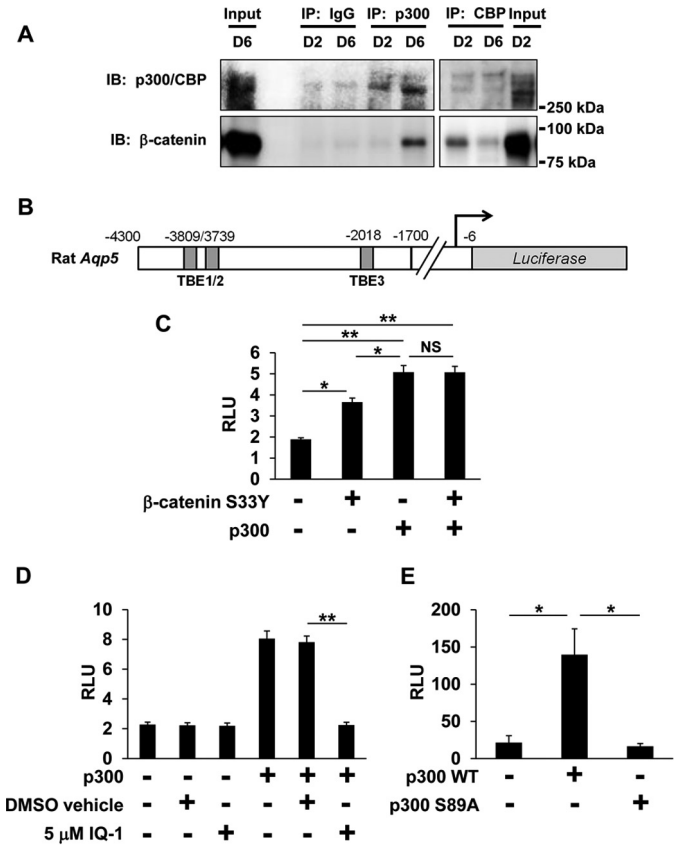

FIGURE 1. **Co-activator usage changes dynamically during AEC differentiation and p300 regulates *Aqp5-Luc* activity.** *A,* representative co-immunoprecipitation of nuclear extracts at day 2 (*D2*) and day 6 (*D6*) during AEC differentiation demonstrates increased p300/β-catenin interaction in AT1-like cells (*D6*) with concurrent reduction in CBP/β-catenin interaction. Immunoblotting (*IB*) indicates that immunoprecipitated p300 and CBP (~300 kDa) levels are comparable during differentiation. $n \geq 2$. β-Catenin molecular mass is ~92 kDa. *B,* schematic of *Aqp5* luciferase (*Aqp5-Luc*) promoter construct. 4.3-kb of the rat *Aqp5* promoter is fused upstream of a luciferase reporter in pGL2 basic vector. *Dark gray boxes* represent conserved putative TCF/LEF binding elements (*TBE*) predicted by rVista. *C,* MLE-15 cells were transiently co-transfected with *Aqp5*-Luc and constitutively active β-catenin (β-catenin S33Y) with or without p300 expression constructs. $n = 3$. *, $p < 0.05$; **, $p < 0.001$. *D,* MLE-15 cells were co-transfected with *Aqp5-Luc* and p300 expression plasmids and concurrently treated with IQ-1 (5 μM) or DMSO for 24 h. $n = 3$. **, $p < 0.001$. *E,* MLE-15 cells were transiently co-transfected with *Aqp5-Luc* and p300 WT or p300 S89A expression vectors. $n = 3$. *, $p < 0.05$. *Bars* represent mean ± S.E. *RLU*, relative luciferase units.

(*Luc*) reporter gene (26) that contains several TCF elements potentially responsive to Wnt signaling (Fig. 1*B*). Transient co-transfection of mouse lung epithelial (MLE-15) cells with the *Aqp5-Luc* construct and expression vectors for p300 and/or constitutively activated β-catenin (β-catenin S33Y) increased *Aqp5-Luc* activity (Fig. 1*C*). The effect of p300 was greater than that of activated β-catenin, whereas addition of both β-catenin and p300 did not further increase the effect of p300 on *Aqp5-Luc* activity (Fig. 1*C*), suggesting that β-catenin may be saturated in this system and p300 is key to driving Wnt/β-catenin differentiation-related gene activation. This effect was similar whether constitutively active β-catenin (S33Y) or wild-type β-catenin was used (data not shown). To determine whether p300/β-catenin interaction is important for transcriptional activation of the *Aqp5* promoter, we utilized IQ-1, a small molecule that acts indirectly to reduce phosphorylation of residue

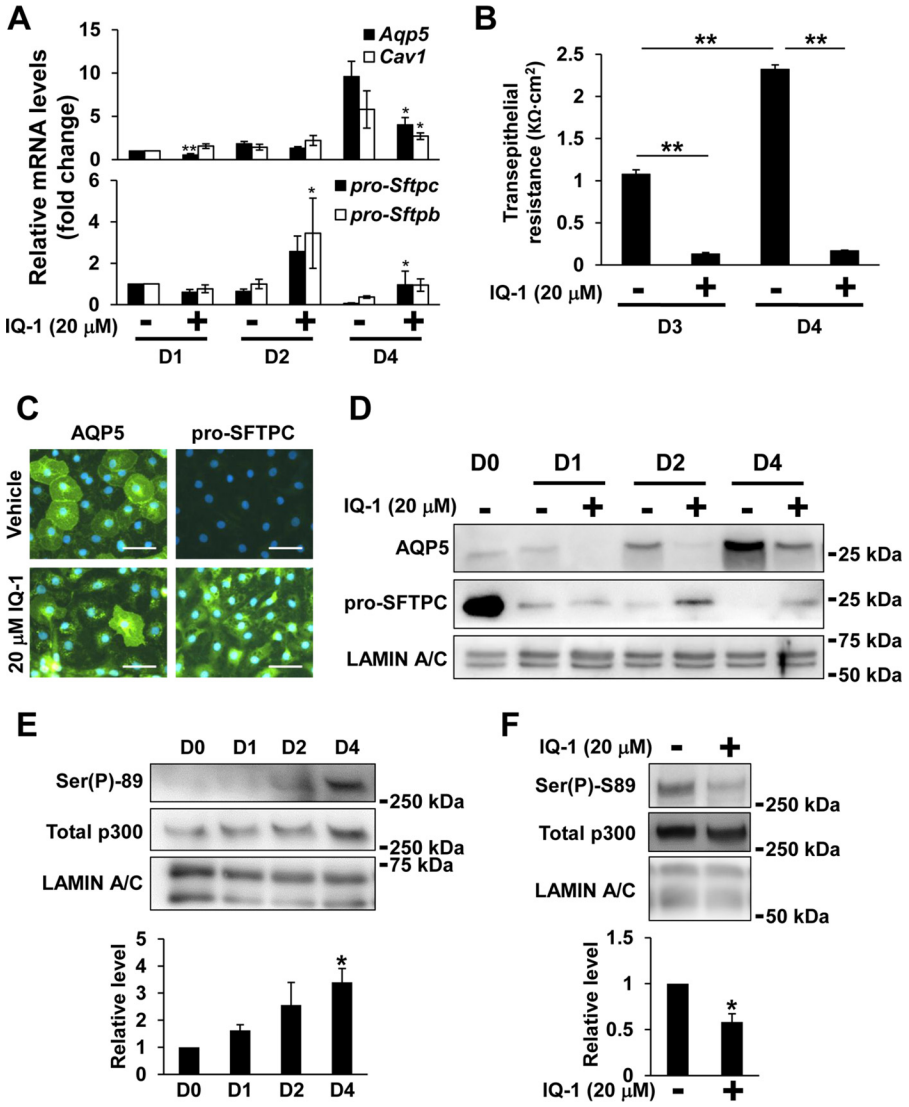

FIGURE 2. **Inhibition of p300/β-catenin interactions inhibit normal AEC transdifferentiation.** *A,* qRT-PCR analysis of AT1 cell markers *Aqp5* and *Cav1* (*top*) and AT2 cell markers *pro-Sftpc* and *pro-Sftpb* (*bottom*) as functions of time in AEC cultivated *in vitro* with IQ-1 (20 μM (+)) or vehicle (DMSO (−)) from the time of plating and harvested at the indicated times. $n = 5$. **, $p < 0.01$; *, $p < 0.05$, compared with vehicle sample at the same time point. *B,* transepithelial electrical resistance across primary rAT2 cell monolayers grown on polycarbonate filters exposed to IQ-1 (20 μM (+)) or vehicle (DMSO (−)) from day 0. $n = 3$. **, $p < 0.001$. *C,* representative immunofluorescence image of primary rat AT2 cells treated with IQ-1 (20 μM) or vehicle (DMSO). Nuclei are labeled with DAPI (*blue*). $n = 3$. *Scale bars* = 50 μm. *D,* representative Western blot of AQP5 (28 kDa) from AEC cultivated *in vitro* with IQ-1 (20 μM) (+) or vehicle (DMSO (−)) at the indicated times. The AT2 cell marker is pro-SFTPC (24 kDa). Lamin A/C (55 and 62 kDa) is loading control. $n = 3$. *E,* representative Western blot and quantitation of phosphorylated p300 at serine 89 (Ser(P)-89, ~300 kDa) during differentiation of primary AEC on days 0–4. Lamin A/C is loading control. $n = 4$. *, $p < 0.05$. *F,* representative Western blot and quantification of Ser(P)-89 levels in primary rat AEC exposed to IQ-1 (20 μM (+)) or vehicle (DMSO (−)) in culture for 2 days from the time of plating. Lamin A/C is loading control. $n = 4$. *, $p < 0.05$. *Bars* represent mean ± S.E.

serine 89 of p300 (Ser(P)-89) thereby inhibiting p300/β-catenin interaction (9). IQ-1 abolished p300-driven *Aqp5-Luc* activity (Fig. 1*D*). Consistent with the effects of IQ-1, co-transfection of a p300 expression vector containing a non-phosphorylatable S89A mutation similarly failed to increase *Aqp5-Luc* activity (Fig. 1*E*). These findings suggest that p300/β-catenin interactions are necessary for p300 transcriptional activation of this marker of AT1 cell differentiated phenotype.

p300/β-Catenin Interaction Promotes AEC Differentiation in Vitro Accompanied by Increased Ser-89 Phosphorylation—We exposed primary rat AEC to IQ-1 to inhibit p300/β-catenin interaction during AT2 to AT1-like cell differentiation *in vitro*. IQ-1 (20 μM) significantly inhibited up-regulation of mRNA for the AT1 cell markers *Aqp5* and *Cav1* (Fig. 2*A*, *top*) and promoted retention of mRNA for the AT2 cell markers *pro-Sftpc*, *pro-Sftpb*, and *pro-Sftpa* (Fig. 2*A*, *bottom*, and data not shown). Although both pro-*Sftpb* and pro-*Sftpc* showed increases following IQ-1 treatment relative to vehicle (DMSO)-treated controls, these reached statistical significance on different days (days 2 and 4, respectively), perhaps due to variability in cell preparations. All vehicle (DMSO)-treated primary AEC formed monolayers by day 4 with transepithelial electrical resistance (Rt) ≥2 kΩ cm^2. IQ-1 exposure decreased Rt (Fig. 2*B*), consistent with inhibition of differentiation. Removal of IQ-1 at day 4 resulted in full recovery of Rt (data not shown). Immunofluorescence and Western analysis results were consistent with gene expression data determined by qRT-PCR (Fig. 2, *C* and *D*). Expression of Ser(P)-89 increased steadily through day 4 (Fig.

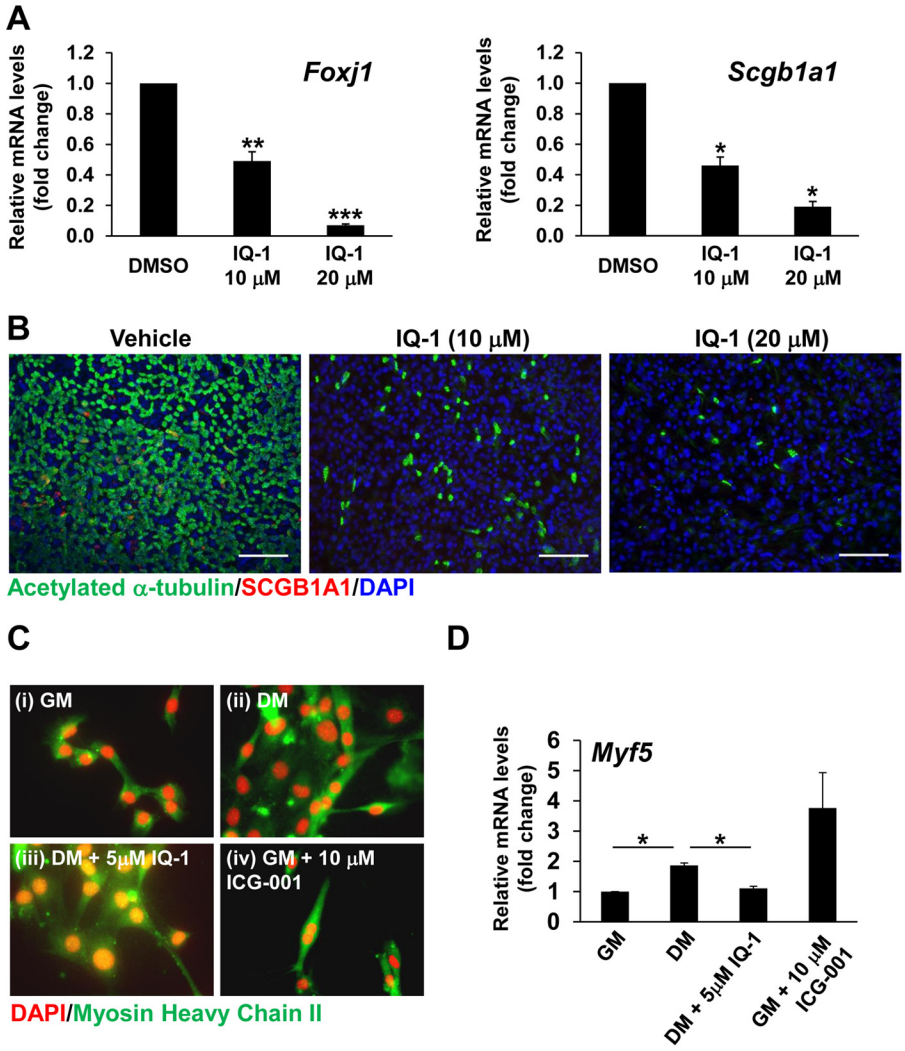

FIGURE 3. **Inhibition of p300/β-catenin interactions inhibit hTEC and myoblast differentiation.** *A,* expression of ciliated cell marker *Foxj1* and club cell marker *Scgb1a1* RNA in primary hTEC exposed to vehicle (DMSO) or IQ-1. *Bars* are mean ± S.E. of triplicate samples from independent experiments using cells of two donors and cultured at air-liquid interface for 24 days. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$. *B,* representative photomicrographs of ciliated marker acetylated α-tubulin (*green*) and club cell marker SCGB1A1 (*red*), with DAPI counterstain (*blue*) using the conditions as described in *A*. *Scale bar* = 100 μm. $n = 2$. *C* and *D,* C2C12 cell differentiation in the presence of IQ-1 and ICG-001. *C*, *i,* immunofluorescence of C2C12 cells cultured in growth medium (*GM*) under subconfluent conditions. *ii*, after 5 days in DM, cells undergo fusion and become multinucleated as seen by myosin heavy chain II (*green*)/DAPI (*red*) staining as they form myotubes. *iii*, cells treated with 5 μM IQ-1/DM resist fusion and remain distinct entities even though they are in direct contact with one another. *iv*, cells in GM + 10 μM ICG-001 become multinucleated and extend to form myotubes in the absence of DM. $n = 3$. *D,* qRT-PCR for *Myf5* mRNA expression from extracts corresponding to the same experimental conditions as in *C*. *Bars* represent mean ± S.E. $n = 3$. *, $p < 0.05$ relative to GM or DM as indicated by *horizontal bars*.

2*E*), further supporting a role for Ser(P)-89 in increasing the p300/β-catenin interaction observed during differentiation. IQ-1 significantly reduced levels of Ser(P)-89 by ~40% at day 2 (Fig. 2*F*), similar to previous observations in embryonic stem cells (9). These data suggest that the p300/β-catenin interaction is necessary for AEC transdifferentiation *in vitro* and that this interaction is modulated by phosphorylation of p300 at Ser-89.

Modulation of p300/β-Catenin Interaction Regulates Differentiation of Other Epithelial and Non-epithelial Cell Types—We explored the effects of IQ-1 on differentiation of hTEC at air-liquid interface over 3 weeks into airway ciliated and secretory cells (club and mucous). IQ-1 (10–20 μM) inhibited differentiation into both ciliated and secretory cell lineages in a dose-dependent manner as reflected by reduced *FOXJ1* and secretoglobin1a1 (*SCGB1A1*) expression, respectively (Fig. 3*A*). Immunofluorescence also demonstrated that IQ-1 inhibited cilia formation and differentiation of hTEC, as determined by dramatically reduced acetylated α-tubulin expression and reductions in secretory cell markers SCGB1A1 and MUC5AC, relative to vehicle (DMSO)-treated cells (Fig. 3*B* and data not shown). We also evaluated effects of IQ-1 on differentiation of mouse C2C12 adult myoblast satellite cells which, when grown in differentiation medium (DM), exhibit cell elongation, fusion, and multinucleation (Fig. 3*C*). Exposure of C2C12 cells to IQ-1 disrupted differentiation in the presence of differentiation media, preventing cell fusion and multi-nucleation (Fig. 3*C*). C2C12 myotubes express myogenic marker *Myf5* (a gene regulated by WNT ligands (29)) upon differentiation. IQ-1-treated C2C12 cells had ~40% reduction in *Myf5* expression relative to growth in DM alone (Fig. 3*D*). ICG-001, a small molecule that specifically inhibits CBP/β-catenin interactions, can promote

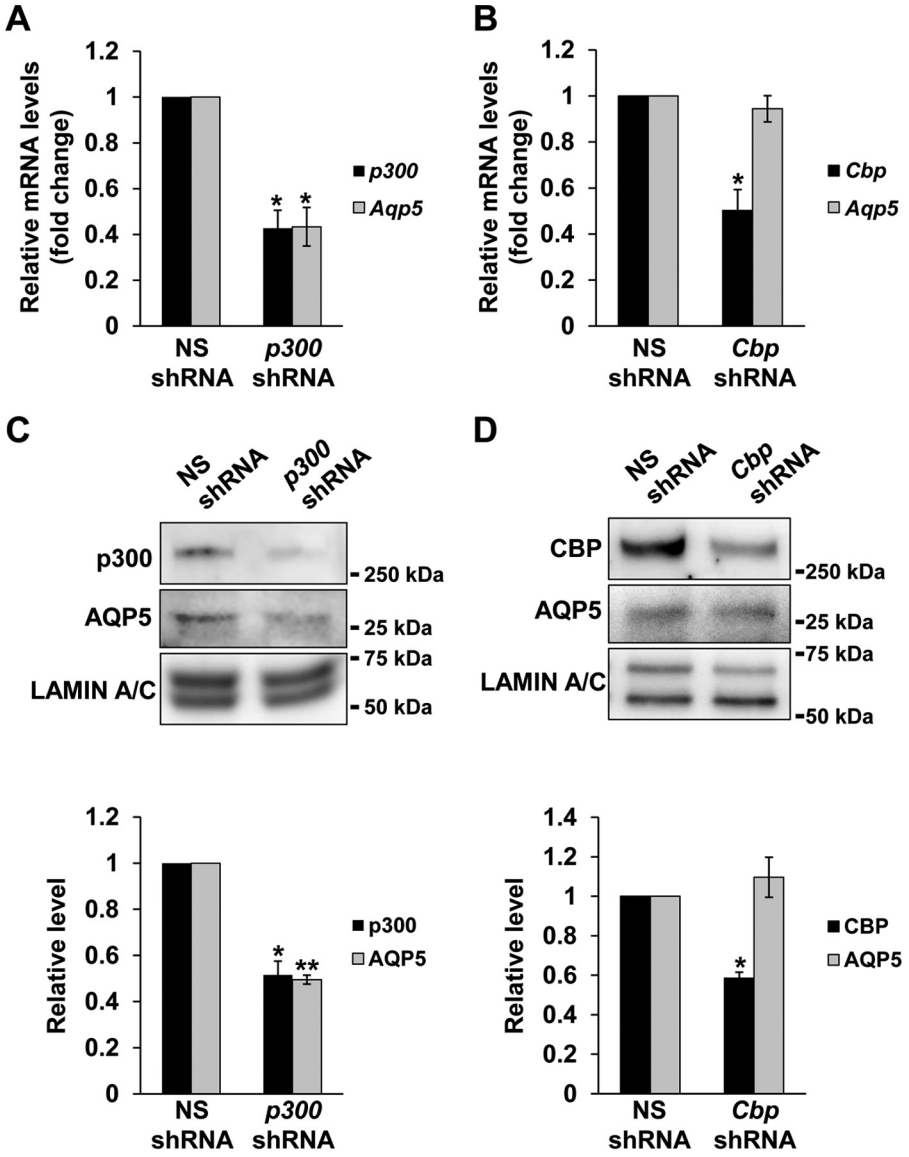

FIGURE 4. **Knockdown of p300 but not CBP in primary cells decreases expression of AT1 cell differentiation marker *Aqp5*.** Primary rat AEC were transduced on day 2 in culture with *p300* shRNA or pGIPZ non-silencing (*NS*) control virus or *Cbp* shRNA or pLKO.1 non-silencing (NS) control virus. Cells were harvested for RNA and protein 72 h posttransduction (on day 5 in culture). *A,* qRT-PCR of *p300* and *Aqp5* mRNA levels following transduction with *p300* shRNA lentivirus compared with pGIPZ NS control virus. $n = 4$. *, $p < 0.01$. *B,* qRT-PCR of *Cbp* and *Aqp5* mRNA expression following transduction with *Cbp* shRNA lentivirus compared with pLKO.1 NS control lentivirus. $n = 4$. *, $p < 0.05$. *C,* Western analysis of p300 and AQP5 protein following transduction with *p300* shRNA lentivirus compared with pGIPZ NS controls. *Top panel* shows a representative Western blot. $n = 3$. *, $p < 0.05$; **, $p < 0.005$. *D,* Western analysis of CBP and AQP5 protein following transduction with *Cbp* shRNA lentivirus compared with pLKO.1 NS vector controls. *Top panel* shows a representative Western blot. *Bottom panel* shows quantitation. $n = 3$. *, $p < 0.01$. *Bars* represent mean ± S.E.

p300/β-catenin interaction (8) and differentiation of stem/progenitor cells. C2C12 myoblasts in growth medium, which normally maintains a proliferative state, underwent differentiation upon exposure to ICG-001 as shown by fusion, multinucleation, and expression of myogenic markers (Fig. 3, *C* and *D*). These results support a model in which p300/β-catenin, but not CBP/β-catenin, interaction is a critical mechanism driving adult stem/progenitor cell differentiation.

Knockdown of p300 Confirms IQ-1 Effects on p300/β-Catenin Interactions—To further investigate mechanisms regulating p300/β-catenin interactions, we focused on the primary AEC differentiation model. We first confirmed specificity of IQ-1 by performing silencing of p300 or *Cbp* using lentiviral-mediated shRNA during differentiation of rat AEC in primary culture. Following p300 knockdown, both p300 mRNA and protein were reduced by 50–60%, with a concomitant decrease in *Aqp5* expression (Fig. 4, *A* and *C*). In marked contrast, *Cbp*-specific shRNA similarly reduced CBP levels by ~50% but did not affect AQP5 expression (Fig. 4, *B* and *D*). These findings are consistent with results of IQ-1 exposure and confirm the importance of p300/β-catenin, but not CBP/β-catenin interactions, for AEC differentiation.

PKC Signaling Regulates Differentiation and Phosphorylation of p300 at Serine 89—We next sought to elucidate endogenous upstream mechanisms that promote p300/β-catenin interactions during differentiation. PKC, a known downstream effector of non-canonical WNT5a signaling (30, 31), was previously shown to phosphorylate p300 at serine 89 (32) and has been

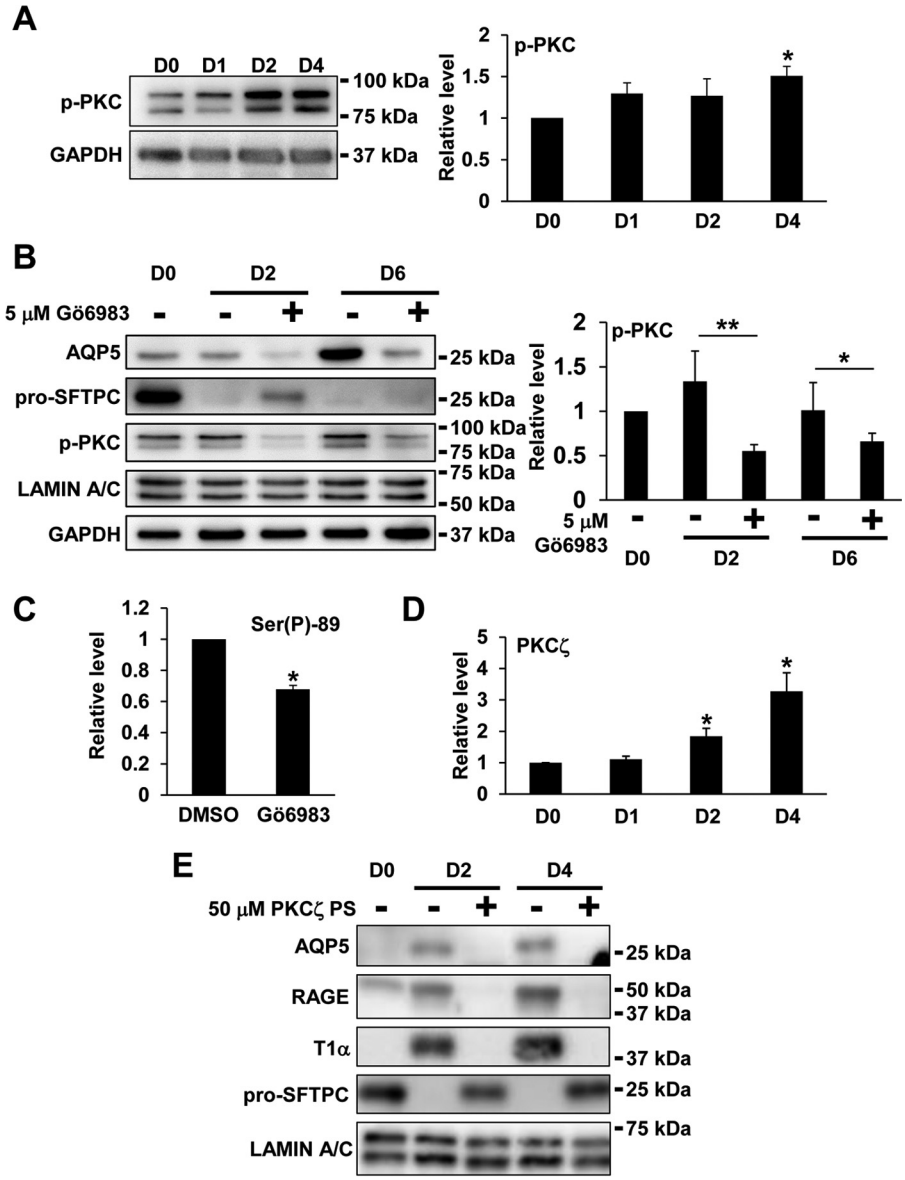

FIGURE 5. **PKC increases during AEC differentiation and inhibition of PKC signaling inhibits AEC differentiation.** *A,* representative Western blot and quantitative analysis of pan phospho-PKC levels (*p-PKC*, 77–88 kDa) in cultured primary rat AEC during transdifferentiation from day 0 (*D0*) to *D4*. $n = 4$. *, $p < 0.05$. GAPDH (37 kDa) was used a loading control. *B*, representative Western blot of primary AEC exposed to Gö6983 (5 μM (+)) *versus* vehicle (DMSO (−)). Quantification of p-PKC is shown to the *right*, normalized to D0. $n = 4$. *, $p < 0.05$; **, $p < 0.005$. *Bars* represent mean ± S.E. *C*, quantitation of Western blot analysis of Ser(P)-89 in primary rat AT2 cells exposed to Gö6983 (5 μM) for 1 h. $n = 3$. *, $p < 0.01$. *Bars* represent mean ± S.E. *D,* quantitation of PKCζ levels by Western analysis. $n = 4$. *, $p < 0.05$. *Bars* represent mean ± S.E. *E,* representative Western blot of AT1 and AT2 cell markers during differentiation of rAT2 cells treated with vehicle (DMSO (−)) or PKCζ pseudosubstrate (50 μM; *PS* (+)). Molecular weight of T1α is 40 kDa and RAGE is 50 kDa. $n = 3$.

implicated in differentiation of several tissues during development (33), although whether these effects involve changes in p300/β-catenin interactions is unknown. Activated pan phospho-PKC (p-PKC) protein levels increased significantly by day 4 of AEC differentiation in primary culture (Fig. 5*A*). PKC inhibitor Gö6983 reduced both PKC phosphorylation and AT1 cell marker expression by day 2 in culture (Fig. 5*B*) and reduced phosphorylation of serine 89 of p300 within 1 h of treatment (Fig. 5*C*). PKC phosphorylation was not affected by IQ-1 (data not shown), consistent with IQ-1 acting downstream of PKC. Inhibition of CAMKII, another kinase regulated by WNT5a, did not inhibit AEC differentiation (data not shown) supporting the notion that our observations were specific to PKC. Analysis of expression of various PKC isoforms revealed a significant increase in PKCζ during AEC differentiation (Fig. 5*D*). Consistent with a role for PKCζ in regulation of AEC differentiation, inhibition of this isoform using a specific pseudosubstrate inhibitor delayed acquisition of AT1 cell markers (AQP5, RAGE, and T1α) and promoted retention of AT2 cell marker pro-SFTPC (Fig. 5*E*), in a manner similar to IQ-1 and Gö6983. Although not ruling out involvement of additional isoforms, these data suggest that PKC (potentially PKCζ) is an important upstream regulator of p300/β-catenin interaction during AEC differentiation through modulation of Ser(P)-89 levels.

AEC Differentiation Is Disrupted in Wnt5a$^{-/-}$ Mice—Based on our results implicating PKC signaling in AEC differentiation and that WNT5a is known to activate PKC (30, 31), we investigated the role of WNT5a as an upstream regulator of PKC act-

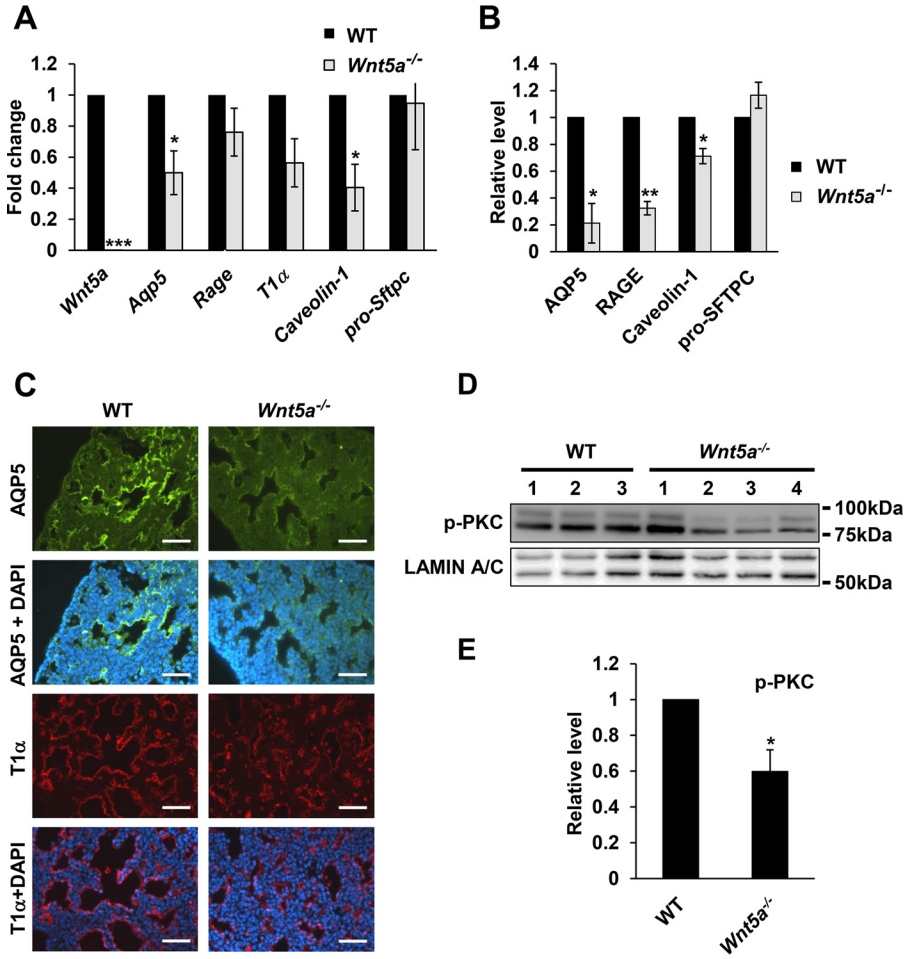

FIGURE 6. ***Wnt5a*$^{-/-}$ mice exhibit reduced expression of AT1 cell differentiation markers and activated PKC.** *A,* qRT-PCR of AT1 cell markers *Aqp5*, *Rage*, *T1α*, and *Cav-1* and AT2 cell marker *pro-Sftpc* at E18.5. WT: $n = 3$. *Wnt5a*$^{-/-}$: $n = 4$. *, $p < 0.05$; ***, $p < 0.0001$ compared with WT. *B,* Western blot quantification of AQP5, RAGE, CAV-1, and pro-SFTPC in *Wnt5a*$^{-/-}$ E18.5 mouse lung homogenates compared with WT littermates. WT, $n = 3$. *Wnt5a*$^{-/-}$, $n = 4$. *, $p < 0.05$; **, $p < 0.001$. *C,* immunofluorescence of AQP5 (*green*) and T1α (*red*) in lungs of *Wnt5a*$^{-/-}$ mice at E18. Nuclei are stained with DAPI (*blue*). *Scale bars* = 50 μm. $n = 2$. *D,* Western blot and *E,* quantitation of p-PKC in *Wnt5a*$^{-/-}$ E18.5 lung homogenates. WT, $n = 3$. *Wnt5a*$^{-/-}$, $n = 4$. *, $p < 0.05$. *Bars* represent mean ± S.E.

ing to enhance p300/β-catenin interaction in AEC differentiation. Given that *Wnt*$^{-/-}$ mice have delayed lung maturation as indicated by decreased airspace size and increased epithelial cellularity (21), we investigated AEC differentiation defects in these mice by analyzing AT1 cell marker RNA and protein expression. At E18.5, a developmental point at which AT1 cell marker expression is present despite the fact that fully differentiated AT1 cells are not yet evident, lungs of *Wnt5a*$^{-/-}$ mice had reduced levels of *Aqp5*, *Rage*, *T1α*, and *Cav-1* mRNA and AQP5, RAGE, and CAV-1 protein relative to WT littermates (Fig. 6, *A* and *B*), and reduced levels of AQP5 and T1α protein by immunostaining of paraffin-embedded lung sections (Fig. 6*C*). Expression of *pro-Sftpc* mRNA or pro-SFTPC protein was not changed in extracts from *Wnt5a*$^{-/-}$ mice relative to WT littermates (Fig. 6, *A* and *B*). *Wnt5a*$^{-/-}$ mouse lung extracts showed reduced levels of p-PKC relative to WT littermates (Fig. 6, *D* and *E*), supporting a role for WNT5a as an upstream regulator of PKC in the context of AEC differentiation during lung development.

Regulation of p300/β-Catenin Interactions and Differentiation by WNT5a in Adult AEC—In our *in vitro* adult AEC differentiation model, *Wnt5a* mRNA expression increased between days 1 and 4 in primary culture (Fig. 7*A*), concurrent with upregulation of *Aqp5*. Exposure of primary AT2 cells to recombinant WNT5a (100 ng/ml) for 24 h from the time of plating significantly increased AQP5 protein, suggesting acceleration of AT1 cell differentiation (Fig. 7*B*). Effects of WNT5a were abrogated by treatment with the PKC inhibitor Gö6983, supporting a role for PKC in WNT5a-mediated activation of AT1 cell marker expression (Fig. 7*C*). To test the effects of *Wnt5a* knockdown on expression of AT1 cell markers, we used the E10 mouse lung epithelial cell line that has AT1 cell features. Lentiviral-mediated *Wnt5a* shRNA reduced *Wnt5a* mRNA levels by ~95% and *Aqp5* levels by ~70% (Fig. 7*D*). Large-scale *Wnt5a* knockdown in E10 cells also reduced Ser(P)-89 protein levels by ~50% in nuclear extracts (*input*, Fig. 7, *E* and *F*). To test effects of *Wnt5a* knockdown on p300/β-catenin interactions, we performed co-immunoprecipitation using a Ser(P)-89 antibody and immunoblotted for β-catenin. *Wnt5a* knockdown resulted in a 60–70% reduction in p300/β-catenin interaction as assessed by the ratio of absolute intensity of β-catenin bands to Ser(P)-89 immunoprecipitated bands (Fig. 7, *E* and *G*). Similar reductions in p300/β-catenin interaction were observed using an antibody for total p300 (Fig. 7*G*). Although we cannot rule

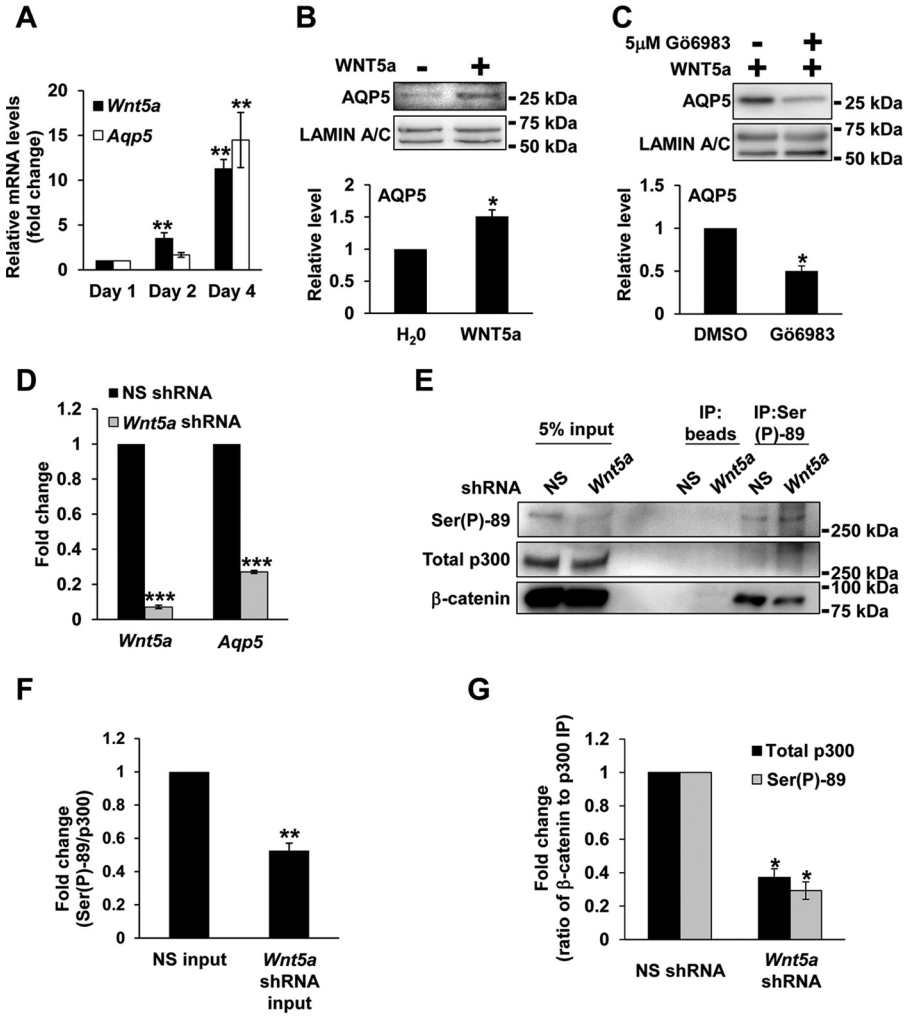

FIGURE 7. **WNT5a regulates Ser(P)-89 expression, p300/β-catenin interactions, and AQP5 expression.** *A,* qRT-PCR for *Wnt5a* performed on rat AEC in primary culture harvested on days 1, 2, and 4 during *in vitro* transdifferentiation. *Aqp5* is shown as control to confirm AT1 cell transdifferentiation. $n \geq 3$. **, $p <$ 0.001 relative to day 1. *B,* representative Western blot and quantification of AQP5 protein expression at 24 h post-plating following exposure to recombinant WNT5a (100 ng/ml) *versus* H_2O vehicle control from D0. $n = 3$. *, $p < 0.05$. *C,* representative Western blot and quantification of AQP5 protein expression at 24 h post-plating in the presence of recombinant WNT5a (100 ng/ml) and Gö6983 (5 μM (+)) or vehicle (DMSO (−)). Values were normalized to WNT5a vehicle control. $n = 3$. *, $p < 0.05$. *D,* qRT-PCR for *Wnt5a* and *Aqp5* mRNA following lentivirus-mediated shRNA knockdown of *Wnt5a* in the E10 mouse epithelial cell line. $n = 3$. ***, $p < 0.0001$. *E,* representative co-immunoprecipitation of nuclear extracts harvested 72 h after transduction with pLKO.1 non-silencing (*NS*) shRNA or *Wnt5a* shRNA. 135–150 μg of extract was immunoprecipitated (*IP*) with an antibody recognizing p300 phosphorylated at serine 89 (Ser(P)-89) followed by immunoblotting for Ser(P)-89 and β-catenin. Blots were stripped and re-probed for total p300. $n = 3$. *F,* quantification of Ser(P)-89 expression relative to total p300 levels in 5% input samples used in *D* following knockdown of *Wnt5a*, normalized to pLKO.1 NS control. $n = 4$. **, $p < 0.005$. *G,* β-catenin pulldown relative to p300 following *Wnt5a* knockdown calculated as the amount of co-immunoprecipitated β-catenin divided by immunoprecipitated Ser(P)-89 or total p300 as shown in *E*. $n = 3$. *, $p < 0.01$. *Bars* represent mean ± S.E.

out the possibility that knockdown of *Wnt5a* affects p300/β-catenin interactions through additional regulatory mechanisms, these results support involvement of WNT5a in regulation of the p300/β-catenin interaction through modulation of Ser(P)-89 levels, in turn demonstrating the importance of Ser(P)-89 downstream of WNT5a in differentiation of adult progenitor cells.

Discussion

Understanding molecular mechanisms, in particular integration of multiple signal transduction pathways and environmental cues that regulate normal adult somatic stem/progenitor cell maintenance and differentiation, is critical to understanding aberrant differentiation after tissue injury or in cancer. The complex Wnt signaling pathway regulates both stem/progenitor cell maintenance and differentiation, although mechanisms that account for these divergent functions have not been clearly established. We describe a novel paradigm in which the traditionally non-canonical Wnt ligand, WNT5a, via activation of PKC (potentially PKCζ) increases p300 phosphorylation at Ser-89, thereby increasing p300/β-catenin interaction to promote adult progenitor cell differentiation. Using primary distal lung AEC as a model of adult somatic progenitor cell differentiation, we demonstrate that p300/β-catenin interactions increase during AT2 to AT1 cell differentiation, whereas the CBP/β-catenin interactions decrease. p300 increases transcriptional activity of the *Aqp5* promoter, a gene expressed by AT1 cells *in situ* and up-regulated during transdifferentiation of AT2 cells to an AT1 cell-like phenotype *in vitro*. Using hTEC in primary culture as a model of adult proximal airway epithelial cell differentiation,

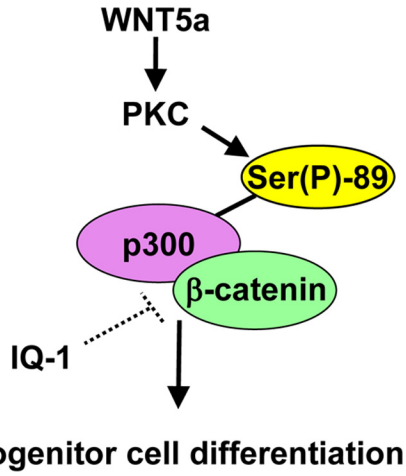

FIGURE 8. **Model for role of p300/β-catenin in adult progenitor cell differentiation.** Phosphorylation of p300 at residue serine 89 (Ser(P)-89) enhances p300/β-catenin interactions to promote progenitor cell differentiation. The small molecule inhibitor IQ-1 reduces Ser(P)-89 levels via indirect mechanisms thereby disrupting p300/β-catenin interactions and inhibiting differentiation of AEC, hTEC, and C2C12 cells. Ser(P)-89 expression is regulated by PKC. In turn, WNT5a activates PKC signaling, increases Ser(P)-89 expression and promotes p300/β-catenin interactions to drive differentiation of progenitor cells. These findings support a model in which p300/β-catenin interactions promote differentiation of adult progenitor cells in a PKC- and WNT5a-dependent manner.

and C2C12 myoblasts as a model of adult mesenchymal cell differentiation, we demonstrate that disruption of the p300/β-catenin interaction similarly inhibits transition to a more differentiated phenotype. Phosphorylation of p300 at serine 89, a modification previously shown to enhance p300/β-catenin avidity (9), increases during AEC differentiation. Furthermore, Ser(P)-89 is regulated in a PKC-dependent manner downstream of WNT5a, previously implicated in lung development and epithelial cell maturation (21). Knockdown of WNT5a leads to reduced Ser(P)-89 levels and p300/β-catenin interactions, and decreased expression of AT1 cell differentiation markers, establishing a novel mechanism linking this non-canonical Wnt ligand to β-catenin-dependent signaling and cellular differentiation (Fig. 8). Utilizing three unique models (AEC, hTEC, and C2C12 myoblasts) that facilitate monitoring of temporal changes in phenotype during normal differentiation in the absence of tissue injury/repair, we demonstrate the importance of differential β-catenin co-activator interactions (CBP *versus* p300) for regulation of stem/progenitor cell-progeny relationships in adult cells. This paradigm appears to be generalizable to both epithelial and non-epithelial adult somatic stem/progenitor cells. In support of the universality of this model, similar β-catenin/co-activator interactions have been shown to regulate differentiation of other stem/progenitor cells including mouse and human embryonic stem cells (9, 34), cardiovascular progenitors (35, 36), neuronal progenitors (37), hepatoblasts and hepatic tumor initiating cells (38), and leukemia cells (39). This report extends these studies to elucidate a novel intersection between non-canonical and canonical signaling arms, further highlighting the complexity of Wnt signaling.

p300 and CBP are highly homologous histone Kat3 acetyltransferases that are largely viewed as interchangeable despite considerable evidence indicating that they are not functionally redundant (7). *p300* knock-out leads to heart defects (40), whereas *Cbp* heterozygous mice exhibit growth retardation and craniofacial abnormalities (41, 42). Specific inhibition of the CBP/β-catenin interaction leads to decreased transcription of a subset of Wnt target genes, including *Survivin* and *CCND1* (8, 10), whereas p300/β-catenin interactions are critical to the expression of other Wnt/TCF target genes (*e.g. WISP1*) (43). Using a combination of approaches in conjunction with adult differentiation model systems, we provide further strong support for divergent roles of p300 and CBP in adult stem/progenitor behavior, with p300/β-catenin interactions driving differentiation of adult stem/progenitor cells and CBP/β-catenin promoting progenitor maintenance/self-renewal. Consistent with this model, inhibition of p300/β-catenin interactions with IQ-1 decreases differentiation of multiple cell types (Figs. 2 and 3) (35). IQ-1 inhibits p300/β-catenin interactions by decreasing Ser(P)-89 levels as a result of inhibiting protein phosphatase 2A activity, as previously described (9). Inhibition of protein phosphatase 2A is thought to prevent dephosphorylation of adjacent Ser(P)-90 thereby inhibiting phosphorylation of Ser-89 and reducing p300/β-catenin interaction. In support of results with IQ-1, knockdown of p300 but not CBP affected AQP5 expression in AEC (Fig. 4), whereas mutant p300 did not transcriptionally activate *Aqp5-Luc*. These studies support functionally distinct roles of co-activators p300 and CBP, and specifically differential interaction of β-catenin with these Kat3 co-activators, in both embryonic and adult progenitor cell fate decisions.

Regulation of adult stem/progenitor cell differentiation is particularly important in the context of tissue injury and repair. Dichotomous effects of p300 *versus* CBP interactions with β-catenin in adult stem/progenitor cells helps reconcile apparently contradictory conclusions of studies showing a requirement for β-catenin-dependent signaling for normal lung epithelial cell differentiation (43, 44) and evidence for up-regulation of epithelial Wnt signaling in fibrotic lung and kidney disease (45–48), with improved outcomes in models of injury/fibrosis following inhibition of β-catenin signaling (49, 50). In fibrotic lung diseases such as idiopathic pulmonary fibrosis, intermediate AEC states have been observed in damaged lungs, suggesting that impaired differentiation (concurrent with increased proliferation) of AEC progenitors may contribute to disease pathogenesis (49, 51–53). Consistent with the notion of impaired AEC differentiation in idiopathic pulmonary fibrosis, we previously demonstrated co-localization of β-catenin with CBP in nuclei of abnormal hyperplastic AT2 cells in lungs of idiopathic pulmonary fibrosis patients, whereas our current results suggest a requirement for p300/β-catenin interaction in driving normal differentiation (Fig. 1*A*) (54). These results strongly support a model in which differential interactions of p300 *versus* CBP with β-catenin regulate dichotomous roles of Wnt signaling in determining stem/progenitor cell fates.

WNT ligands traditionally signal through distinct and independent canonical or non-canonical pathways, a selection thought to be context-, ligand-, and receptor-dependent (55). Recent studies suggest, however, that classification of Wnt signaling as purely canonical or non-canonical represents an oversimplification, because there are increasing examples of cross-

talk between these two arms. In this study, we identified a novel mechanism underlying the intersection between canonical and non-canonical pathways and showed that non-canonical WNT5a signals through PKC to influence canonical β-catenin-dependent signaling via enhanced p300/β-catenin interaction (Fig. 8). Knockdown of *Wnt5a* and inhibition of PKC resulted in reduced phosphorylation of p300 at Ser-89 (Figs. 7, *E* and *F*, and 5*C*, respectively) and reduced expression of AT1 cell differentiation markers (Figs. 7*D* and 5, *B* and *E*, respectively). Consequently, knockdown of *Wnt5a* reduced p300/β-catenin interaction in AEC (Fig. 7, *E* and *G*), supporting the importance of the p300/β-catenin interaction in regulation of differentiation-related genes. Traditionally non-canonical WNT ligands (*e.g.* WNT5a) cooperate with canonical Wnt ligands (*e.g.* WNT3a) to drive neuronal differentiation (56), whereas WNT5a has been shown to require β-catenin and PKC signaling to drive endothelial cell differentiation (57), supporting the intersection of these two arms in regulating functional outcomes critical for tissue homeostasis and repair. WNT5a has been implicated in differentiation of chondrocytic, adipocytic, osteogenic, and neuronal stem/progenitors during development (58–61) as well as in adult cardiomyocyte differentiation from murine stromal vascular cells (62) and axonal differentiation through activation of atypical PKC (33). Whereas these latter studies attributed differentiation solely to non-canonical pathway activation, our findings link WNT5a/PKC to p300/β-catenin interaction and transcription of a specific subset of Wnt target genes. Using a combination of inhibitor and knockdown approaches, and studies in *Wnt5a*$^{-/-}$ mice, we have elucidated a novel PKC-dependent pathway downstream of WNT5a that modulates β-catenin-dependent gene expression through regulation of Ser(P)-89 to promote β-catenin/p300 interaction and stem/progenitor cell differentiation. These findings highlight a growing body of evidence demonstrating intersection of canonical and non-canonical Wnt signaling pathways in regulation of cell fate decisions.

Despite significant evidence supporting the involvement of this novel signaling axis in adult progenitor differentiation, we note that signaling pathways are complex. There are likely other mechanisms regulating both p300/β-catenin interaction and adult progenitor cell differentiation. Additional signaling pathways likely compensated to drive differentiation of AEC in our assays, given that small molecule inhibition of PKC signaling or p300/β-catenin interaction was incomplete. Multiple kinases are known to phosphorylate p300 at serine 89 including salt-inducible kinase and AMP-activated protein kinase (63, 64). To our knowledge, links between WNT5a signaling and these kinases have not been previously demonstrated, prompting us to focus on PKC. Inhibition of PKC signaling abrogated WNT5a-enhanced expression of AQP5 (Fig. 7*C*), supporting the notion that at least at early time points, PKC signaling is necessary to activate expression of differentiation markers downstream of WNT5a. Although we cannot exclude the possibility that other PKC isoforms are also involved in regulating p300/β-catenin interactions, data showing an increase in PKCζ levels accompanying differentiation (Fig. 5*D*) and inhibition of acquisition of AT1 cell markers (Fig. 5*E*) by the PKCζ pseudosubstrate implicate this PKC isoform in regulation of AEC differentiation. We also note that knockdown of *Wnt5a* in E10 cells appeared to increase efficiency of p300 pulldown in those samples (Fig. 7*E*), and cannot rule out that knockdown of *Wnt5a* has other effects on p300 stability or induces structural changes affecting antibody interactions. Notwithstanding these caveats, these studies identify a novel signaling axis important for adult stem/progenitor cell differentiation. Elucidation of pathways regulating differential β-catenin co-activator interactions have important implications for design of therapies to modulate adult progenitor cell behavior in response to Wnt/β-catenin-dependent signaling and promote their differentiation in fibrosis and other diseases.

Author Contributions—M. E. R. designed, performed, analyzed experiments and wrote the manuscript. B. Z. designed experiments, analyzed data, and edited the manuscript. N. S., M. S., C. L., C. N., J. P., and Y. L. performed and analyzed experiments. P. M., S. L. B., and E. D. C. designed experiments and contributed to manuscript preparation. M. K. and Z. B. conceived the study, designed experiments, reviewed and analyzed data, and wrote and edited the manuscript.

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