ORIGINAL ARTICLE

Loss of p73 Expression Contributes to Chronic Obstructive Pulmonary Disease

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Abstract

Rationale: Multiciliated cell (MCC) loss and/or dysfunction is common in the small airways of patients with chronic obstructive pulmonary disease (COPD), but it is unclear if this contributes to COPD lung pathology.

Objectives: To determine if loss of p73 causes a COPD-like phenotype in mice and explore whether smoking or COPD impact p73 expression.

Methods: $p73^{floxE7-E9}$ mice were crossed with Shh-Cre mice to generate mice lacking MCCs in the airway epithelium. The resulting $p73^{\Delta airway}$ mice were analyzed using electron microscopy, flow cytometry, morphometry, forced oscillation technique, and single-cell RNA sequencing. Furthermore, the effects of cigarette smoke on p73 transcript and protein expression were examined using *in vitro* and *in vivo* models and in studies including airway epithelium from smokers and patients with COPD.

Measurements and Main Results: Loss of functional p73 in the respiratory epithelium resulted in a near-complete absence of MCCs in $p73^{\Delta airway}$ mice. In adulthood, these mice spontaneously developed neutrophilic inflammation and emphysema-like lung remodeling and had progressive loss of secretory cells. Exposure of normal airway epithelium cells to cigarette smoke rapidly and durably suppressed p73 expression *in vitro* and *in vivo*. Furthermore, tumor protein 73 mRNA expression was reduced in the airways of current smokers ($n = 82$) compared with former smokers ($n = 69$), and p73-expressing MCCs were reduced in the small airways of patients with COPD ($n = 11$) compared with control subjects without COPD ($n = 12$).

Conclusions: Loss of functional p73 in murine airway epithelium results in the absence of MCCs and promotes COPD-like lung pathology. In smokers and patients with COPD, loss of p73 may contribute to MCC loss or dysfunction.

Keywords: COPD; ciliated cell; p73; cigarette smoke

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At a Glance Commentary

Scientific Knowledge on the Subject: p73 is a transcription factor required for multiciliated cell (MCC) differentiation. To better understand if the loss of MCCs contributes to lung pathology in chronic obstructive pulmonary disease (COPD), we generated mice lacking p73 in the respiratory epithelium.

What This Study Adds to the Field: Loss of p73 in the airways results in multiple COPD-relevant phenotypes, including loss of MCCs and secretory cells, neutrophilic inflammation, and emphysema-like lung remodeling. Furthermore, p73 is suppressed by cigarette smoke *in vitro* and *in vivo* and is reduced in the airways of smokers and patients with COPD.

Chronic obstructive pulmonary disease (COPD) is a common and often fatal disorder associated with long-term exposure to cigarette smoke (CS) or other inhaled irritants (1). Mucus plugging is common in the small airways of patients with COPD with or without chronic bronchitis and contributes to disease-defining airflow obstruction (2, 3). Although mucus plugging is believed to primarily result from mucus hypersecretion from goblet cell hyperplasia (4), reduced numbers of multiciliated cells (MCCs) and defects in cilia length and function have been described in smokers and patients with COPD (5–14) and may contribute to defects in mucociliary clearance in these groups (15–18). Through the metachronal beating of their cilia, MCCs normally move mucus and particles entrapped in mucus from small to large airways, where they are then expectorated (19). Thus, loss or impairment of MCCs may contribute to inadequate mucociliary clearance and mucus plugging in COPD. However, little is known regarding the mechanisms of MCC dysfunction in smokers or patients with COPD or the downstream consequences of MCC loss.

p73 (tumor protein 73) is a member of the p53 family of sequence-specific transcription factors (p53, p63, and p73) with known roles in regulating the cell cycle, apoptosis, cell–cell junctions, wound healing, DNA repair, and cellular differentiation (20–24). Studies using p73 null ($p73^{-/-}$) mice led to the discovery that p73 is expressed in MCCs and a subset of basal cells and is required for MCC development (25–28). Mice lacking p73 have a nearly complete absence of MCCs throughout the body and many resulting phenotypes, including chronic infection and inflammation of the lungs and sinuses and substantial postnatal mortality (26, 27, 29). Similar phenotypes are observed in humans with homozygous loss-of-function mutations in human tumor protein 73 (TP73) (30). However, it is unknown if p73 contributes to MCC loss or dysfunction in smokers or patients with COPD or if loss of p73 in the airways specifically contributes to COPD pathogenesis.

Here, we generated mice lacking p73 in the airway epithelium to study the impact of MCC loss on lung pathology *in vivo*. We found mice lacking p73 in the airways have a near-complete loss of MCCs and spontaneously develop emphysema-like parenchymal remodeling, chronic neutrophilic inflammation, and progressive loss of $SCGB1A1^{+}$ (Secretoglobin family 1A member 1) secretory cells. In addition, we show that CS suppresses p73 expression *in vitro* and *in vivo* and that p73 is reduced in the airways of smokers and patients with COPD. Together, these data suggest CS-mediated loss of p73 may contribute to COPD-relevant phenotypes, including MCC loss/dysfunction, neutrophilic inflammation, and emphysema-like lung remodeling, and secretory cell loss, and may continue in patients with COPD after smoking cessation. Some of the results of these studies have been previously reported in the form of abstracts (31, 32).

Methods

Oversight

Institutional Animal Care and Use Committee approval. All mouse experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center (Institutional Animal Care and Use Committee #V1800148 and M1800069-00).

Institutional review board approval. The explanted lungs of patients with COPD undergoing lung transplantation were obtained after informed consent according to an institutional review board (IRB)-approved protocol from Vanderbilt University Medical Center (IRB #060165). Control samples were obtained from deceased organ donors and exempted from IRB review. Available clinical and demographic information for human-derived samples is provided in Table E1 in the online supplement.

$p73^{\Delta airway}$ mouse model. The $p73^{floxE7–E9}$ model has been previously described (26). For these experiments, $p73^{floxE7–E9}$ mice were backcrossed a minimum of seven generations onto a C57Bl6/J background. To generate $p73^{\Delta airway}$ mice, $p73^{floxE7–E9}$ mice were bred to mice constitutively expressing Cre recombinase driven by the Sonic hedgehog locus (strain #005622, The Jackson Laboratory) (33) to generate C57Bl6/J mice lacking full-length p73 in the airway epithelium. For all experiments, controls consisted of age-matched littermates homozygous for the $p73^{floxE7–E9}$ allele but not expressing Cre recombinase.

Data availability. Single-cell RNA sequencing (scRNA-seq) data from $p73^{\Delta airway}$ mice will be available in the Gene Expression Omnibus database under GSE240096. Transcriptional data on airway brushings used in our *in silico* analyses are available at GSE37147 (34) and https://data.mendeley.com/datasets/7r2cwbw44m/1) (35).

Results

Targeted p73 Deletion in the Airways Results in MCC Loss, Emphysema-like Parenchymal Remodeling, and Neutrophilic Inflammation

To investigate the impact of p73 deletion in the respiratory epithelium, we bred mice with loxP sites flanking exons 7–9 of *Trp73* ($p73flox^{E7–E9}$ mice) (26) to Shh-Cre mice. The resulting Cre^{+} progeny, which we termed $p73^{\Delta airway}$ mice, had complete loss of full-length *Trp73* expression in the lungs but continued to produce a truncated version of p73 protein, which has previously been shown to be functionally inactive (26) (Figures E1A and E1B). However, there was also a slight reduction in full-length p73 in the skin and a total loss of full-length p73 in the esophagus, suggesting that loss of p73 is not entirely specific to the airway epithelium in this model (Figure E1B). However, apart from mild runting (Figure E1C), 6-month-old $p73^{\Delta airway}$ mice lacked many of the systemic phenotypes previously observed

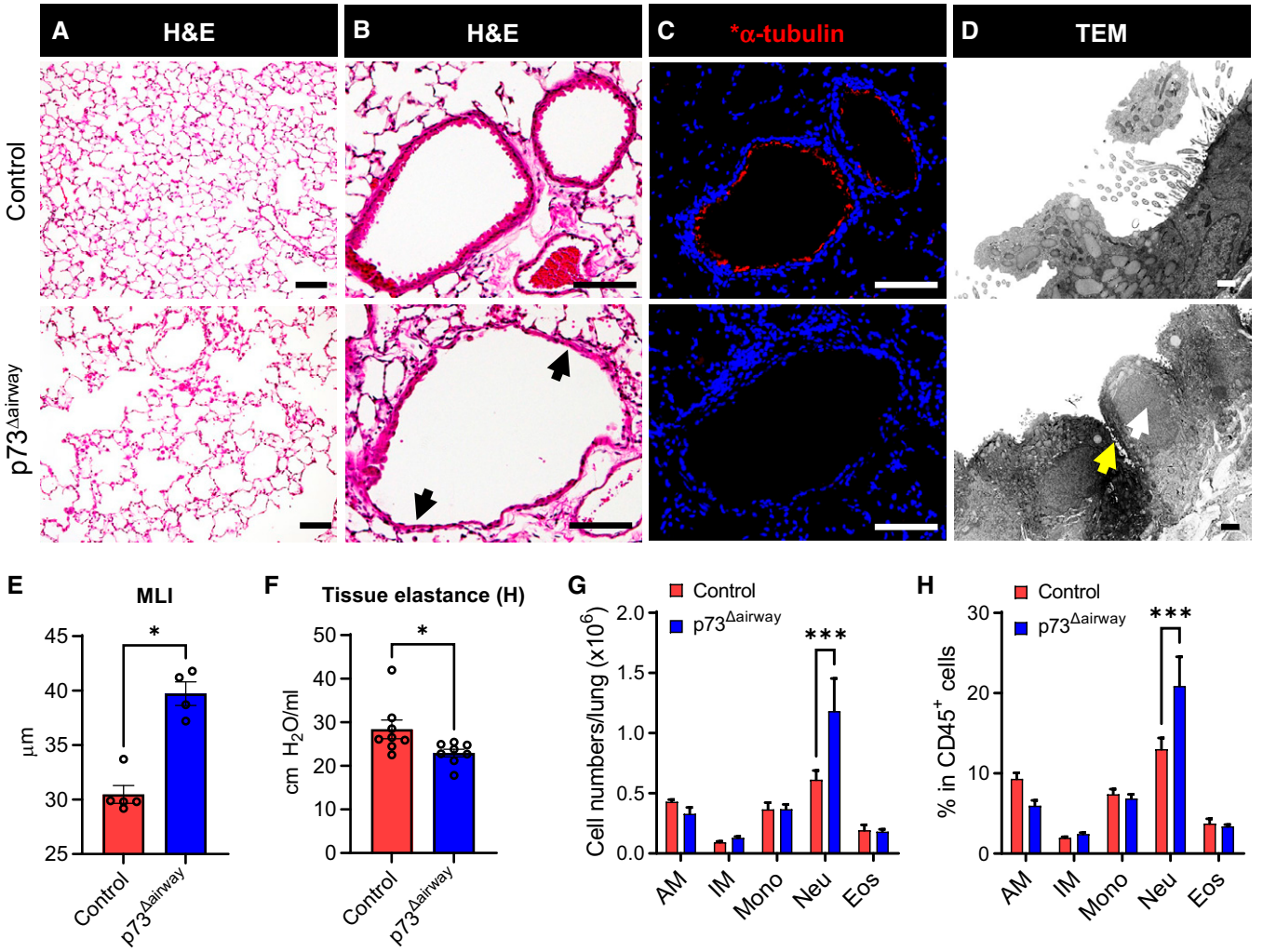

Figure 1. Targeted p73 deletion in the airways results in multiciliated cell (MCC) loss, emphysema-like lung remodeling, and neutrophilic inflammation. All experiments in this figure were performed on adult (4–7 months old) $p73^{\Delta airway}$ mice and littermate control mice. (*A*) Low-magnification view of lungs of $p73^{\Delta airway}$ mice shows emphysema-like lung remodeling but no overt pneumonitis. Scale bars, 100 μm. (*B*) The airways of $p73^{\Delta airway}$ mice contain flattened, dysmorphic epithelial cells and areas without overlying epithelial cells (black arrows). Scale bars, 100 μm. (*C*) Representative images of immunostaining for acetylated α-tubulin (*α-tubulin) showing a near-complete absence of MCCs in the airways of $p73^{\Delta airway}$ mice. Scale bars, 100 μm. (*D*) TEM micrographs showing absent MCCs, loss of cell–cell junctions (yellow arrow), and apically oriented nuclei (white arrow) in airway epithelial cells from $p73^{\Delta airway}$ mice. Scale bars, 2 μm. (*E*) MLI in $p73^{\Delta airway}$ and littermate control mice. $n = 4$–5 mice/group. $^*P < 0.05$; Mann-Whitney *U* test. (*F*) Tissue elastance (H) in $p73^{\Delta airway}$ mice and littermate control mice. $n = 8$ mice/group. $^*P < 0.05$; Mann-Whitney *U* test. (*G* and *H*) Neutrophil numbers (*G*) and percentage among $CD45^+$ cells (*H*) in the left lungs of adult (4–7 months old) $p73^{\Delta airway}$ mice and littermate control mice. $^{***}P < 0.001$; two-way ANOVA with Šidák's multiple comparison correction. AM = alveolar macrophages; Eos = eosinophils; H&E = hematoxylin and eosin; IM = interstitial macrophages; MLI = mean linear intercept; Mono = monocytes; Neu = neutrophils; TEM = transmission electron microscopy.

in $p73^{-/-}$ mice (26, 27), such as reduced fertility and postnatal lethality. $p73^{\Delta airway}$ mice did not develop overt pneumonitis (Figure 1A) but had a structurally abnormal epithelium characterized by flattened, dysmorphic epithelial cells and areas of basement membrane without overlying epithelium (Figure 1B). Immunostaining for the MCC marker acetylated α-tubulin (*α-tubulin) indicated a near-complete absence of MCCs in the airways of $p73^{\Delta airway}$ mice (Figure 1C). Transmission electron microscopy confirmed the loss of MCCs and indicated $p73^{\Delta airway}$ mice also have abnormal cell–cell junctions and a general loss of polarity, including abnormally positioned nuclei and secretory organelles (Figure 1D).

Low-magnification images of the lung parenchyma of 6-month-old $p73^{\Delta airway}$ mice indicated possible emphysema-like remodeling (Figure 1A). To evaluate this further, we quantified emphysema using mean linear intercept and forced oscillation technique. We found that by 6 months of age, $p73^{\Delta airway}$ mice had an increase in mean linear intercept and a reduction in tissue elastance by forced oscillation technique, both indicative of emphysema-like lung remodeling (Figures 1E and 1F). Because emphysema can be driven by immune/inflammatory cell–derived proteases in patients with COPD and in COPD animal models (36, 37), we

determined if p73$^{\Delta airway}$ mice have increased lung immune/inflammatory cells. Using established flow cytometry protocols (38, 39) (Figure E2A), we quantified major myeloid and T-cell subsets in the lungs of p73$^{\Delta airway}$ mice. We noted an approximately twofold increase in neutrophils in the lungs of p73$^{\Delta airway}$ mice and an increase in the percentage of neutrophils among CD45^{+} cells (Figures 1G and 1H). In contrast, numbers of other major myeloid and T-cell subsets did not differ between p73$^{\Delta airway}$ mice and littermate control mice, and differences in the percentages among CD45^{+} cells were minor (Figures E2B and E2C). Together, these data indicate that p73$^{\Delta airway}$ mice have nearly a complete absence of MCCs in the lungs and spontaneously develop emphysema-like lung remodeling and neutrophilic inflammation, with minor impacts on other immune/inflammatory cell types.

Loss of p73 in the Airway Epithelium Results in Progressive Loss of SCGB1A1^{+} Secretory Cells

We next focused on the impact of p73 deletion on the airway epithelium. Light microscopy indicated a potential reduction in the number of cells covering the airway basement membrane in 6-month-old p73$^{\Delta airway}$ mice (Figure 1A). To quantify this, we counted the number of airway epithelial cells per 1 mm basement membrane in both young adult (2-month-old) and adult (6-month-old) p73$^{\Delta airway}$ mice and age-matched littermate control mice, focusing on smaller airways that fit entirely inside a single field of view at 200-fold magnification. The number of DAPI^{+} cells per 1 mm basement membrane was reduced by 36% and 38% in 2- and 6-month-old p73$^{\Delta airway}$ mice, respectively (Figure 2A). Immunostaining for SCGB1A1 in 2-month-old p73$^{\Delta airway}$ mice and littermate control mice indicated most of the remaining cells in the airways of p73$^{\Delta airway}$ expressed SCGB1A1 (Figure 2B, top panels). These cells covered most of the airway but assumed a wider, flattened morphology (Figure 2B, insets). In contrast, 6-month-old p73$^{\Delta airway}$ mice had extensive areas in which no SCGB1A1^{+} cells were present (Figure 2B, bottom panels). Quantification of the percentage of SCGB1A1^{+} cells among DAPI^{+} cells

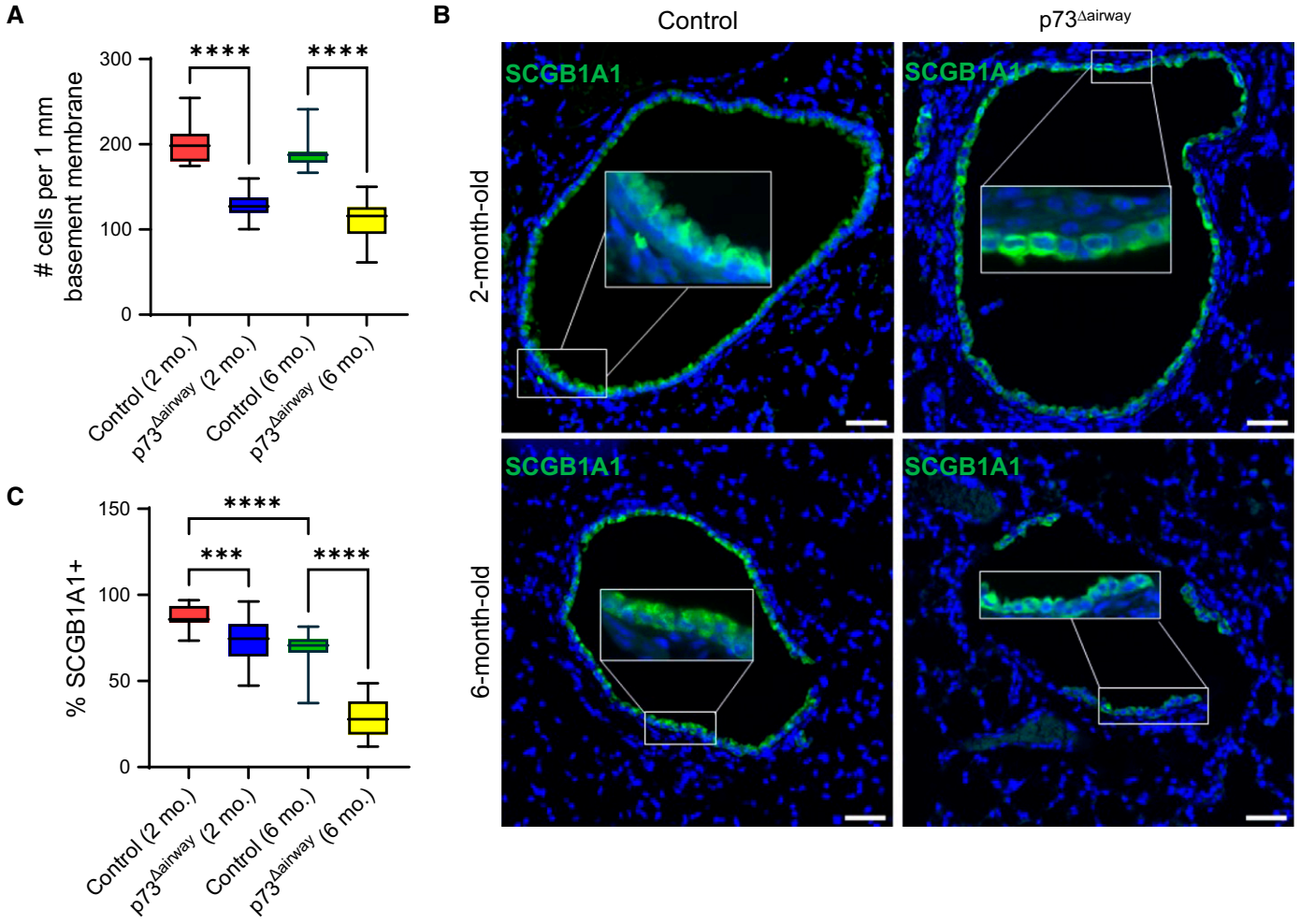

Figure 2. Deletion of p73 in the airway epithelium results in progressive loss of SCGB1A1^{+} secretory cells. (*A*) Quantification of DAPI^{+} cells per 1 mm basement membrane in 2- and 6-month-old p73$^{\Delta airway}$ mice and littermate control mice. $n = 4$–5 mice/group and 5–13 airways/mouse. ****$P < 0.0001$; one-way ANOVA with Tukey's multiple comparisons test. (*B*) Representative images of SCGB1A1 immunostaining in 2- and 6-month-old p73$^{\Delta airway}$ mice and littermate control mice. Scale bars, 50 μm. Inset, 2× magnification. (*C*) Percentage of SCGB1A1^{+} cells among total DAPI^{+} cells in 2- and 6-month-old p73$^{\Delta airway}$ mice and littermate control mice. $n = 4$–5 mice/group and 6–19 airways/mouse. ***$P < 0.001$ and ****$P < 0.0001$; one-way ANOVA with Tukey's multiple comparisons test.

confirmed a dramatic reduction in the percentage of SCGB1A1^{+} cells in 6-month-old p73$^{\Delta airway}$ mice and a more minor reduction in the percentage of these cells in 2-month-old mice (Figure 2C).

Loss of p73 in the Airway Epithelium Increases Cell–Cell and Cell–Basement Membrane Gene Expression in *Scgb1a1*$^{+}$ Cells and Reduces Expression of microRNAs

We next examined if structural changes in the airway epithelium of p73$^{\Delta airway}$ mice were accompanied by changes in gene expression. We performed scRNA-seq on whole lungs from six p73$^{\Delta airway}$ mice and six littermate control mice using split-pool ligation-based transcriptome sequencing (40). Two-month-old mice were selected for study, rather than 6-month-old mice, to maximize cellular recovery, given the dramatic reduction in airway epithelial cells in 6-month-old p73$^{\Delta airway}$ mice (Figure 2A). After filtering out poor-quality cells (Figure E3A and Supplemental Methods), the final dataset included 2,100 *Epcam*$^{+}$ (epithelial cell adhesion molecule) cells from p73$^{\Delta airway}$ mice and 3,809 *Epcam*$^{+}$ cells from control mice. These were grouped by gene expression using unsupervised, graph-based clustering (Leiden), manually annotated based on expression of canonical markers (Figure E3B) and visualized by Uniform Manifold Approximation and Projection (Figure 3A). As expected, there were few mature MCCs or club cells in p73$^{\Delta airway}$ mice (Figure 3B). The MCCs that remained expressed *Trp73* (Figure 3C) and were transcriptionally similar to MCCs from control mice (Figure 3D), suggesting they escaped Cre recombinase activity. In the alveolar epithelium, gene expression was similar between p73$^{\Delta airway}$ mice and control mice. There were no differentially expressed genes (DEGs) for alveolar type I (AT1) and transitional alveolar type II (AT2) cells and just 22 DEGs for immature AT2s and 62 DEGs for mature AT2s (Figure 3D). Significant DEGs for all cell types are provided in Table E2.

We next focused on potential mechanisms of SCGB1A1^{+} cell loss using our scRNA-seq data. Club, goblet, and bronchioalveolar stem cells (BASC)-like cells all expressed varying amounts of *Scgb1a1* (Figure E3B). Although gene expression in club and goblet cells was similar between p73$^{\Delta airway}$ mice and control mice (Figure 3D), there were 197 DEGs between BASC-like cells from p73$^{\Delta airway}$ mice and control mice. We then calculated DEGs among all *Scgb1a1*$^{+}$ secretory cell populations combined for p73$^{\Delta airway}$ and control mice and performed pathway analyses on the calculated DEGs using Enrichr (41). In p73$^{\Delta airway}$ mice, *Scgb1a1*$^{+}$ cells were enriched in pathways related to cell–cell and cell–basement membrane attachment, including focal adhesions, extracellular matrix (ECM)–receptor interactions, regulation of the actin cytoskeleton, adherens junctions, and tight junctions (Figure 3E). These changes were driven by widespread upregulation in integrin, laminin, collagen, and other attachment genes (Figure 3F). We did not observe an increase in genes related to senescence or apoptosis, and there was no terminal deoxynucleotidyl transferase dUTP nick end labeling staining, a marker of apoptosis, in p73$^{\Delta airway}$ mice or control mice (Figure E4). Taken together, these data indicate that, in mice, loss of p73 in the airway epithelium has modest effects on the alveolar niche but results in upregulation of genes associated with cell–cell and cell–basement membrane attachment on the remaining secretory cell populations. Despite upregulation in attachment genes, however, SCGB1A1^{+} cells are lost over time.

Because microRNAs (miRNAs) in the miR-34/449 family have been shown to play critical roles in multiciliogenesis in mice (42), we also investigated miRNA expression in p73$^{\Delta airway}$ mice and littermate control mice. We pooled EPCAM-enriched epithelial cells isolated from the lungs of two 6-month-old p73$^{\Delta airway}$ mice and compared miRNA expression to pooled cells from two age-matched littermate control mice. We noted a marked global reduction in small RNAs, including miRNAs, in p73$^{\Delta airway}$ mice (Figures E5A and E5B). The top 10 most highly expressed miRNAs were shared between p73$^{\Delta airway}$ mice and control mice, arguing against large shifts in the overall composition of miRNAs. However, we did note reduced expression of some miRNAs associated with multiciliogenesis, particularly miR-34c-5p (839 reads per million in control mice vs. 77 reads per million in p73$^{\Delta airway}$ mice), which could have contributed to reduced multiciliogenesis in these mice. The fold-change values for all miRNAs tested are available in Table E3.

CS Exposure Reduces p73 Expression *In Vitro* and *In Vivo*

Chronic exposure to tobacco smoke is a common cause of COPD, particularly in higher-income countries (43), and has known detrimental effects on MCC function and cilia length (5, 6, 8, 9, 11–13). Therefore, we determined the effect of CS on p73 expression *in vitro* and *in vivo*. To determine the effects of acute CS exposure, we treated differentiated murine tracheal epithelial cells (MTECs) with 2.5% CS extract (CSE) or 2.5% vehicle (phosphate-buffered saline, PBS) for 0.5–24 hours and performed immunofluorescent staining for p73 and *α-tubulin. Both p73 and *α-tubulin expression were decreased as early as 30 minutes after CSE exposure and remained reduced until 24 hours after exposure (Figures 4A and 4B). Because p63 (tumor protein 63) is an important regulator of basal cell function (44) and has substantial sequence homology with p73 (45), we also assessed p63 protein abundance across the same time course of CSE treatment (Figure 4C). We observed a slight increase in p63 expression above baseline in PBS-treated controls (Figure 4D). However, p63 expression was markedly increased above baseline and above PBS-treated controls in CSE-treated cells after 0.5 and 1 hour before returning to baseline at 4 hours (Figure 4D).

We next evaluated the relationship between CS exposure and p73 expression in human cells in a subacute model by exposing primary human small airway epithelial cells (HSAECs) to mainstream CS once daily for 5 days. To accomplish this, we adapted the SIU24 *in vivo* CS system (Promech) for *in vitro* use in air–liquid interface culture (Figure 4E). In this model, *TP73* was significantly reduced after 1 day of CS exposure and remained reduced after 5 days of treatment (Figure 4F). HSAECs (passage 5) exposed to CS had a trend toward decreased *TP63* expression 1 day after exposure, which returned to baseline after 5 days of exposure (Figure 4G). Together, these data indicate acute or subacute exposure to CS suppresses *TP73* expression *in vitro* and that p73 and p63 respond differently to CS exposure.

To determine the effect of chronic CS exposure on p73 abundance *in vivo*, we treated wild-type C57Bl6/J mice with mainstream CS for 1 or 3 months and measured p73 expression in murine bronchioles by immunostaining. We noted reductions in numbers of p73^{+}, α-tubulin^{+}, and FOXJ1^{+} cells after 1 and 3 months of CS exposure (Figure 5A). Furthermore, we measured *Trp73* expression in whole-lung lysates by qRT-PCR after 1 week, 1 month, and 3 months of CS exposure and found

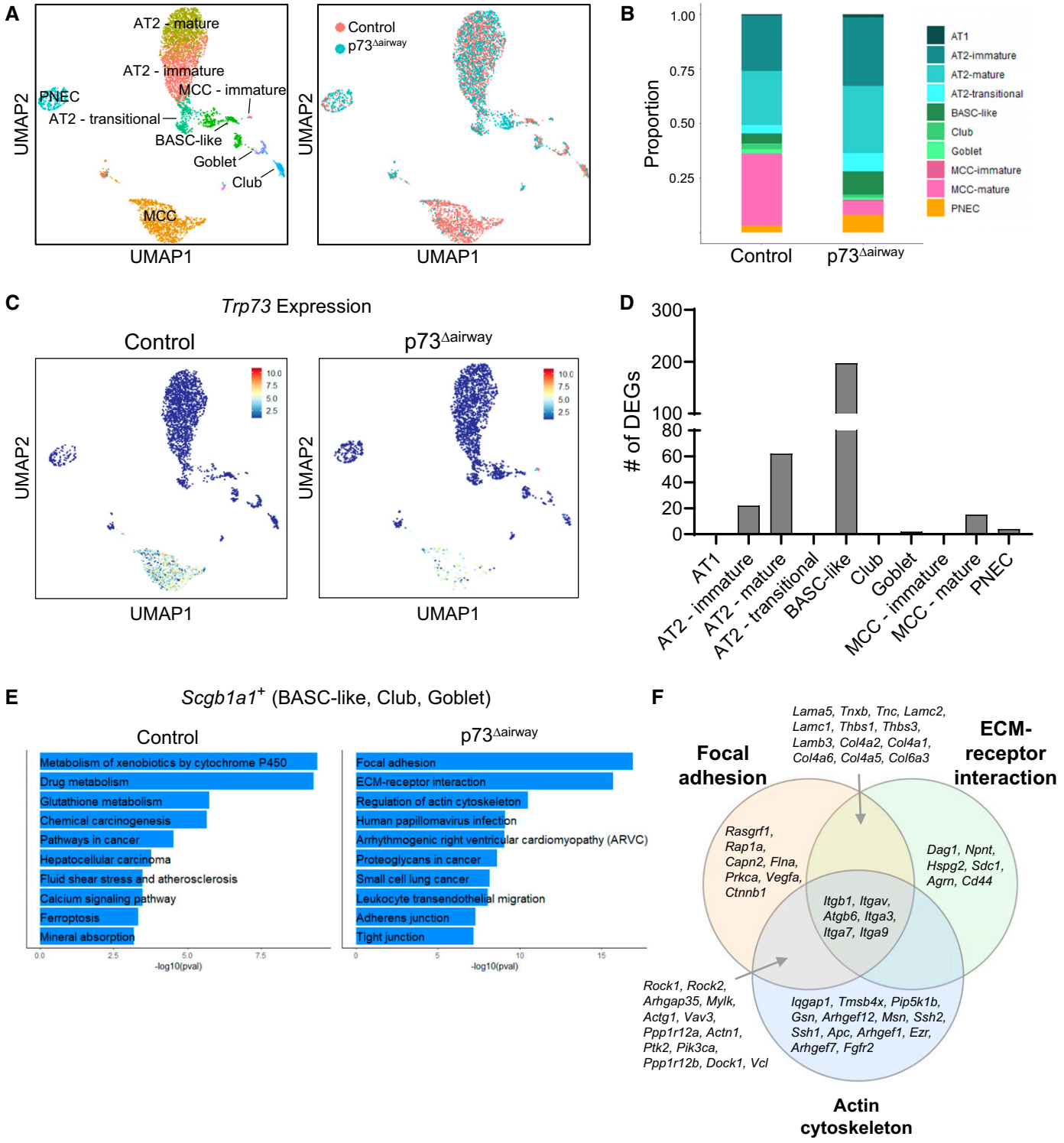

Figure 3. Loss of p73 in the airway epithelium increases cell–cell and cell–basement membrane gene expression in *Scgb1a1*$^{+}$ cells. (*A*) UMAP colored by annotated cell type (left panel) or genotype (right panel). (*B*) Proportion of each cell type originating from p73$^{\Delta airway}$ or control mice. (*C*) Feature plot showing *Trp73* expression in MCCs from p73$^{\Delta airway}$ and control mice. (*D*) Number of significant differentially expressed genes (DEGs) between p73$^{\Delta airway}$ and control mice for each cell type. Significance was defined as >0.25-fold expression and adjusted *P* value < 0.05 (Wilcoxon rank sum test) in genes expressed in at least 25% of the cells in the cluster. (*E*) Pathways enriched among DEGs in *Scgb1a1*$^{+}$ cells from p73$^{\Delta airway}$ and control mice (KEGG 2019 mouse). (*F*) Venn diagram showing genes that contributed to the focal adhesion, ECM–receptor interaction, and actin cytoskeleton pathways in *E*. AT1 = alveolar type I; AT2 = alveolar type II; BASC = bronchioalveolar stem cells; ECM = extracellular matrix; MCCs = multiciliated cells; PNEC = pulmonary neuroendocrine cells; UMAP = Uniform Manifold Approximation and Projection.

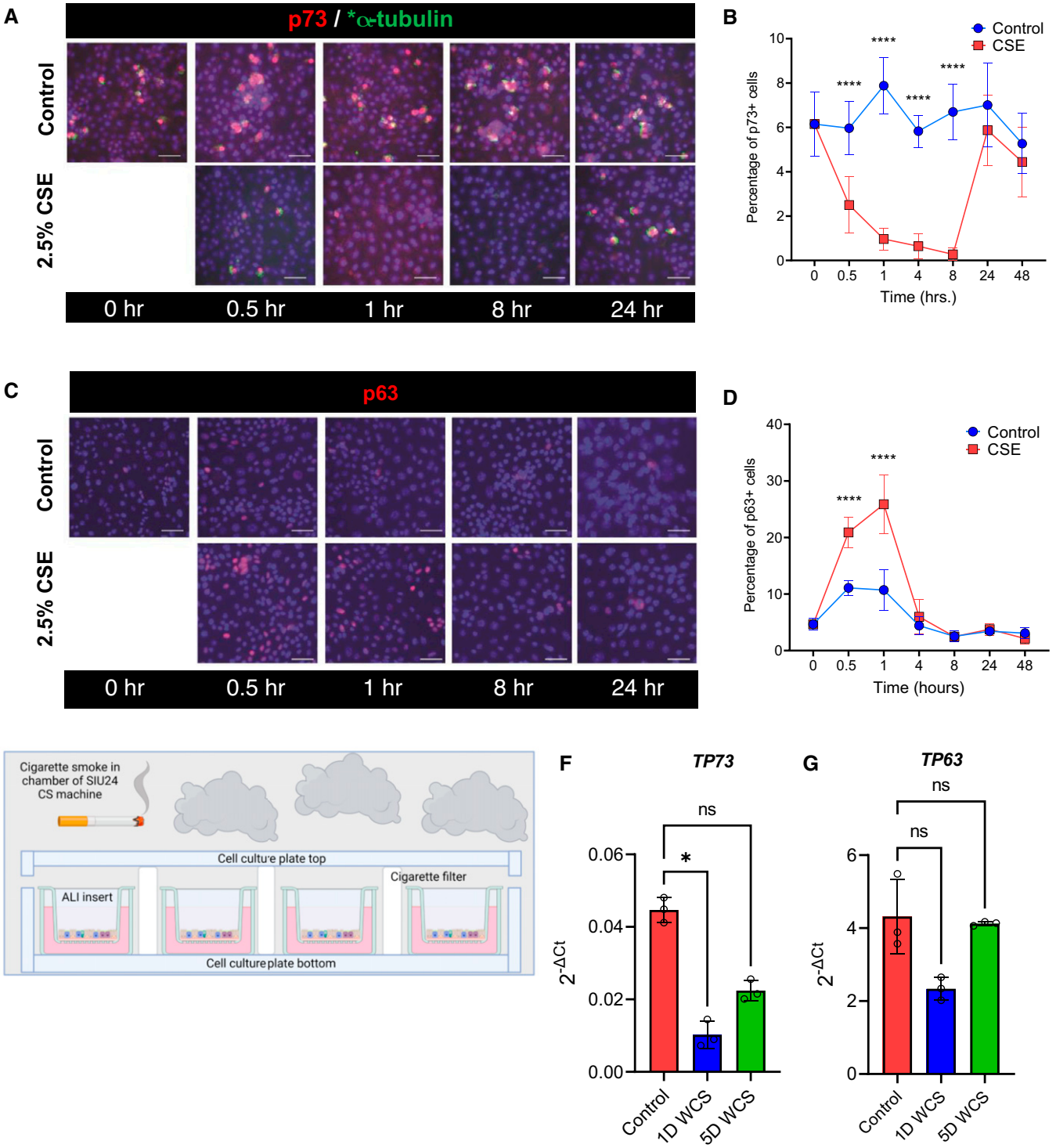

Figure 4. Acute cigarette smoke suppresses p73 expression *in vitro*. (*A*) Murine tracheal epithelial cells (MTECs) were differentiated in air–liquid interface (ALI) culture and then treated with 2.5% cigarette smoke exposure (CSE) or 2.5% phosphate-buffered saline (PBS) (control). Representative immunostaining for p73 (red) and acetylated α-tubulin (*α-tubulin) (green) in both groups at the indicated time points. (*B*) Quantification of $p73^+$ cells in 12 fields of view for each time point shown in *A*. $^{****}P < 0.00001$ compared with CSE-treated cells at the same time point; Student's *t* test. (*C*) Representative immunostaining for p63 (red) in MTECs treated with 2.5% CSE or 2.5% PBS (control) at the indicated time points. (*D*) Quantification of $p63^+$ cells in 12 fields of view for each time point shown in *C*. $^{****}P < 0.00001$ compared with CSE-treated cells at the same time point; Student's *t* test. (*E*) Adaptation of the SIU24 *in vivo* cigarette smoke machine for *in vitro* use. The apical side of human small airway epithelial cells (HSAECs) in ALI culture was exposed to mainstream cigarette smoke by lifting the top of

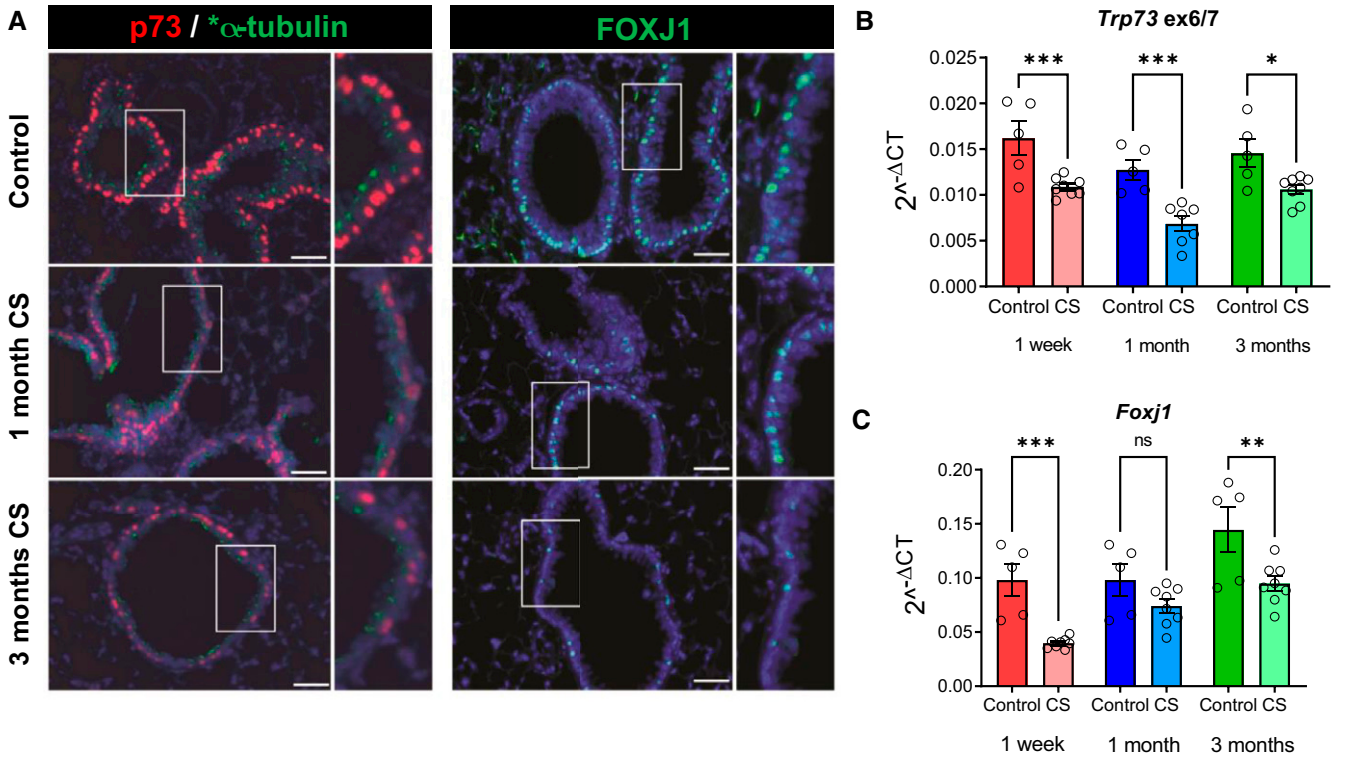

Figure 5. Chronic cigarette smoke (CS) exposure suppresses p73 expression *in vivo*. (*A*) Representative immunostaining for p73 (red) and acetylated α-tubulin (*α-tubulin) (green, left panel) and FoxJ1 (green, right panel) in mice exposed to mainstream CS for 1 or 3 months and unexposed control mice. All immunostaining and micrographs were taken at the same time with the same exposure settings. Data represent quantification of at least three fields of view from three or four inserts performed in triplicate (e.g., a minimum of 27 fields of view per time point). (*B* and *C*) qRT-PCR analysis using primers for *Trp73* (*B*) and *Foxj1* (*C*) in whole-lung lysates from mice exposed to mainstream cigarette smoke for 1 or 3 months and unexposed control mice. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; two-way ANOVA with Tukey's multiple corrections test. ns = not significant.

significantly reduced expression at each time point (Figure 5B). Similarly, *Foxj1* (forkhead box protein J1) was significantly reduced after 1 week and 3 months of exposure (Figure 5C). These data indicate chronic CS treatment reduces p73 expression and downstream MCC markers *in vivo*.

p73 Is Reduced in the Airways of Smokers and Former Smokers with COPD

Because p73 is a critical mediator of MCC development (26, 27) and was reduced by CS *in vitro* and *in vivo*, we hypothesized that MCC loss/dysfunction in smokers would be associated with p73 loss in human airway epithelial cells. To test this, we analyzed publicly available datasets that included annotation of smoking status. In a dataset consisting of bulk RNA-sequencing on bronchial brushings (GSE37147) (34), we found reduced expression of *TP73* in current smokers ($n = 82$) compared with former smokers ($n = 69$) (Figure E6A). To investigate how smoking affects *TP73* mRNA expression in MCCs specifically, we examined MCCs from smokers ($n = 96$ cells) and nonsmokers ($n = 2{,}499$ cells), all without known respiratory disease, in an existing scRNA-seq dataset (35) (Figure E6B). Expression of *FOXJ1* was significantly decreased in MCCs from current smokers, whereas *TP73* and other markers of early ciliogenesis (*CCDC78*, *MYB*) trended toward reduced expression in MCCs from smokers (Figure E6C). Together, these data suggest that smoking reduces *TP73* and other factors associated with ciliogenesis in human lungs.

Because MCCs are reduced in patients with COPD after smoking cessation (14), we also investigated if p73 expression is attenuated in small (<2 mm) airways of patients with COPD. We immunostained small airways from COPD lung explants (all former smokers) and deceased organ donors without known lung disease whose lungs were rejected for transplantation (control subjects) for p73 and the canonical basal cell marker p63 (Table E1 and Figure 6A). $p63^+$ cells were increased in patients with COPD (Figure 6B). In contrast, the percentage of $p73^+$ MCCs among all $DAPI^+$ airway epithelial cells was reduced from 42% in control small airways to 24% in COPD airways (Figure 6B). Furthermore, the percentage of $p63^+$ and $p73^+$ cells decreased from 13% in control small airways to 9% in COPD small airways (Figure 6B). Together, these data indicate $p73^+$ cells are reduced in COPD airways, and despite their sequence homology there are divergent changes in $p63^+$ and $p73^+$ cells in the small airways of patients with COPD.

Figure 4. (*Continued*). the cell culture plate. Medium was changed immediately after each exposure to minimize smoke exposure to the basolateral side of each insert. (*F*) *TP73* or (*G*) *TP63* expression was measured by qRT-PCR in HSAECs treated with mainstream CS for 1 or 5 days. $^*P < 0.05$; Kruskal-Wallis test with Dunn's multiple comparison correction. ns = not significant; WCS = whole cigarette smoke.

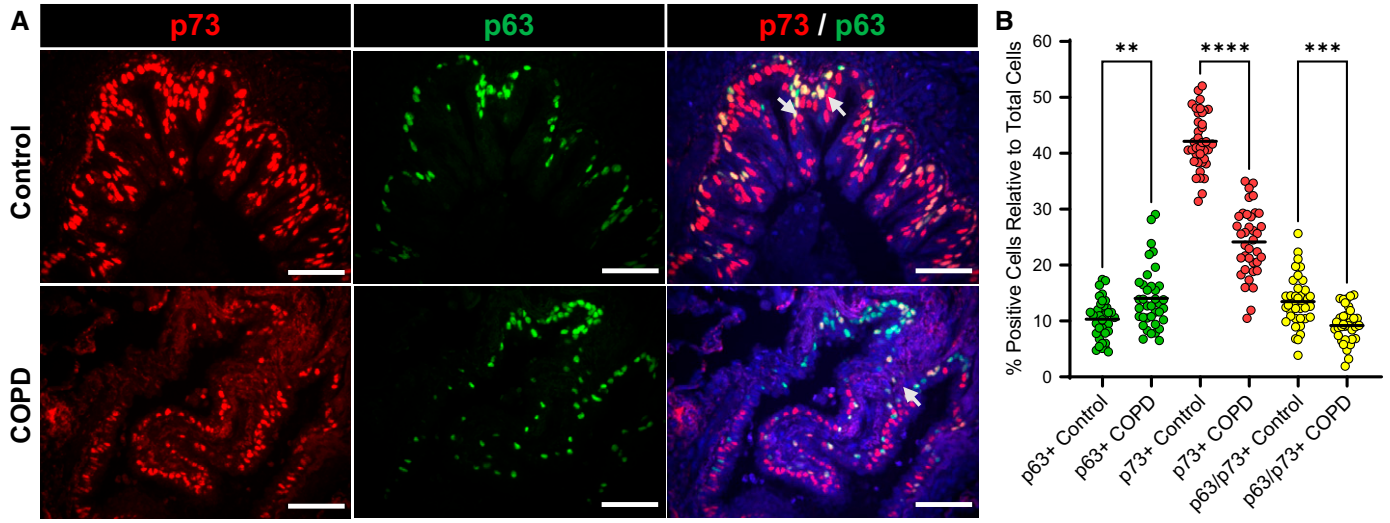

Figure 6. p73^{+} cells are reduced and p63^{+} cells are increased in the airways of patients with chronic obstructive pulmonary disease (COPD). (*A*) Representative images of immunostaining for p73 (red), p63 (green), and DAPI (blue) in small (<2 mm) airways from patients with COPD and deceased organ donors without COPD (control subjects). (*B*) Quantification of p73^{+} and p63^{+} as a percentage of total epithelial cells indicated by DAPI staining. At least three images were analyzed for 11 patients with COPD and 12 control subjects. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$; one-way ANOVA with Šidák's multiple comparison correction.

Discussion

Reduced numbers of MCCs and defects in cilia length and function are well-documented in smokers and patients with COPD (5–10, 12–14). Furthermore, smoking is associated with reduced mRNA expression of genes related to MCC differentiation and structure (8, 11, 46). Here we show that p73 is reduced in the small airways of smokers and patients with COPD, is suppressed by CS *in vitro* and *in vivo*, and is sufficient to generate COPD-relevant phenotypes in mice, including neutrophilic inflammation, emphysema-like lung remodeling, and progressive loss of secretory cells. Collectively, our results suggest p73 provides a mechanistic link between smoking and/or airway inflammation and reduced MCC function and suggest loss of p73 may contribute to lung pathology in smokers and patients with COPD.

We discovered that mice lacking functional p73 in the airway epithelium have nearly a complete absence of MCCs and spontaneously develop neutrophilic inflammation and emphysema-like lung remodeling. These results are consistent with the well-established relationship between neutrophil-derived proteases and emphysema in COPD animal models (36). However, the specific mechanism(s) through which loss of p73 drives neutrophilic accumulation require additional investigation. We observed areas of exposed basement membrane in aged p73$^{\Delta airway}$ mice and increased expression of ECM genes in younger mice, similar to that previously observed in skin in *p73*$^{-/-}$ mice (24). Degradation of ECM components releases bioactive fragments called matrikines, which are potent neutrophil chemotactic factors (47) and could contribute to neutrophilic inflammation in p73$^{\Delta airway}$ mice. Furthermore, although we did not observe activation of inflammatory signaling pathways in the epithelium of 2-month-old p73$^{\Delta airway}$ mice, it is possible that progressive loss of barrier function in older p73$^{\Delta airway}$ mice prompts release of neutrophil chemotactic factors by airway epithelial cells. This loss of barrier function could relate to a general loss of cells covering the airway or a specific loss of secretory cells, which are known to be critical regulators of airway homeostasis (48). Finally, many effects could be the direct result of loss of ciliated cells and impaired mucociliary clearance in these mice.

Loss of secretory cells and secretory cell products is increasingly recognized as an important contributor to COPD pathology (48). We observed a reduced percentage of SCGB1A1^{+} secretory cells in p73$^{\Delta airway}$ mice, which worsened as these mice aged. This finding was unexpected, because p73 is not expressed in secretory cells in mice (26). Furthermore, secretory cell loss appeared to occur despite upregulation of genes related to cell–cell and cell–basement membrane attachment in p73$^{\Delta airway}$ mice. It is possible that, over time, attempts by secretory cells to adhere to the basement membrane begin to fail, resulting in secretory cell loss. Alternatively, it is possible that the proliferative capacity of secretory cells diminishes over time as they continually divide in an attempt to cover the epithelium. Finally, it is important to note that secretory cell loss also occurred with aging in control (wild-type) mice, which could occur through shared or distinct mechanisms to p73$^{\Delta airway}$ mice. Additional time course experiments will be required to more fully define the mechanisms of secretory cell loss in p73$^{\Delta airway}$ and control mice and to determine whether these mechanisms are also true in humans, given species-specific differences in the distribution of Scgb1a1^{+} cells between humans and mice.

We found that CS suppressed p73 *in vitro* and *in vivo* and that p73 was reduced in bronchial brushings from smokers. Prior studies in mice indicate p73 coordinates a transcriptional program required for ciliogenesis through direct binding and activation of MCC genes, including the transcription factors *Cdkn1a*, *Myb*, *Rfx3*, *Traf3ip1*, and the canonical MCC transcription factor *Foxj1* (26). Thus, CS-mediated loss of p73 could explain the well-documented reduction in genes associated with ciliogenesis

in smokers and the observation that overexpression of *FOXJ1* rescues CS-induced defects in cilia *in vitro* (11, 46). Whether loss of p73 impacts mucociliary clearance *in vivo* and whether restoration of p73 rescues impairments in MCC function *in vivo* are important topics for future study. In addition, it would be interesting to induce goblet cell hyperplasia in $p73^{\Delta airway}$ mice to determine if loss of MCC function directly contributes to formation of mucus plugs in this model.

Prior studies indicate that p73 expression is dynamic and context dependent. Ultraviolet radiation, cisplatin, paclitaxel, doxorubicin, and sorbitol have all been shown to upregulate p73 by increasing expression of *TP73*, enhancing the stability of p73 protein, or both (49, 50). In contrast, nuclear factor-κB (NF-κB) pathway activation increases ubiquitination and proteasomal degradation of p73 and inhibits p73 activity through NF-κB–mediated expression of Mdm2 (51, 52). CS is a complex stimulus with wide-ranging and cell-specific effects on gene expression in the airway epithelium (53). Thus, there could be species (mouse vs. human), location (small vs. large airways), and model-specific differences in CS-mediated p73 loss, and additional studies will be required to clarify these mechanisms. Nonetheless, the consistency with which CS suppresses p73 across multiple model systems suggests a robust relationship.

We previously reported that $p73^{-/-}$ mice have reduced numbers of $p63^+$ basal cells in the trachea. In contrast, we observed increased numbers of $p63^+$ basal cells in COPD small airways, despite reduced numbers of $p73^+$ cells. Despite their structural similarities (45), p73 and p63 have different functions and different mechanisms of regulation. Indeed, we observed divergence in the response of these two transcription factors to acute CS exposure in MTECs. Additional studies will be required to understand the differential regulation of p63 and p73 in response to CS and in COPD. In addition, it is important to note that although basal cell hyperplasia has previously been reported in COPD, it is not uniform across all studies (14, 54) and may differ depending on the stage of COPD being studied and the composition of the control group (never-smokers, former smokers, etc.). Finally, although our studies suggest that $p63^+$ and $p73^+$ basal cells are present in both COPD and control airways, we do not provide functional characterization of these cells, and it is unclear whether they continue to have the pluripotency typical of basal cells or are restricted to an MCC fate.

Our study has several important limitations. First, we used a constitutive Cre model, and it is possible that loss of p73 during lung development contributed to the pathologic phenotypes described. In addition, Cre recombinase activity was not entirely limited to the airway epithelium in our model, and it is possible that loss of p73 in other tissues contributed to the observed phenotypes. However, these mice lacked many of the phenotypes that make $p73^{-/-}$ mice difficult to study, including perinatal lethality and reduced fertility. Third, our experimental design did not allow us to distinguish phenotypes resulting from the loss of p73 specifically from phenotypes resulting from the loss of ciliated cells. Fourth, although we showed that CS suppressed p73 in HSAECs and in murine small airways, our *in silico* analysis was performed on gene expression data obtained from large airway brushings. Thus, it remains unknown whether CS suppresses p73 expression in human small airways.

In summary, we report that loss of p73 is common in the airways of patients with COPD and smokers, that CS suppresses p73 *in vitro* and *in vivo*, and that mice lacking p73 spontaneously develop a COPD-like phenotype. Thus, p73 may link smoking and COPD to MCC dysfunction and contribute to COPD lung pathology. ■

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