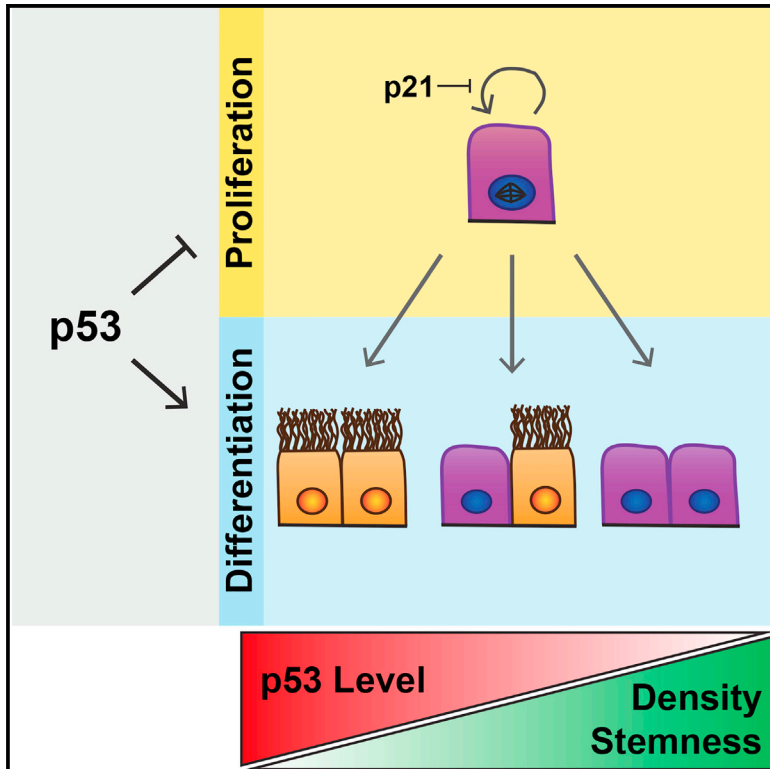Report

p53 Regulates Progenitor Cell Quiescence and Differentiation in the Airway

Graphical Abstract

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In Brief
Through genetic manipulation of p53 copy number in vivo and in vitro, McConnell et al. show that p53 is a key regulator of airway epithelial cell density and composition. This is due to the ability of p53 to control proliferation and differentiation of progenitor cells.

Highlights
- p53 regulates proliferation and differentiation of airway epithelial progenitors

- Loss of p53 increases progenitor self-renewal, multipotency, and proliferation

- An extra copy of p53 promotes terminal differentiation and decreased proliferation

- Single-cell RNA-seq reveals that p53 alters molecular phenotype of progenitors

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p53 Regulates Progenitor Cell Quiescence and Differentiation in the Airway

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SUMMARY

Mechanisms that regulate progenitor cell quiescence and differentiation in slowly replacing tissues are not fully understood. Here, we demonstrate that the tumor suppressor p53 regulates both proliferation and differentiation of progenitors in the airway epithelium. p53 loss decreased ciliated cell differentiation and increased the self-renewal and proliferative capacity of club progenitors, increasing epithelial cell density. p53-deficient progenitors generated a pseudostratified epithelium containing basal-like cells in vitro and putative bronchioalveolar stem cells in vivo. Conversely, an additional copy of p53 increased quiescence and ciliated cell differentiation, highlighting the importance of tight regulation of p53 levels. Using single-cell RNA sequencing, we found that loss of p53 altered the molecular phenotype of progenitors and differentially modulated cell-cycle regulatory genes. Together, these findings reveal that p53 is an essential regulator of progenitor cell behavior, which shapes our understanding of stem cell quiescence during homeostasis and in cancer development.

INTRODUCTION

Epithelial tissues consist of closely packed functional cells that are replaced by resident stem or progenitor populations at rates that vary between tissues. Here, we investigate how these progenitor cells are regulated in a slowly replacing tissue, the airway epithelium of the lung. The mammalian airway epithelium varies in composition between species and according to airway location. Intralobar-conducting bronchi and bronchioles of the mouse lung are composed of secretory and ciliated cells. Tight control over the proportions and abundance of these cell types is essential for effective mucociliary clearance of inhaled particulates and microorganisms.

Club cells, previously known as Clara cells, are secretory cells that express the protein *Scgb1a1* (also known as CCSP or CC10)

(Rackley and Stripp, 2012). They function as regional progenitors due to their ability to self-renew and differentiate into ciliated cells (Hogan et al., 2014). Club cells have a low level of turnover during homeostasis, yet how this quiescence is maintained is still poorly understood (Rawlins et al., 2009). Developmentally important pathways, such as Wnt, BMP, Notch, JAK/STAT, and Hippo/Yap, have been shown to regulate various populations of lung stem and progenitor cells, directly influencing epithelial composition (Hogan et al., 2014). However, very few studies have identified novel integrators of these pathways that control the critical balance between progenitor cell renewal and differentiation.

Trp53 (p53) is a tumor suppressor and one of the most commonly mutated genes in cancer (Kandoth et al., 2013). Germline loss of p53 leads to Li-Fraumeni syndrome, a cancer predisposition disorder, and somatic mutations in p53 are clinically associated with shortened survival time (Malkin, 2011). In addition to its classical functions in regulating cell fate following cellular stress, there is growing evidence that p53 regulates progenitor cells in a variety of developing and adult tissues (Armesilla-Diaz et al., 2009; Cicalese et al., 2009; Li et al., 2015; Liu et al., 2009; Meletis et al., 2006; Tosoni et al., 2015). These studies highlight an essential role for p53 using rapidly dividing models. However, it is not clear what role, if any, p53 plays in regulating progenitor cell behavior in a quiescent tissue, such as the lung.

Here, we show that p53 regulates the composition of postnatal airway epithelium by maintaining quiescence and regulating differentiation of resident progenitor cells during homeostasis. Genetic manipulation of p53 copy number altered both the density and the cellular composition of the airway epithelium. Furthermore, p53 regulated the multipotency of club progenitors. Using single-cell RNA sequencing (RNA-seq), we discovered that p53 loss changes the proportions of subtypes of cells and alters expression of cell-cycle regulators. Together, our findings reveal key roles for p53 in the homeostatic regulation of airway epithelial progenitor cells.

RESULTS

p53 Regulates Proliferation and Cell Death In Vitro

To examine the contribution of p53 in regulating club progenitor cells, we used a loss-of-function model coupled with lineage tracing to assess in vitro clonogenic potential. *Scgb1a1-CreER^TM; Rosa26-mT/mG* mice with either a wild-type or
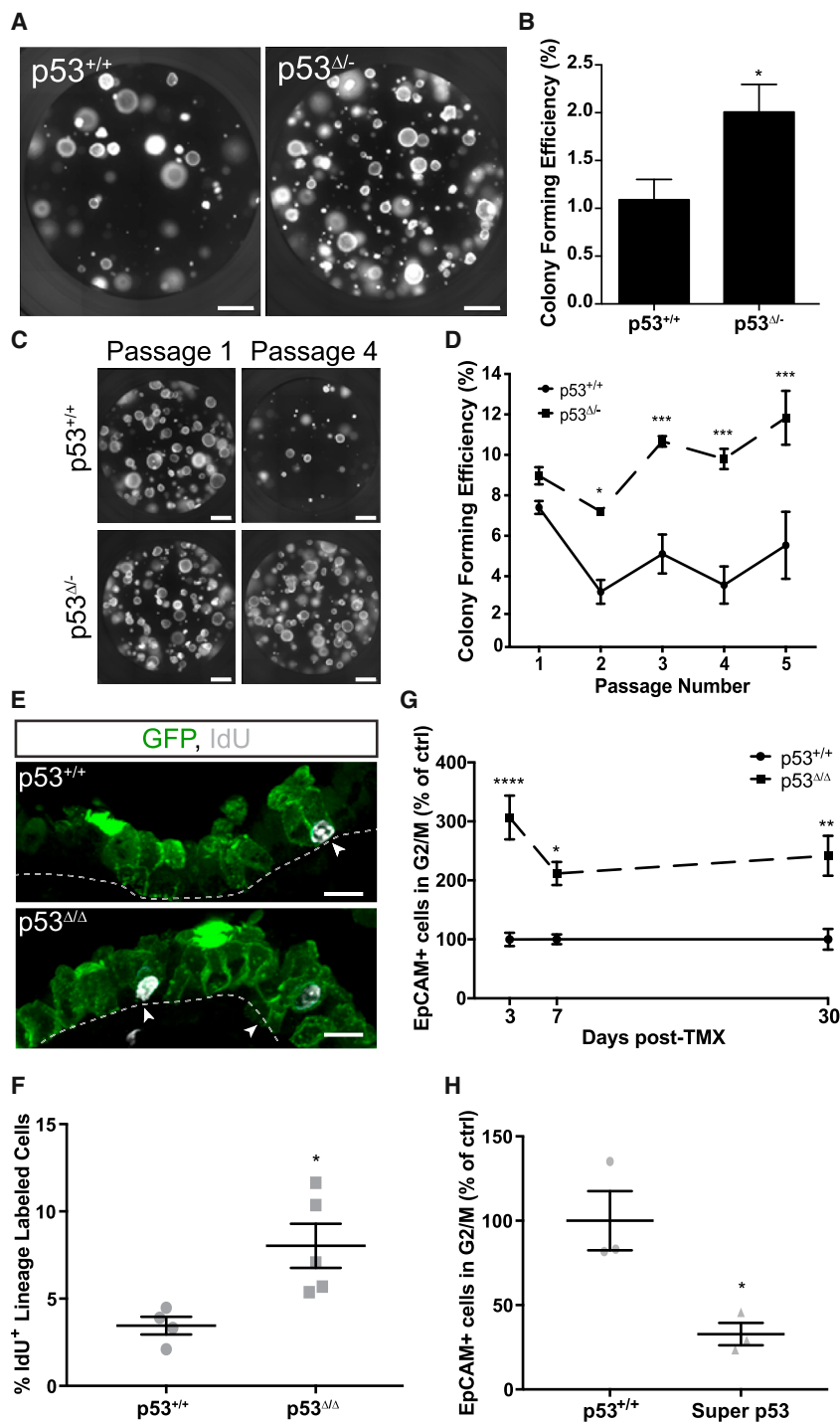
Figure 1. p53 Regulates Proliferation

(A) Fluorescent images of GFP+ passage 0 (P0) after 7 days in culture. Cells were isolated from $Scgb1a1\text{-}CreER^{TM}$; $Rosa26\text{-}mT/mG$; $p53^{+/+}$ or $p53^{\Delta/-}$ mice.

(B) Colony-forming efficiency (percentage of colonies of total number of cells seeded) at P0 (n = 3−4 mice).

(C) Fluorescent images of GFP+ colonies after 7 days in culture at passages 1 and 4. Cells were isolated from $Scgb1a1\text{-}CreER^{TM}$; $Rosa26\text{-}mT/mG$; $p53^{+/+}$ or $p53^{\Delta/-}$ mice.

(D) Colony-forming efficiency at various passages (n = 3 mice).

(E) Immunofluorescent (IF) staining for GFP-lineage tag and IdU. Arrowheads indicate IdU+ cells; a dashed line is shown at the basement membrane. Analysis was performed in $Scgb1a1\text{-}CreER^{TM}$; $Rosa26\text{-}mT/mG$; $p53^{+/+}$ or $p53^{\Delta/\Delta}$ mice.

(F) Percentage of IdU-positive GFP-lineage-tagged cells (n = 4–5 mice).

(G) Percentage of isolated epithelial cells from $Scgb1a1\text{-}CreER^{TM}$; $p53^{\Delta/\Delta}$ mice in G2/M relative to wild-type control (ctrl) as indicated by propidium iodide (PI) staining (n = 3–5 mice). TMX, tamoxifen.

(H) Percentage of isolated epithelial cells from Super p53 mice in G2/M relative to wild-type control, as indicated by propidium iodide (PI) staining (n = 3 mice).

Scale bars represent 1 mm in (A) and (C) and 10 μm in (E). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. All data shown represent mean ± SEM. See also Figures S1 and S2.

($p53^{+/+}$) and express GFP in club cells. Lineage-tagged GFP+ club progenitors were sorted and co-cultured with p53-sufficient fibroblasts in a 3D Matrigel culture system (Figure S1A). After 7 days, p53-deficient cells generated significantly more colonies than p53-sufficient cells (Figures 1A and 1B). Additionally, p53-deficient cells generated significantly more colonies across multiple passages (Figures 1C and 1D). Considering that p53 is a well-known regulator of cell proliferation and survival, we assessed the impact of altered p53 status on colony-forming ability in vitro. p53 loss resulted in significantly more Ki67-positive lineage-tagged cells (Figures S1B and S1C). Additionally, we found significantly fewer cleaved-caspase-3-positive apoptotic

$p53^{flox/-}$ allele were used. All cells in the body of $Scgb1a1\text{-}CreER^{TM}$; $Rosa26\text{-}mT/mG$; $p53^{flox/-}$ mice are heterozygous for p53. Upon tamoxifen injection, the loxP sites around the remaining p53 allele (indicated by $p53^{flox}$) are recombined, yielding club cells that are deficient for p53 ($p53^{\Delta/-}$) and are genetically lineage tagged with GFP. Control $Scgb1a1\text{-}CreER^{TM}$; $Rosa26\text{-}mT/mG$; $p53^{+/+}$ tamoxifen-treated mice are sufficient for p53

cells in cultures of p53-deficient cells (Figures S1D and S1E). Furthermore, using flow cytometry at the time of passage, we detected significantly more lineage-tagged cells staining positive for 7AAD, a DNA-binding dye that identifies dead cells, in p53-sufficient cultures than in p53-deficient cultures at late passages (Figures S1F and S1G). These results suggest that p53 inhibits progenitor cell proliferation and survival in vitro.

p53 Maintains Quiescence of Club Cells In Vivo

We next sought to determine whether altering the p53 level would affect epithelial proliferation in vivo in a normally quiescent tissue such as the lung. FACS (fluorescence-activated cell sorting)-enriched total epithelial cells from tamoxifen-treated *Scgb1a1-CreER^TM; p53^{flox/flox}* mice (p53^{Δ/Δ} mice) and wild-type controls were stained with propidium iodide and analyzed for DNA content to measure cell-cycle phase via flow cytometry (Figure S1H). p53-deficient mice had significantly more epithelial cells in G2/M at 3 days following tamoxifen exposure compared to p53-sufficient controls (Figure 1G). No change in proliferation existed in non-tamoxifen-treated p53^{Δ/Δ} mice (Figure S1I). Additionally, *Scgb1a1-CreER^TM; Rosa26-mT/mG; p53^{+/+}* or *p53^{flox/flox}* mice were treated with a single dose of tamoxifen and then were given iododeoxyuridine (IdU) drinking water for 7 days to assess proliferation. p53-deficient mice had significantly more GFP-positive-lineage-labeled cells that incorporated IdU as compared to controls, further supporting the idea that p53 inhibits proliferation in a cell-autonomous manner in vivo (Figures 1E and 1F).

We also assessed cell cycle in mice carrying one transgenic p53 allele in addition to the two endogenous alleles, referred to as "Super p53" mice (García-Cao et al., 2002). In contrast to our findings with p53-deficient mice, Super p53 mice with three copies of p53 had significantly fewer epithelial cells in G2/M compared to *p53^{+/+}* controls (Figure 1H). These findings were further reinforced when bromodeoxyuridine (BrdU) incorporation was used to measure proliferation. Flow cytometry for BrdU revealed that Super p53 mice had significantly fewer proliferating cells compared to control (Figure S1J). Together, these results indicate that altering p53 copy number tightly regulates quiescence of epithelial progenitor cells in the homeostatic airway.

Airway Epithelial Cell Density Is Regulated by p53 in a Dose-Dependent Manner

Considering that p53 gene dose regulates proliferation in the airway epithelium, we next sought to determine whether altered proliferation led to changes in cell density. To test this, we used *Scgb1a1-CreER^TM; Rosa26R-Confetti; p53^{+/+}, p53^{flox/−},* or *p53^{flox/flox}* mice (p53^{+/+}, p53^{Δ/−}, or p53^{Δ/Δ} mice, respectively). Tamoxifen exposure in these mice induces recombination at the *Confetti* allele (specifically in club cells), which genetically tags all club cells and their progeny (lineage tag) with either nuclear GFP, cytoplasmic YFP (yellow fluorescent protein), membrane CFP (cyan fluorescent protein), or cytoplasmic RFP (red fluorescent protein). GFP-, YFP-, and CFP-lineage-labeled cells were identified by a GFP antibody, and RFP-lineage-labeled cells were identified by an RFP antibody. Cell density was determined by quantifying the number of lineage-tagged cells per unit basement membrane at 2, 30, and 70 days post-tamoxifen exposure. p53 loss significantly increased the number of lineage-tagged cells per unit basement membrane compared to p53-sufficient controls (Figures 2A and 2B). Additionally, total cell density along the airway epithelium was significantly increased in p53^{Δ/−} and p53^{Δ/Δ} mice (Figures S1K and S1L). Similar findings were observed using whole-mount imaging, in which p53 loss significantly increased both the number of lineage-labeled patches per unit area and the total airway epithelial cell density (Figures 2D–2G and S1M). Next, we assessed the number of confetti-lineage-tagged cells per unit basement membrane in *Scgb1a1-CreER^TM; Rosa26-Confetti; Super p53* mice at 70 days post-tamoxifen. Super p53 mice had significantly fewer lineage-tagged cells per unit basement membrane, as well as significantly fewer nuclei per unit area compared to wild-type controls (Figures 2C, 2F, and 2G). Taken together, these data demonstrate that p53 copy number determines the density of airway epithelial cells during homeostasis.

## p53 Levels Influence Clonal Behavior of Progenitor Cells

To assess the ability of a single progenitor cell to clonally expand, we administered a low dose of tamoxifen (1 × 5 mg/kg) to *Scgb1a1-CreER^TM; Rosa26R-Confetti; p53^{+/+}* or *p53^{flox/flox}* mice, referred to as p53^{+/+} or p53^{Δ/Δ} mice, and quantified the size of GFP, RFP, or YFP patches. p53 loss resulted in a significant increase in the percentage of clones containing three or more cells compared to control (Figures S2A and S2B). Additionally the number of cells per patch was significantly increased, with p53-deficient cells generating patches twice as large as that in control (Figure S2C). *Scgb1a1-CreER^TM; Rosa26R-Confetti; Super p53* mice had a significant decrease in the number of cells per patch, indicating that tight control over p53 levels is essential to maintain correct progenitor pool size (Figures S2D–S2G).

p53 Regulates Differentiation of Club Progenitor Cells

Given that club cells generate ciliated cells, we tested the hypothesis that p53 regulates club to ciliated cell differentiation in the airway epithelium. To do this, we assessed the number of lineage-tagged ciliated cells, indicated by FoxJ1 staining, following tamoxifen exposure in *Scgb1a1-CreER^TM; Rosa26R-Confetti; p53^{+/+}, p53^{flox/−},* or *p53^{flox/flox}* mice (p53^{+/+}, p53^{Δ/−}, or p53^{Δ/Δ} mice, respectively). Loss of p53 in club cells led to the generation of significantly fewer ciliated cells as compared to control (Figures 3A and 3B; Figures S3A and S3B). To test whether increasing the p53 gene dose would influence ciliated cell differentiation, we quantified the number of FoxJ1+ cells in the airway epithelium of Super p53 mice and found significantly more ciliated cells compared to controls (Figures 3C and S3C). Conversely, we found that p53 loss led to an increase in club cell generation, while an extra copy of p53 decreased club cell numbers (Figures 3D and 3E). These results demonstrate that the number of p53 gene copies directly regulates club-to-ciliated cell differentiation in vivo.

p53 Loss of Function Alters Differentiation Potential

We hypothesized that p53 loss might also affect differentiation in vitro. Cultures were stained for markers of various airway cell types, including p63 and keratin 5 (K5), which traditionally mark basal cells, a stem cell population located in the trachea and proximal conducting airway of mice. Basal cells are not derived from *Scgb1a1*-expressing cells under homeostatic conditions in the postnatal mouse. Surprisingly, culture of lineage-labeled GFP+ cells from tamoxifen-treated *Scgb1a1-CreER^TM; Rosa26-mT/mG; p53^{flox/−}* mice yielded organoids composed of a pseudostratified epithelium containing a significant number of ciliated cells, marked by α-tubulin, as well as p63-and K5-positive lineage-tagged basal-like cells (Figures 3F–3K and S3D–S3F). This indicates that an *Scgb1a1*-expressing progenitor was able to
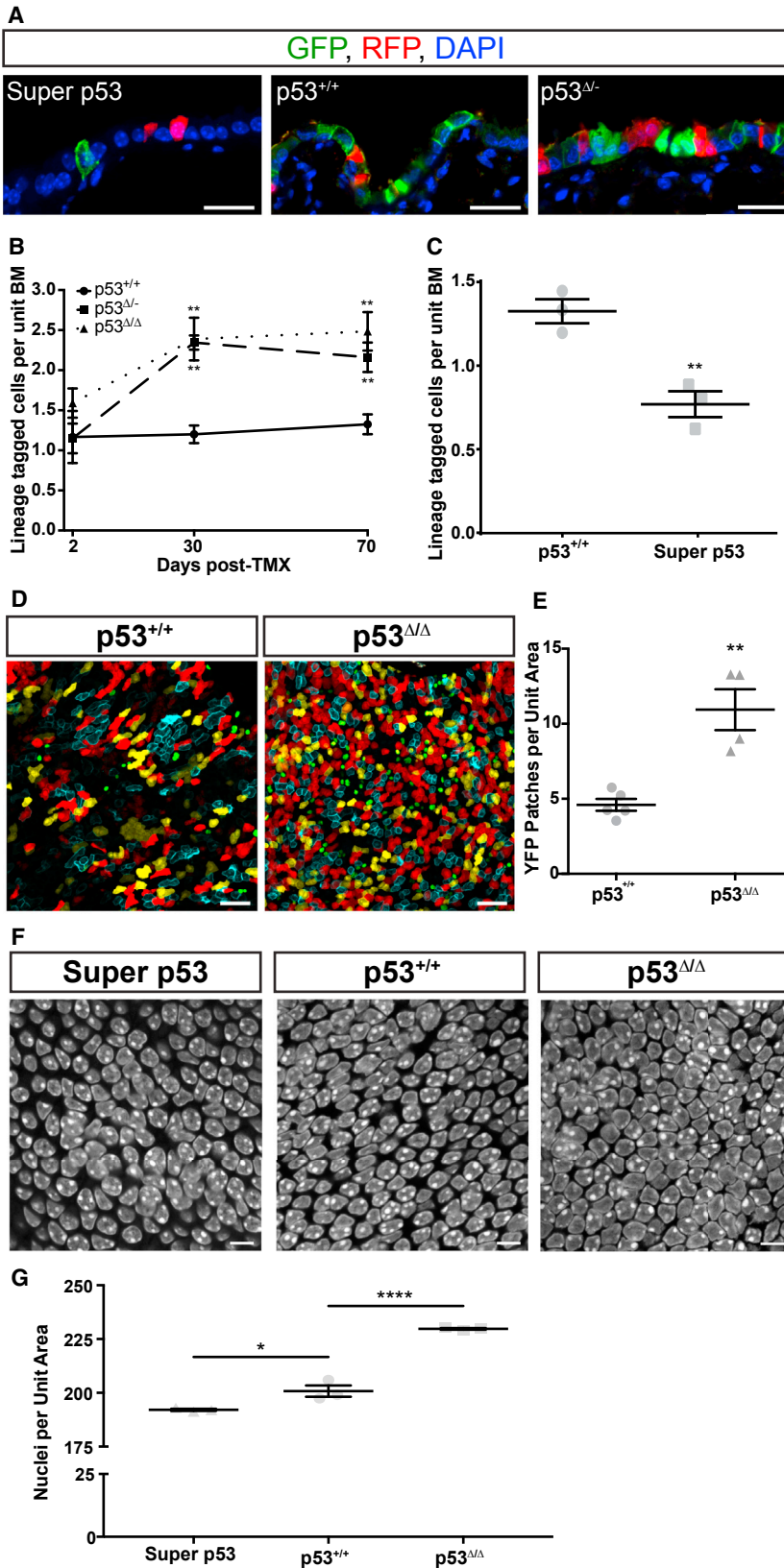
Figure 2. p53 Regulates Epithelial Density

(A) Immunofluorescent (IF) staining for confetti-lineage tag (nuclear GFP, cytoplasmic YFP, and membrane CFP are indicated in green, and cytoplasmic RFP is indicated in red) and DAPI in blue in *Scgb1a1-CreER^TM; Rosa26R-Confetti; p53^{+/+}, p53^{Δ/−}*, or *Super p53* mice 70 days post-tamoxifen.

(B) Quantification of the number of lineage-tagged cells per unit basement membrane (BM) in *Scgb1a1-CreER^TM; Rosa26R-Confetti; p53^{Δ/−}* or *p53^{Δ/Δ}* mice (p53^{Δ/−} or p53^{Δ/Δ}, respectively) at 2, 30, and 70 days post-tamoxifen (post-TMX) (n = 3–4).

(C) Quantification of the number of lineage-tagged cells per unit basement membrane in *Scgb1a1-CreER^TM; Rosa26R-Confetti; Super p53* mice at 70 days post-tamoxifen (n = 3).

(D) Whole-mount image of native confetti fluorescence in *Scgb1a1-CreER^TM; Rosa26R-Confetti; p53^{+/+}* or *p53^{Δ/Δ}* mice at 70 days after 600 mg/kg tamoxifen.

(E) Quantification of the number of YFP patches per unit area at 70 days after 600 mg/kg tamoxifen (n = 4–5).

(F) Whole-mount DAPI staining along airways of Super p53, wild-type, and p53-deficient mice.

(G) Number of nuclei per unit area (n = 3).

BM and area are measured in pixels and square pixels, respectively. Scale bars represent 20 μm in (A), 50 μm in (D), and 10 μm in (F). *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$. All data shown represent mean ± SEM.

See also Figures S1 and S2.

generate a basal-like cell in vitro, following p53 loss. Interestingly, only about 40% of colonies containing p63-positive cells also contained K5-positive cells, showing discordance between these two basal cell markers in our in vitro system (Figures S3G and S3H). Taken together, these data indicate that p53 loss alters the differentiation potential of club cells in vitro (Figure 3L).

p53 Regulates the Proportion of Progenitors in the Airway

To better understand the cellular and molecular mechanisms driving p53-dependent regulation of progenitor cell fate, we used single-cell RNA-seq. Lineage-tagged GFP+ cells from tamoxifen-treated *Scgb1a1-CreER^TM; Rosa26-mT/mG* mice with a *p53^flox/flox* or *p53^+/+* allele (p53$^{\Delta/\Delta}$ and p53$^{+/+}$, respectively) were sorted at day 70, and single-cell sequencing was performed using a Fluidigm C1 system. After quality control and normalization, transcriptomes of 64 p53$^{+/+}$ and 64 p53$^{\Delta/\Delta}$ cells were analyzed using principal-component analysis and an unsupervised heatmap of the top 250 protein coding genes with the highest expression variation across all cells (Figures S3I and S3J). Surprisingly, clustering patterns observed in the principal-component analysis and heatmap suggest that cellular heterogeneity between *Scgb1a1*-lineage-labeled cells, rather than p53 status, represent the principal determinants of molecular variability (Figure 3M). We saw segregation between four clusters of cells; each cluster containing various markers of proximal or distal club cell subtypes. p53 loss altered the proportion of cells that fell in each cluster (Figure 3N).

Considering that p53 loss expanded differentiation potential in vitro, RNA-seq data were mined for alterations in other progenitor types. Bronchioalveolar stem cells (BASCs) are a controversial progenitor type marked by the co-expression of *Scgb1a1* and *Sftpc* and were proposed to generate both airway and alveolar cell types (Kim et al., 2005). We identified airway epithelial cells (*Sox2^+*) (Gontan et al., 2008) that express both *Scgb1a1* and *Sftpc*, which increased in abundance when p53 was lost (Figure 3O). Additionally, the number of lineage-labeled Scgb1a1$^+$Sftpc$^+$ cells per terminal bronchiole is significantly increased at 70 days post-tamoxifen in p53$^{\Delta/\Delta}$ mice, further supporting the notion that p53 regulates multipotency of club progenitors (Figures 3P and 3Q). These data show that p53 loss does not radically change the gene expression profile of club progenitor cells but, instead, may regulate the proportions of regionally distinct subtypes of progenitor cells within the *Scgb1a1*-lineage-labeled population.

p53 Loss Increases the Number of Cycling Cells

To gain further insights into mechanisms by which p53 regulates progenitor behavior, we analyzed pathways that were altered in p53$^{\Delta/\Delta}$ cells using the DAVID online pathway analysis tool (Huang et al., 2009a, 2009b). The most significantly enriched pathway in p53$^{\Delta/\Delta}$ cells was cell cycle (Figure S4A). Expression levels of genes frequently enriched in the G2/M cell-cycle phase were increased in p53-deficient cells (Figure 4A). Both the total number and expression levels of cell-cycle-promoting genes were significantly increased in p53$^{\Delta/\Delta}$ cells (Figures 4B and 4C). Conversely, p53$^{\Delta/\Delta}$ cells expressed significantly reduced levels of cell-cycle-inhibitor genes compared to p53-sufficient

cells (Figures 4D and 4E). Notably, we found reduced expression of the cell-cycle inhibitor p21 (Cdkn1a), whose expression is normally upregulated following stress-induced p53 activation (Figure S4E). As a control for random variability in gene expression between genotypes, we evaluated the number of housekeeping genes expressed in each cell and found no significant difference between genotypes (Figure S4B). Finally, the expression of pro-apoptotic genes was also significantly lower in p53$^{\Delta/\Delta}$ cells as compared to control (Figures S4C and S4D). Additionally, we performed RNA-seq on sorted GFP+ cells from *Scgb1a1-CreER^TM; Rosa26-mT/mG; p53^flox/flox* or *p53^+/+* mice and verified that the expression of p53 and traditional p53 pathway members decreased in p53$^{\Delta/\Delta}$ cells (Figure 4F). Together, these findings reinforce the notion that p53 maintains quiescence of club progenitor cells by regulating genes that are normally associated with stress-activated p53 signaling.

p21 Regulates Proliferation, but Not Differentiation, of Club Progenitors

To further explore roles for p21 as a downstream target of p53 in regulating progenitor cell quiescence, we determined whether p21 deletion phenocopied cell cycle and density phenotypes observed with p53 loss of function. To assess proliferation, the number of FACS-isolated epithelial cells from p21$^{+/-}$ and p21$^{-/-}$ mice in G2/M phase was measured by propidium iodide (PI) staining. p21$^{-/-}$ mice, but not p21$^{+/-}$ mice, contained significantly more cells in G2/M (Figures 4G and S4F). We quantified the number of nuclei per unit basement membrane in p21$^{+/-}$ and p21$^{-/-}$ mice and found that p21 loss resulted in increased cell density in both genotypes compared to control (Figures 4H and S4G). To determine whether p21 also regulates differentiation, we quantified the percentage of FoxJ1-positive ciliated cells in the airway epithelium. Interestingly, there was no significant change in the number of ciliated cells in p21-deficient mice (Figures 4I and S4H). These results suggest that quiescence, but not differentiation, of club progenitor cells is regulated by p21 (Figure 4J). This finding partially phenocopies p53 loss of function and suggests that other p53 targets regulate differentiation of club progenitor cells.

DISCUSSION

In this study, we demonstrate that p53 critically regulates progenitor cell behavior to control cell density and composition in airways. We use cell-type-specific p53 knockouts as well as Super p53 mice to show that regulation of progenitor cell behavior occurs in a gene dose-dependent manner. These data illustrate that changes in baseline expression of p53 are important determinants of progenitor cell fate.

Previous studies show that p53 regulates self-renewal and differentiation of neural, mammary, hematopoietic, and nephron stem and progenitor cells (Armesilla-Diaz et al., 2009; Cicalese et al., 2009; Li et al., 2015; Liu et al., 2009; Meletis et al., 2006; Tosoni et al., 2015). However, these previous studies have been performed under conditions of rapid cell expansion in vitro or during development. As such, these studies leave gaps in our understanding of roles for p53 in regulating stem and progenitor cells in quiescent tissues under homeostatic
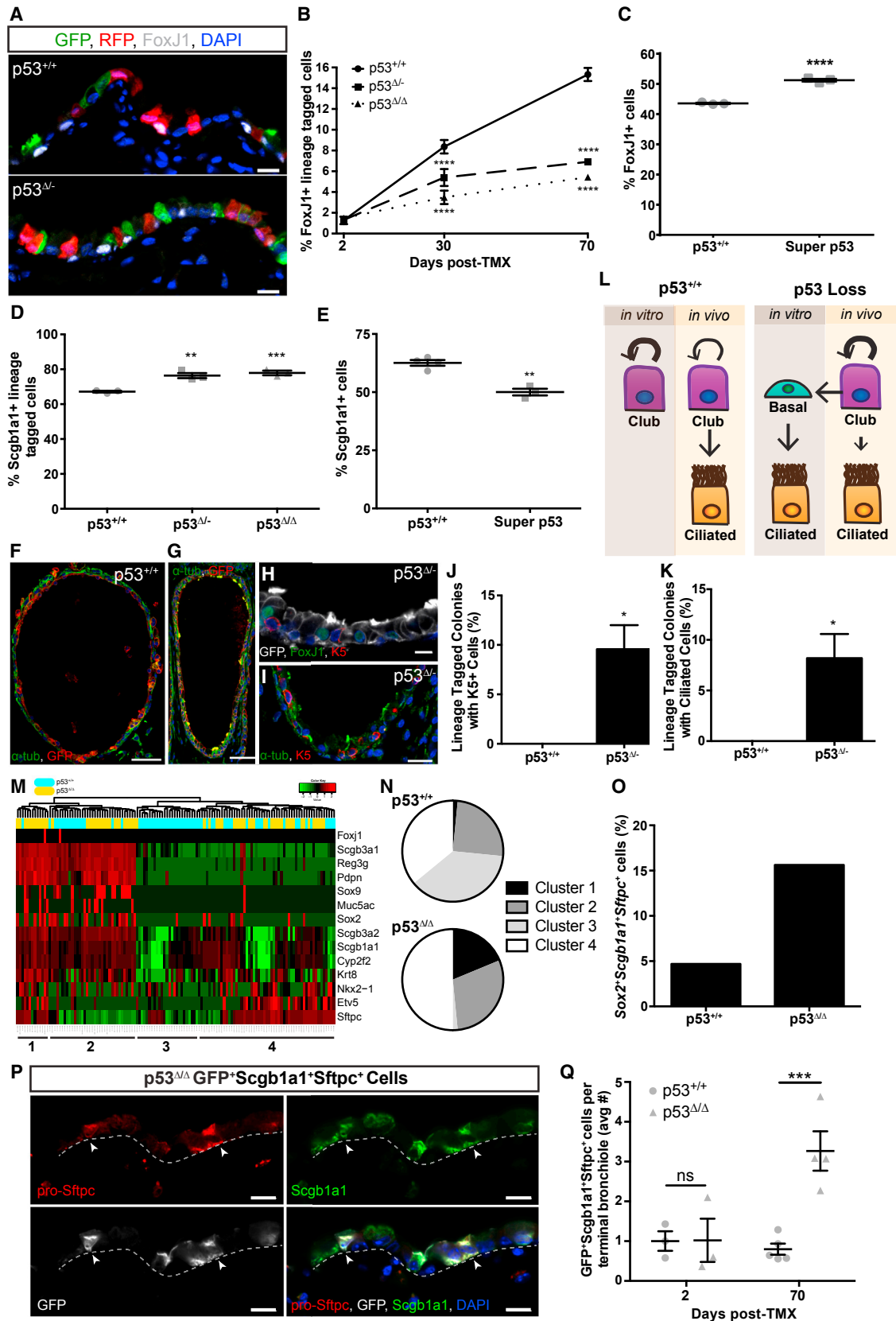
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conditions. Our finding that p53 controls quiescence and differentiation in the homeostatic lung provides key insights into progenitor cell regulation and complements previously published results.

We show that an extra copy of p53 promoted ciliated cell differentiation and decreased proliferation, leading to decreased epithelial cell density. Furthermore, we found a dose-dependent effect on p53 levels in the ability of club progenitors to clonally expand. This demonstrates that tight control of p53 is essential to maintain the proper number of progenitor cells that contribute to epithelial maintenance under homeostatic conditions. Precise regulation of secretory-to-ciliated cell ratios is essential for effective mucociliary clearance and host defense.

Surprisingly, club cells with p53 loss showed altered differentiation potential in vitro, yielding organoids composed of a pseudostratified epithelium containing ciliated cells as well as p63- and K5-expressing basal-like cells. Previous reports have described the dedifferentiation of club cells to basal cells following basal cell deletion and Yap overexpression, indicating that club cells have an inherent plasticity that is suppressed under homeostatic conditions (Tata et al., 2013; Zhao et al., 2014). In contrast to these findings, we never observed lineage-labeled basal cells in vivo. However, our in vitro data indicate that the differentiation potential of club cells is suppressed by both p53-dependent cell-autonomous and microenvironmental factors. Furthermore, organoids derived from p53-deficient club cells included p63-positive epithelial cells that were negative for K5. Organoids containing p63+ K5− epithelial cells were also observed following culture of p53-sufficient club cells, but with a significantly lower frequency than observed following culture of p53-deficient club cells. Discordance in p63 and K5 expression may reflect immaturity and the relatively high rate of cell proliferation among basal cells derived from p53-deficient club cells. The finding that ciliated cells were only observed in association with K5-positive cells and that the appearance of

K5-positive cells preceded that of ciliated cells (data not shown), suggests that ciliated cells are basal cell derived and that basal-to-ciliated cell differentiation is not influenced by p53 status. These data are in contrast to observations in vivo, wherein p53 deficiency promoted club cell renewal in preference to ciliated cell differentiation.

Scgb1a1-expressing cells were previously thought to be a relatively uniform pool of quiescent progenitors (Rawlins et al., 2009). However, using single-cell RNA-seq, we observed heterogeneity between various subtypes of cells within the *Scgb1a1*-lineage-labeled population. We identified lineage-traced cells that co-expressed *Scgb1a1* and *Sftpc*, the molecular phenotype described for putative BASCs. Furthermore, the number of Scgb1a1⁺Sftpc⁺ cells increased with p53 loss, implicating p53 as a regulator of this progenitor state. However, the functional role of these progenitor cells in repairing the airway and alveoli has yet to be determined. The potential for p53 loss to expand the pool of "active" progenitors was supported by the observation of an increase in the number of cycling cells and a decrease in cell-cycle inhibitors, particularly p21, following p53 loss. Mice with germline loss of p21 function phenocopied effects of conditional p53 loss on epithelial cell proliferation, but not differentiation. Even though we cannot exclude the potential for non-cell-autonomous effects of germline p21 deficiency, these results support the notion that proliferation and differentiation in club cells are regulated by distinct downstream targets of p53. Candidate pathways that may mediate the effects of p53 on cellular differentiation include Notch, a known target of p53 and a pathway that has been shown to modulate club-to-ciliated-cell transdifferentiation (Lafkas et al., 2015).

Recent literature has demonstrated that cancers often arise from resident stem or progenitor cells (Blanpain, 2013; Tomasetti and Vogelstein, 2015). Here, we show that the loss of a tumor suppressor leads to an increase in self-renewal and proliferation of progenitor cells. These conditions could lead to preneoplastic

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Figure 3. p53 Regulates Differentiation

(A) Immunofluorescent (IF) staining for confetti-lineage tag (nuclear GFP, cytoplasmic YFP, and membrane CFP are indicated in green, and cytoplasmic RFP is indicated in red) and FoxJ1 in white in *Scgb1a1-CreER^{TM}; Rosa26R-Confetti; p53$^{+/+}$* or *p53$^{\Delta/-}$* mice at 70 days post-tamoxifen.

(B) Quantification of the percentage of FoxJ1-positive confetti-lineage-tagged cells in *Scgb1a1-CreER^{TM}; Rosa26R-Confetti; p53$^{\Delta/-}$* or *p53$^{\Delta/\Delta}$* mice (p53$^{\Delta/-}$ or p53$^{\Delta/\Delta}$, respectively) at 2, 30, and 70 days post-tamoxifen (post-TMX) (n = 3–4).

(C) Quantification of the percentage of FoxJ1-positive nuclei out of total nuclei in the airway epithelium in Super p53 mice (n = 3).

(D) Percentage of Scgb1a1-positive confetti-lineage-tagged cells in *Scgb1a1-CreER^{TM}; Rosa26R-Confetti; p53$^{\Delta/-}$* or *p53$^{\Delta/\Delta}$* mice (p53$^{\Delta/-}$ or p53$^{\Delta/\Delta}$, respectively) at 70 days post-tamoxifen (n = 3).

(E) Percentage of Scgb1a1-positive cells out of total airway epithelial cells in Super p53 mice (n = 3–4).

(F and G) IF of a p53$^{+/+}$ (F) or p53$^{\Delta/-}$ (G) colony with GFP (red) and α-tubulin to visualize cilia (green).

(H) IF staining of basal cell marker K5 (red), ciliated cell marker FoxJ1 (green), and GFP (white).

(I) IF staining of basal cell marker K5 (red) and α-tubulin to visualize cilia (green).

(J and K) Percentage of lineage-tagged colonies containing K5-positive cells (J) or ciliated cells (K) (n = 3).

(L) p53 controls ciliated-cell differentiation in vivo and differentiation potential in vitro.

(M) Unsupervised heatmap showing cell-subtype-specific genes, segregated by four secondary clades (clusters).

(N) Pie chart depicting the percentage of cells that fall into the four clusters in (M).

(O) Percentage of single cells that co-express *Sox2*, *Scgb1a1*, and *Sftpc*.

(P) Immunostaining for Sftpc in red, Scgb1a1 in green, and GFP in white in *Scgb1a1-CreER^{TM}; Rosa26-mT/mG; p53$^{\Delta/\Delta}$* mice at 70 days post-tamoxifen. Arrowheads indicate co-expressing cells; a dashed line is shown at the basement membrane.

(Q) Number of lineage-tagged Scgb1a1⁺Sftpc⁺ cells per terminal bronchiole in *Scgb1a1-CreER^{TM}; Rosa26-mT/mG; p53$^{+/+}$* or *p53$^{\Delta/\Delta}$* mice at 2 or 70 days post-tamoxifen (TMX). avg, average.

DAPI is indicated in blue. n = 64 cells per genotype for (M)–(O). Scale bars represent 10 μm in (A), (H), and (P); 20 μm in (I); and 50 μm in (F) and (G). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant. All data shown represent mean ± SEM.

See also Figure S3.
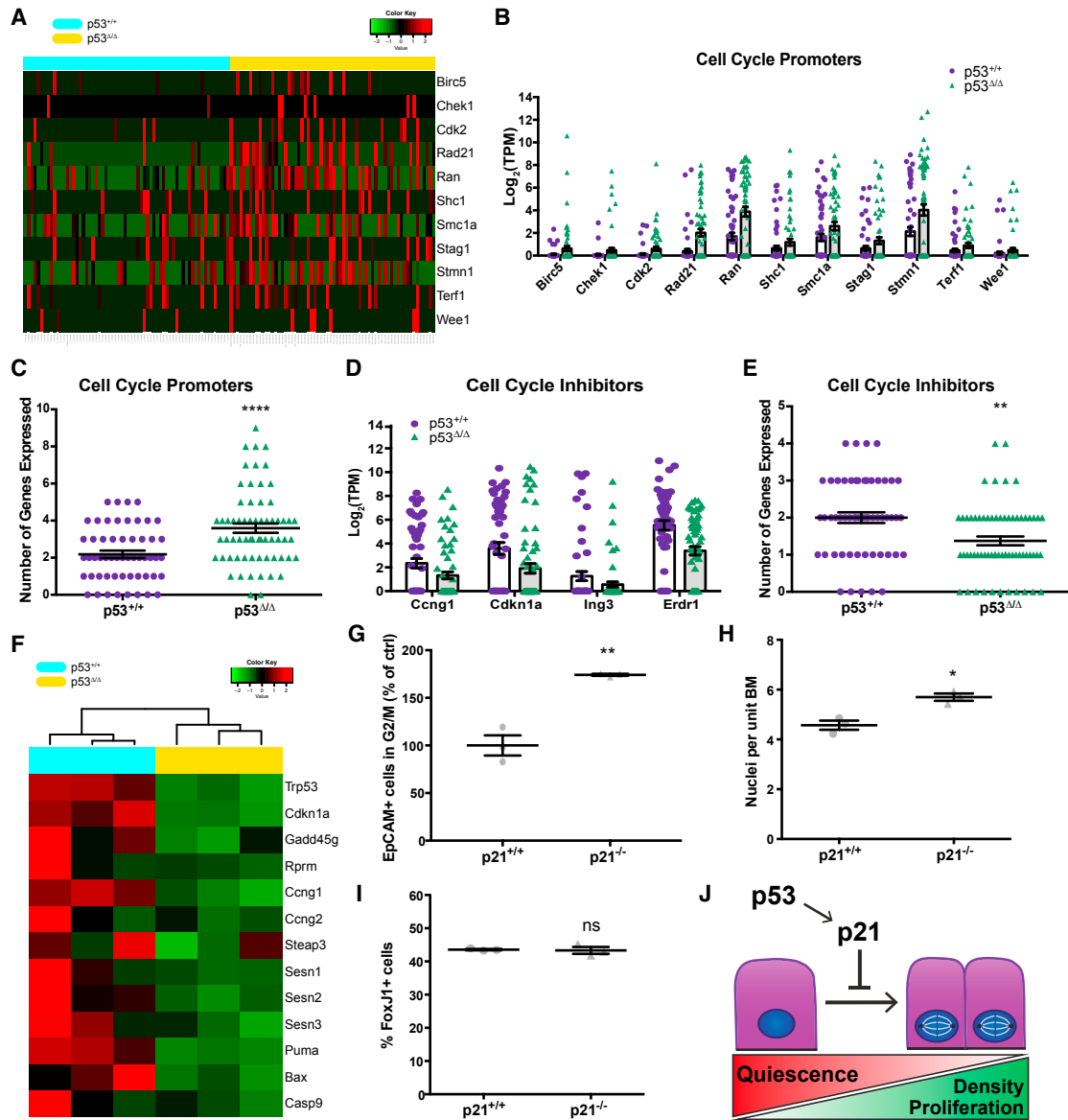
Figure 4. p53-Deficient Progenitors Have Increased Cell-Cycle Progression and Decreased Cell-Cycle Inhibition in a p21-Dependent Manner

(A) Supervised heatmap showing cell-cycle-promoting genes, which have higher expression in p53-deficient cells.

(B) Expression of cell-cycle-promoting genes is significantly higher when p53 is lost. *p = 0.0157, two-way ANOVA.

(C) Scatterplot depicting the total number of cell-cycle promoters expressed (Log_2(transcripts per million reads [TPM]) > 1) per cell.

(D) Expression of cell-cycle inhibitors are lower when p53 is lost.

(E) Scatterplot depicting the number of cell-cycle inhibition genes expressed (Log_2(TPM) > 1) per cell.

(F) Unsupervised heatmap showing p53 pathway genes obtained from population RNA-seq performed on isolated *Scgb1a1*-lineage-labeled cells from *Scgb1a1-CreER^{TM}; Rosa26-mT/mG; p53^{+/+}* or *p53^{Δ/Δ}* mice (p53^{+/+} or p53^{Δ/Δ}, respectively) (n = 3).

(G) Percent total isolated epithelial cells from p21 knockout mice in G2/M relative to control (n = 3).

(H) Number of nuclei per unit basement membrane in the airway epithelium of p21 knockout mice (n = 3).

(I) Percent FoxJ1-positive nuclei in the airway epithelium of p21 knockout mice (n = 3).

(J) p53 controls proliferation and density through p21.

*p < 0.05; **p < 0.01; ****p < 0.0001; ns, not significant. All data shown represent mean ± SEM.

See also Figure S4.

lesions, either alone or in combination with additional injuries or mutations. Interestingly, a high number of p53 copies in elephants has been correlated with a lower cancer risk (Abegglen et al., 2015; Sulak et al., 2015). We discovered that an additional copy of p53 reduced the proliferation rate and increased terminal differentiation, both of which likely promote tumor suppression.

Together, our data demonstrate that p53 plays an essential role as a tumor suppressor by regulating progenitor cell behavior in the lung.

EXPERIMENTAL PROCEDURES

Mice

Scgb1a1-CreER^TM; Rosa26-mT/mG and *Scgb1a1-CreER^TM; Rosa26R-Confetti* mice were previously described (Farin et al., 2015). These mice were crossed to p53^flox mice (The Jackson Laboratory, stock number 008462), p53^{−/−} mice (The Jackson Laboratory, stock number 002101), or Super p53 tg mice (García-Cao et al., 2002), which were provided by Manuel Serrano, to generate experimental animals. Detailed explanation of mouse strains are in the Supplemental Experimental Procedures. Mice were injected with tamoxifen in corn oil: 3 × 200 mg/kg (body weight), 1 × 250 mg/kg (body weight), or 1 × 5 mg/kg (body weight) for high-dose, RNA-seq, and low-dose experiments, respectively. All mice were maintained and treatments were carried out according to IACUC (Institutional Animal Care and Use Committee)-approved protocols.

Immunofluorescence Staining, Imaging, and Quantification

Immunofluorescence imaging was performed on fixed lung tissue embedded in paraffin and processed as previously described (Chen et al., 2012). The antibodies used, imaging, and quantification details are given in the Supplemental Experimental Procedures.

Cell-Cycle Analysis

Airway epithelial cells (EpCAM^+, CD31/34/45^−, and 7AAD^−) were sorted using a MoFlo XDP cell sorter (Beckman Coulter), fixed with 70% ice-cold ethanol, treated with RNase A (19101, QIAGEN), and stained with PI (P4170, Sigma). The Supplemental Experimental Procedures detail the BrdU treatment and analysis. Stained cells were analyzed on an LSRFortessa (BD Biosciences) flow cytometer.

In Vitro Sultures

Airway epithelial cell isolation and flow cytometry were performed as previously described (Farin et al., 2015). The Supplemental Experimental Procedures contain details regarding culture conditions and serial passaging. Colony-forming efficiency was performed after 7 days in vitro, and immunofluorescence analysis was performed after 14 days in vitro.

RNA-Seq

Details on single-cell and population RNA-seq methodology and analysis are provided in the Supplemental Experimental Procedures.

Statistical Analysis

Data were analyzed and compared between groups using a two-tailed, unpaired Student's t test, a one-way ANOVA, or a two-way ANOVA with post hoc analysis (Prism, GraphPad). A $p < 0.05$ was considered statistically significant and is presented as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, or $****p < 0.0001$.

ACCESSION NUMBERS

The accession number for the single-cell and population RNA-seq data reported in this paper is GEO: GSE78045.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.11.007.

AUTHOR CONTRIBUTIONS

A.M.M. designed and performed experiments and wrote the manuscript. A.R.Y. and A.S.S. performed experiments. C.Y., Y.W., and J.T. performed bio-informatics analysis for single-cell and population RNA-seq. D.G.K. provided input on experiments and essential mouse strains and edited the manuscript. B.R.S. co-designed experiments and co-wrote the manuscript.

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