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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Notch3-Jagged signaling controls the pool of undifferentiated airway progenitors

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ABSTRACT

Basal cells are multipotent airway progenitors that generate distinct epithelial cell phenotypes crucial for homeostasis and repair of the conducting airways. Little is known about how these progenitor cells expand and transition to differentiation to form the pseudostratified airway epithelium in the developing and adult lung. Here, we show by genetic and pharmacological approaches that endogenous activation of Notch3 signaling selectively controls the pool of undifferentiated progenitors of upper airways available for differentiation. This mechanism depends on the availability of Jag1 and Jag2, and is key to generating a population of parabasal cells that later activates Notch1 and Notch2 for secretory-multiciliated cell fate selection. Disruption of this mechanism resulted in aberrant expansion of basal cells and altered pseudostratification. Analysis of human lungs showing similar abnormalities and decreased NOTCH3 expression in subjects with chronic obstructive pulmonary disease suggests an involvement of NOTCH3-dependent events in the pathogenesis of this condition.

KEY WORDS: Notch, Basal cells, p63, Airway differentiation, Progenitor cells, Lung regeneration, Jagged, COPD

INTRODUCTION

Basal cells are multipotent epithelial progenitors recognized largely by expression of p63 and intermediate filament keratins, such as Krt5 and Krt14 in multiple organs, including the skin, upper digestive, respiratory and urinary tracts (Yang and McKeon, 2000; Rock and Hogan, 2011; Hegab et al., 2011; Pignon et al., 2013; Fuchs, 2008; Kurita et al., 2004). In the adult human lung, these cells are seen throughout the airway epithelium from the trachea to the distal bronchioles; in mice, basal cells are found predominantly in the trachea, submucosal glands and extrapulmonary airways (Rock and Hogan, 2011; Hegab et al., 2011). Lineage studies have shown that these cells self-renew and generate secretory and multiciliated epithelial cell phenotypes crucial for homeostasis of the conducting airways. Still, little is known about how basal cells transition from the proliferative uncommitted state to differentiation in the developing and the adult airway epithelium. Studies in different tissues, including the human lung, have identified a non-basal intermediate cell with some ultrastructural features of basal cells but no defined features of the typically differentiated cellular phenotypes of the airway epithelium (Donnelly et al., 1982; Mercer et al., 1994; Breuer and Zajicek, 1990). The topographic nuclear position in between the basal and luminal cell led them to be named as suprabasal or parabasal cells. These cells have also been shown to have limited proliferative capacity and were identified as early progenitors of the airway epithelium. The process that gives rise to these cells resembles that described during the generation of the epidermis and is strongly dependent on Notch signaling. In the skin, Notch is required for repression of basal cell genes and commitment of basal to the suprabasal cell phenotype early in epidermal development, initiating a genetic program of differentiation (Blanpain et al., 2006). In the adult lung, Notch signaling has been shown to promote the transition of basal cells into a population of early epithelial progenitors and later to drive differentiation to secretory cell fate (Rock et al., 2011).

Although these studies clearly implicate Notch in the process, they raise multiple questions about how these events occur as the epithelial progenitors undergo differentiation. Are these regulated by a single Notch signal acting through different thresholds that determine initially suprabasal cell fate and later secretory differentiation? Are parabasal cells progenitor cells but presumably already committed precursors to a particular cell fate? Is there more than one Notch signaling event regulating the stepwise transition of the basal cells to the differentiated airway epithelium? If so, what specific Notch receptors and ligands are differentially involved?

Here, we have used a combination of genetic and pharmacological approaches to investigate these issues. Analysis of adult airway progenitors in air-liquid interface (ALI) cultures revealed a population of undifferentiated Notch3-activating epithelial progenitors interspersed with p63+ basal cells. We provide evidence that selective activation of endogenous Notch3 is crucial to control the pool of p63+ airway progenitors and generate a population of parabasal cells that will later activate Notch1 and Notch2 for secretory-multiciliated cell fate selection. Loss of Notch3 signaling markedly expanded the population of basal cells both in the developing and adult lung. The presence of similar abnormalities in NOTCH3 and basal cells in human subjects with chronic obstructive pulmonary disease suggests an involvement of NOTCH3 in the pathogenesis of this disease.

RESULTS

Inhibition of Notch signaling promotes expansion of basal and multiciliated cells

Notch signaling regulates cell fate decisions in the developing and the adult airway epithelium (Rock et al., 2011; Tsao et al., 2009, 2011; Morimoto et al., 2010; Xing et al., 2012; Guseh et al., 2009). To gain further insights into this process, we analyzed airway progenitors from adult murine tracheas growing in air-liquid

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interface (ALI) cultures under Notch-deficient conditions using a pharmacological or a genetic approach.

Airway progenitors were expanded in Transwell cultures for 4 days and then treated with vehicle control (DMSO) or the gamma secretase inhibitor DAPT (50 µM) 3 days prior to induction of ALI until harvest at days 0, 4, and 8 (Fig. 1A). DAPT-treated cultures showed the expected suppression of the secretory phenotype and increase in number of multiciliated cells (Fig. 1B). Surprisingly, Ki67 labeling revealed an increasing number of proliferating cells in sharp contradiction to the increase in the population of multiciliated cells, known to be non-proliferative (Fig. 1C) (Rawlins et al., 2007). Immunofluorescence analysis for Krt5 and p63 showed that by day 8, DAPT-treated cultures had abundant labeling with nearly eightfold more p63+ basal cells than controls cultures (Fig. 1B,D). Morphometric analyses revealed that the increase in p63+ cells by disruption of Notch preceded the initiation of multiciliated cell differentiation and was already detected in the airway progenitor cells prior to the establishment of ALI at day 0 (Fig. 1E). Moreover, double Ki67/p63 immunofluorescence showed twice as many p63+ cells labeled with Ki67 at days 0 and 4, suggesting that a Notch-mediated mechanism that restricts proliferation of basal cells occurred largely prior to or as these cells started differentiating (Fig. 1F).

The expansion in p63+ cells was similarly observed in airway epithelial progenitors isolated from adult *Rbpj*$^{f/f}$ tracheas transduced at the time of plating with a lentivirus expressing Cre-recombinase (Fig. 1G). p63/cre double-labeled cells were significantly increased in cultures from *Rbpj*$^{f/f}$ compared with wild-type mice (Fig. 1H,I). The inability to form secretory cells in DAPT or in *Rbpj*$^{f/f}$-lenti-Cre conditions suggested that the excessive number of multiciliated cells seen at later stages did not result from conversion of club (Clara) cells to multiciliated cells, but could be arising directly from the basal cells.

We reasoned that this mechanism could also serve to regulate the pool of immature p63+ basal cells in the developing airways. Previous studies have not identified significant changes in basal cells in Notch-deficient mice during embryonic development. However, it was unclear whether regions typically enriched in basal cells, such as the trachea, had been examined (Tsao et al., 2009, 2011; Morimoto et al., 2010). We compared the abundance and

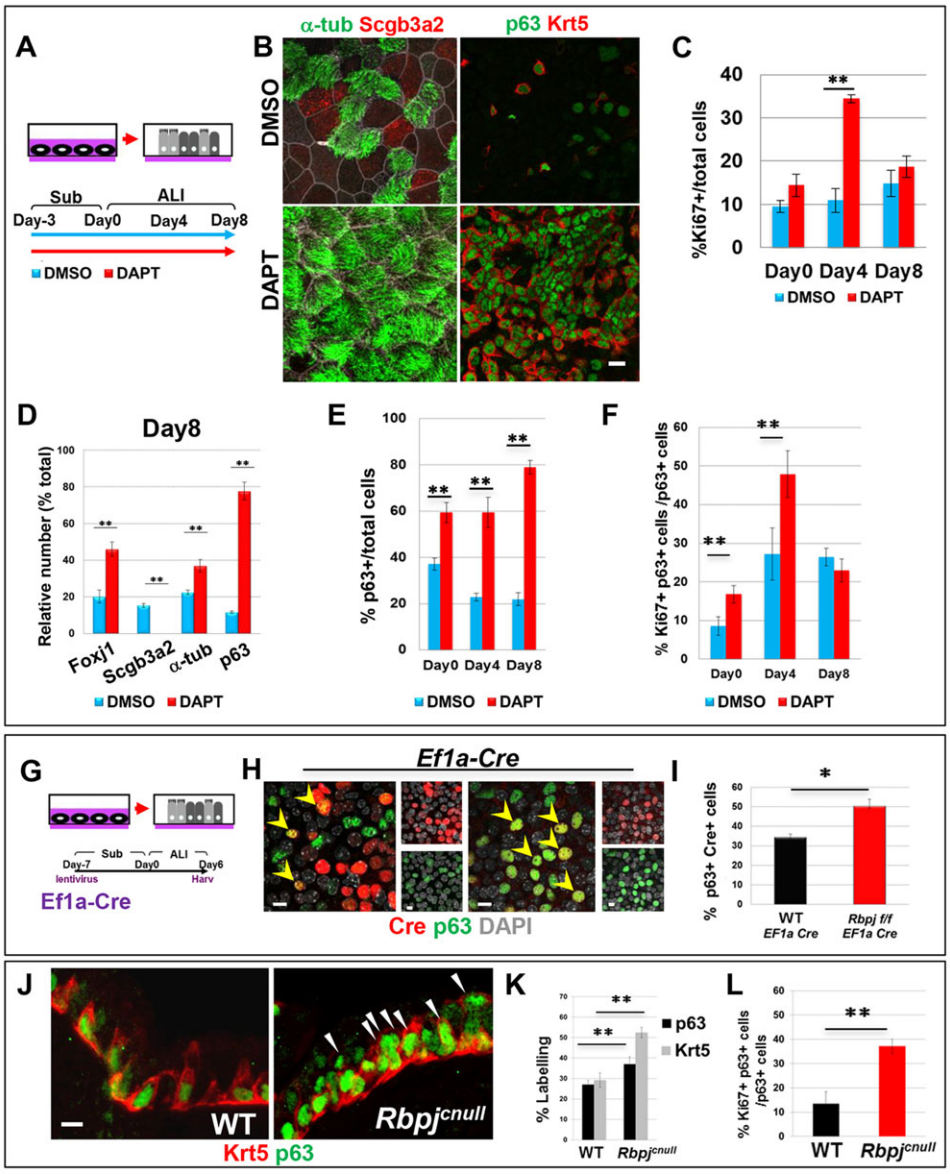

Fig. 1. Notch signaling restricts the pool of p63+ airway progenitors *in vitro* and *in vivo*. (A) Experimental design. Adult airway progenitors cultured under submerged condition (Sub) and subsequently in air-liquid interface (ALI) treated with DMSO (control, blue) or DAPT (γ-secretase inhibitor; red) from day –3 to ALI day 8. (B) Increased number of multiciliated cells (acetylated α-tubulin (α-tub) and abolishment of secretory cells (Scgb3a2+). Expansion of basal cells (p63+ Krt5+) in DAPT compared with control. (C) Relative number of Ki67+ cells per total DAPI-labeled cells (%) at each time point. (D) Relative number of cells labeled with each markers at ALI day 8 (% total). (E) Relative number of p63+ cells per total DAPI-labeled cells (%), or (F) Ki67 +p63+ cells per total p63+ cells at each time point. (G) Experimental design summary: Ef1α-Cre lentivirus-mediated disruption of Notch signaling in adult wild-type or *Rbpj*$^{f/f}$ airway progenitors. (H) Immunofluorescence for Cre, p63 or DAPI at ALI day 6: there is an increase in the population of p63-Cre double-labeled cells (arrowheads). (I) Relative number of p63+ cells in Cre-labeled cells at ALI day 6. (J) Immunofluorescence for p63 and Krt5 in E18.5 wild-type and *Rbpj*^{cnull} tracheal epithelium; arrowheads indicate expanded basal cell population. Morphometric analysis of (K) p63+ or Krt5+ cells, and (L) p63+Ki67+ cells per p63+ cells in E18.5 wild-type and *Rbpj*^{cnull} mice. Data are mean±s.e.m. from five or six fields per sample, n=3. Student's t-test *P<0.05 or **P<0.01. Scale bars: 10 µm.

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distribution of basal cells in the trachea and extrapulmonary airways of E18.5 wild-type and *Rbpj*^{cnull} mice. p63+ Krt5+ single or double-labeled cells were identified in multiple layers of the airway epithelium of *Rbpj*^{cnull} mutants, in contrast to the distribution in wild type (Fig. 1J). Quantitative analysis confirmed the increase in number of these cells (Fig. 1K), and showed that they proliferate to a greater extent in *Rbpj*^{cnull} compared with wild type (Fig. 1L). Thus, Notch signaling could be acting as gatekeeper, controlling the size of the progenitor cell pool available for differentiation.

To better understand how timing of endogenous Notch activation ultimately influenced the abundance of basal cells, we disrupted Notch signaling with DAPT at different stages in ALI cultures and examined the effect at day 8 (supplementary material Fig. S1A,B). Under control conditions (DMSO), we found that basal cells (p63+) represented around 10% of all day 8 cells, while multiciliated and secretory cells averaged around 20% each (You et al., 2002). Disrupting Notch signaling when these progenitors were still expanding and throughout the culture period (day –3 to day 8) altered their behavior dramatically. No secretory cells were present and 76% of all cells remained as basal cells, while the others became multiciliated cells. Disruption of Notch signaling for a shorter period after the progenitors became confluent and differentiation started (day 0 to day 3 post-ALI) still resulted in expansion of basal cells, although to a lesser degree. Later (DAPT from day 3 to day 8), the effect on basal cells was no longer seen but there was a prominent increase in multiciliated cells. These results are in agreement with previous observations in tracheosphere cultures (Rock et al., 2011) and they collectively indicate that Notch signaling is active prior to differentiation, regulating the size of the p63+ progenitor cell pool. They also suggest that another subset of progenitor cells exist that are interspersed with the p63+ cells and activate endogenous Notch signaling.

Notch3 is selectively activated in a population of p63-negative undifferentiated progenitor cells

To look for evidence of activation of specific Notch receptors during this process, we used immunofluorescence with antibodies that recognize selectively the Notch1, Notch2 or Notch3 C-terminus intracellular domains (ICD) and their subcellular localization. Specificity of these antibodies has been confirmed in Notch-null mice (see below) (Tsao et al., 2009, 2011; Morimoto et al., 2010, 2012). Immunofluorescence of day 0 cultures showed no evidence of activated Notch1 or Notch2 at this stage; Notch1 expression was very weak and signals for both receptors were absent from the nucleus. By contrast, prominent nuclear Notch3 expression was abundantly detected at day 0, suggesting that Notch3 is selectively active in undifferentiated progenitor cells (Fig. 2A-C). By day 8, strong nuclear Notch1 was evident and Notch2 signals were no longer restricted to the cell membrane. The Notch1 and Notch2 activation at later stages was consistent with the reported role of these receptors in secretory-multiciliated cell fate selection (Tsao et al., 2009, 2011; Morimoto et al., 2010, 2012). Nuclear Notch3

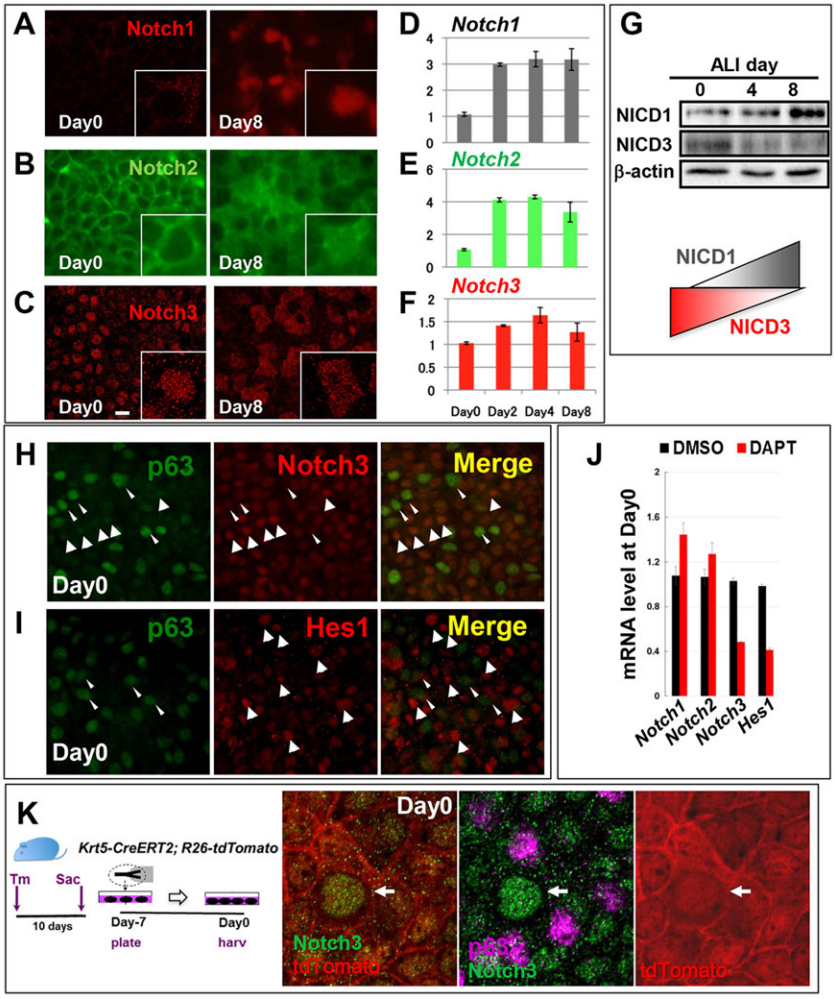

Fig. 2. Notch3 is selectively activated in p63-negative undifferentiated airway progenitors. (A-C,G) At ALI day 0, nuclear signals are prominent for Notch3 but not for Notch2 or Notch1, which appear only later during differentiation. (A-C) Immunofluorescence of ALI day 0 and day 8 cells using antibodies against Notch1 (cleaved NICD1: Val1744) (A), the C-terminus of Notch2 (B) and the C-terminus of Notch3 (C) proteins. (D-F) Real-time RT-PCR of *Notch1*, *Notch2* and *Notch3* mRNA at ALI days 0, 2, 4, 8 (data are mean±s.e.m., *n*=3 per group). (G) Western blot confirming predominant activation of Notch3 at ALI day 0 and Notch1 at day 8; β-actin is used as an internal control. (H,I) Immunofluorescence at day 0 showing that Notch3 is activated in the nuclei of p63-negative cells and Hes1-expressing cells. (J) Real-time PCR of *Notch1*, *Notch 2*, *Notch3* and *Hes1* in ALI day 0 cultures treated with DMSO (control) or DAPT for 72 h showing that only *Notch3* and *Hes1* are differentially expressed between controls and DAPT at this stage. (K) Lineage analysis of adult *Krt5-CreERT2; R26-tdTomato* mice treated with tamoxifen (Tm, 10 mg/kg i.p.) 10 days prior to the sacrifice; airway progenitors cultured for 7 days to confluence; immunofluorescence for Notch3, Td Tomato and p63 showing Notch3 signals (arrows) in lineage-labeled tdTomato+ cells at ALI day 0. Scale bar: 10 µm.

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staining continued to be observed at day 8, although signals appeared to be less prominent than at earlier stages. qPCR showed that the overall temporal pattern of expression of Notch mRNAs correlated with the changes observed by immunofluorescent staining. *Notch1* and *Notch2* mRNAs increased from day 0-4 as these cells initiated differentiation, unlike *Notch3* (Fig. 2D-F). The differences in the temporal pattern of activation of these receptors were best exemplified by comparing Notch1 and Notch3, as illustrated by western blot analysis of days 0, 4 and 8 cell homogenates from these cultures (Fig. 2G).

Previous studies in differentiating basal cells supported a model in which an initial Notch-dependent event generates a population of multipotent p63-negative progenitor with features of transit amplifying cells (Rock et al., 2011). To investigate this issue, we looked at the distribution of Notch and p63+ cells in the undifferentiated airway progenitors at ALI day 0. As expected, p63-expressing cells were abundantly present at this stage; however, many of the cells were not labeled with p63. Strikingly, we found that the p63-negative cell population expressed endogenous NICD3 and the Notch target Hes1 in the nucleus. The Notch3/Hes1+ and the p63+ cell populations seem to exist at roughly equal numbers and with relatively little overlap (Fig. 2H,I). This strongly suggested that Notch3 mediates a Notch-dependent event that generates the undifferentiated p63-negative progenitors. Additional evidence of the relevance of endogenous Notch3 was the finding that at this stage the DAPT-mediated expansion of p63+ cells was selectively associated with downregulation of *Notch3* mRNA but not *Notch1* or *Notch2* mRNAs (Fig. 2J). Moreover, this decrease in Notch3 expression best correlated with the Hes1 downregulation at this stage by qPCR (Fig. 2J). Interestingly, Hes1 gain of function is known to inhibit expansion of p63+ basal cells (Rock et al., 2011).

We reasoned that the Notch3-expressing cells originated from the basal cell population, presumably as they expanded to form a confluent monolayer. We performed lineage-tracing analysis using a *Krt5-CreERT2-R26 tdTomato* reporter mouse, known to label basal cells and their descendants (Rock et al., 2009). Tamoxifen (single dose 150 mg/kg body) was administered to adult mice 10 days prior to sacrifice; epithelial progenitors were isolated from tracheas and cultured to confluence (day 0), as before. Double immunofluorescence and confocal analysis of these cultures showed that all Notch3-expressing cells were also tdTomato positive (Fig. 2K).

Endogenous Notch3 activation identifies parabasal cells in developing and adult airways

To find how the observations above related to the role of Notch3 *in vivo*, first we investigated the potential sites of Notch3 activation in the developing and the adult airway epithelium and compared these with the sites of p63 expression. Notch3 expression in the lung has been described mostly in the mesenchymal compartment; less is known about its expression and activation in the epithelium (Li et al., 2009; Ghosh et al., 2011; Domenga et al., 2004; Xu et al., 2010; Ito et al., 2000). At E14.5, p63 is expressed in columnar cells of the trachea and main bronchi both at basal and luminal positions in the pseudostratified epithelium. At this stage, we found weak Notch3 cytoplasmic and nuclear signals in some cases overlapping with p63 in basal cells. At subsequent stages, these signals become increasingly segregated, with Notch3 cells occupying a preferential parabasal position by E16.5 and onwards (Fig. 3A).

Studies using tracheosphere assays identified a population of p63-negative putative adult epithelial progenitors (EPs) in the pseudostratified epithelium that expresses Krt8 and activates Notch signaling during differentiation from the basal cells (Rock et al., 2011, 2009). We reasoned that the parabasal epithelial progenitors referred to in these studies are the Notch3-labeled population identified here. Indeed, confocal analysis of confluent adult airway epithelial cultures (day 0 ALI) revealed a similar architectural organization consistent with the idea of a pseudostratified epithelium in which p63-positive cells lay underneath a population of p63-negative Krt8+ progenitor cells that activates Notch3 signaling prior to differentiation (Fig. 3B, right panel). Krt8 does not mark exclusively the parabasal cells and has been also reported in luminal differentiated cells (Rock et al., 2009). These patterns were also seen during development, as clearly illustrated in E18.5 airways (Fig. 3B, left panel) and its functional significance is intriguing in the embryonic lung, because during development the contribution of p63-expressing cells to differentiation of airway epithelium has not been established.

Notch3-mediated regulation of airway progenitor cell expansion *in vitro* and *in vivo*

Our data strongly suggested that Notch3 could be the receptor selectively mediating the control of the p63+ progenitor cell pool. Thus, we analyzed airways of *Notch3*$^{-/-}$ mice. Previous reports showed defects in lung vascular smooth muscle but apparently no other lung abnormality, being viable throughout adulthood (Li et al., 2009; Domenga et al., 2004). No information is available about the impact of Notch3 deficiency on the ability of the airway progenitors to expand or reconstitute the epithelium *in vivo* or *in vitro*.

Gross morphology and histological analysis of *Notch3*$^{-/-}$ mice did not reveal major abnormalities. However, immunofluorescence and quantitative analysis for p63 revealed expansion of basal cells in tracheal epithelium by E14.5 (Fig. 3C). This phenotype was also identified in the adult lung (Fig. 3D-K) and became even more noticeable when cultures of *Notch3*$^{-/-}$ and wild-type adult airway epithelial progenitors were compared (Fig. 3D). The marked expansion of basal cells at ALI day 0 was further supported by a nearly fivefold increase in number of Ki67+p63+ double-labeled cells in *Notch3*$^{-/-}$ mice relative to wild type (Fig. 3E,F). The expansion of basal cells was consistent with our findings of Notch-deficient signaling seen cultures of *Rbpj*$^{f/f}$ lentiCre or DAPT-treated epithelial cells (Fig. 1). Assessment of markers of multiciliated and secretory cells did not reveal differences in the balance of these cell types between adult *Notch3*$^{-/-}$ and wild type (data not shown). This further supported the idea of a selective program of Notch3 activation acting early in airway progenitor cells to restrict expansion and presumably allow initiation of differentiation.

Immunofluorescent staining of adult *Notch3*$^{-/-}$ airways showed expansion of the population of basal (p63+) and parabasal/luminal Krt8+ cells. Quantitative analysis confirmed the increased number of p63+ cells and that the epithelium of Notch3-deficient mice had increased pseudostratification compared with wild type (Fig. 3G,H,I). Interestingly, we found that the expansion of the p63+ basal cells was accompanied by the appearance of a population of Krt5+p63− parabasal cells (Fig. 3J). The significance of this observation remains to be clarified. Nevertheless, it suggests that in upper airways, Notch3 contributes to control the architectural arrangement of the pseudostratified epithelium by regulating not only the pool of p63+ basal cells, but presumably other rare populations of undifferentiated progenitors, such as the Krt5+p63− cells. The parabasal nature of these cells was further supported by their expression of both Krt5 and Krt8. This population of double-labeled cells was increased in *Notch3*$^{-/-}$ mice and, concurrent with the increase in Krt5/p63+ cells,

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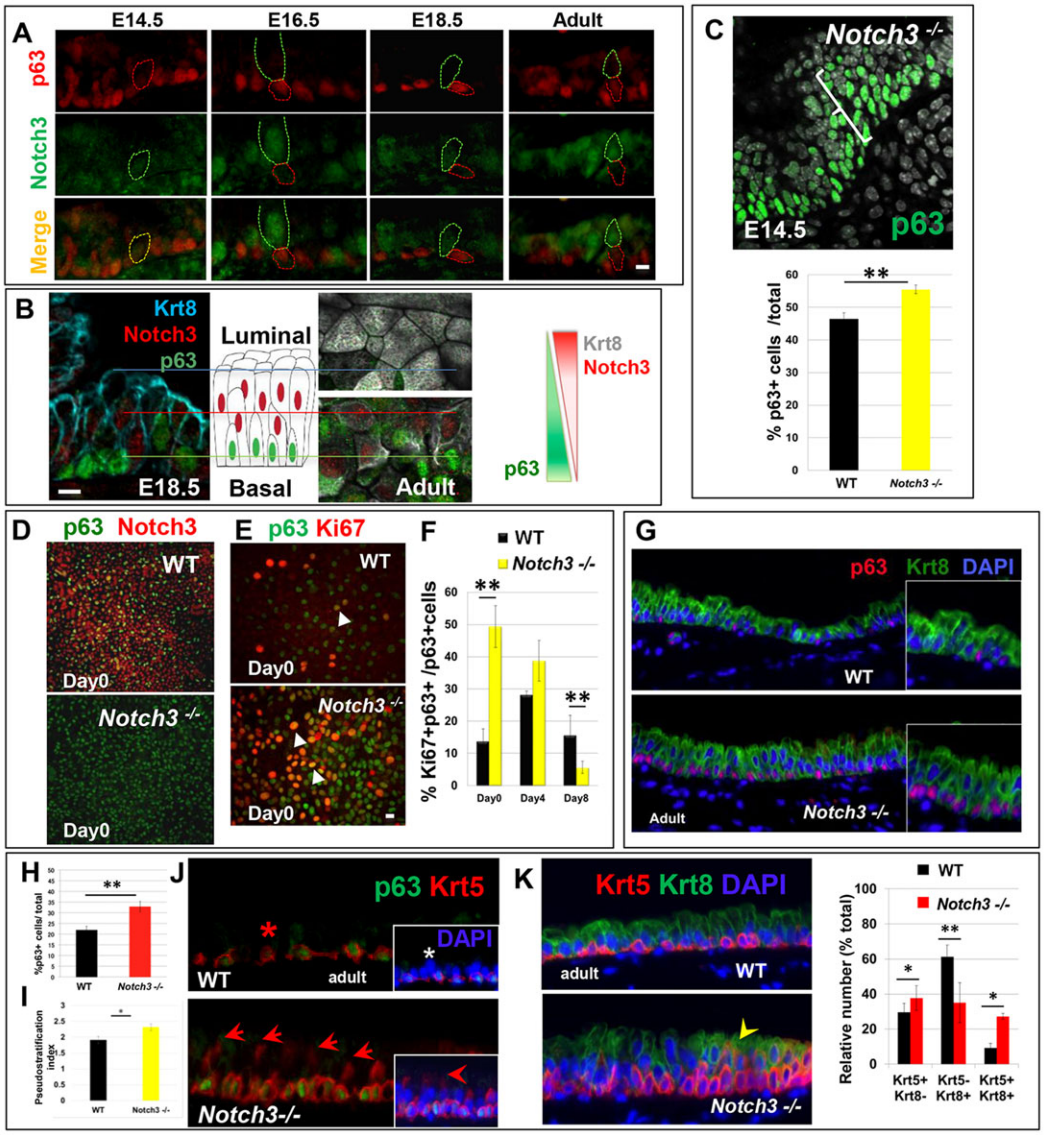

Fig. 3. Endogenous Notch3 activation identifies parabasal cells and restricts the pool of p63+ cells *in vivo* and *in vitro*. (A) Immunofluorescence of Notch3 and p63 in developing and adult tracheal epithelium; nuclear Notch3 and p63 overlapping extensively at E14.5 (dashed line, yellow), signals becoming more segregated later (>E16.5). (B) Nuclear Notch3+ cells occupy a parabasal position and express Krt8 in developing (left, E18.5) and adult (right, ALI Day 0) airways. Diagram represents confocal image depicting luminal (top) and basal (bottom) views of airway epithelium. There is enrichment of p63+ in basal layer and Notch3+Krt8+ in parabasal layers; horizontal lines indicate the comparison of E18.5 and adult (ALI Day 0) epithelium. (C) Notch3 inactivation expands the p63+ pool in the embryonic (E14.5) airways of *Notch3*$^{-/-}$ mice. (D,E) Immunofluorescent staining of ALI cultures at day 0 showing significant increase in the p63+ population and in Ki67+p63+ double-labeled cells (arrowheads) in *Notch3*$^{-/-}$ cultures compared with wild-type cultures. (F) Increased cell proliferation in cultures of *Notch3*$^{-/-}$ no longer seen after ALI day 4. (G,H) Adult *Notch3*$^{-/-}$ airways showing expansion of the population of basal (p63+) and luminal (Krt8+) cells, (I) increased pseudostratification and (J) the appearance of Krt5+p63– parabasal cells (arrows) not seen in wild type (asterisks). In the inset in J, arrowhead indicates Krt5 and DAPI in parabasal cells. (K) *Notch3*$^{-/-}$ airways have increased number of Krt5+ single and Krt5+Krt8+ double-labeled cells and decreased number of Krt8+Krt5– cells. Data are mean±s.e.m., analysis of six fields (n=3 per group; *P<0.05, **P<0.01, Student's t-test). Scale bars: 8 µm.

contributes to the increase in pseudostratification seen in the airways of these mutants (Fig. 3K).

Notch3 activation in adult airway progenitors does not prevent differentiation

Our finding of preferential activation of Notch3 in undifferentiated adult progenitors raised the possibility that, in contrast to Notch1 and Notch2, activation of this receptor could be incompatible with differentiation. Interestingly, analysis of late-stage ALI cultures and embryonic lungs revealed scattered nuclear Notch3 labeling in both Scgb1a1+ cells and Foxj1+ cells, presumably representing parabasal cells transiently undergoing differentiation into secretory and ciliated cells, respectively (data not shown). From this observation, however, we could not ensure that, at late stages, endogenous Notch3 was activated at sufficient levels to influence differentiation. To address this issue, we examined the effect of artificially maintaining Notch3 activated throughout the period of expansion and differentiation of the adult airway progenitors in ALI cultures.

A previous report showed that, when activated Notch3 is conditionally expressed early in the embryonic lung epithelium *in vivo* (Sftpc-NICD3) the lung epithelium remains undifferentiated, halted in an early developmental stage (Dang et al., 2003). Based on this, we predicted that activating Notch3 constitutively in adult airway progenitors would maintain these cells in an uncommitted

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state, ultimately arresting differentiation. Thus, at the time of plating, we transduced adult airway epithelial progenitors from *Notch3*$^{-/-}$ mice with a lentivirus expressing HA-tagged NICD3 or control GFP constructs. *Notch3*$^{-/-}$ was used to minimize potential overexpression effects of superimposing exogenous with wild-type endogenous Notch3. Confocal microscopy reveals that NICD3 transduction resulted in a major reduction in the proportion of p63-positive cells in infected cells tagged by GFP or HA (from 70% in controls to only 10% in NICD3-transduced cultures) (Fig. 4A,B).

Unexpectedly, Scgb3a2 immunofluorescence showed multiple NICD3-HA-Scgb3a2 double-labeled cells (mean±s.e.m.: 28±5.2%, *n*=3) already present at day 0, well before differentiation occurs (Fig. 4D). No Scgb3a2 labeling was present in GFP-control transduced cells (Fig. 4D,F). By day 8, the majority of the NICD3-transduced cells were double labeled with Scgb3a2 (mean±s.e.m.: 92±2.1%, *n*=3), whereas only a relatively smaller number of Scgb3a2-GFP double labeling in control cells were present (mean±s.e.m.: 28±0.7%, *n*=3) (Fig. 4E,F). Similar experiments transducing NICD3-HA in wild-type cells confirmed the effect seen in mutants and showed an even greater increase in number of Scgb3a2+ cells, likely due to the additional contribution of endogenous Notch3 (day 0, mean±s.e.m.: 50±7.3%, *n*=3) (Fig. 4C,F).

Our data indicated that constitutive Notch3 activation in adult airway progenitors led to precocious excessive secretory cell differentiation in contrast to the maintenance of the undifferentiated status, as reported in the embryonic lung (Dang et al., 2003). Moreover, timing and levels of endogenous Notch3 seem to be tightly regulated in adult airway progenitors to control the pool of basal cells and to prevent precocious induction of differentiation. Together our data are consistent with a model in which regulated activation of Notch3 in undifferentiated progenitors controls the pool of p63+ basal cells available to differentiate later, when Notch1 and Notch2 trigger the secretory-multiciliated cell phase decision. Secretory differentiation, however, can be triggered by prolonged or increased expression of Notch3.

Jag1 and Jag2 have partially overlapping functions in restricting basal cell expansion

Preferential Notch ligand-receptor binding depends on multiple factors, including biological context and cell type (D'Souza et al., 2008; Kopan and Ilagan, 2009). We reasoned that p63+ airway progenitors were a source of Notch ligands activating Notch3 signaling in neighbor cells, ultimately controlling expansion of the p63 basal cell population to initiate differentiation. A relationship between Notch3 and jagged 1 (Jag1) has been reported in the context of cancer cell survival and growth (Konishi et al., 2007; Sansone et al., 2007). To gain insights into this relationship in our system, we investigated expression of Jag ligands in the proximal airway epithelium at the onset of differentiation in regions associated with p63 during development and in adult progenitors cultured under ALI conditions. *In situ* hybridization analysis of *Jag1* showed epithelial signals as early as E14.5 in luminal and basal-located cells enriched in p63+ cells. Around this stage *Jag2* signals were stronger and more clearly associated with basal cells (Fig. 5A). Later, expression of both ligands extended to intrapulmonary airways in basal and multiciliated cells. In adult airways, *Jag2* has been reported as the most differentially expressed ligand in basal cells (Rock et al., 2011). Our immunofluorescent

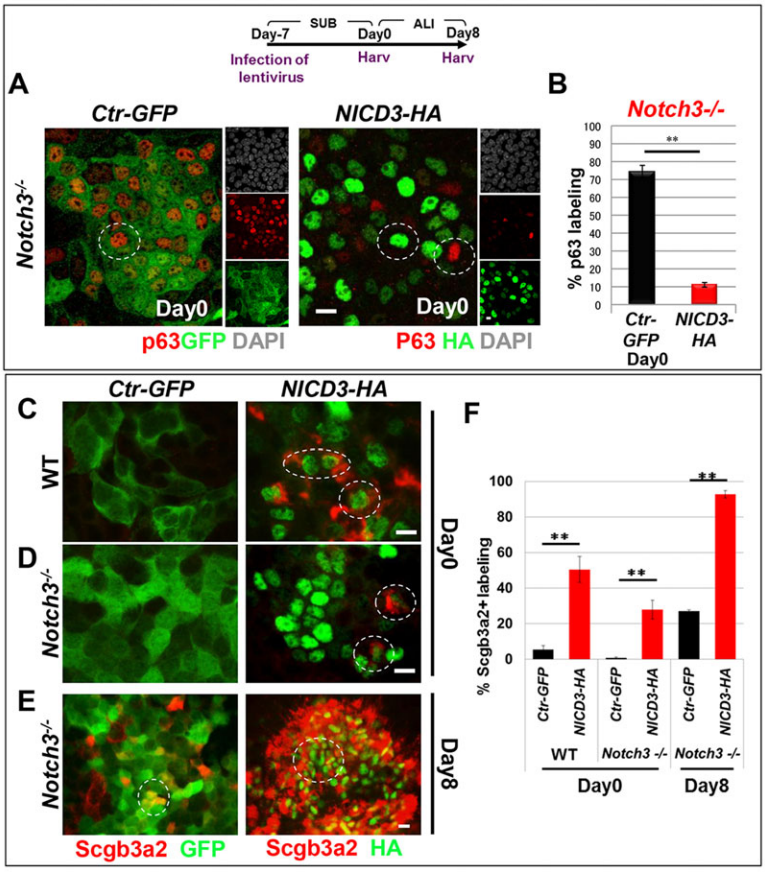

Fig. 4. Constitutive activation of Notch3 restricts the number and precociously differentiated p63+ cells. Diagram shows lentiviral gene transduction of control (Ctr-*GFP*) or *NICD3-HA* in airway epithelial progenitors from wild-type and *Notch3*$^{-/-}$ mice. (A) Immunofluorescence/confocal analysis at day 0 showing major reduction in the p63+ cell population in *Notch3*$^{-/-}$ transduced with NICD3 (DAPI, GFP, HA show transduction efficiency): no co-expression of p63 and HA (circled areas indicate distinct nuclear signals) in contrast to the extensive double labeling in day 0 Ctr-GFP cultures (circled area indicates cytoplasmic GFP and nuclear p63). (C,D) NICD3 transduction induces precocious Scgb3a2 expression and the secretory cell phenotype in *Notch3*$^{-/-}$ and wild-type cultures at day 0. (E) By ALI day 8, *Notch3*$^{-/-}$ cultures transduced with NICD3 showed extensive areas of Scgb3a2+HA+ double labeling, suggesting prominent induction of the secretory phenotype (circled areas) compared with *Ctr-GFP* cultures. (B,F) Morphometric analysis of the percentage of p63 (B) or Scgb3a2 (F) cells double-labeled with HA (*NICD3-HA* lentivirus) or GFP (*Ctr-GFP* lentivirus) in ALI cultures from *Notch3*$^{-/-}$ or wild-type airway progenitors. Data are mean±s.e.m. of the percentage labeling in six fields at day 0 or day 8, *n*=3 per group; $^{**}P<0.01$, Student's *t*-test. Scale bars: 8 µm.

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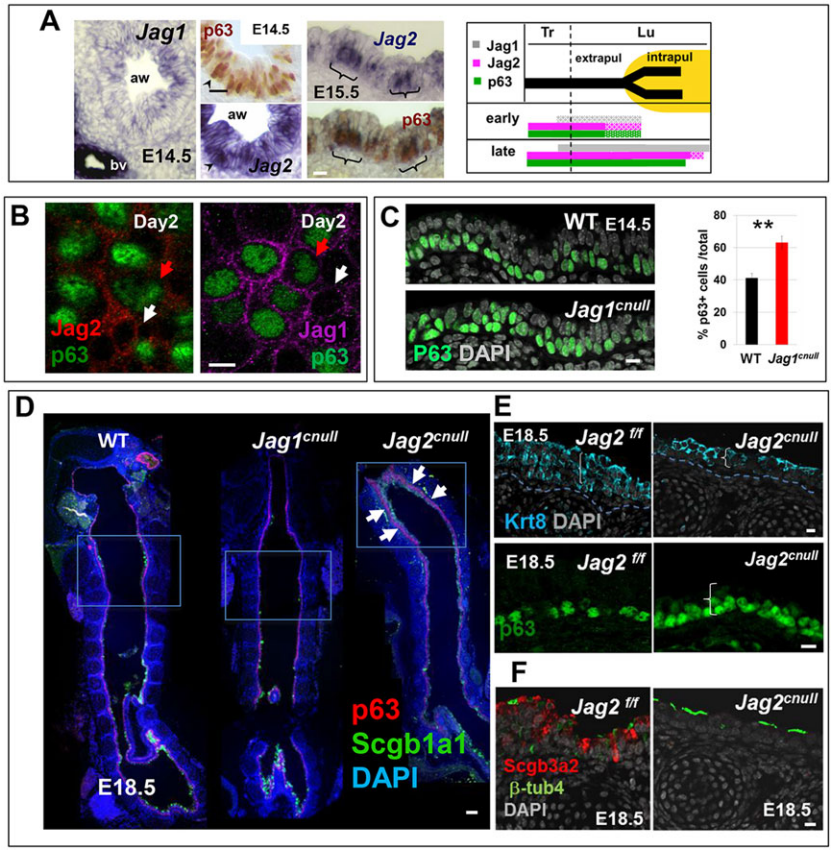

Fig. 5. Jag1 and Jag2 have partially overlapping functions in restricting basal cell expansion. (A) Expression pattern of *Jag1* and *Jag2* by *in situ* hybridization and p63 by immunohistochemistry in developing airways. Stronger signals for *Jag2* compared with *Jag1* in E14.5-E15.5 proximal airways initially in all epithelial cells and then overlapping with p63 at E15.5 (double *in situ* hybridization/immunohistochemistry; aw, airway; bv, blood vessels). Diagram summarizes temporal-spatial patterns from the panels above and data not shown. Proximal-distal domains of expression in trachea (Tr), extrapulmonary and intrapulmonary proximal airways of the lung (Lu). Solid and dotted bars represent strong and weak signals, respectively, as revealed by *in situ* hybridization or immunohistochemistry. (B) Jag1 and Jag2 are expressed in adult p63+ airway progenitors in culture (ALI day 2). (C) p63 immunofluorescence and morphometric analysis (% labeling) showing expansion of basal cells in the E14.5 trachea and extrapulmonary airways of $Jag1^{cnull}$ mice. (D) Double p63-Scgb1a1 immunofluorescence in E18.5 wild-type $Jag1^{cnull}$ and $Jag2^{cnull}$ tracheas showing local (squares) expansion of p63+ cells in $Jag2^{cnull}$ mice (arrows) not present in wild type or $Jag1^{cnull}$. (E) In E18.5 $Jag2^{cnull}$ airways, expansion of p63+ cells is accompanied by reduced thickness of the Krt8-labeled cell layer, and (F) suppression of the secretory (Scgb3a2+) phenotype with expansion of multiciliated cell population (β-tubulin+). Data are mean±s.e.m. of the percentage of p63+ cells/total cells, n=3 per group; $^{**}P$<0.01, Student's t-test. Scale bars: 10 µm in A-C,E,F; 100 µm in D.

analysis of adult airway progenitors in culture showed that both Jag1 and Jag2 are strongly expressed in the cell surface of p63+ cells (Fig. 5B). We could not detect expression of Dll ligands other than in neuroendocrine cells using *in situ* hybridization (Guha et al., 2012).

We asked whether these Jag ligands contributed equally to mediate the Notch effects in restricting the pool of the p63+ cell population. Thus, first we investigated the effect of inactivating Jag1 independently in the airway epithelium using mice carrying Jag1 floxed alleles and the ShhCre line. Our approach differed from that of a previously reported *Jag1$^{f/f}$; Sftpc-rtTA;Tet-O-Cre* transgenic mice (Zhang et al., 2013); here, ShhCre induces recombination much earlier, at the onset of lung development (Harris et al., 2006). Analysis of $Jag1^{cnull}$ mutants confirmed the imbalance between secretory and multiciliated cells previously seen in other Notch-deficient models (Tsao et al., 2009, 2011; Morimoto et al., 2010; Zhang et al., 2013). Moreover, we found increased number of p63+ cells in trachea and extrapulmonary airways already at E14.5, a phenotype not reported in the *Jag1$^{f/f}$; Sftpc-rtTA;Tet-O-Cre* mice (Fig. 5C). Interestingly, the expansion in p63+ cells was no longer seen in E18.5 $Jag1^{cnull}$ but was clearly present at E18.5 in $Jag2^{cnull}$ mice (*Jag2$^{f/f}$*; *ShhCre*). This expansion occurred predominantly in the upper trachea (Fig. 5D) and at the expense of luminal cells, as suggested by the reduced thickness of the Krt8-labeled cell layer in $Jag2^{cnull}$ airways (Fig. 5E). $Jag2^{cnull}$ mice also showed the imbalance between secretory and multiciliated cells seen in $Jag1^{cnull}$ (Fig. 5F). Overall, these data suggested that Jag2 contributes more prominently than Jag1 to restrict the expansion of p63+ cells mediated by Notch signaling *in vivo*. During development, this function appears to be regulated in a temporally and regionally distinct fashion, with Jag1 being less important than Jag2 at later stages and Jag2 being more relevant in upper trachea.

DISCUSSION

Here, we provide evidence of a mechanism mediated by selective activation of endogenous Notch3 that regulates the pool of progenitor cells available for differentiation in the murine tracheal and proximal airways (Fig. 6). We have identified Notch3 as the receptor selectively activated to control the balance of basal and luminal progenitors during differentiation. This mechanism is dependent on the availability of Jag1 and Jag2, and is key to generating a population of Notch3-expressing parabasal cells that later activates Notch1 and Notch2 for secretory-multiciliated cell fate selection in the pseudostratified epithelium.

Our results are consistent with the idea that proximal airway progenitors modulate the activity of specific Notch receptors individually or in combination, presumably in response to developmental or environmental stimuli to alter the abundance of specific cellular phenotypes. For example, selective expansion of the pool of basal cells can be achieved by downregulation of endogenous Notch3 (Kent et al., 2011). Once expanded, these cells can be converted into parabasal cells by local activation of Notch3 signaling and subsequently differentiate. Increase in the secretory cell pool requires activation of endogenous Notch signaling. Although forced expression of Notch3 induces secretory differentiation, evidence from stepwise inactivation of Notch receptors in the airway epithelium reveals that Notch2 and Notch1, but not Notch3, are key in promoting club cell differentiation (Morimoto et al., 2012). Basal and multiciliated cells can be collectively expanded by inhibiting all Notch receptors, as in our $Rbpj^{cnull}$. This could be relevant in establishing the local balance of cellular phenotypes in

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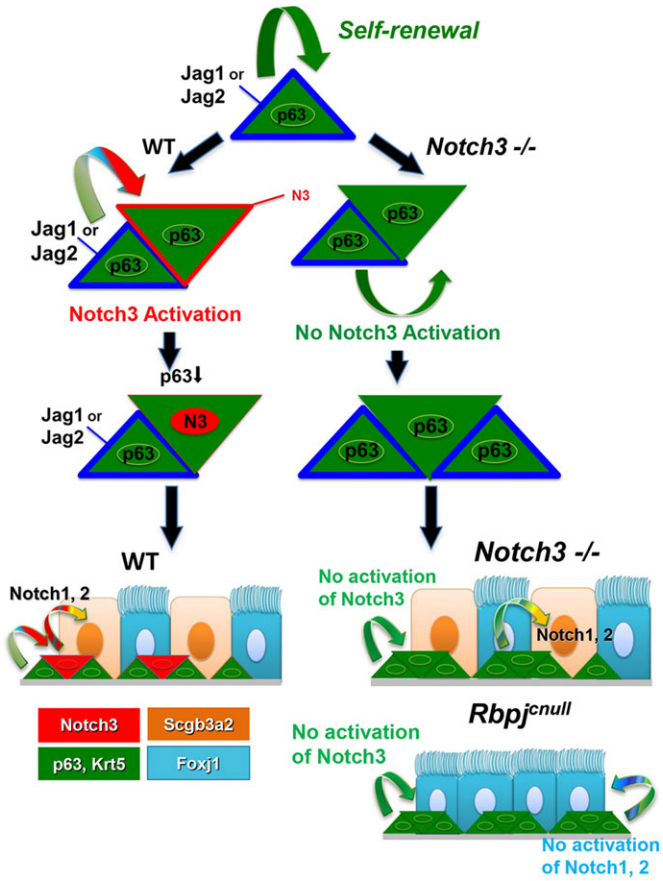

Fig. 6. Summary of results and proposed model of Notch regulation of airway epithelial progenitor cell expansion and differentiation. Under homeostatic conditions, basal cells express Jag ligands but do not activate Notch signaling for self-renewal and continue to expand. Once the pool of p63+ basal cells is properly expanded, Notch3 becomes selectively expressed in the cells occupying a parabasal position and Notch3 signaling is activated by Jag2 and/or Jag1 from the adjacent basal cells. This pool of Notch3+ progenitor cells remains undifferentiated until later, when signaling by Notch1 and Notch2 is turned on for secretory-multiciliated cell differentiation. The balance of basal and parabasal cells is properly achieved by modulating endogenous levels of Notch3 activation selectively, but differentiation is not dependent on Notch3. The basal and multiciliated cell pools can be collectively expanded by inhibiting signaling by all Notch receptors (as in the *Rbpj*^{cnull} mice). Diagrams summarize these conclusions in light of the Notch genetic models.

the proximal epithelium, as both basal and multiciliated cells are regionally enriched in proximal airways. Expansion of multiciliated cells can be achieved by disruption of the mechanism of cell fate selection mediated by Notch2-Notch1, but with little Notch 3 contribution.

NICD3 transduction greatly reduced the p63+ cell pool in *Notch3*$^{-/-}$ epithelial cultures, suggesting that it rescued the basal cell expansion phenotype of these mutants. Nevertheless, high thresholds of NICD3 activation also induced precocious differentiation of these progenitors into secretory Scgb3a2-expressing cells. Thus, timing and levels of Notch activation may bypass the effect of selective Notch activation.

The expression pattern of Jag ligands and the expansion of p63+ cells *Jag1*^{cnull} and *Jag2*^{cnull} airways strongly suggested that these ligands activate endogenous Notch3 in the basal cell compartment. How this mechanism initiates is unclear. We propose that p63+ airway progenitors are largely Jag-expressing cells and, in the absence of Notch signaling, have uninhibited proliferation. As basal cells expand, extensive cell-cell communication triggers a bias that leads to the appearance of Notch3-expressing cells and activation of Notch signaling. This ultimately leads to suppression of p63 and acquisition of a Notch3-expressing parabasal cell phenotype primed for further differentiation. p63-negative–Notch3-positive cells maintain Jag expression in p63-positive basal cells. This balance is maintained until activation of other Notch receptors is initiated to trigger differentiation.

Our data suggest that Notch3 deficiency appears to alter the overall architecture of the airway epithelium by expanding the basal cell layer. This raises the issue of whether under this or similar abnormal conditions the pseudostratified epithelium converts into a truly stratified epithelium. Stratification is influenced by multiple mechanisms, including changes in polarity, mitotic spindle orientation or integrin β1 levels, among others (Tang et al., 2011; Chen and Krasnow, 2012).

Interestingly, expansion of the basal cell compartment (basal cell hyperplasia) in association with metaplastic changes in the airway epithelium are among the hallmark airway abnormalities of smokers and individuals with chronic obstructive pulmonary disease (COPD) (Demoly et al., 1994; Khuri et al., 2001). NOTCH2 and NOTCH3 have been reported by qPCR analysis as the most abundant Notch receptors in airways of healthy humans, and NOTCH3 was the receptor most downregulated in smokers and individuals with COPD (Tilley and Harvey, 2009). These intriguing observations led us to examine whether we could correlate our observations in animal studies with pathological changes in human airways from individuals with COPD. Indeed, analysis of lung sections (main bronchus) from adult non-smoker healthy transplant donor showed strong expression of NOTCH3 in parabasal cells immediately adjacent to the P63-expressing layer of basal cells. As observed in the adult mouse lung, expression of these markers was largely segregated but overlapping in some cells (supplementary material Fig. S2A). Remarkably, analysis of COPD lungs showed NOTCH3 expression was markedly reduced in areas associated with increased density of P63-labelled cells and squamous metaplasia (supplementary material Fig. S2B).

Mechanisms controlling the balance of p63 and Notch3-expressing cell populations could presumably be relevant in the pathogenesis of human chronic respiratory conditions. Our results are consistent with those reported in the mammary gland and bladder in which the growth of p63+ cells is inhibited by Notch signaling (Bouras et al., 2008). Aberrant expansion of stem/progenitor cell populations contribute to tumorigenesis (Chiche et al., 2013; Lim et al., 2009). Thus, failure of mechanisms that prevent abnormal expansion of p63+ basal cells in the airway epithelium could also play a role in lung cancer.

MATERIALS AND METHODS

Culture of airway epithelial progenitor cells in air-liquid interface (ALI)

Primary cultures of mouse airway epithelial progenitors were performed as previously described (You et al., 2002; Mahoney et al., 2014). Briefly, mouse tracheal epithelial cells (mTECs) were isolated from adult (8- to 12-weeks old) wild-type, *Rbpj*$^{f/f}$ or *Notch3*$^{-/-}$ mice, cultured onto Transwell dishes (Corning) under submerged conditions in mTEC/plus media, 10% FCS, with retinoic acid (RA) until confluence (7 days), then under air-liquid interface (ALI) conditions (day 0 ALI) in differentiation media (mTEC/serum free, RA) up to 8 days (day 8 ALI). For Notch pharmacological inhibition, cells were cultured with DAPT (50 µM, Sigma) or DMSO (Sigma, D8418) media. For basal cell lineage tracing, adult *Krt5-CreERT2; Rosa26-tdtomato* mice were treated with tamoxifen (Tm) (150 mg/kg) 10 days prior to sacrifice. Airway progenitors from tracheas were cultured as

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above and analyzed. All experiments were approved by Columbia University Institutional Animal Care and Use Committee.

Lentiviral-mediated gene transduction

Lentiviral-mediated gene transduction was performed in cultured airway epithelial progenitors from mutant mice using *pHAGE-EF1α-Cre-w* lentivirus (a gift from Dr Darrell N. Kotton, Boston University, MA, USA). This construct has the nuclear localization sequence (NLS) at the Cre N terminus. *pLenti-CMV-Hyg* lentivirus carrying an HA-tagged NICD3 or a control EGFP under the CMV promoter were generated as previously described (Cui et al., 2013). Lentivirus was concentrated to 1×10^9 PFU/ml and transduction was performed at the time of plating (~30 MOI) in mTEC/plus+RA media+Rho kinase inhibitor (Y-27632 5 µM, Sigma). Transduction efficiency was 30-70%. Results were normalized to HA, GFP or Cre expression to take into account differences in transduction efficiency.

Immunofluorescence and immunohistochemistry

Cells were fixed (in 4% paraformaldehyde for 10 min at room temperature) and stored (at 4°C in PBS). Samples were incubated with primary antibodies (2 h), secondary antibody conjugated with Alexa488, 567 or 647 (1:300) with NucBlue Fixed Cell ReadyProbes Reagent (DAPI) (Life Technology) for 1 h. After washing, samples were mounted with ProLong Gold antifade reagent (Invitrogen, P36962) for immunofluorescent analysis as described previously (Tsao et al., 2009, 2011; Mahoney et al., 2014). Immunohistochemistry was carried out in 5 µm paraffin wax-embedded sections from wild-type, $Rbpj^{cnull}$, $Notch3^{-/-}$, $Jag1^{cnull}$ or $Jag2^{cnull}$ mice at various developmental stages using ABC or MOM kit (Vector Laboratories) according to the manufacturer's protocol. When necessary, antigen retrieval was performed using Unmasking Solution (Vector Laboratories #H-3300) and microwave or Tris-EDTA buffer (1 mM EDTA/Tris-HCl at pH8.3) for 15 min at 110°C in a pressure cooker. Sections from human main bronchus from healthy donors or unidentified individuals with chronic pulmonary obstructive disease during lung transplantation were also stained. Antibodies used were: anti-Scgb3a2 (a gift from Dr S. Kimura, NIH, Bethesda, MD, USA; 1:1000), anti-Krt5 (Covance, #PRB-160P, 1:500), anti-Foxj1 (eBioscience, #2A, 1:100), anti- acetylated α-tubulin (Abcam, ab125356, 1:1000), anti-acetylated α-tubulin (Sigma, T7451, 1:2000), anti-acetylated β-tubulin IV (Abcam, ab11315, 1:500), anti-p63 (Santa Cruz, #4E4, 1:100), anti-p63 (H-137) (Santa Cruz, sc-8343, 1:100), anti-deltaNp63 goat (Santa Cruz, sc-8609, 1:50), anti-GFP chicken pAb (Abcam, ab13970, 1:1000), anti-Krt8 (Abcam, ab107115, 1:2000), anti-Scgb1a1 goat (Santa Cruz, sc-9772, 1:500), anti-Ki67 (B&D, #550609, 1:100), anti-Hes1 (Cell Signaling, #11988, 1:100), anti-Notch3 Rb (Cell Signaling, #5276, 1:50), anti-cleaved-Notch1 (Cell Signaling, #2421, 1:100), anti-Notch2 (Cell Signaling #4530, 1:100), anti-Rbpj (Cell Signaling, #5313, 1:100), Jag1 Rb mAb (Cell Signaling, #2155, 1:50), Jag2 Rb mAb (Cell Signaling, #2210, 1:50), anti-HA (Cell Signaling, #2367, 1:100 and #3724, 1:100), anti-Cre (Millipore, MAB3120, 1:100) and anti-Cre rabbit pAb (Millipore, #69050, 1:200). F-actin and nucleus were visualized by Alexa Fluor 647 phalloidin (Life Tech, A22287) and NucBlue Fixed Cell ReadyProbes Reagent (Life Tech, R37606), respectively. Images were acquired using a Nikon Labophot 2 microscope equipped with a Nikon Digital Sight DS-Ri1 charge-coupled device camera or on a Zeiss LSM700 confocal laser scanning microscope.

In situ hybridization

In situ hybridization was performed in frozen sections (5-7 µm) using digoxigenin-UTP-labeled *Jag1* or *Jag2* riboprobes, as previously described (Tsao et al., 2008, 2009, 2011). Sections were briefly postfixed and immunohistochemistry was performed on the same sections as for colocalization studies.

Morphometric analysis

To determine the relative number of specific cell populations in airway epithelial cultures, six non-overlapping random fields per condition were analyzed (20× magnification). For each field, we counted the number of cells immunostained with specific antibodies and normalized by the total number of epithelial cells (DAPI-labelled nuclei). A minimum of three replicates (animal or experimental condition) was used for analysis. Results were represented in graphs by mean±s.e.m. Statistical analysis was performed (Student's *t*-test), differences were considered significant if $P<0.05$. Morphometric analysis of immunohistochemistry in E14.5-E18.5 lungs was performed similarly. The percentage of epithelial cells labeled with each antibody was determined by counting cells in equivalent regions (within the anterior-posterior axis) of the trachea from control and mutants animals at 63× magnification. For each maker, ten fields were analyzed in three animals per group. Counting was facilitated by the identification of the basal lamina with anti-laminin and DAPI. To estimate changes in pseudostratification in the airway epithelium due to Notch deficiency we stained adult tracheal sections of wild-type or $Notch3^{-/-}$ mice with DAPI and determined the number of labeled nuclei per surface of basal lamina (pseudostratification index). To minimize variability, only regions associated with cartilage rings and at roughly equivalent levels along the anterior-proximal axis of the trachea were analyzed (based on distance from the carina; three or four regions/trachea, $n=3$ per group). Data are represented as mean±s.e.m.; Student's *t*-test, differences are significant if $P<0.05$.

Quantitative real-time PCR

Quantitative real-time PCR was performed as reported (Mahoney et al., 2014) using total RNA (RNeasy Qiagen,) reverse-transcribed (Superscript III, Invitrogen), ABI 7000 (Applied Biosystems, CA) and Taqman probes for Notch1, Notch2, Notch3, Hes1 and β-actin (Assays-on-Demand, Applied Biosystems). The relative concentration of the RNA for each gene to β-actin mRNA was determined using the equation 2−DCT, where DCT=(CT mRNA−CT beta-actin R).

Western blot analysis

Western blot analysis was performed as reported previously (Tsao et al., 2009, 2011; Li et al., 2009). Membranes were blocked with 1% skim milk (1 h, room temperature) and probed with anti-Notch1 ICD Rb mAb (Cell Signaling #4380, 1:100) and Notch3 ICD goat pAb (Santa Cruz, M-134, 1:200). Antigen-antibody complexes were identified with HRP-conjugated secondary antibodies. Enhanced chemiluminescence was detected using LAS 4000 (GE Health Care).

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.M. designed and performed the experiments, analyzed data and wrote the manuscript. J.E.M. performed ALI airway epithelial culture experiments. M.R.S. managed transgenic lines, analyzed *in vivo* data and edited the manuscript. J.R.P. provided $Notch3^{-/-}$ mice and helped with experimental design. A.D.S. and X.V. helped with western blot analysis and discussions. D.B.H. and J.S. helped in the lineage-tracing experiments *in vivo*. H.Z. generated lentivirus for Notch3 gain-of-function and rescue experiments. W.V.C. led the investigation, devised the project, and wrote and edited the manuscript.

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Supplementary material

Supplementary material available online at
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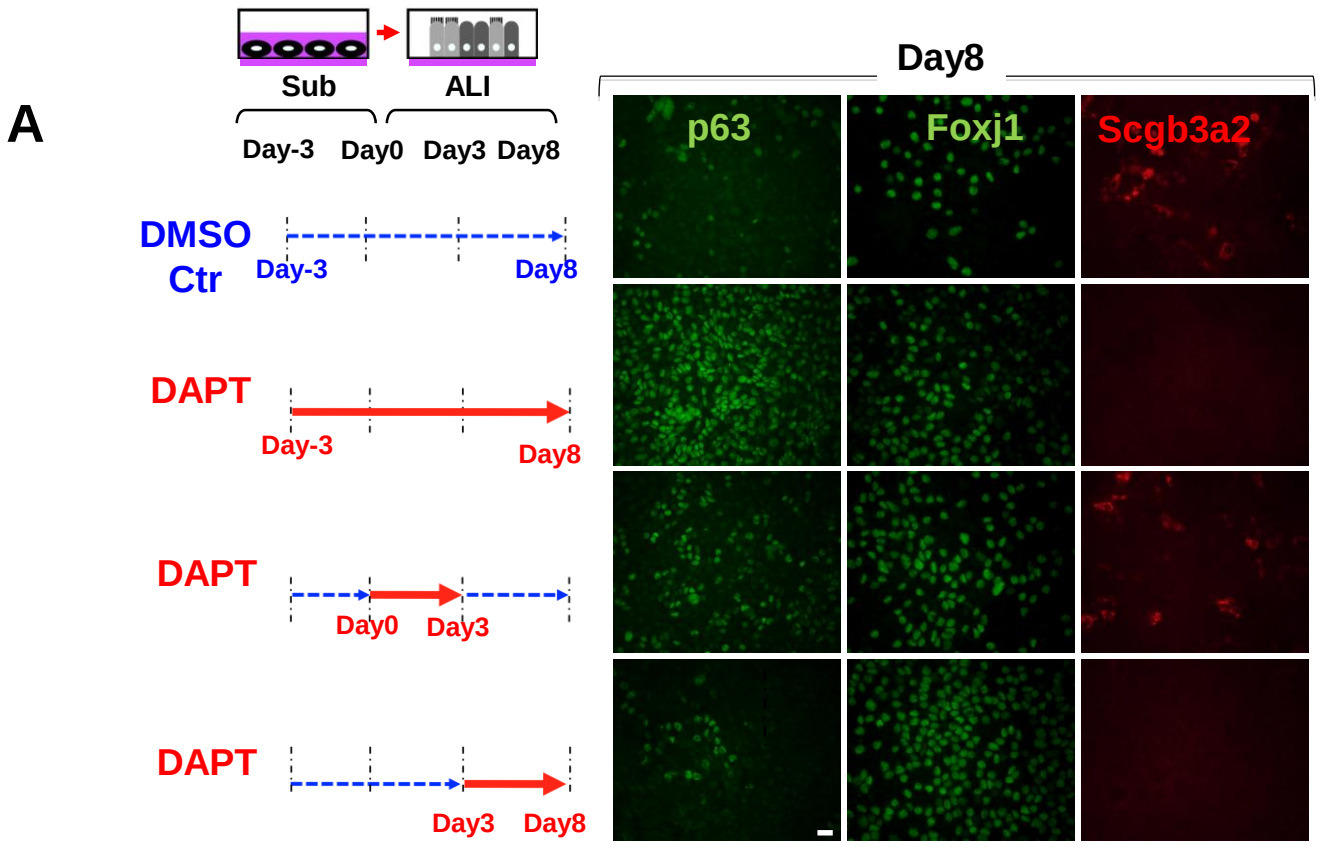
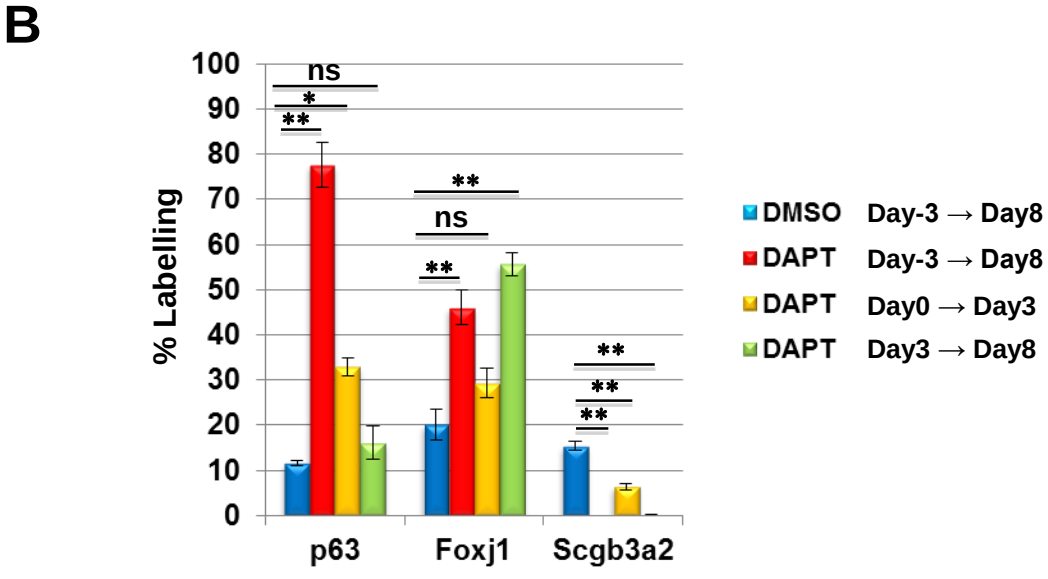
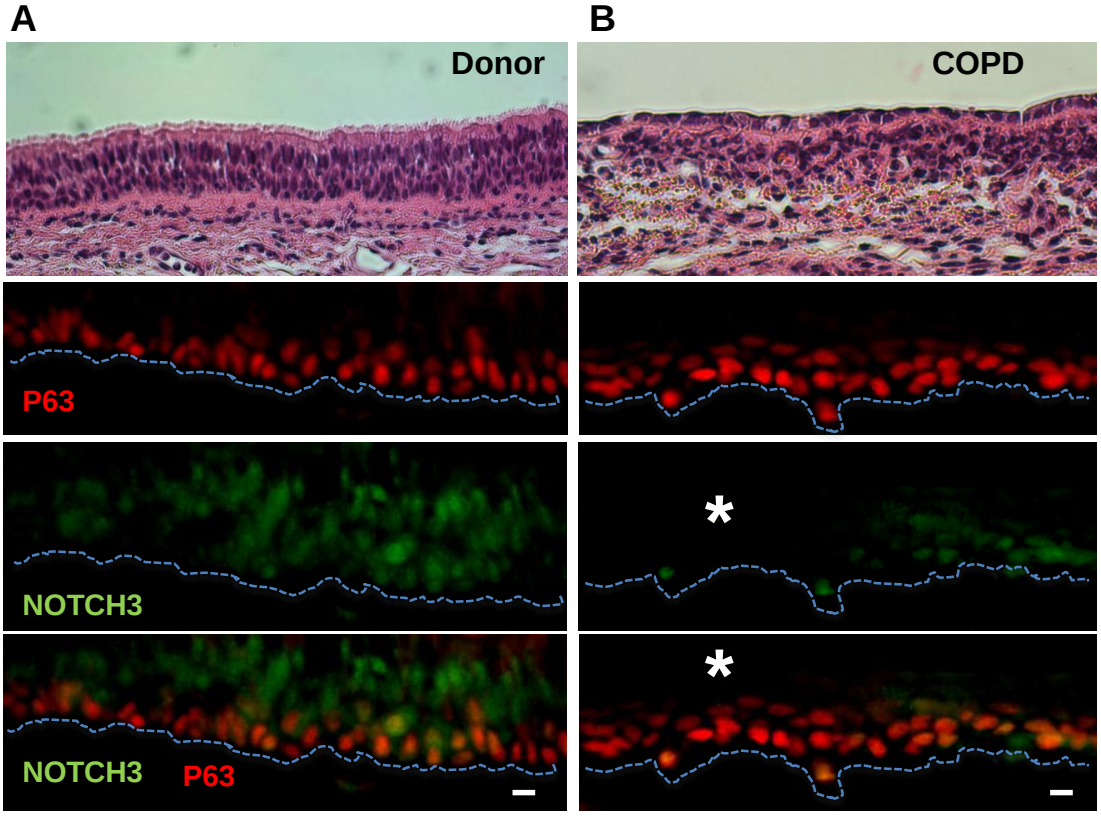




Supplemental Figure 1. Disrupting Notch signaling in cultured adult airway progenitors at different stages of expansion and differentiation results in distinct effects. (A) Airway epithelial progenitors cultured under submerged (Sub) and then air-liquid interface (ALI) conditions in DMSO (Ctr: control, blue) or DAPT (red) for different periods from 3 Days prior to ALI (Day-3) to 8 Days after ALI. All cultures analyzed at Day8 by immunofluorescence (p63, Foxj1, Scgb3a2). (B) Morphometric analysis of ALI Day8 cultures in each condition: relative number of cells expressing each marker per total cells (%) in Ctr or DAPT treated cells (n=3, each 6 fields +/- SEM). Asterisks, significance at $p< 0.05$* and $p< 0.01$** (Student's T test; ns: no significance). Scale bar in A, 20 μm



Supplemental Figure 2. NOTCH3 is expressed in parabasal cells of human airways and is inhibited in COPD. HE and immunofluorescence staining of adult human airway (main bronchus) from (A) normal donor, showing P63 and NOTCH3 expression in basal and parabasal cells, respectively (B) COPD patient showing areas of unbalance in these cell populations with marked decrease in the NOTCH3-expressing parabasal cells relative to the P63+ basal cells (asterisks). Scale bar in A-B, 10 μm.