Published in final edited form as:
Cell Stem Cell. 2015 February 5; 16(2): 184–197. doi:10.1016/j.stem.2015.01.002.

Injury induces direct lineage segregation of functionally distinct airway basal stem/progenitor cell subpopulations

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Summary

Following injury, stem cells restore normal tissue architecture by producing the proper number and proportions of differentiated cells. Current models of airway epithelial regeneration propose that distinct cytokeratin 8-expressing progenitor cells, arising from $p63^+$ basal stem cells, subsequently differentiate into secretory and ciliated cell lineages. We now show that immediately following injury, discrete subpopulations of $p63^+$ airway basal stem/progenitor cells themselves express Notch pathway components associated with either secretory or ciliated cell fate commitment. One basal cell population displays intracellular Notch2 activation and directly generates secretory cells; the other expresses c-myb and directly yields ciliated cells. Furthermore, disrupting Notch ligand activity within the basal cell population at large disrupts the normal pattern of lineage segregation. These non-cell autonomous effects demonstrate that effective airway epithelial regeneration requires intercellular communication within the broader basal stem/progenitor cell population. These findings have broad implications for understanding epithelial regeneration and stem cell heterogeneity.

Introduction

The murine tracheal epithelium and much of the human airway epithelium is composed of two cellular compartments: the basal cell compartment, where basal stem/progenitor cells reside, and the luminal cell compartment, which contains mature secretory cells and ciliated cells (Rock and Hogan, 2011; Rock et al., 2010). Murine lineage tracing experiments have demonstrated that basal cells, as a population, are stem cells since they self-renew and differentiate into ciliated and secretory luminal cells over an extended period of time (Rock

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Author contribution: A.P-S. and B.M.L. equally designed and performed experiments and wrote the manuscript. P.R.T performed the chlorine gas exposure experiments. J.V. optimized some of the staining protocols. B.S., H.M. and R.Z. performed the *ex vivo* experiments and provided some of the *in vivo* samples. J.R. suggested and co-designed the study and co-wrote the manuscript.

et al., 2009; Hogan et al., 2014). However, prior reports also present evidence for heterogeneity within the airway basal cell compartment with regard to both basal cell proliferative and differentiation capacity (Ghosh et al., 2011a, 2011b, 2013a, 2013b; Hong et al., 2004).

In order to further investigate the heterogeneity of basal stem/progenitor cells, we sought to define the expression patterns of early markers of differentiation in the airway epithelium. Current models of the airway epithelial cell lineage hierarchy suggest that basal stem cells, characterized by p63, NGFR and Podoplanin (Pdpn) expression, give rise to uncommitted suprabasal $CK8^+$ $p63^-$ progenitor cells that subsequently segregate into ciliated and secretory cells (Rock et al., 2011, Pan et al., 2014). To our surprise, we have identified mutually exclusive populations of basal cells that express low levels of c-myb and N2ICD (the active Notch2 intracellular domain). After injury, the numbers of these $c\text{-}myb^+$ and $N2ICD^+$ basal cells increases dramatically and very rapidly. As epithelial regeneration ensues, we show that basal cells that express N2ICD will produce mature secretory cells, while the other subset of basal cells that express c-myb will directly give rise to ciliated cells. Thus, basal cells can directly produce either ciliated or secretory cell progeny.

In aggregate, our findings show that basal cells are comprised of a heterogeneous population of stem/progenitor cells. Whether these subpopulations are fixed or occur stochastically and whether they exist within an explicit lineage hierarchy of stem and progenitor cells with different potencies remains to be seen. In general, our results point to the notion that seemingly homogeneous stem/progenitor cell populations in many epithelia are likely much more complex than previously thought.

Results

Expression of Cell Fate Associated Markers in the Airway Basal Cell Compartment

Lineage commitment to either secretory or ciliated cell fates following airway injury is currently thought to involve Notch signaling, and to occur at an early stage of epithelial regeneration in a set of $CK8^+$ partially differentiated luminal progenitor cells that are derived from basal stem cells (Rock and Hogan, 2011; Rock et al., 2011). To our surprise, in the homeostatic airway epithelium, when we utilized tyramide signal amplification protocols for the immunohistochemical detection of Notch signaling pathway components that had previously been associated with secretory or ciliated cell fate choices (Morimoto *et al.* 2010; Morimoto *et al.* 2012), we found expression of these Notch-related proteins in basal cells. This suggested that lineage commitment might be occurring within the basal cell population itself. Specifically, we observed cells expressing basal cell markers (p63, CK5, and Pdpn) and c-myb, a transcription factor acting downstream of Notch signaling that has been demonstrated to have a conserved role in multiciliogenesis (Tan et al., 2013) and which is required for ciliated differentiation (Pan et al., 2014) (Figure 1A-1C). Indeed, $7.4 \pm 1.2\%$ of $p63^+$ basal cells co-expressed c-myb (Figure 1G). Similarly, cells expressing basal cell markers also co-expressed the activated intracellular domain of the Notch2 receptor (N2ICD), an essential transcription factor for secretory cell fate specification in the embryonic lung (Morimoto et al., 2012) (Figure 1D-1F). In this case, $5.0 \pm 0.4\%$ of basal cells expressing p63 at steady state also expressed N2ICD (Figure 1H). We did not observe

any basal cell that expressed both c-myb and N2ICD. Surprisingly, most of the cells that co-expressed c-myb or N2ICD and basal cell markers, did not express the differentiation marker CK8 (Figure 1B, 1C, and 1F). We hypothesized that the presence of these Notch signaling components in homeostatic basal cells might reflect a process in which some basal stem/progenitor cells are directly undergoing differentiation into either the secretory or ciliated cell lineages. This hypothesis was further supported by the presence of rare basal cells that expressed N2ICD or c-myb, as well as the differentiation marker CK8 (Figure 1E, yellow arrow). Since there is a very low rate of turnover in the normal homeostatic airway epithelium (Kauffman, 1980; Rock et al., 2009), we sought to test our hypothesis concerning basal cell lineage commitment in an injury model in which rapid cell proliferation and differentiation is required for airway epithelial regeneration.

Segregation of Basal Stem/Progenitor Cells into Two Functionally Distinct Populations of Cells Following Injury

We used the well-characterized sulfur dioxide model (SO_2) of injury to accelerate cell turnover and differentiation in the airway epithelium. SO_2 injury causes the loss of differentiated luminal cells, prompting the remaining basal stem/progenitor cells to replicate and then differentiate (Figure 2A). As early as 24 hpi, we observed the mutually exclusive expression of N2ICD and c-myb in a large fraction (19 ± 2.5% and 34 ± 4.9% respectively) of p63-expressing basal cells (Figure 2B, 2F and 2G). N2ICD and c-myb expression continued to be present in 27.9 ± 0.9% and 35.5 ± 1.9% of basal cells respectively, at 48 hpi (Figure 2C, 2F, 2G) and their expression was again mutually exclusive (Figure 2H). We confirmed this pattern of expression using CK5 to identify basal stem/progenitor cells (Figure S1A and S1B). We next analyzed even earlier time points to determine when these two markers were first induced following injury. Basal cells expressing c-myb or N2ICD were detected as early as six hours after SO_2 exposure (Figure 2D-2G and Figure S1C and S1D). Indeed, 29.9 ± 5.6% and 36 ± 8% of the $p63^+$ basal cells were expressing c-myb or N2ICD, respectively (Figure 2F and 2G). Of note, this time point was so early that occasional suprabasal cells remained prior to their sloughing. At 12 hpi, all suprabasal cells were sloughed, and a single layer of basal cells was observed, in which 43 ± 6% and 34.8 ± 8.7% of $p63^+$ cells expressed N2ICD and c-myb respectively (Figure 2D-2G). The percentage of $N2ICD^+$ basal cells decreased to 23.1 ± 4% of $p63^+$ cells in the next six hours while that of $c\text{-}myb^+$ cells increased to represent 37.9 ± 5.1 % of the basal cell compartment (Figure 2D-2G and Figure S1C and S1D). These results demonstrate an exceptionally early and dynamic segregation of basal stem/progenitor cells into two new subpopulations in response to injury.

Following SO_2 exposure, the remaining basal cells are known to undergo proliferation to restore the airway epithelium (Rawlins et al., 2007; Rock et al., 2011). Using BrdU incorporation and Ki67 staining, we first detected proliferating basal cells at 12 hpi (Figure S1E). Proliferation reached a maximum at 18 hpi at which time approximately 90% of basal cells were proliferating (Figure S1E). This rate of proliferation was maintained at 24 hpi and started to gradually decrease at 36 hpi as a suprabasal layer of cells started forming (data not shown). Thus, the segregation of basal stem/progenitor cells into two new subpopulations occurs prior to the onset of their replication. Additionally, both $c\text{-}myb^+$ $p63^+$ and $N2ICD^+$

p63$^+$ populations of basal cells undergo proliferative events following their segregation, since 90% of p63$^+$ cells are positive for BrdU and Ki67. Although c-myb is known to suppress apoptosis in other systems (Pattabiraman et al., 2013), we do not see evidence of any apoptosis even in those cells that are not expressing c-myb (data not shown), suggesting that c-myb is functioning as a differentiation factor rather than a factor that prevents cell death.

To confirm the segregation of basal stem/progenitor cells in a second injury model, we exposed C57BL6 mice to chlorine gas (Musah et al. 2012). This injury causes the loss of greater numbers of basal cells in addition to a complete loss of ciliated and secretory cells. Remarkably, we observed that within the pool of surviving p63$^+$ cells, a large fraction of cells strongly expressed N2ICD or c-myb as early as 1 hpi (Figure S2A and S2B). Of note, regardless of the injury model, post-injury N2ICD$^+$ and c-myb$^+$ basal cells expressed higher levels of Notch signaling components than their homeostatic counterparts.

To strengthen the association of c-myb and N2ICD expression with specific functional cell fate specification, we characterized their expression patterns at 72 hpi and 5 dpi, as these time points are associated with the appearance of markers of fully specified ciliated (FoxJ1) and secretory (Scgb1a1) cells respectively (Figure 3A-3C). Analysis at 72 hpi revealed that 98.5 ± 0.6% of cells that expressed the ciliated transcription factor FoxJ1, also co-expressed c-myb (Figure 3B and 3D). Similarly, 92.5 ± 2.5% of cells that expressed the secretory cell marker Scgb1a1 at 5 dpi co-expressed N2ICD (Figure 3C and 3E). N2ICD is also detected in the earliest secretory cell progenitors at 72 hpi, when secretory cell differentiation is first evidenced by SSEA1 expression (Figure 3F). Once again, c-myb and N2ICD expression was mutually exclusive (Figure 3G). By 72 hpi, the percentages of basally located p63$^+$ cells that express c-myb or N2ICD are reduced to their homeostatic numbers. Indeed, the abundance of c-myb and N2ICD expression was restricted to the suprabasal layer containing committed ciliated and secretory cell progenitors respectively (Figure 3G). We confirmed that N2ICD$^+$ cells were distinct from c-myb and FoxJ1 expressing cells (Figure 3G and 3H). Conversely, c-myb was not expressed in Scgb1a1$^+$ cells (Figure 3I). In aggregate, these results further support the conclusion that basal stem/progenitor cells segregate into lineage-specific N2ICD$^+$ and c-myb$^+$ subpopulations that will then, in turn, give rise to secretory and ciliated cell progenitor cells which will subsequently differentiate into mature secretory cells and ciliated cells respectively.

Airway regeneration involves a continuum of differentiating cell types

In order to even more deeply scrutinize the time course of differentiation during airway regeneration, we assessed the dynamic co-expression of CK8 (previously associated with suprabasal progenitor cells, (Rock et al., 2011)) and the basal cell marker CK5. CK5 is expressed, as expected, in the basal cell monolayer at baseline and at 24 hpi (Figure S3A). In contrast, CK5 and CK8 are co-expressed in both the basal and suprabasal layers at 48-72 hpi (Figure S3A). By the time we first detect markers of terminal differentiation (FoxJ1 at approximately 60 hpi and Scgb1a1 at 5 dpi), these markers are co-expressed with CK8 as expected, and these committed cells were largely seen in the suprabasal layer (Figure S3B). By 5 dpi, CK5 and CK8 are again fully segregated and are found in their previously

established basal and suprabasal homeostatic expression patterns (Figure S3A). Thus, we demonstrate that during the process of regeneration, there is a continuum of differentiation in which markers previously associated exclusively with suprabasal or basal cell layers, are transiently co-expressed. Importantly, the earlier noted segregation of N2ICD and c-myb expression first seen in the monolayer of $p63^+$ basal cells (Figure 2B, 2D-2G, S1A and S1C-S1D) occurs well before any expression of CK8 (Figure S3C). In aggregate, these data suggest that c-myb and N2ICD are indeed the earliest determinants of cell fate and that they first appear within the basal stem/progenitor cell population itself prior to the expression of CK8.

To further clarify the nature of the differentiation process, we next focused on the precise time points when c-myb^+ $p63^+$ and $N2ICD^+$ $p63^+$ cells are not yet expressing more markers of differentiating ciliated and secretory cells. As noted above, CK8 first appears at 48 hours, and at this time, we see that N2ICD and c-myb are expressed in this new population of $CK8^+$ cells (Figure S3D). In fact, as further evidence for a continuum of differentiation, at 48 hpi, some cells are co-expressing p63 and CK8 together with N2ICD or c-myb (Figure S3E and S3F). Indeed, some weakly-positive p63-expressing cells are seen in a suprabasal location at 48 hpi, presumably as they are losing their basal cell identity and differentiating into suprabasal early progenitors (Figure S3E). In contrast to the decrement in levels of p63 that is seen as this continuum of differentiation ensues, the levels of expression of N2ICD and c-myb progressively increase as differentiation proceeds (Figure S3D, S3E and S3F). This higher expression of c-myb in ciliated cell progenitors allows the detection of this marker without signal amplification at this specific time point (data not shown), consistent with the previously reported expression patterns of c-myb during airway epithelial regeneration (Tan et al., 2013; Pan et al. 2014).

Notch signaling pathway components are active in basal stem/progenitor cells during early airway regeneration

The Notch pathway has previously been shown to regulate epithelial cell fate in the embryonic lung (Tsao et al., 2009; Guseh et al., 2009; Morimoto et al., 2010). Notch signaling is also activated following injury-induced regeneration in the adult airway (Rock et al., 2011). In this context, in addition to describing the expression pattern of N2ICD above, we also assessed the expression of the active intracellular domain of the other Notch receptors expressed in the airway epithelium, N1ICD and N3ICD (Rock et al., 2011). Nuclear N1ICD staining was detected in the majority of basal cells at 24 hpi and 36 hpi (Figure S4A, left panels), while N3ICD was barely and weakly detected in some non-basal cells at 24 hpi and in a few suprabasal $p63^+$ cells that likely represent transitional differentiating cells at 36 hpi (Figure S4A, right panels). Thus, in contrast to segregated N2ICD expression, Notch1 signaling appears to be active in all basal stem/progenitor cells following injury while N3ICD is expressed in very few basal cells.

With respect to Notch target genes, Hey1 and HeyL gene expression have previously been shown to increase after injury (Rock et al., 2011). So, we assessed their protein expression at an early time point after injury. At 24 hpi, Hey1 was detected in all basal stem/progenitor cells, perhaps acting downstream of N1ICD (Figure S4B). However, HeyL was only

detected in the subset of basal cells (Figure S4B) that correspond to N2ICD$^+$ p63$^+$ cells (data not shown), suggesting that HeyL is the Notch target gene acting downstream of N2ICD to commit cells towards a secretory cell fate following injury.

Notch Pathway Inhibition Disrupts the Segregation of Basal Stem/Progenitor Cells

In order to directly test whether basal cell segregation is contingent on Notch signaling, we abrogated canonical Notch pathway activity in the basal cell compartment using *CK5-rtTA;tet(O)cre;RBPJk$^{fl/fl}$* mice (hereafter referred to as CK5-RBPJk$^{fl/fl}$). Doxycycline administration led to a basal cell-specific deletion of RBPJk, an essential transcription factor necessary for canonical Notch signaling (Morimoto et al., 2010; Tsao et al., 2009) (Figure 4A). We first verified that RBPJk was efficiently deleted exclusively in basal stem/progenitor cells (Figure S5A and S5B). Since RBPJk is ubiquitously expressed in the airway epithelium, any cell that lacks RBPJk must have originally arisen from a basal cell. Stated otherwise, the lack of RBPJk in any differentiating or mature cell is equivalent to a lineage trace of that cell from a basal stem/progenitor cell in which RBPJk was previously deleted.

We next exposed control and experimental CK5-RBPJk$^{fl/fl}$ mice to SO_2 injury and assessed the fate of basal stem/progenitor cells at early time points after injury. We confirmed the efficient SO_2-induced removal of CK8-expressing luminal cells in both control and mutant mice (Figure S5C). At 24 hpi, we observed a disruption in the normal segregation of basal stem/progenitor cells into N2ICD$^+$ and c-myb$^+$ populations. After RBPJk removal, the proportion of c-myb$^+$ basal cells increased (71.1 ± 3.4%) as compared to control mice (51.7 ± 2.2%) (Figure 4B and 4D). This was accompanied by a concomitant decrease in the percentage of N2ICD$^+$ basal cells (5.1 ± 2.2%) in mutant mice as compared to control mice (18.8 ± 1.3%) (Figure 4C and 4E). Additionally, basal cells expressing N2ICD at 24 hpi in mutant mice were always basal cells in which RBPJk had not been removed (Figure S5D). Furthermore, p63 immunostaining revealed no change in the overall fraction of cells that were p63$^+$ basal stem/progenitor cells following injury (84.8 ± 3.0% of total epithelial cells in controls *vs*. 81.9 ± 6.1% in mutants) (Figure S5E), demonstrating that although the total numbers of basal cells remained the same, their segregation into subpopulations was altered. Additionally, proliferation rates of basal cells following SO_2 injury were unaltered as assessed by Ki67 immunostaining (89.0 ± 5.1% of total epithelial cells in controls *vs*. 87.0 ± 5.6% in mutants) (Figure S5F and S5G). These results suggest a model in which the proper segregation of basal stem/progenitor cells requires canonical Notch signaling within the basal cell population itself (Figure 4F).

Prior models of the airway lineage hierarchy have suggested that Notch signaling is required for the formation of the population of suprabasal CK8$^+$ early progenitor cells (Rock and Hogan, 2011; Rock et al., 2011). Surprisingly, in CK5-RBPJk$^{fl/fl}$ mice treated with doxycycline, at 48 hpi when CK8$^+$ cells first appear, we see the continued production of CK8$^+$ luminal cells many of which are negative for CK5 and p63 (Figure 4G). Indeed, the fraction of CK8$^+$ cells remained unchanged when compared to controls (44.4 ± 2.0% of total epithelial cells in controls *vs*. 46.74 ± 3.5% in mutants) (Figure 4H). Given this surprising result, we verified the absence of RBPJk in CK8$^+$ early progenitor cells in the regenerating epithelium (Figure 4G and 4I), which confirmed that canonical Notch activity is dispensable

for the production of $CK8^+$ luminal cells *per se*. Instead, the disruption of canonical Notch signaling in basal stem/progenitor cells promoted an increase in the fraction of the c-myb expressing subset of basal cells at the expense of N2ICD expressing basal cells. Thus, the lack of Notch activity in basal stem/progenitor cells favors ciliated cell lineage commitment and differentiation. Taken together, these data suggest that Notch signaling is not required for the differentiation of a $CK8^+$ progenitor population, but rather to segregate basal stem/progenitor cells into functionally distinct subpopulations at the level of the basal cell population itself.

Functional Consequences of the Disrupted Segregation of Basal Stem/Progenitor Cells into Subpopulations

Because Notch pathway deletion increased the population of basal cells that expressed c-myb, we hypothesized that these cells would exclusively give rise to excess mature ciliated cells. To test this hypothesis, SO_2 was administered to CK5-$RBPJk^{fl/fl}$ mutant mice and we assessed the cell fate distribution in the epithelium at 7 dpi, a time point well after the initial appearance of mature differentiated ciliated and secretory cell markers (Figure 5A). When compared to control mice, CK5-$RBPJk^{fl/fl}$ mutant mice given doxycycline prior to injury showed a decrease in the percentage of cells expressing the mature secretory cell marker Scgb1a1 (50.5 ± 3.5% of total epithelial cells in controls *vs*. 29.2 ± 1.1% in mutants) as well as the secretory cell marker SSEA1 (52.5 ± 6.3% of total epithelial cells in controls *vs*. 23.6 ± 3.2% in mutants) (Figure 5B and 5C). Additionally, the $Scgb1a1^+$ secretory cells that remain in mutant mice are in fact $RBPJk^+$ (Figure 5B, white arrow) reaffirming the notion that canonical Notch signaling is necessary for secretory cell fate specification. In contrast to the decrement in the proportion of secretory cells, there was the predicted corresponding proportionate increase in the percentage of cells expressing the mature ciliated cell markers FoxJ1 (39.0 ± 0.9% of total epithelial cells in controls *vs*. 63.7 ± 2.2% in mutants) and acetylated tubulin (40.3 ± 3.8% of total epithelial cells in controls *vs*. 60.7 ± 0.8% in mutants) (Figure 5D and 5E). Furthermore, at 14 dpi when the airway epithelium is normally fully regenerated, we continued to observe the above noted lineage shift in experimental CK5-$RBPJk^{fl/fl}$ mutant mice (Figure 5F and 5G). These results demonstrate that canonical Notch signaling is required for the generation of secretory cells after injury, but dispensable for the generation of ciliated cells. Furthermore, it appears that the basal cells that did not commit to generate secretory cell progeny instead generated ciliated cell progeny.

Disruption of Notch ligand expression in basal cells also promotes direct ciliated cell differentiation at the expense of secretory cell differentiation

Since the Notch signaling pathway is known to act through direct cell to cell interactions, and since basal cells express different Notch ligands (Rock et al., 2011), we sought to assess whether some basal cells send Notch ligands to neighboring basal cells to activate N2ICD, the canonical Notch signaling effector we identified above. To remove basal cell Notch ligands, we generated *CK5-rtTA; tet (O) Cre; Mindbomb1*$^{fl/fl}$ mice (hereafter referred to as CK5-$Mib1^{fl/fl}$). In these mice, doxycycline administration causes the deletion of an E3 ubiquitin ligase, Mindbomb1 (Mib1), which is required for the normal endocytic processing of all Notch ligands (Koo et al., 2005), specifically in basal stem/progenitor cells. CK5-

$Mib1^{fl/fl}$ mice were then subjected to SO_2 injury and their airways were collected at different time points to test whether the disruption of a Notch-activating signal emanating from basal cells would recapitulate the cell autonomous phenotype associated with RBPJk removal (Figure 6A).

Indeed, we again observed a significant increase in the percentage of c-myb^+ basal cells in mice with basal cell-specific Mib1 removal (71.6 ± 5.8%) compared to control mice (44.5 ± 6.1%) at 24 hpi (Figure 6B and 6D). We also observed a corresponding decrease in $N2ICD^+$ basal cells in Mib1 mutant mice (7.0 ± 1.8%) compared to control mice (18.4 ± 1.6%) (Figure 6C and 6E). Thus, our data suggests that some basal stem/progenitor cells provide an essential ligand stimulus to instruct the differentiation of other basal stem/progenitor cells. As a consequence of the deletion of Notch ligand activity, CK5-$Mib1^{fl/fl}$, experimental airways displayed a decrease in $Scgb1a1^+$ cells (60.1 ± 2.9% of total epithelial cells in controls *vs* 29.6± 3.2% in mutants) and an increase in both acetylated tubulin (31.1 ± 4.4% of total epithelial cells in controls *vs* 56.20 ± 1.9% in mutants) and $FoxJ1^+$ cells (30.0 ± 4.6% of total epithelial cells in controls *vs* 62.27 ± 4.2% in mutants) at 7 dpi (Figure 6F and 6G). These results demonstrate that the removal of an intercellular Notch signal emanating from some basal cells recapitulates the phenotype associated with the cell autonomous abrogation of canonical Notch signaling within basal cells. Thus, the population of basal stem/progenitor cells at large uses intercellular Notch signaling to segregate distinct functional subpopulations of cells following injury.

We next assessed the effect of the loss of Notch signaling on mucous cell differentiation. We did not detect any substantive difference in the numbers of $FoxA3^+$ or $Muc5AC^+$ mucus-secreting cells when RBPJk or Mib1 was deleted prior to regeneration (Figure S6A and S6B). Since prior reports indicated that N1ICD overexpression led to increased mucous cell differentiation (Guseh et al., 2009; Rock et al., 2011) and we have now implicated N2ICD in secretory cell fate determination, we analyzed the effect of N2ICD overexpression in murine air-liquid interface (ALI) cultures. The general pattern of c-myb and N2ICD expression seen after *in vivo* injury was also seen during the differentiation of ALI cultures. Basal cells in culture begin to express N2ICD and c-myb as early as 7 hours after plating and these Notch components continue to be expressed when an air-liquid interface is created (d0) and during the initial steps of the differentiation process (d1 ALI) (Figure S7A). When N2ICD was overexpressed (Figure S7B and S7C), the number of cells expressing c-myb was decreased (Figure S7C) and as a result, fewer ciliated cells (acetylated tubulin) and more secretory cells (Scgb1a1 and Muc5AC) were observed 8 days after air-liquid interface creation (Figure S7D).

In aggregate, our data suggest a new working model for the lineage hierarchy of the airway epithelium following injury (Figure 7). We demonstrate that a functional segregation of basal stem/progenitor cells is induced almost immediately following injury. Furthermore, we show the existence of mutually exclusive subpopulations of c-myb^+ and $N2ICD^+$ basal cells that subsequently undergo lineage commitment into mature ciliated or secretory cell fates. Surprisingly, the deletion of essential components of the canonical Notch signaling pathway in basal cells does not prevent their differentiation into suprabasal $CK8^+$ cells as previously predicted, but rather results in the production of ciliated cell progeny at the expense of

secretory cell progeny. Furthermore, we show evidence those basal cells directly produce ciliated cells without the need for a secretory cell intermediate.

Discussion

We have identified subsets of basal cells that themselves express ciliated or secretory cell associated fate determinants, prior to the formation of $CK8^+$ luminal cells. Prior studies point to the direct differentiation of a subset of basal cells into the ciliated cell lineage (Rawlins et al., 2009; Ghosh et al., 2011b) and our demonstration of a population of c-myb^+ $p63^+$ basal cells is consistent with these findings. Here, we now show that the c-myb expression commences in a population of $p63^+$ basal cells which will invariantly replicate. In contrast, prior work has described the expression of c-myb in a $p63^-$ ciliated progenitor cell that does not self-renew (Pan et al., 2014). In essence, the mere use of signal amplification protocols allowed us to detect low levels of c-myb and N2ICD expression in basal stem/progenitor cells and this allowed us to resolve a very early step in the cell fate commitment process. Of note, we see that levels of both c-myb and N2ICD increase as cell differentiation ensues, allowing the detection of the transient expression of c-myb during airway cell differentiation without amplification specifically at 48hpi. Without signal amplification, N2ICD and c-myb at other time points after injury or at homeostasis are not detected. Whether injury-induced and homeostatic c-myb^+ and $N2ICD^+$ basal cell subpopulations are identical cell types remains to be resolved. It will be also interesting to determine how injury-based mechanisms involved in basal cell segregation differ or correspond to those used homeostatically to regulate these subpopulations.

In addition, we show that Notch-mediated intercellular communication within the basal cell stem/progenitor cell compartment is necessary for their injury-induced segregation. This may occur by a mechanism akin to lateral inhibition as previously implicated in *Xenopus* mucociliary epithelium (Deblandre et al., 1999; Dubaissi and Papalopulu, 2011; Dubaissi et al., 2014). However, we note that there are 3 subpopulations of airway basal cells ($p63^+$, $p63^+$ c-myb^+, and $p63^+$ $N2ICD^+$) and that conventional lateral inhibition mechanisms usually involve 2 cell types, suggesting that more complex mechanisms may be at work. Indeed, injury results in an influx of immune cells and alterations in stromal cells that may participate in Notch signaling modulation (Tadokoro et al., 2014). And why is segregation not occurring in the homeostatic epithelium? Part of the answer may lie in other Notch components that are expressed in secretory and ciliated cells (N3ICD) or in basal cells (N1ICD) which might prevent the basal to basal cell interactions that are necessary for segregation. Furthermore, a recent study demonstrates a requirement for IL-6/STAT3 activity during regeneration after SO_2 injury, and demonstrates that Stat3 signaling is required for ciliated cell differentiation presumably through the inhibition of Notch pathway (Tadokoro et al., 2014). It will be of interest to study the molecular interactions between P-STAT3, N2ICD and c-myb.

Airway stem/progenitor cell subpopulations have been previously reported (Ghosh et al., 2011a, 2011b, 2013a, 2013b; Hong et al., 2004). Our study now defines a heterogeneity based upon the presence of functionally relevant proteins that participate in cell fate commitment. Since injury provokes such a rapid induction of N2ICD or c-myb protein

expression within the basal stem/progenitor cell population at large, one does wonder whether there may be an underlying heterogeneity of homeostatic basal stem/progenitor cells in which some subsets are primed to rapidly transcribe and/or translate c-myb or activate N2ICD formation and nuclear translocation.

Interestingly, prior studies pointed to the existence of unipotent and bipotent basal progenitor cells in the post-injury airway epithelium (Ghosh et al., 2011b; Hong et al., 2004). Specifically, these studies pointed to two types of bipotent progenitor cells: one that produces clones restricted to ciliated and basal cell fates and a second that produces secretory and basal cell fates. More surprisingly, the authors suggested the existence of unipotent basal cell progenitors that only generate other basal cells (Ghosh et al., 2011b; Hong et al., 2004). Intriguingly, we now provide a possible mechanistic basis for this interesting observation. The three subpopulations of regenerating basal cells ($N2ICD^+$ $p63^+$, $c\text{-}myb^+$ $p63^+$ and single-positive p63 cells) would nicely correspond to the bipotent and unipotent progenitor cells that the aforementioned studies described.

Murine lineage tracing experiments have demonstrated that basal cells, as a population, self-renew over the long term and differentiate into ciliated and secretory luminal cells (Rock et al., 2009; Hogan et al., 2014), but to our knowledge no clonal analysis has been performed *in vivo* to definitively conclude that basal cells are clonally multipotent over an extended period of time. The subsets of basal cells that we have identified here may be lineage committed, but this remains hypothetical. Indeed, the potency of the induced $c\text{-}myb^+$ or $N2ICD^+$ stem/progenitor basal cells is still unknown since we haven't addressed (1) whether they are committed to a certain lineage as single stem/progenitor cells over time, (2) whether they are interconvertible between one another, and (3) if they can interconvert into putative multipotent stem cells. It is for this reason that we have elected to use the term stem/progenitor cell to refer to all subsets of basal cells until definitive clonal lineage data is obtained. However, multiple groups, have shown that single airway basal cells cultured *ex vivo* have the potential to act as clonal multipotent stem cells that produce both ciliated and secretory cells (Rock et al., 2009; Gosh et al., 2011a; Tata et al., 2013a), but none, including our own group, have performed the requisite clonal lineage tracing experiments *in vivo*. Indeed, these studies only reflect a potential of single basal-derived cells *in vitro*, and do not necessarily reflect their *in vivo* behavior as a sheet of interacting basal cells that coordinately respond to injury. Recent studies also suggest that human basal progenitor cells maintain airway homeostasis through a neutral drift model in which all progenitor cells are equipotent (Teixeira et al., 2013). This study is not necessarily at odds with our results since our basal cell subpopulations may be stochastically interconvertible and furthermore those observations were conducted on human patients instead of mice. Additionally, many complex hierarchies of stem and progenitor cells can obey a neutral drift pattern.

Further investigation at the single cell level using clonal analysis will be necessary to determine the identity and potential of different basal cell subpopulations. It took many years to sort out the lineage hierarchies in the hematopoietic system and in the epidermis and the work is still not entirely complete. It may be that many seemingly homogeneous stem/progenitor cell compartments are much more complex than we had imagined.

Experimental Procedures

See Supplemental Experimental Procedures for an extended and detailed description of the materials and methods used.

Mice and Injury Models

CK5-rtTA (Diamond et al., 2000), *tet(O)cre* (JAX stock number 006224), *RBPJk*$^{fl/fl}$ (Tanigaki et al., 2002) and *Mindbomb1*$^{fl/fl}$ (Koo et al., 2007) mice were previously described. Doxycycline induction, SO_2 and chlorine injury models have been previously described (Tata et al., 2013b; Tata et al., 2013a; Musah et al., 2012). All protocols were approved by the MGH Subcommittee on Research Animal Care in accordance with NIH guidelines.

Immunofluorescence, Microscopy and Imaging

Immunofluorescent detection has been described previously (Kim et al., 2012; Pardo-Saganta et al., 2013; Tata et al., 2013a). Briefly, the detection of Notch2 intracellular domain and of c-myb required signal amplification. The primary antibodies used were rabbit anti-Notch2 (1:2000; D67C8, Cell Signaling) and rabbit anti-c-myb (1:3000; sc-519, Santa Cruz Biotechnology). Images were generated using an Olympus FluoView FV10i confocal microscope (Olympus Corporation) and ImageJ (NIH) and Adobe Photoshop Creative Suite 5 (Adobe).

Cell culture and Viral Transduction

Mouse tracheal basal cells were obtained and cultured as previously described (Pardo-Saganta et al. 2013; Tata et al. 2013a; Zhao et al. 2014). Lentiviral vectors expressing murine N2ICD were generated from 3XFlagNICD2 plasmid (Addgene, 20184).

Statistical Analysis

The standard error of the mean was calculated from the average of at least 3 independent tracheal samples per condition unless otherwise mentioned. Data was compared among groups using the Student's t-test (unpaired, two-tailed test). A *p*-value of less than 0.05 was considered significant. Analysis was performed with Prism software (Graphpad Prism version 5.0a).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Adam Glick for providing the *CK5rtTA* mice, Brigid Hogan for providing *Mib1 floxed* mice (originally from Young-Yun Kong who kindly offered to share the mice), and Ben Z Stanger for providing the *RBPJk floxed* mice. We also thank Barry Stripp for providing the goat anti-SCGB1A1 antibody and Joseph Pearlberg for providing the lentiviral donor vectors. We wish to extend our thanks to all of the members of the Rajagopal Laboratory for their constructive criticisms and comments and for valuable discussion and support. This research was supported by the New York Stem Cell Foundation (J.R. is a New York Stem Cell Foundation-Robertson

Investigator), by an NIH-NHLBI Early Career Research New Faculty (P30) award (5P30HL101287-02), an R01 (R01HL118185) from NIH/NHLBI (to J.R.), and a Harvard Stem Cell Institute (HSCI) Junior Investigator Grant (to J.R.).

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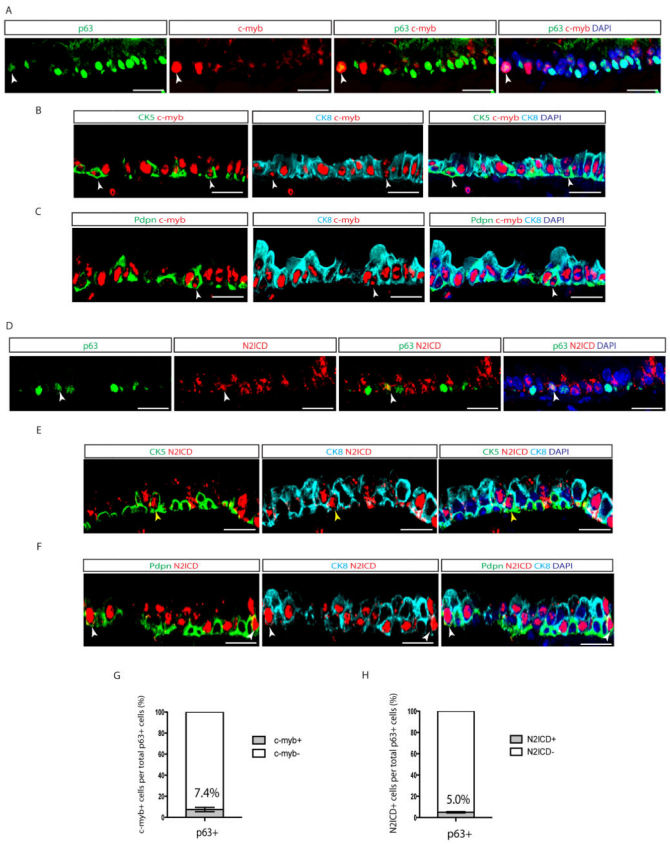

Figure 1. Notch pathway components are expressed in homeostatic basal cells

a, Immunostaining for the basal cell marker p63 (green) and c-myb (red) on sections of wild type (WT) airway epithelium. b, Immunostaining for the basal cell marker CK5 (green), c-myb (red) and CK8 (cyan) on WT tracheal sections. c, Immunostaining for the basal cell marker Pdpn (green), c-myb (red) and CK8 (cyan) on WT tracheal sections. a-c, white arrows point to c-myb^{+} basal cells. d, Immunostaining for p63 (green) and N2ICD (red) on WT tracheal sections. e, Immunostaining for CK5 (green), N2ICD (red) and CK8 (cyan) on WT tracheal sections. f, Immunostaining for Pdpn (green), N2ICD (red) and CK8 (cyan) on WT tracheal sections. d-f, white arrows point to N2ICD^{+} basal cells. e, yellow arrow points to a N2ICD^{+} CK5^{+} cell that co-expresses CK8 g, Quantification of the percentage of c-myb^{+} cells per total p63^{+} cells in wild type airway sections (n=3). h, Quantification of the percentage of N2ICD^{+} cells per total p63^{+} cells in WT airway sections (n=3). Data shown in the graphs are means ± S.E.M. Nuclei stained with DAPI (blue). Scale bar, 20μm.

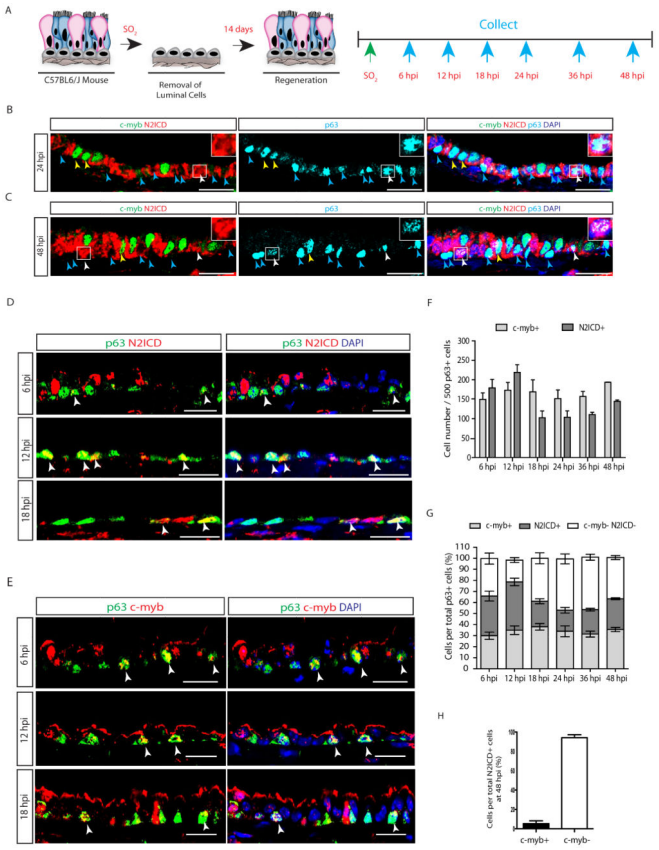

Figure 2. Enhanced segregation of basal stem/progenitor cells after airway injury

a, Schematic representation of SO_2-induced airway injury and subsequent tissue collection from WT mice. Ciliated cells, secretory cells and basal stem/progenitor cells are shown in blue, pink and gray colors respectively. b,c, Immunostaining for c-myb (green), N2ICD (red) and p63 (cyan) at 24 hpi (b) and 48 hpi (c). Yellow arrows point to c-myb$^+$ p63$^+$ cells, white arrows to N2ICD$^+$ p63$^+$ cells, and blue arrows to p63$^+$ c-myb$^-$ N2ICD$^-$ cells. Higher magnification images of individual N2ICD$^+$ p63$^+$ cells are shown in the insets. d,e, Immunostaining for N2ICD (d) or c-myb (e) (red), and p63 (green) at 6 hpi, 12 hpi and 18 hpi. Arrows point to N2ICD$^+$ p63$^+$ cells (d) or c-myb$^+$ p63$^+$ cells (e). f, g, Quantification of the total number of c-myb$^+$ p63$^+$ cells, N2ICD$^+$ p63$^+$ cells, and c-myb$^-$ N2ICD$^-$ p63$^+$ cells per total p63$^+$ cells during the regeneration process (n=3-6). Graph in f represents absolute numbers of each cell population per 500 p63$^+$ cells while g shows their percentage as a fraction of p63$^+$ cells. h, Quantification of the percentage of c-myb$^+$ p63$^+$ cells (black bar) or c-myb$^-$ p63$^+$ cells (white bar) per total N2ICD$^+$ p63$^+$ cells at 48 hpi (n=4). Nuclei stained with DAPI (blue). Of note, some background for N2ICD at 12hpi (d) or c-myb at 12 and 18hpi (e) is observed in the surface of the remaining epithelium. Data shown in the graphs are means ± S.E.M. Scale bar, 20μm. See also Figure S1, S2 and S3.

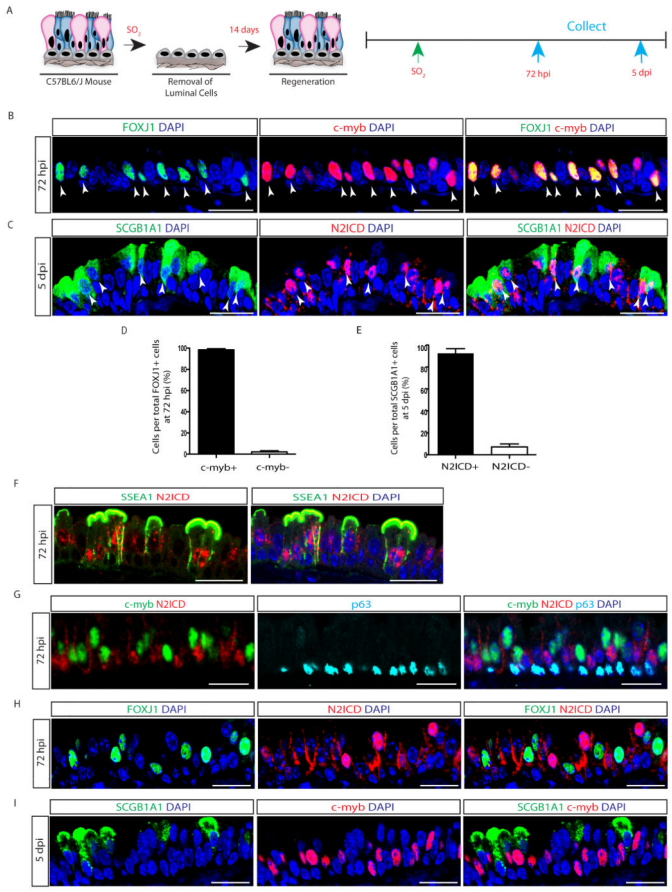

Figure 3. Functional segregation of basal stem/progenitor cells into differentiated cells after injury

a, Schematic representation of SO_2 injury in WT mice. b, Immunostaining for FoxJ1 (green) and c-myb (red) at 72 hpi. White arrows point to FoxJ1$^+$ c-myb$^+$ cells. c, Immunostaining for Scgb1a1 (green) and N2ICD (red) at 5 dpi. White arrows point to Scgb1a1$^+$ N2ICD$^+$ cells. d, Quantification of the percentage of c-myb$^+$ cells (black bar) or c-myb$^-$ cells (white bar) per total FoxJ1$^+$ cells (n=3). e, Quantification of the percentage of N2ICD$^+$ cells (black bar) or N2ICD$^-$ cells (white bar) per total Scgb1a1$^+$ cells (n=3). f, Immunostaining for SSEA1 (green) and N2ICD (red) at 72 hpi. g, Immunostaining for c-myb (green), N2ICD (red) and p63 (cyan) at 72 hpi. h, Immunostaining for FoxJ1 (green) and N2ICD (red) at 72 hpi. i, Immunostaining for Scgb1a1 (green) and c-myb (red) at 5 dpi. Note the membrane staining of Notch2 receptor, in addition to the detection of the intracellular domain in the nucleus, in some cells in c, g and h. Nuclei stained with DAPI (blue). Data shown in the graphs are means ± S.E.M. Scale bar, 20μm.

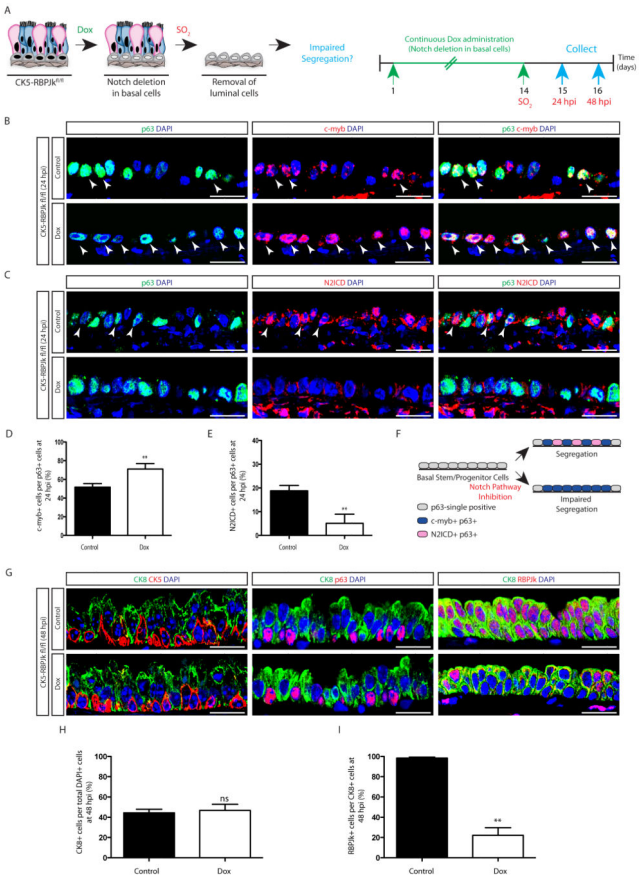

Figure 4. Notch signaling inhibition alters the segregation of basal stem/progenitor cells into functional subpopulations but does not prevent the formation of CK8^{+} luminal cells

a, Schematic representation of SO_2 injury and tissue collection of CK5-RBPJk$^{fl/fl}$ mice. b, c, Immunostaining for p63 (green), and c-myb (b) or N2ICD (c) (red) on either control (upper panels) or doxycycline (Dox) treated (lower panels) CK5-RBPJk$^{fl/fl}$ mice at 24 hpi. Arrows point to c-myb^{+} p63^{+} cells (a) or N2ICD^{+} p63^{+} cells (b). d, e, Quantification of the percentage of c-myb^{+} cells (d) or N2ICD^{+} cells (e) per total p63^{+} cells in control (black bars) or Dox treated (white bars) CK5-RBPJk$^{fl/fl}$ mice (n=3). f, Schematic summary of the consequences of RBPJk loss on basal cell segregation. g, Immunostaining for CK8 (green) (left and middle panels) in combination with CK5 (red) (left panels), p63 (red) (middle panels) or RBPJk (red) (right panels) in either control (upper panels) or Dox treated (lower panels) CK5-RBPJk$^{fl/fl}$ mice at 48 hpi. h, i, Quantification of CK8^{+} cells per total DAPI^{+} cells (h) or RBPJk^{+} cells per total CK8^{+} cells (i) in control (black bars) or Dox treated (white bars) CK5-RBPJk$^{fl/fl}$ mice (n=3). Nuclei stained with DAPI (blue). ** p<0.01; ns: not significant. Data shown in the graphs are means ± S.E.M. Scale bar, 20μm. See also Figure S4, S5 and S7.

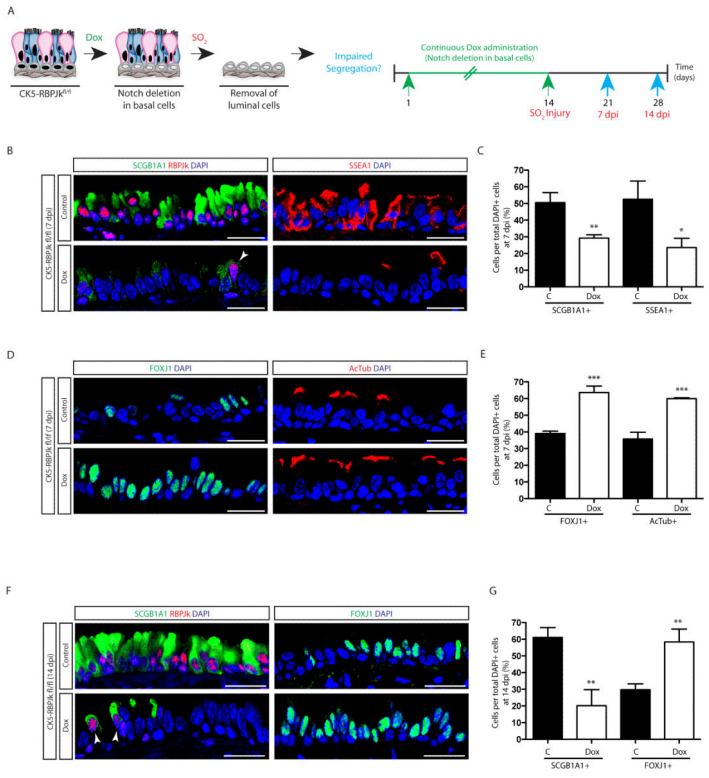

Figure 5. Canonical Notch signaling loss in basal cells promotes ciliated cell differentiation at the expense of secretory cell differentiation

a, Schematic representation of SO_2 injury and tissue collection of CK5-RBPJk$^{fl/fl}$ mice. b, Immunostaining for Scgb1a1 (green) in combination with RBPJk (red) (left panels) and for SSEA1 (red) (right panels) on either control (upper panels) or Dox treated (lower panels) CK5-RBPJk$^{fl/fl}$ mice at 7 dpi. White arrow points to a Scgb1a1^{+} RBPJk^{+} cell. c, Quantification of the percentage of Scgb1a1^{+} and SSEA1^{+} cells per total DAPI^{+} cells in control (black bars) or Dox treated (white bars) CK5-RBPJk$^{fl/fl}$ mice at 7 dpi (n=3). d, Immunostaining for FoxJ1 (green) (left panels) and acetylated tubulin (AcTub) (red) (right panels) on either control (upper panels) or Dox treated (lower panels) CK5-RBPJk$^{fl/fl}$ mice at 7 dpi. e, Quantification of the percentage of FoxJ1^{+} and AcTub^{+} cells per total DAPI^{+} cells in control (black bars) or Dox treated (white bars) CK5-RBPJk$^{fl/fl}$ mice at 7 dpi (n=3). f, Immunostaining for Scgb1a1 (green) in combination with RBPJk (red) (left panels) or FoxJ1 (green) (right panels) on either control (upper panels) or Dox treated (lower panels) CK5-RBPJk$^{fl/fl}$ mice at 14 dpi. White arrows point to Scgb1a1^{+} RBPJk^{+} cells in Dox treated CK5-RBPJk$^{fl/fl}$ mice. g, Quantification of the percentage of Scgb1a1^{+} and FoxJ1^{+} cells per total DAPI^{+} cells in control (black bars) or Dox treated (white bars) CK5-RBPJk$^{fl/fl}$ mice at 14 dpi (n=3). Nuclei stained with DAPI (blue). * p<0.05; ** p<0.01;*** p<0.001; Data shown in the graphs are means ± S.E.M. Scale bar, 20μm. See also Figure S6 and S7.

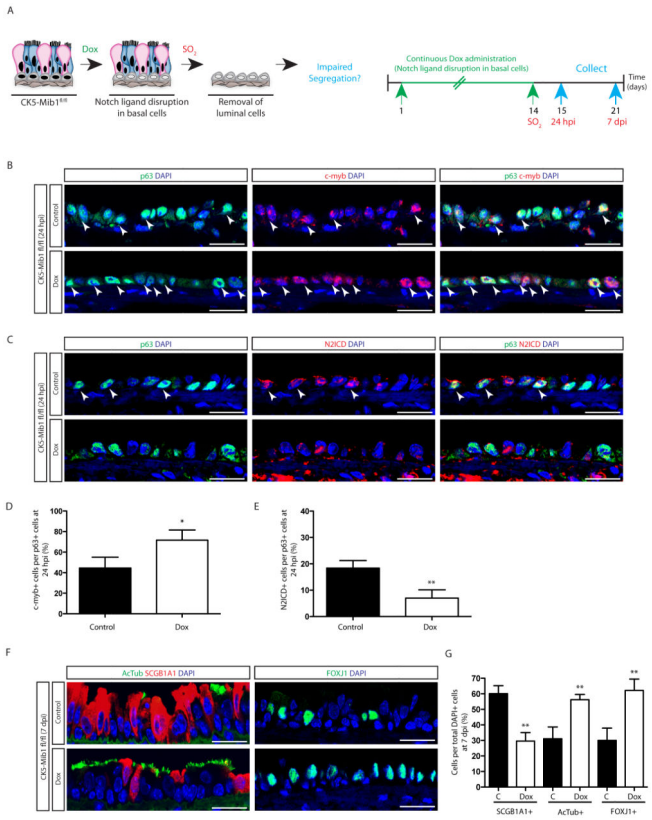

Figure 6. Disruption of Notch ligand expression in basal cells prevents secretory cell formation and promotes direct differentiation into a ciliated cell lineage

a, Schematic representation of SO_2 injury and tissue collection of CK5-Mib1$^{fl/fl}$ mice. b, c, Immunostaining for p63 (green), and c-myb (red) (b) or N2ICD (red) (c) on either control (upper panels) or doxycycline (Dox) treated (lower panels) CK5-Mib1$^{fl/fl}$ mice at 24 hpi. White arrows point to c-myb^{+} p63^{+} (b) or N2ICD^{+} p63^{+} (c) cells. d, e, Quantification of the percentage of c-myb^{+} cells (d) or N2ICD^{+} cells (e) per total p63^{+} cells in control (black bars) or Dox treated (white bars) CK5-Mib1$^{fl/fl}$ mice (n=3) f, Immunostaining for AcTub (green) in combination with Scgb1a1 (red) (left panels) and for FoxJ1 (green) (right panels) on either control (upper panels) or Dox treated (lower panels) CK5-Mib1$^{fl/fl}$ mice at 7 dpi. g, Quantification of the percentage of Scgb1a1^{+}, AcTub^{+} and FoxJ1^{+} cells per total DAPI^{+} cells in control (black bars) or Dox treated (white bars) CK5-Mib1$^{fl/fl}$ mice at 7 dpi (n=3). Nuclei stained with DAPI (blue). *p<0.05; ** p<0.01; Data shown in the graphs are means ± S.E.M. Scale bar, 20μm. See also Figure S6 and S7.

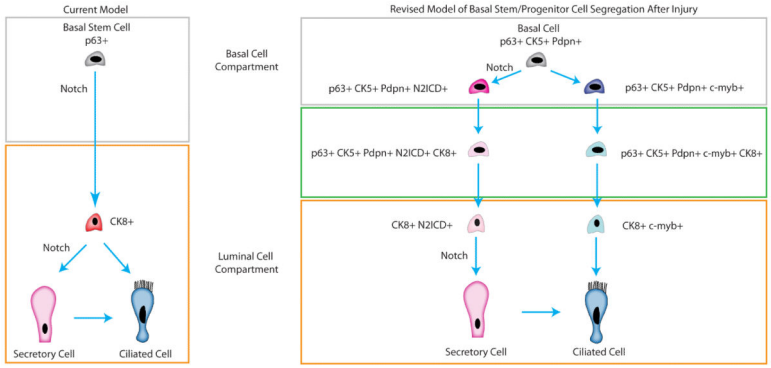

Figure 7. Revised model of the airway epithelial lineage hierarchy induced by injury

Basal cells expressing p63, CK5 and Pdpn segregate into a N2ICD$^+$ population and a c-myb$^+$ population in response to injury. During regeneration, a continuum of differentiation is observed and basal cell markers and markers of differentiation are co-expressed between 48-72 hpi. N2ICD$^+$ basal cells and c-myb$^+$ basal cells subsequently express the differentiation marker CK8 as basal cell markers are gradually lost. Suprabasal N2ICD$^+$ CK8$^+$ cells then give rise to secretory cells while c-myb$^+$ CK8$^+$ cells give rise to ciliated cells.