Notch signaling controls the balance of ciliated and secretory cell fates in developing airways

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Although there is accumulated evidence of a role for Notch in the developing lung, it is still unclear how disruption of Notch signaling affects lung progenitor cell fate and differentiation events in the airway epithelium. To address this issue, we inactivated Notch signaling conditionally in the endoderm using a Shh-Cre deleter mouse line and mice carrying floxed alleles of the Pofut1 gene, which encodes an O-fucosyltransferase essential for Notch-ligand binding. We also took the same conditional approach to inactivate expression of Rbpjk, which encodes the transcriptional effector of canonical Notch signaling. Strikingly, these mutants showed an almost identical lung phenotype characterized by an absence of secretory Clara cells without evidence of cell death, and showed airways populated essentially by ciliated cells, with an increase in neuroendocrine cells. This phenotype could be further replicated in cultured wild-type lungs by disrupting Notch signaling with a gamma-secretase inhibitor. Our data suggest that Notch acts when commitment to a ciliated or non-ciliated cell fate occurs in proximal progenitors, silencing the ciliated program in the cells that will continue to expand and differentiate into secretory cells. This mechanism may be crucial to define the balance of differentiated cell profiles in different generations of the developing airways. It might also be relevant to mediate the metaplastic changes in the respiratory epithelium that occur in pathological conditions, such as asthma and chronic obstructive pulmonary disease.

KEY WORDS: Notch, Pofut1, Rbpjk (Rbpj), Cell fate, Lung development, Airway differentiation, Ciliated cell, Clara cell, Neuroendocrine cell

INTRODUCTION
The respiratory system represents a major interface between the body and the external environment, serving functions as diverse as mucociliary clearance, fluid and electrolyte homeostasis, surfactant production and gas exchange. To exert these functions the respiratory epithelium harbors a wide variety of cell phenotypes differentially distributed from the tracheobronchial region (proximal) to the alveoli (distal), including basal, ciliated secretory and neuroendocrine cells in airways and type I and type II pneumocytes in alveoli (Weibel, 1984; Rawlins and Hogan, 2006; Franks et al., 2008). In the embryo, these cells arise from developmental programs that initially specify proximal and distal lung progenitors, and later on drive prespecified cells to differentiate towards specific cellular phenotypes. Although factors such as Titf1 (Nkx2-1), Foxa2, β-catenin, Gata and Sox family members, have been implicated in these programs, little is known about how cell fates are regulated and diversity is achieved in the respiratory epithelium (Cardoso and Lu, 2006; Maeda et al., 2007; Warburton et al., 2008).

Studies in species from Drosophila to humans implicate Notch signaling in the control of cell fate decisions, in the establishment of asymmetries and in the timing of differentiation during development. These effects have been widely reported in various organs, including the lung (van Es et al., 2005; Guilmot et al., 2008; Murtough et al., 2003; Liu et al., 2007; Okamura and Saga, 2008; Gridley, 2007; Collins et al., 2004). Four Notch receptors (Notch1-4) and five ligands [jagged 1 and 2 (Jag1 and 2) and delta-like 1, 3 and 4 (Dll1, 3 and 4)] have been identified in mammals. Ligand-receptor interactions via cell-cell contact trigger a series of enzymatic events, which ultimately results in gamma-secretase cleavage of the Notch intracellular domain (NICD) and NICD binding to the transcriptional effector Rbpjk (Rbpj). This leads to activation of the Notch downstream target genes Hes and Hey, which then exert their biological effects (Radtke and Raj, 2003).

There is evidence that Notch components are already present during the initial stages of lung development, and that Notch is dynamically activated at the tips of lung epithelial buds (Tsao et al., 2008; Post et al., 2000; Kong et al., 2004). The effects of pharmacological inhibition of Notch signaling in foregut or lung explant cultures suggest that Notch is crucial to control the balance of proximal and distal cell fates and for proper development of the proximal progenitors of the developing airways (Tsao et al., 2008). In transgenic mice expressing a constitutively active Notch3 targeted to the distal lung epithelium, distal progenitors fail to differentiate and remain immature (Dang et al., 2003). Insights into how Notch signaling influences progenitor cell fate during the acquisition of specific cell phenotypes come from the analysis of mice deficient in Hes1, one of the Notch targets. Hes1 deficiency in the lungs results in an increased number of neuroendocrine cells, with a relatively small decrease in the number of secretory cells (Ito et al., 2000). Although informative, these observations do not reflect the full role of Notch in the developing lung, as inactivation of Hes1 does not lead to global disruption of Notch signaling. Thus, the question still remained as to whether preventing signaling by all Notch receptors could affect developmental events not yet revealed by these previous approaches.

Here we investigate this issue using three distinct Notch loss-of-function approaches in the murine lung. The protein O-fucosyltransferase 1 (Pofut1) catalyzes the reaction that attaches O-fucose to the EGF repeats of Notch (Okajima and Matsuda, 2006).
**Pofut1** is ubiquitously expressed during organogenesis, including in the developing lung (Shi and Stanley, 2003; Tsao et al., 2008). Studies in which **Pofut1** has been deleted systemically or selectively in different organs reveal that this modification is crucial for efficient Notch-ligand binding and Notch-mediated signaling. Although O-fucosylation has also been implicated in other pathways, there is substantial biochemical and genetic evidence to suggest that during development, the Pofut1 requirement is essentially circumscribed to the Notch pathway (Sasamura et al., 2007; Shi and Stanley, 2003; Okamura and Saga, 2008; Guilmot et al., 2008).

To perturb ligand-receptor interactions and block Notch-dependent events upstream of all transcriptional targets in the lung epithelium, we inactivated **Pofut1** using a conditional knockout approach in mice. Analysis of these mutants revealed a substantial differentiation defect, in which airways are completely devoid of the Clara cell secretory lineage and are overpopulated with ciliated cells and neuroendocrine cells. We show that this defect can be replicated by deleting the Notch transcriptional effector *Rbpjk* in the lung epithelium in vivo and by pharmacological disruption of Notch signaling in lung explant cultures. Our results support a major role for Notch in establishing the balance between ciliated and secretory cell fates during airway differentiation.

**MATERIALS AND METHODS**

**Animals and genotyping**

For the **Pofut1** model, **Pofut1**+/− mice (Shi and Stanley, 2003) were mated to *Shh*Cre/+ mice (Harfe et al., 2004) to generate **Pofut1**−/−;*Shh*Cre/+ offspring. **Pofut1**−/−;*Shh*Cre/+ were then crossed to **Pofut1**F/F (Shi et al., 2005) to generate mice with conditional **Pofut1** deletion of both (**Pofut1**+/−;*Shh*Cre/+ or one (**Pofut1**−/−;*Shh*Cre/+ and **Pofut1**−/−) allele, or with intact **Pofut1** alleles (**Pofut1**+/+). For the *Rbpjk* model, a similar approach was taken using *Shh*Cre/+;*Rbpjk*+/− and *Rbpjk*−/− mice (Han et al., 2002) (BioResource Center, Tsukuba, Japan) to generate conditional deletion of *Rbpjk*, as above. Mice were genotyped by PCR as described (Han et al., 2002; Harris et al., 2006; Shi et al., 2005). All protocols were approved by IACUC, Boston University School of Medicine.

**Lung organ cultures**

Litters containing the various genotypes were isolated at E11.5 from **Pofut1**F/F females (crossed to **Pofut1**+/−;*Shh*Cre/+ males) and dissected lungs were cultured for 48 hours in DMEM containing 1% fetal bovine serum (FBS) at 37°C, as previously described (Tsao et al., 2008). Embryonic tissue was collected for genotyping to allow comparisons between control and mutants animals at 40X magnification. For each mutant, ten fields were analyzed in three to five animals per group. At E14.5, only large
proximal airways were analyzed, as epithelial differentiation was restricted to this area. At E18.5, large (main/lobar bronchi), medium (second to third generation) and small (bronchioles down to terminal bronchiole) airways were analyzed. Data were represented as mean±s.e. Statistical analysis was performed using Student’s t-test; differences were significant at P<0.05. For the analysis of neuroendocrine cells, we counted Pgp9.5-positive cells in 40 random fields of E18.5 lung sections at 40× magnification in control and Pofut1 mutants (three animals per group) and the total number of labeled cells per group plotted as a bar chart.

RESULTS
Conditional deletion of Pofut1 disrupts Notch signaling and leads to neonatal death and lung defects

To investigate the role of Notch in the developing lung and circumvent the vascular defects and early embryonic lethality of the systemic deletion of Pofut1, we inactivated Pofut1 conditionally in the lung epithelium using a Shh-Cre deleter mouse line. Previous studies using Shh-Cre;R26R reporter mice have shown efficient Cre-mediated recombination in Shh-expressing tissues, including the respiratory field of the foregut endoderm and the lung epithelium (Harris et al., 2006; Harfe et al., 2004).

Genotyping of litters derived from Pofut1+/–;ShhCre/+ crossed to Pofut1F/F mice (n>30 altogether) at E11.5, E14.5, E18.5 and at birth (P0) revealed Pofut1F/F;ShhCre/+ (conditional deletion of both Pofut1 alleles, termed Pofut1cnull), Pofut1F/F;ShhCre/+ and Pofut1F/F (conditional deletion of a single Pofut1 allele) and Pofut1F/F (no deletion of Pofut1, termed control) offspring at an expected Mendelian distribution. Real-time PCR analysis of lung epithelial and mesenchymal tissues isolated by microdissection at E11.5 confirmed selective disruption of Pofut1cnull which showed that Pofut1 mRNA selectively in the epithelium of Pofut1cnull animals (Fig. 1A), consistent with previous studies using the Shh-Cre mice. Nevertheless, downregulation of Notch signaling, as suggested by a decrease in expression of Notch downstream targets, was observed consistently only from E14.5 onwards. At E14.5, when we could no longer separate individual lung layers, PCR analysis of whole-lung homogenates showed a substantial reduction in expression of Hes and Hey genes in the mutants (Fig. 1B). The residual expression seen for Pofut1 (~20%) and for Hes and Hey genes (~50%) in E14.5 Pofut1cnull whole lungs was likely to represent transcripts from the mesenchyme. Epithelial disruption of Notch signaling was further suggested by in situ hybridization, which showed that Hes1 signals were almost abolished in the airways of E18.5 Pofut1cnull mice (Fig. 1C–F).

Analysis of the Pofut1cnull mutants at birth showed pups that were grossly normal, although already smaller than their littermates. Within the initial 2-3 weeks of postnatal life the mutants failed to thrive (Fig. 2A) and died; in examining more than ten litters, only 1 out of 17 Pofut1cnull pups survived until P28. Mice carrying a single Pofut1 allele behaved identically to controls with respect to all features analyzed in our study (n>3 for all parameters). A detailed characterization of the postnatal phenotype of the Pofut1cnull mutants will be reported elsewhere. Preliminary analysis of these lungs at P7, P14 and P21 revealed a dramatic attenuation of the airway epithelium that was particularly obvious in medium-sized airways and in terminal bronchioles. By P21, instead of the typical bronchiolar epithelium of controls, Pofut1cnull lungs showed airways lined by a thin metaplastic squamous epithelium, with scattered cell debris and isolated foci of inflammatory cells, including macrophages (Fig. 2B,C). The overall changes were reminiscent of those reported in lungs injured by naphthalene exposure (Stripp et al., 1995; Park et al., 2006). Interestingly, these changes were not present in the lungs at birth or at prenatal stages. At E18.5, a cuboidal epithelium was clearly present in the airways of both groups (Fig. 2D,E). Thus, these changes occurred within the initial week of postnatal life. We concluded that Shh-Cre-mediated deletion of Pofut1 led to alterations in the lung and possibly other organs that are incompatible with early postnatal life.

Conditional deletion of Pofut1 does not perturb distal lung formation

Inhibition of Notch signaling in lung organ cultures has been shown to alter proximal-distal patterning and cell fate in airways undergoing branching morphogenesis (Tsao et al., 2008; Kong et al., 2004). We asked whether disrupting Pofut1 selectively in the lung epithelium resulted in similar defects. When we analyzed the lungs of Pofut1mice at E11.5, E14.5 and E18.5, we found no obvious differences in gross morphology, including size, compared with control lungs. Also, no difference in the branching pattern of airways was detected when E11.5 lungs from control and mutant embryos were cultured for 24 and 48 hours (see Fig. S1A-F in the supplementary material; data not shown). This was not surprising,

Fig. 1. Conditional deletion of Pofut1 and expression of Notch target genes in the mouse lung. (A) Real-time PCR of isolated E11.5 lung epithelium (Epi) and mesenchyme (Mes) shows a marked decrease in Pofut1 mRNA selectively in the epithelium of Pofut1cnull mutants relative to the control; however, expression of Notch targets, such as Hes1, is unchanged in both epithelium and mesenchyme. (B-F) Disruption of Notch signaling as suggested by downregulation of Pofut1 and Notch target genes (Hes1, Hes5, Hey1 and Hey2) in E14.5 whole-lung homogenates of Pofut1cnull mutants (B, real-time PCR), and by loss of Hes1 signals [arrowhead in controls (Ctr)] in the airway epithelium of E18.5 Pofut1cnull lungs (C-F, in situ hybridization). (C,D) Bronchus, (E,F) Bronchiole. Scale bar: 40 μm.
Pofut1 conditional deletion prevents the formation of Clara cells

Next, we investigated airway epithelial differentiation. In the developing murine airways, Clara cells can be recognized at E16.5 by expression of the Clara cell secretory product CC10 [also termed Scgb1a1 (Reynolds et al., 2002)]. We assessed CC10 expression by immunohistochemistry in control lungs at birth and at E18.5 and found the typical pattern of staining in secretory cells throughout the airways. Strikingly, no signals were detected in Pofut1\textsuperscript{cnull} mutants despite the preserved integrity of the lung epithelium (Fig. 3A-D). In situ hybridization confirmed that CC10 transcripts were also absent in these mutants (data not shown). We asked whether disruption of Notch interfered with the specification or survival of the Clara cell lineage in the airways of these mutants. First, we assessed the expression of claudin 10 (Cldn10) and secretoglobin 3A2 (Scgb3a2, also known as Ugrp1), two further markers of this lineage (Reynolds et al., 2002; Zemke et al., 2009; Kurotani et al., 2008). Immunostaining confirmed strong signals in secretory cells of E18.5 control lungs; however, as observed for CC10, both epitopes were absent from E18.5 mutants (Fig. 3E-H).

We explored the possibility that Clara cells could have been initially specified, but needed Notch signaling to survive. Comparison of the pattern of caspase 3 and TUNEL staining in E14.5, E18.5 and P0 lungs revealed no difference between control and mutants (data not shown), arguing against apoptosis as a mechanism contributing to the Pofut1\textsuperscript{cnull} phenotype. Thus, our data suggested that Pofut1 deletion in the epithelium severely impairs the capacity of proximal progenitors to differentiate into Clara cells.

We then investigated the possibility that disruption of Notch could have altered the secretory cell program and led Clara cell precursors to differentiate into mucin-producing goblet cells. Conditional inactivation of Pofut1 or Rbpjk in the intestinal epithelium is known to result in a marked increase in the goblet cell population (van Es et al., 2005; Guilmeau et al., 2008). Goblet cells are not normally present in the embryonic murine lung, but can be found postnatally in conditions that lead to inflammation (Evans et al., 2004; Rogers, 2003). We stained lung sections of E14.5 and E18.5 control and mutants with Periodic Acid Schiff (PAS) or Alcian Blue and found no evidence of mucin-producing cells (data not shown). However, similar analysis in tracheal sections from control and mutants sacrificed at birth revealed scattered PAS/Alcin Blue-positive cells in both groups, suggesting that goblet cell fate is not lost in the mutants (see Fig. 6C-F).

Conditional disruption of Rbpjk results in the same defects as in Pofut1\textsuperscript{cnull} mutants

We examined whether the inability to form Clara cells in the Pofut1\textsuperscript{cnull} mutant could be reproduced by disrupting Notch function in vivo in an independent fashion. We used Shh-Cre mice to delete expression of the crucial Notch canonical transcriptional effector Rbpjk (Rbpsuh) in the developing lung epithelium (Han et al., 2002) (see also Materials and methods). Analysis of mice in which both Rbpjk alleles were conditionally deleted (Rbpjk\textsuperscript{f/f};Shh\textsuperscript{Cre/+}) revealed at birth (n=6) and prenatally (E14.5, n=6; E18.5, n=8) a phenotype that was remarkably similar to that seen in the Pofut1\textsuperscript{cnull} lungs. No CC10- or Scgb3a2-expressing cells were detected in Rbpjk\textsuperscript{cnull} mice at E18.5 (Fig. 3I-L). Subsequent characterization of the Rbpjk\textsuperscript{cnull} phenotype revealed further similarities consistent with the Pofut1\textsuperscript{cnull} model for all parameters analyzed here.

Airways from Pofut1\textsuperscript{cnull} or Rbpjk\textsuperscript{cnull} mutants are overpopulated by ciliated cells

Next, we investigated the impact of Notch inactivation in the development of the ciliated cell lineage. During normal murine development, ciliated cells are first recognized in the trachea and lobar bronchi by morphological criteria and by β-tubulin expression
at ~E16 (Toskala et al., 2005; Rawlins et al., 2007). Ciliated cell differentiation proceeds in a proximal-to-distal pattern, as the lung matures. By E18.5, ciliated cells are present interspersed with secretory cells throughout the respiratory epithelium of airways from the trachea to the terminal bronchiole. We stained E18.5 lungs with a β-tubulin antibody and confirmed this pattern in controls (Fig. 4A,C). By contrast, airways of Pofut1<sup>−/−</sup> mutants seemed to almost exclusively comprise β-tubulin-expressing ciliated cells (Fig. 4B,D). The identity of these cells was further confirmed by immunostaining for Foxj1, a transcription factor crucial for acquisition of ciliated cell phenotype (Fig. 4E,F) (Brody et al., 2000). Analysis of Foxj1 in E18.5 Rbpjk<sup>−/−</sup> lungs revealed labeling in 80-85% of the epithelial cells at all levels in mutants. *, P<0.05. Scale bar: 15 μm. 

Fig. 4. Epithelial disruption of Notch signaling dramatically increases the number of ciliated cells in airways. (A-H) Immunohistochemistry for β-tubulin (A-D) and Foxj1 (E-H) shows a significant increase in labeling (arrowheads) in E18.5 airways from Pofut1<sup>−/−</sup> (A-F) and Rbpjk<sup>−/−</sup> (G,H) mice, compared with controls. (I) Quantification of Foxj1-positive cells in large, medium and small airways of E18.5 control and Pofut1<sup>−/−</sup> lungs reveals labeling in 80-85% of the epithelial cells at all levels in mutants. *, P<0.05. Scale bar: 15 μm.

Fig. 3. Epithelial disruption of Notch signaling ablates Clara cells in the airways. (A-D) Immunohistochemistry for CC10 at birth (P0) (A,B) and at E18.5 (C,D) shows typical cytoplasmic expression associated with Clara cells (red arrow) in control airways (A,C), but reveals no CC10 signals in the epithelium of Pofut1<sup>−/−</sup> lungs (B,D). (E-H) Cldn10 (E,F) and Scgb3a2 (G,H), used as additional markers for the secretory lineage, were also absent from Pofut1<sup>−/−</sup> lungs at E18.5 by immunostaining. (I-L) Lungs of Rbpjk<sup>−/−</sup> mice revealed the same defect, as shown by the absence of CC10 and Scgb3a2 signals in airways of E18.5 mutants (I,L), as compared with controls (I,K). White arrowheads indicate negative cells. Scale bar: 40 μm.
The aberrant epithelial differentiation of Pofut1<sup>−/−</sup> or Rbpjk<sup>−/−</sup> mutants is also reproduced by pharmacological inhibition of Notch in vitro

To provide additional evidence that abnormal epithelial differentiation is linked to Notch loss-of-function, we used a classic gamma-secretase inhibitor to disrupt Notch in a lung organ culture assay. We cultured wild-type lungs at ~E13.0 (instead of E11.5) in control and DAPT-containing (50 μM) media, and extended the culture period for 4 days (Weinmaster and Kopan, 2006; Tsao et al., 2008); this enabled the examination of differentiation events that normally occur at a relatively late stage in vitro. Analysis of these lungs confirmed the patterning abnormalities and decreased expression of Notch targets reported previously (data not shown) (Tsao et al., 2008). We then assessed the expression of markers of epithelial differentiation and found that in the proximal airways of DAPT-treated explants, the majority of the epithelium consisted of ciliated cells. In this group, Foxj1 immunostaining was present in almost all cells, in contrast to controls, in which positive and negative cells were interspersed in the airway epithelium (Fig. 5A,B,E,F). Foxj1-expressing cells showed phenotypic features of ciliated cells and expressed β-tubulin (Fig. 5C,G). By contrast, immunostaining for the secretory cell marker Scgb3a2 was almost negative in DAPT-treated lungs (Fig. 5D,H), consistent with the inhibition of the secretory cell program observed in our in vivo models. Thus, both in vitro and in vivo models strongly support a role for Notch in lung epithelial differentiation.

Effect of Notch disruption on the differentiation of other respiratory lineages

There is evidence that neuroendocrine (NE) differentiation is under Notch control in the lung epithelium. An excessive number of NE cells is found in mice deficient in the Notch effector Hes1 (Ito et al., 2000). Conversely, reduced NE cell number has been reported in transgenic mice expressing an activated Notch1 driven by the calcitonin (Cgrp; Calca) promoter (Shan et al., 2007). This prompted us to investigate whether NE differentiation was affected in lungs of Pofut1<sup>−/−</sup> mice. In the developing murine lung, NE cells are recognized at E16.5 by expression of the neural markers Cgrp and Pgp9.5 (Uch1) (reviewed by Linnolia, 2006). Immunostaining of control and mutant lungs at E14.5 did not reveal Cgrp or Pgp9.5 signals in the lung epithelium. However, at E18.5, NE cells were strongly labeled by these markers in both groups, and quantitative analysis of Pgp9.5 showed that more NE cells were present in the mutants (Fig. 6A,B) (control=3 and Pofut1<sup>−/−</sup>=14 Pgp9.5-labeled cells in 40 random fields, n=3 animals per group). The relative increase in NE cells correlated well with the Hes1 downregulation that we observed in these mutants at E18.5 (Fig. 1C-F), and is in agreement with the phenotype reported in Hes1-null lungs (Ito et al., 2000).

We also examined whether the distribution or number of basal cells was influenced by Pofut1 deficiency. Immunohistochemical analysis of p63, a marker of basal cells in the respiratory epithelium, did not reveal any difference between the control and Pofut1<sup>−/−</sup> mutants (Daniely et al., 2004) (data not shown). As described above and shown in Fig. 6C-F, disruption of Notch did not prevent goblet cells from forming in the trachea of mutants at birth. Together, our data implicate Notch in the balance between at least three distinct epithelial lineages in the embryonic lung.

The aberrant epithelial differentiation of Pofut1 and Rbpjk mutants is accompanied by a substantial decrease in cell proliferation

During organogenesis, Notch can regulate the balance between progenitor cell pools and their differentiating progenies (Radke and Raj, 2003; Okamura and Saga, 2008). To determine whether...
conditional disruption of Notch signaling influences the proliferation status of a particular pool of epithelial cells, we performed Ki67 immunostaining in lung sections of the Pofut1 mutants. In E18.5 controls, Ki67-labeled cells comprised ~25-30% of the epithelium at all airway levels (Fig. 7A-E). Ki67 signals were essentially associated with non-ciliated cells, consistent with previous reports (Fig. 7A,C) (McDowell et al., 1985; Otani et al., 1986; Rawlins et al., 2007). By contrast, Ki67 labeling was significantly reduced in the lung epithelium of E18.5 Pofut1null mutants relative to controls, and averaged 15%, 10% and 5% in large, medium and small airways, respectively (P<0.05) (Fig. 7E). By contrast, Ki67 labeling was significantly reduced in the lung epithelium of E18.5 Pofut1null mutants relative to controls, and averaged 15%, 10% and 5% in large, medium and small airways, respectively (P<0.05) (Fig. 7E). Analysis of E18.5 Rbpjknull mutants confirmed the changes in Ki67 seen in the Pofut1 model and showed an even more severe reduction in labeling (Fig. 7F-H). Presumably, this is a consequence of the large number of ciliated cells present in the mutants. In both Pofut1null and Rbpjknull mutants, we could not determine precisely what types of cells were proliferating. Most of them appeared undifferentiated, although some resembled NE cells or immature ciliated cells, as suggested by their morphology and marker analysis in serial sections. These results were further confirmed by PCNA immunostaining (not shown).

**Notch restricts commitment to a ciliated cell fate**

Next, we asked whether epithelial disruption of Pofut1 could have led to a precocious commitment to the ciliated cell program and to exhaustion of the progenitor cell pool required for secretory cell differentiation. To address this question, we assessed Foxj1 and
Ki67 expression in controls and Pofut1<sup>cnull</sup> mutants at E14.5, prior to ciliated differentiation, when commitment to a ciliated cell fate has just initiated in proximal airways (Rawlins et al., 2007). Foxj1 immunostaining revealed scattered epithelial labeling in the trachea and main bronchi of E14.5 controls (Fig. 8A). Interestingly, quantitative analysis of Foxj1 labeling in proximal airways (main and lobar bronchi) showed significantly more positive cells in E14.5 Pofut1<sup>cnull</sup> lungs than in controls at the same site (Fig. 8B,G). Nevertheless, there was no evidence that in the mutants, Foxj1-expressing cells were more mature (no β-tubulin detected) or that the ciliated cell program had advanced towards more distal airways. Ki67 epithelial labeling in proximal airways was essentially similar in E14.5 control and mutant lungs (Fig. 8C,D,G). Since previous BrdU studies in Foxj1-EGP mice have shown that ciliated cell precursors do not proliferate (Rawlins et al., 2007), the Ki67-positive cells we observed presumably represented uncommitted proximal progenitors that were still available for differentiation. Thus, at this stage, Notch does not seem to be crucial for the expansion of proximal progenitors.

We tested whether we could identify early precursors of the secretory lineage in this pool of E14.5 proximal progenitors. Although Cldn10 and Scgb3a2 have been reported in the lung at relatively early stages, their relationship to putative secretory cell progenitors prior to E16.5 remains elusive (Zemke et al., 2008; Kurotani et al., 2008). We stained E14.5 lungs with an anti-Cldn10 antibody and found signals throughout the entire airway epithelium, except for the distal buds, in both control and Pofut1<sup>cnull</sup> lungs (not shown). By contrast, Scgb3a2 immunostaining revealed strong labeling in discrete groups of proximal epithelial cells (Fig. 8E,F). This pattern differed greatly from the more uniform Cldn10 distribution and suggested that, at E14.5, Scgb3a2-positive cells could represent early secretory cell precursors. Quantitative analysis showed a trend towards a decrease in the number of Scgb3a2-expressing cells in the Pofut1<sup>cnull</sup> mutants, which, although not statistically significant, correlated inversely with the increase in the proportion of Foxj1-labeled cells (Fig. 8F,G). These data raise the possibility that although secretory cell differentiation is abrogated in Pofut1<sup>cnull</sup> mutants, the initial stages of the secretory cell program might take place in the absence of Notch. Interestingly, we have evidence that early markers of ciliated and secretory cell fate can be co-expressed at the onset of differentiation. For example, at E15.5, immunofluorescence/confocal analysis showed Foxj1/Scgb3a2 single- and double-labeled cells in proximal regions where this process occurs, whereas labeling was absent in more distal regions (Fig. 8H-J). Thus, our results suggest that Notch acts when commitment to a ciliated or non-ciliated cell fate takes place, restricting ciliated fate in these cells.

**Effect of Notch disruption on Sox2 expression**

Notch interactions with Sox family transcription factors have been shown to regulate developmental programs in a number of systems (Kiernas et al., 2006; Dabdoub et al., 2008; Okamura and Saga, 2008). In the developing lung, Sox2 is dynamically expressed in non-branching regions of the airways and marks cells initially committed to a proximal cell fate (Ishii et al., 1998; Que et al., 2007; Gontan et al., 2008). Pharmacological inhibition of Notch in the early lung interferes with the establishment of proximal cell fate, resulting in a substantial reduction in the Sox2 expression domain in the forming airways (Tsao et al., 2008).

We hypothesized that the Notch effects in proximal progenitors could be accomplished by changes in Sox2 that are potentially relevant to the Pofut1<sup>cnull</sup> phenotype. Sox2 immunohistochemistry in E14.5 control lungs showed signals throughout the respiratory epithelium, as consistent with previous reports, in ~65% of all epithelial cells (Fig. 9A). At E14.5, Sox2 labeling of Pofut1<sup>cnull</sup> and control lungs was essentially similar in both the pattern and number of labeled cells at all levels (*, P<0.05) in Pofut1<sup>cnull</sup> as compared with control lungs. Scale bar: 40 μm.
expressing progenitor cells retain their ability to proliferate and assume different proximal cell fates. This is in agreement with studies in the developing spinal cord showing that Sox2 maintains the proliferative capacity and the pan-neural properties of progenitor cells by inhibiting their terminal differentiation (Graham et al., 2003). Later, as a differentiation program that includes Notch signaling, including Hes1 targets.

**DISCUSSION**

Here we provide novel evidence from three distinct models that Notch signaling plays a major role in controlling the number of ciliated and secretory cells during airway differentiation. Targeted disruption of Notch-ligand interactions in Pofut1<sup>−/−</sup> mice, or targeted inactivation of Notch canonical signaling in Rbpjk<sup>−/−</sup> mice, resulted in a remarkably similar phenotype characterized by complete ablation of the Clara cell secretory lineage and airways overpopulated with ciliated cells. The presence of the same differentiation defect in the proximal airways of lung explants in which Notch cleavage was prevented by gamma-secretase inhibitor, further supports the idea that these defects resulted from abrogation of Notch signaling in the developing airway epithelium. Moreover, our findings support conclusions from previous reports implicating Hes1-mediated canonical Notch signaling in restricting NE differentiation (Ito et al., 2000; Collins et al., 2004; Shan et al., 2007). The phenotype we report is, however, more severe and involves multiple lineages, consistent with a broader disruption of Notch signaling, including Hes1 targets.

How crucial is Notch signaling for induction of the secretory lineage in the lung? The presence of Scgb3a2-labeled cells in Pofut1<sup>−/−</sup> lungs at early but not late developmental stages suggests that putative secretory cell precursors might be initially specified but cannot differentiate further in the absence of Notch. To confirm whether Scgb3a2-expressing cells in the E14.5 lung truly represent secretory cell precursors would require a lineage study, which is beyond the scope of the present work. Nevertheless, the substantial increase in the population of ciliated cells without any evidence of...
increased cell proliferation or cell death in our mutants supports the idea that, during normal development, Notch selectively suppresses the ciliated and the NE cell programs in proximal progenitors to allow secretory cell differentiation. This is consistent with observations reported in other developing systems, in which Notch also plays a prominent role in balancing different cell fates (Hayes et al., 2007; Stubbs et al., 2006; Deblandre et al., 1999). For example, functional studies in the Xenopus embryo suggest that initially, all ectodermal cells of the layer that gives rise to the ciliated cells in the epidermis express a factor that is instructive to the ciliated cell program. The role of Notch is to silence this factor in a subset of cells that is not permitted to differentiate into the ciliated phenotype. This appears to involve a classical mechanism of Notch-mediated lateral inhibition in which transient expression of the ligand Delta selectively in ciliated cell precursors prevents them from activating Notch. Meanwhile, Notch activity in neighboring cells fosters non-ciliated cell differentiation. Disruption of canonical Notch signaling in this system results in a dramatic increase in the number of ciliated cells, as in our mouse mutants. Conversely, constitutive activation of Notch signaling in the Xenopus skin by expression of a Notch intracellular domain inhibits ciliated cell differentiation (Deblandre et al., 1999).

Evidence of a similar mechanism controlling ciliated cell differentiation during zebrafish kidney development and neuronal differentiation during Xenopus neurogenesis suggests that this role of Notch has been highly conserved (Liu et al., 2007; Wettstein et al., 1997). How does this relate to the mammalian lung? Interestingly, the overall mechanism controlling ciliated cell fate in the developing Xenopus skin seems to be analogous to the one we have described for the mouse lung epithelium. In both cases, expression of a Notch ligand in progenitor cells is associated with the ciliated cell phenotype. However, our data suggest that instead of Dll1, the most probable ligand implicated in this process is Jag1. Future studies will explore these observations further.

The function of Clara cells is not fully understood, in part owing to the lack of adequate experimental models. Much of what is currently known about the role of Clara cells in airway homeostasis has been inferred from analysis of CC10-null mice. These mice appear to phenocopy pathological aspects described in conditions such as chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis (Stripp et al., 2002). However, these studies focused on CC10 instead of on the Clara cell itself.

Although we have not focused on postnatal development, our model nevertheless offered a unique opportunity to observe the impact of a lack of Clara cells on airway homeostasis during the initial weeks of life. We found histological evidence of epithelial damage and an abnormal adaptive response of the epithelium to the postnatal environment in Pofut1cnull lungs. A striking feature of these airways was the flattening of the epithelium, which is reminiscent of the metaplastic changes reported in naphthalene-injured lungs after Clara cell ablation (Stripp et al., 1995; Rawlins and Hogan, 2006). In the naphthalene model, the squamous metaplastic cells derived from the ciliated cells spread beneath the injured Clara cells to maintain the integrity of the airway epithelium and eventually lead to repair (Park et al., 2006). Analysis of CC10-null mice shows that loss of CC10 by itself may already lead to extensive epithelial damage if animals are exposed to ozone (Johnston et al., 1999). We speculate that the absence of Clara cells in our mutants results in changes in the epithelium and its lining fluid that greatly increase its susceptibility to damage by environmental agents such as oxygen.

In conclusion, our study provides strong in vivo evidence that Notch signaling is crucial to balance different cellular phenotypes in the developing airway. It is noteworthy that the differentiation profile of the airway epithelium can be profoundly altered in the adult lung under chronic inflammatory conditions. Thus, it is possible that changes in Notch might also play a major role in pathological responses of the respiratory epithelium in conditions such as asthma and COPD. Consistent with this hypothesis, a recent analysis of the airway transcriptome in human subjects has shown substantial differences in the expression of Notch components associated with smoking and COPD (Tilley et al., 2009).

**Note added in proof**

Since completion of this article, a complementary Notch gain-of-function approach has been published showing aberrant formation of mucous cells and decreased ciliated cells (Guseh et al., 2009). We thank Pamela Stanley, Tasuku Honjo and Cliff Tabin for the mouse mutants; Steven Brody, G. Singh, S. Katyal and Shiko Kimura for providing valuable antibodies; Felicia Chao and Mary Williams for thoughtful discussions; and Leah Cushing, Fengzhi Shao and Ann Hinds for their help in performing some of the experiments. This work was supported by grants from NIH/NHLBI (PO1 HL47049 to W.V.C.) and by a NHRI-Taiwan-Physician Scientist Award (PS9402 to P.-N.T.). Deposited in PMC for release after 12 months.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/13/2297/DC1

**References**


Notch regulates lung progenitor cell differentiation


Supplementary Figure 1
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