

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

Po-Nien Tsao^{1,3,*}, Michelle Vasconcelos^{1,4,*}, Konstantin I. Izvolsky¹, Jun Qian¹, Jining Lu¹ and Wellington V. Cardoso^{1,2,†}

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; Guilmeau et al., 2008; Murtaugh 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).

¹Pulmonary Center, Department of Medicine, and ²Department of Pathology, Boston University School of Medicine, Boston, MA 02118, USA. ³Department of Pediatrics, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan. ⁴Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP 05508-900, Brazil.

*These authors contributed equally to this work

†Author for correspondence (e-mail: wcardoso@bu.edu)

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; Guilmeau 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*^{F/F};*Shh*^{Cre/+}) or one (*Pofut1*^{F/+};*Shh*^{Cre/+} and *Pofut1*^{F/-}) allele, or with intact *Pofut1* alleles (*Pofut1*^{F/+}). For the *Rbpjk* model, a similar approach was taken using *Shh*^{Cre/+}, *Rbpjk*^{+/-} and *Rbpjk*^{F/F} 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 mutant lungs. In another experiment, lungs from CD1 wild-type embryos were isolated at E13.5 and cultured for 4 days in media containing DAPT {N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester} (50 μM, Sigma) or DMSO (control). Cultured lung explants were fixed with 4% paraformaldehyde in PBS overnight at 4°C and processed for immunohistochemistry. At least three explants per group (control, mutant) were analyzed.

Microdissection of lung mesenchyme and epithelium

E11.5 lungs (control, mutant) were isolated from litters of *Pofut1*^{F/F} females (crossed to *Pofut1*^{+/-};*Shh*^{Cre/+} males), incubated in Dispase (B&D; diluted 1:3 in PBS) for 12 minutes at 37°C, then washed with PBS three times and microdissected with fine forceps to separate the epithelium from the mesenchyme. Tissues were transferred to Eppendorf tubes containing RNAlater Stabilization Reagent (Qiagen #1017980) and processed for real-time PCR analysis of *Pofut1* and *Hes* and *Hey* genes (see below). Embryonic tissue was collected for genotyping. Mesenchymal contamination of epithelial tissues was assessed by expression of a collagen gene (*Colla2*).

Riboprobe synthesis and in situ hybridization

In situ hybridization was performed on 10 μm paraffin or frozen sections of lungs from control and mutants (*n*>3 each) using digoxigenin-UTP-labeled *Notch1*, *Jag1*, *Dll1* and *Hes1* riboprobes, as previously described (Lü et al.,

2004; Tsao et al., 2008). The DNA template for the mouse *Hes1* antisense riboprobe was generated by PCR using primers carrying T7 promoter sequences (forward, 5'-TAATACGACTCACTATAGGGGTCGTCAGAGAGAGGT-3' and reverse, 5'-AATTAACCCTCACTAAAGGGTT-CAGCGAGTGCATGAACGA-3'), which produces a PCR product of 568 bp. In some experiments, sections were briefly postfixed and immunohistochemistry was performed on the same sections as for colocalization studies.

Immunohistochemistry

Immunohistochemistry was performed on 5 μm paraffin sections of lungs from control and mutants using the ABC or M.O.M. Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. When necessary, antigen retrieval was performed using Unmasking Solution (Vector Laboratories #H-3300). Sections were incubated with primary antibody at 4°C overnight, biotinylated anti-mouse IgG reagent for 10 minutes at room temperature, then with DAB (3,3'-diaminobenzidine tetrahydrochloride) as the chromogen. Sections were counterstained with Methyl Green for 2 minutes (Tacs #4828-30-18), dehydrated and mounted (media from Shandon, #9999120). The following primary antibodies were used: anti-Scgb3a2 (gift from Dr S. Kimura, NIH), anti-Cldn10 (Zymed #415100), anti-CC10 (gift from Dr Singh and Dr Katyal, University of Pittsburgh), anti-Sox2 (Chemicon #AB5603), anti-Foxj1 (gift from Dr S. Brody, Washington University), anti-β-tubulin (Biogenex #MU178-UC), anti-p63 (Santa Cruz #4E4), anti-Pgp9.5 (Dako #25116), anti-Cgrp (Sigma #C8198), anti-Titf1 (Dako #M3575) and anti-T1alpha (mab 8.1.1, Developmental Studies Hybridoma Bank, University of Iowa). Cell proliferation was assessed using anti-Ki67 (B&D #550609) and the PCNA Staining Kit (Zymed #93-1143). Cell death was investigated by caspase 3 (R&D #AF835) and TUNEL (Apoptosis Detection Kit, Chemicon #S7107) analyses. Immunofluorescence was performed using secondary antibodies conjugated to Alexa Fluor 488 or 598 (Molecular Probes) and analyzed using a Zeiss confocal laser-scanning microscope (LSM510 META), as previously described (Chen et al., 2007; Tsao et al., 2008). Conclusions were based on the analysis of more than three animals per group.

Periodic Acid Schiff (PAS) and Alcian Blue staining

We used the PAS Staining Kit (Sigma) on lung sections from E14.5, E18.5 and neonatal (P0) mice. Control and mutant lungs were preincubated with or without amylase (10 minutes) to distinguish mucin from glycogen, as described in the manufacturer's protocol. We also performed Alcian Blue pH 2.5 staining by incubating sections in 3% acetic acid (3 minutes) and then Alcian Blue (40 minutes at room temperature), counterstaining with Hematoxylin.

Quantitative real-time PCR

Total RNA was isolated from lung tissue from control and mutant mice using the RNeasy Mini Kit (Qiagen #74104) and reverse-transcribed (RT) using Superscript III (Invitrogen #18080-051). We used an ABI 7000 instrument (Applied Biosystems, Foster City, CA, USA) to perform quantitative RT-PCR as previously described (Tsao et al., 2008; Chen et al., 2007). Primers (*Hes1*, *Hes5*, *Hey1*, *Hey2*, *Pofut1*, *Colla2* and beta-actin) were obtained from Assays-on-Demand (Applied Biosystems, Austin, TX, USA). Reactions (25 μl) were performed using the TaqMan Gene Expression Assay (Applied Biosystems, Austin, TX, USA). The relative concentration of the RNA for each gene to beta-actin mRNA was determined using the equation 2^{-DCT}, where DCT=(CT mRNA - CT beta-actin RNA).

Lung histology

High-resolution JB-4 plastic sections (2 μm) were generated from tissue fixed in 2% glutaraldehyde, 1% paraformaldehyde, 0.15 M sodium cacodylate buffer and stained with Toluidine Blue (Ramirez et al., 2003).

Morphometric analyses

The percentage of labeled epithelial cells immunostained for Foxj1, Ki67, Sox2 and Scgb3a2 was determined by counting cells in lung sections from control and mutants animals at 40× magnification. For each marker, 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 \pm 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 \times 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*^{+/-}; *Shh*^{Cre/+} crossed to *Pofut1*^{F/F} mice ($n > 30$ altogether) at E11.5, E14.5, E18.5 and at birth (P0) revealed *Pofut1*^{F/-}; *Shh*^{Cre/+} (conditional deletion of both *Pofut1* alleles, termed *Pofut1*^{cnul}), *Pofut1*^{F/+}; *Shh*^{Cre/+} and *Pofut1*^{F/-} (conditional deletion of a single *Pofut1* allele) and *Pofut1*^{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 *Pofut1* in the epithelium of *Pofut1*^{cnul} 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 *Pofut1*^{cnul} 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 *Pofut1*^{cnul} mice (Fig. 1C-F).

Analysis of the *Pofut1*^{cnul} 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 *Pofut1*^{cnul} 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 *Pofut1*^{cnul} 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, *Pofut1*^{cnul} 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

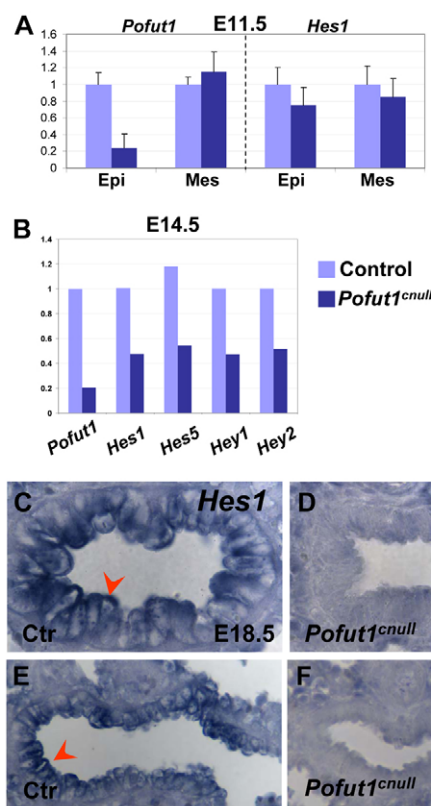


Fig. 1. Conditional deletion of *Pofut1* and expression of Notch target genes in the mouse lung. (A) Real-time PCR of isolated E11.5 lung epithelium (Epi) and mesenchyme (Mes) shows a marked decrease in *Pofut1* mRNA selectively in the epithelium of *Pofut1*^{cnul} mutants relative to the control; however, 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 *Pofut1*^{cnul} mutants (B, real-time PCR), and by loss of *Hes1* signals [arrowhead in controls (Ctr)] in the airway epithelium of E18.5 *Pofut1*^{cnul} lungs (C-F, in situ hybridization). (C,D) Bronchus, (E,F) Bronchiole. Scale bar: 40 μ m.

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 *Pofut1*^{cnul} mice 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,

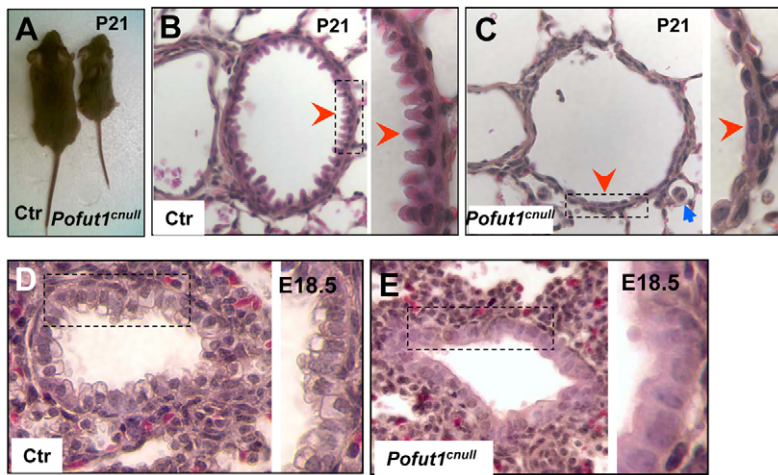


Fig. 2. Postnatal abnormalities of *Pofut1*^{cnul} mice. (A) Mutants fail to thrive and by P21 are much smaller than their control littermates. (B–E) Lung histology (Hematoxylin and Eosin staining) at P21 showing control airways lined by typical cuboidal epithelium (B, arrowhead). Instead, *Pofut1*^{cnul} lungs show airways with a metaplastic squamous epithelium (C, red arrowhead), scattered cell debris and isolated macrophages (blue arrow). At E18.5, a cuboidal epithelium is present in airways of both control (D) and mutant (E) lungs. The boxed areas are shown at higher magnification in the panels to the right.

given the relatively late disruption of Notch signaling in our system as compared with the *in vitro* models mentioned above, and also because we have not disrupted Notch signaling in the mesenchyme, which harbors Notch-responsive patterning genes (Tsao et al., 2008). Moreover, distal differentiation, including formation of alveolar sacs and type I and type II cells, was not affected by *Pofut1* deletion, as assessed by morphological analysis of Hematoxylin and Eosin-stained sections, high-resolution plastic sections and by immunohistochemical assessment of markers such as *Titf1* (thyroid transcription factor 1), *T1alpha* (*Pdpn*) and surfactant-associated protein C (*Sftpc*) (see Fig. S1G–P in the supplementary material). These observations are in agreement with previous *in vitro* data suggesting that Notch is not required for induction of a distal program of differentiation in lung progenitors (Tsao et al., 2008). Interestingly, constitutive activation of Notch *in vivo* in distal progenitors is actually deleterious, as it prevents further differentiation of the distal epithelium (Dang et al., 2003).

***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*^{cnul} 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*^{cnul} 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/Alcian 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*^{cnul} mutants

We examined whether the inability to form Clara cells in the *Pofut1*^{cnul} 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*^{F/F}; *Shh*^{Cre/+}, termed *Rbpjk*^{cnul}) 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*^{cnul} lungs. No CC10- or *Scgb3a2*-expressing cells were detected in *Rbpjk*^{cnul} mice at E18.5 (Fig. 3I–L). Subsequent characterization of the *Rbpjk*^{cnul} phenotype revealed further similarities consistent with the *Pofut1*^{cnul} model for all parameters analyzed here.

Airways from *Pofut1*^{cnul} or *Rbpjk*^{cnul} 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

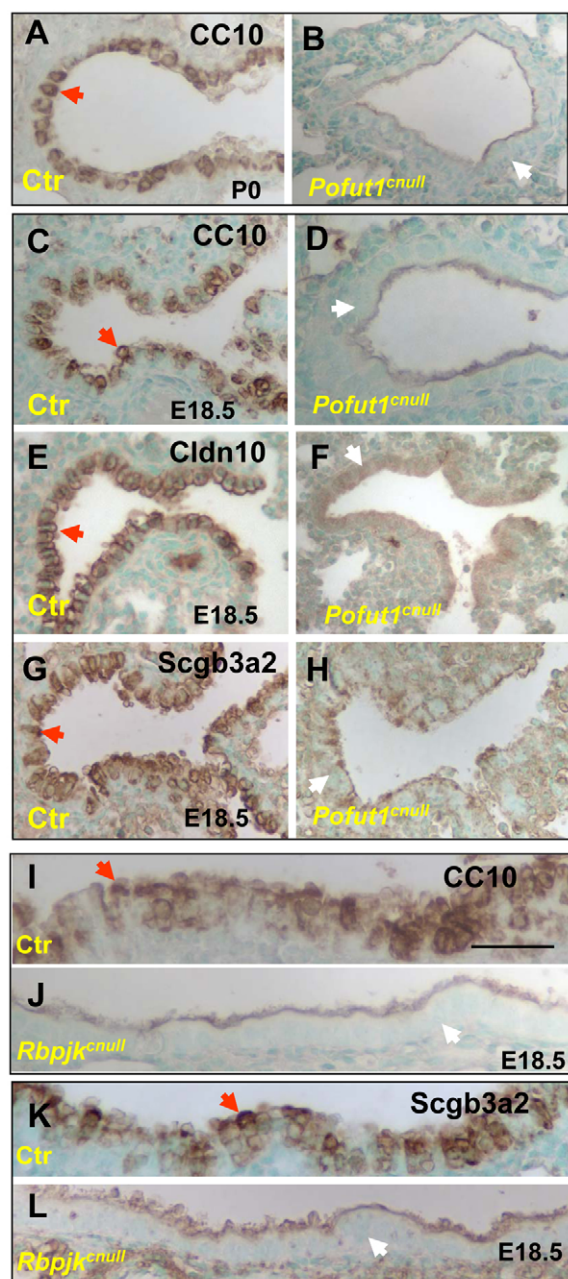


Fig. 3. Epithelial disruption of Notch signaling ablates Clara cells in the airways. (A-D) Immunohistochemistry for CC10 at birth (P0) (A,B) and at E18.5 (C,D) shows typical cytoplasmic expression associated with Clara cells (red arrow) in control airways (A,C), but reveals no CC10 signals in the epithelium of *Pofut1*^{null} 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*^{null} lungs at E18.5 by immunostaining. (I-L) Lungs of *Rbpjk*^{null} mice revealed the same defect, as shown by the absence of CC10 and Scgb3a2 signals in airways of E18.5 mutants (J,L), as compared with controls (I,K). White arrowheads indicate negative cells. Scale bar: 40 μ m.

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

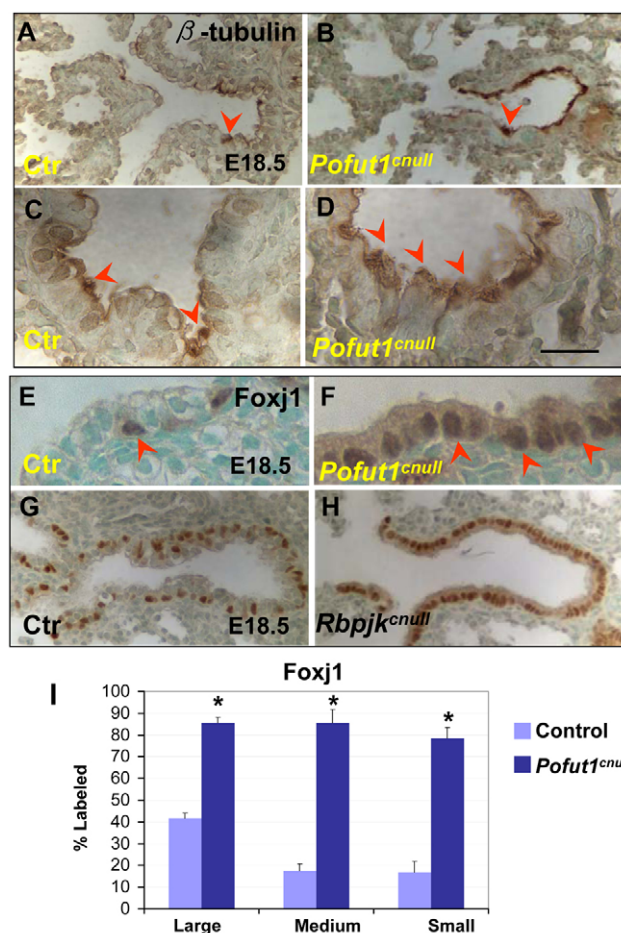


Fig. 4. Epithelial disruption of Notch signaling dramatically increases the number of ciliated cells in airways.

(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*^{null} (A-F) and *Rbpjk*^{null} (G,H) mice, compared with controls. (I) Quantification of Foxj1-positive cells in large, medium and small airways of E18.5 control and *Pofut1*^{null} lungs reveals labeling in 80-85% of the epithelial cells at all levels in mutants. *, $P < 0.05$. Scale bar: 15 μ m.

with a β -tubulin antibody and confirmed this pattern in controls (Fig. 4A,C). By contrast, airways of *Pofut1*^{null} 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*^{null} lungs revealed the same defect (Fig. 4G,H). We asked whether regional differences in the number of ciliated cells normally found throughout the different airway generations could still be observed in *Pofut1*^{null} mutants. Quantitative analysis (Fig. 4I) showed that in E18.5 controls, the percentage of Foxj1-expressing cells averaged 40%, 18% and 17% of the epithelial cells present in the large, medium and small airways, respectively ($n=3-4$). This contrasted sharply with *Pofut1*^{null} lungs, in which Foxj1-positive cells comprised nearly 80% of the population of the airway epithelium, regardless of the airway generation ($n=3$). Thus, Notch signaling might play an important role in the mechanism that controls the number of ciliated cells present in different generations of the developing airways.

The aberrant epithelial differentiation of *Pofut1^{cnul}* or *Rbpjk^{cnul}* 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 μ M) 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

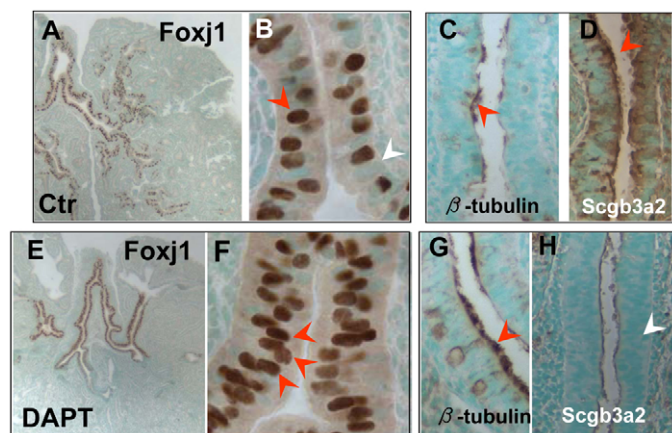


Fig. 5. Pharmacological inhibition of Notch reproduces the aberrant differentiation pattern of the mutants. Wild-type E13.5 mouse lungs cultured for 4 days in (A–D) control and (E–H) DAPT-containing (50 μ M) media. (A,B) Foxj1 immunohistochemistry shows labeling in the proximal epithelium of control lungs (red arrowhead), alternating with negative cells (white arrowhead). (C,D) Expression of β -tubulin (C) and Scgb3a2 (D) further suggests a balanced distribution of ciliated and secretory cells in controls. (E–H) In DAPT-treated lungs, almost all cells in the proximal airways are labeled for Foxj1 (E,F) and β -tubulin (G), but not for Scgb3a2 (H).

Pofut1^{cnul} mice. In the developing murine lung, NE cells are recognized at E16.5 by expression of the neural markers *Cgrp* and *Pgp9.5* (Uchl1) (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^{cnul}*=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^{cnul}* mutants (Danieli 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

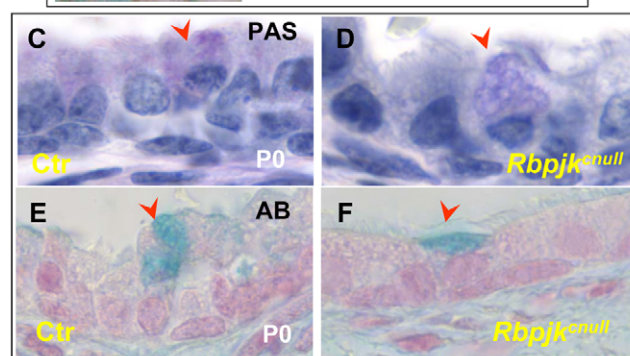
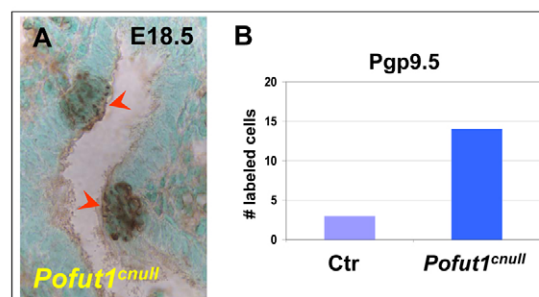


Fig. 6. Effects of Notch disruption on the differentiation of other respiratory lineages. (A,B) Increased number of neuroendocrine (NE) cells in the airways of *Pofut1^{cnul}* mice as shown by *Pgp9.5* immunostaining (A, arrowheads). (B) The total number of *Pgp9.5*-positive cells in 40 random fields of E18.5 lung sections at 40 \times magnification of control and *Pofut1^{cnul}* mutants ($n=3$ animals per group). (C–F) Tracheal sections from control and *Rbpjk^{cnul}* mutants at birth (P0) showing scattered PAS (C,D) and Alcian Blue (AB) (E,F) stained cells in both groups, suggesting that goblet cell fate is not lost in the mutants.

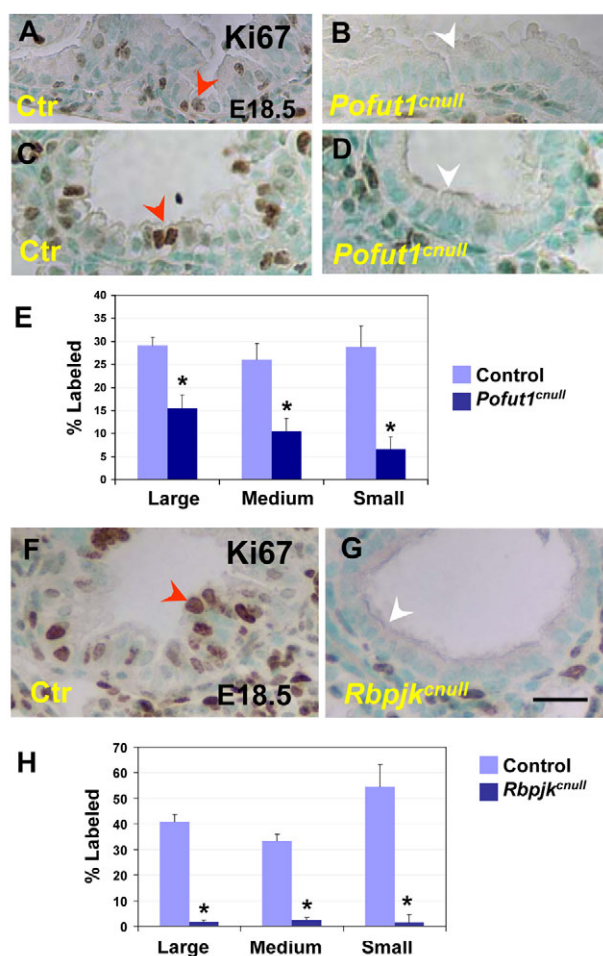


Fig. 7. Aberrant differentiation is accompanied by decreased epithelial proliferation in mutant lungs. Ki67 immunostaining of mouse lungs from E18.5 controls, *Pofut1^{null}* (A-E) and *Rbpjk^{null}* (F-H) shows a significant decrease in the number of labeled epithelial cells (red arrowhead) in large, medium and small airways of mutants, as compared with the respective controls, suggesting a substantial decrease in cell proliferation in both mutants. Bar charts show Ki67-labeled cells in the airway epithelium only; *, $P < 0.05$. Scale bar: 40 μ m.

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 *Pofut1^{null}* 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 *Rbpjk^{null}* 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 *Pofut1^{null}* and *Rbpjk^{null}* 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

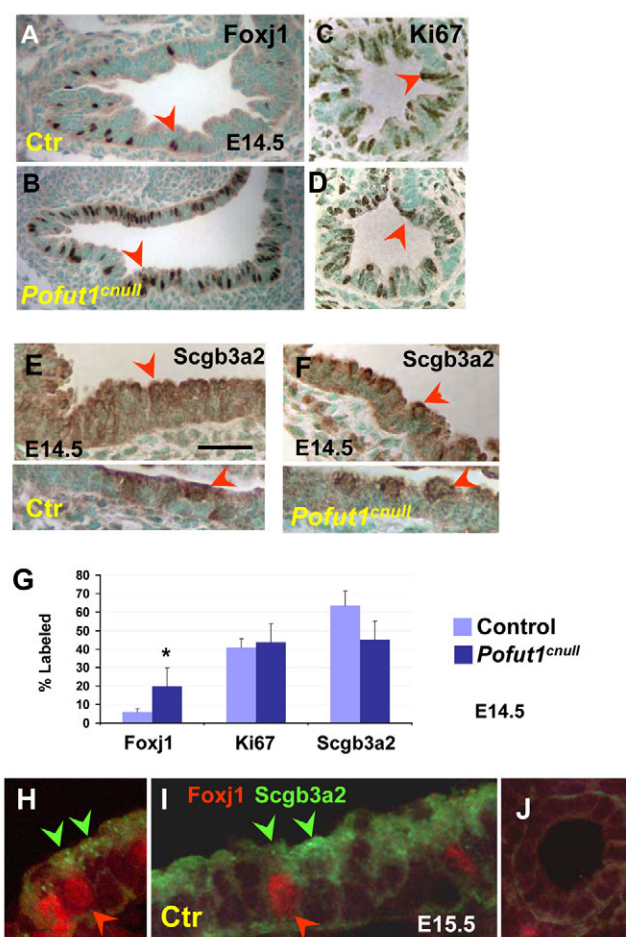


Fig. 8. Early differentiation events in *Pofut1^{null}* mouse lungs. (A,B) At E14.5, Foxj1 immunostaining (arrowhead) is present in scattered ciliated cell precursors that are restricted to the proximal airways of the control (A); labeling is increased in the same region of *Pofut1^{null}* lungs (B). (C,D) Ki67 labeling of the proximal airway epithelium appears similar in E14.5 control (C) and *Pofut1^{null}* (D) lungs. (E,F) Immunohistochemistry for Scgb3a2 at E14.5 shows strong signals in discrete epithelial cells of the proximal airways (arrowheads) in both control (E) and mutant (F), suggesting the presence of secretory cell precursors. (G) Quantitative analysis of epithelial labeling for the three epitopes in E14.5 control and *Pofut1^{null}* proximal airways. In the mutant, there is a significant increase in the percentage of Foxj1-labeled cells (*, $P < 0.05$), a trend towards a decrease in the percentage of Scgb3a2 labeling, and no change in the percentage of Ki67 labeling. (H-J) Double immunofluorescence for Foxj1 (red arrowhead) and Scgb3a2 (green arrowhead) in E15.5 controls shows overlapping and non-overlapping signals (H,I) in airways undergoing commitment to a ciliated or secretory cell fate. No expression is found in distal airways at this time (J). Scale bar: 50 μ m.

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

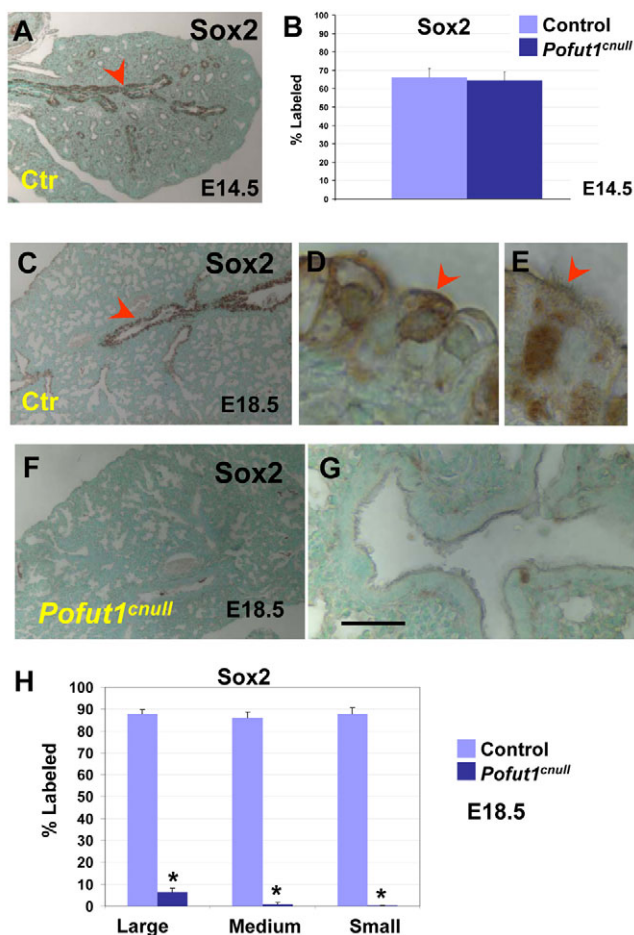


Fig. 9. Effect of *Pofut1* deletion on Sox2 expression. (A) Sox2 immunohistochemistry in E14.5 control mouse lungs shows typical expression throughout the airway epithelium, excluding the distal buds. (B) Quantitative analysis at E14.5 shows Sox2 labeling in ~65% of epithelial cells in both control and *Pofut1^{cnul}* lungs. (C-E) In E18.5 controls, Sox2 is expressed (arrowhead) in the airway epithelium mostly in non-ciliated cells (D), although labeling in scattered ciliated cells can be found in the proximal airways (E). (F,G) In E18.5 *Pofut1^{cnul}* lungs, almost no Sox2 expression is found. (H) Quantitation of Sox2-positive cells in large, medium and small airways at E18.5 shows a substantial reduction in the number of labeled cells at all levels (*, $P < 0.05$) in *Pofut1^{cnul}* as compared with control lungs. Scale bar: 40 μ m.

Ki67 expression in controls and *Pofut1^{cnul}* 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^{cnul}* 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^{cnul}* 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^{cnul}* 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^{cnul}* 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 (Kiernan 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 accompanied by changes in Sox2 that are potentially relevant to the *Pofut1^{cnul}* 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^{cnul}* and control lungs was essentially similar in both the pattern and number of labeled cells (Fig. 9A,B). This suggested that Notch is not required to induce or maintain Sox2 expression in proximal progenitors, at least up to E14.5. By contrast, in E18.5 *Pofut1^{cnul}* mutants, Sox2 expression was almost abolished in the lung (Fig. 9F-H). In E18.5 controls, Sox2 was detected mostly in non-ciliated cells, although a few ciliated cells in the trachea and large airways were also labeled (Fig. 9C-E). In mutants, by contrast, only rare Sox2-expressing cells could be seen in the large airways and these appeared to be ciliated or immature cells.

The changes in the Sox2 expression pattern resembled those seen for Ki67 both at E14.5 (Fig. 9B; Fig. 8G) and E18.5 (Fig. 9H; Fig. 7E, compare with 7H). We speculate that in E14.5 airways, Sox2-

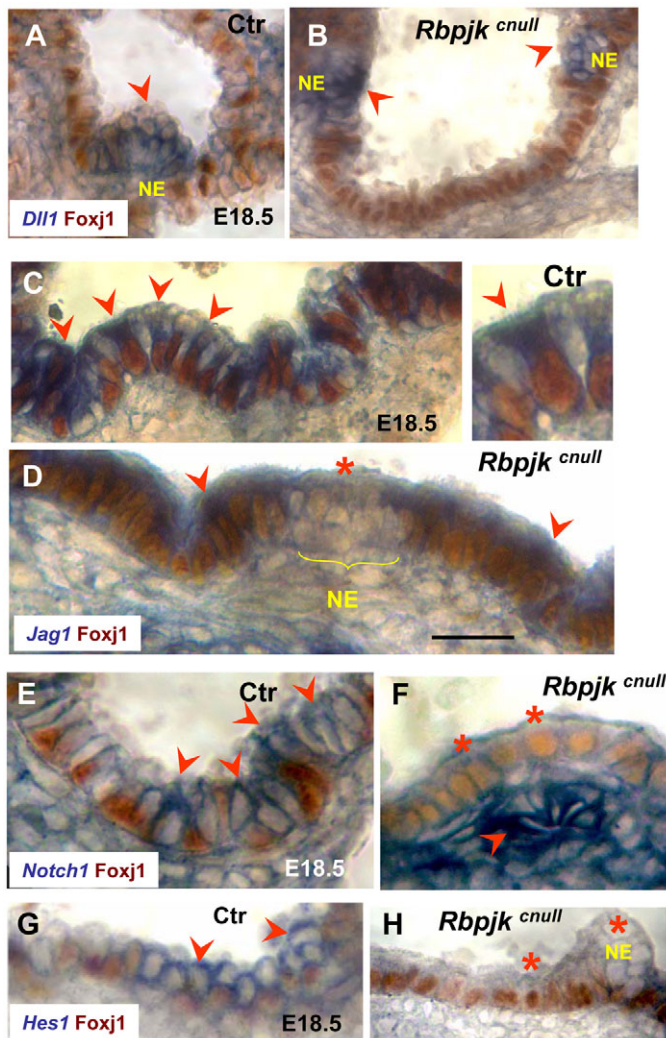


Fig. 10. Notch pathway components in the developing airway epithelium. Double immunohistochemistry for Foxj1 (brown, nuclear staining) and in situ hybridization (blue, cytoplasmic staining, arrowheads) for (A,B) *Dll1*, (C,D) *Jag1*, (E,F) *Notch1* and (G,H) *Hes1* in E18.5 control (A,C,E,G) and *Rbpjk^{null}* (B,D,F,H) mouse lungs. *Dll1* expression in the airway epithelium is restricted to neuroendocrine (NE) cells (A,B), whereas *Jag1* is largely associated with Foxj1-expressing cells in both control (C, inset) and mutants (D, note the abundant *Jag1*/Foxj1 double labeling). Conversely, *Notch1* and *Hes1* are preferentially expressed in Foxj1-negative cells, which are presumably secretory cells (E,G). In mutants, epithelial expression of *Notch1* (F) and *Hes1* (H) is greatly decreased or even abolished (asterisk); compare with the strong *Notch1* signals in the mesenchyme (arrowhead in F). Scale bar: 30 μ m.

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 takes place in the airway, Sox2 expression may be silenced in cells that are differentiating into ciliated cells and NE cells, while remaining in others, such as Clara cells. This mechanism could help establish the proper balance of differentiated cell profiles in

different generations of airways. Presumably, Sox2 expression may still be seen in less mature ciliated cells, as suggested by the analysis of our mutants.

Aberrant differentiation is associated with abnormal expression of Notch components

Data from a variety of developing systems suggest that selective expression of a Notch receptor or ligand might define the Notch activation status and fate of a given cell (Radtke and Raj, 2003). There is evidence that NE cells in the developing airways selectively express the ligand *Dll1*, but not *Notch1* or *Hes1*, and that upon disruption of Notch or *Hes1* signaling the NE cells expand (Beckers et al., 1999; Post et al., 2000; Ito et al., 2000; van Tuyl et al., 2005). We asked whether the expansion of the ciliated cell population observed in our mutants was associated with noticeable changes in the expression of Notch pathway components. We performed double in situ hybridization/immunohistochemistry in E18.5 control and *Rbpjk^{null}* lungs using probes for different Notch components and an anti-Foxj1 antibody. In both groups, *Dll1* expression did not overlap with that of Foxj1 and was restricted to NE cells (Fig. 10A,B). By contrast, *Jag1* was expressed by ciliated cells, in some cases in a salt-and-pepper pattern, and was absent from secretory cells in control lungs. Remarkably, this salt-and-pepper pattern was abolished in mutants and nearly all epithelial cells became *Jag1* positive, consistent with their identity as ciliated cells (Fig. 10C,D). Neither *Jag1* nor Foxj1 signals were present in NE cells. Interestingly, in controls, *Notch1* and *Hes1* were expressed at the highest levels in Foxj1-negative cells, whereas in mutants the expression of both genes was greatly downregulated or nearly absent (Fig. 10E-H). Together, the data suggest that Notch signaling might control the balance of cell fate in the developing airways by generating differences in the levels of receptor/ligand among epithelial progenitor cells.

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^{null}* mice, or targeted inactivation of Notch canonical signaling in *Rbpjk^{null}* 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^{null}* 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 *Pofut1^{cnul}* 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 Chen 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

- Beckers, J., Clark, A., Wunsch, K., Hrabé De Angelis, M. and Gossler, A. (1999). Expression of the mouse Delta1 gene during organogenesis and fetal development. *Mech. Dev.* **84**, 165-168.
- Brody, S. L., Yan, X. H., Wuerffel, M. K., Song, S. K. and Shapiro, S. D. (2000). Ciliogenesis and left-right axis defects in forkhead factor Hfh-4-null mice. *Am. J. Respir. Cell Mol. Biol.* **23**, 45-51.
- Cardoso, W. V. and Lü, J. (2006). Regulation of early lung morphogenesis: questions, facts and controversies. *Development* **133**, 1611-1624.
- Chen, F., Desai, T. J., Qian, J., Niederreither, K., Lü, J. and Cardoso, W. V. (2007). Inhibition of Tgf beta signaling by endogenous retinoic acid is essential for primary lung bud induction. *Development* **134**, 2969-2979.
- Collins, B. J., Kleeberger, W. and Ball, D. W. (2004). Notch in lung development and lung cancer. *Semin. Cancer Biol.* **14**, 357-364.
- Dabdoub, A., Puligilla, C., Jones, J. M., Fritzsche, B., Cheah, K. S., Pevny, L. H. and Kelley, M. W. (2008). Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. *Proc. Natl. Acad. Sci. USA* **105**, 18396-18401.
- Dang, T. P., Eichenberger, S., Gonzalez, A., Olson, S. and Carbone, D. P. (2003). Constitutive activation of Notch3 inhibits terminal epithelial differentiation in lungs of transgenic mice. *Oncogene* **22**, 1988-1997.
- Daniely, Y., Liao, G., Dixon, D., Linnoila, R. I., Lori, A., Randell, S. H., Oren, M. and Jetten, A. M. (2004). Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium. *Am. J. Physiol. Cell Physiol.* **287**, 171-181.
- Deblandre, G. A., Wettstein, D. A., Koyano-Nakagawa, N. and Kintner, C. (1999). A two-step mechanism generates the spacing pattern of the ciliated cells in the skin of *Xenopus* embryos. *Development* **126**, 4715-4728.
- Evans, C. M., Williams, O. W., Tuvim, M. J., Nigam, R., Mixides, G. P., Blackburn, M. R., DeMayo, F. J., Burns, A. R., Smith, C., Reynolds, S. D. et al. (2004). Mucin is produced by clara cells in the proximal airways of antigen-challenged mice. *Am. J. Respir. Cell Mol. Biol.* **31**, 382-394.
- Franks, T. J., Colby, T. V., Travis, W. D., Tuder, R. M., Reynolds, H. Y., Brody, A. R., Cardoso, W. V., Crystal, R. G., Drake, C. J., Engelhardt, J. et al. (2008). Resident cellular components of the human lung: current knowledge and goals for research on cell phenotyping and function. *Proc. Am. Thorac. Soc.* **15**, 763-766.
- Gontan, C., de Munck, A., Vermeij, M., Grosveld, F., Tibboel, D. and Rottier, R. (2008). Sox2 is important for two crucial processes in lung development: branching morphogenesis and epithelial cell differentiation. *Dev. Biol.* **317**, 296-309.
- Graham, V., Khudyakov, J., Ellis, P. and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron* **39**, 749-765.
- Gridley, T. (2007). Notch signaling in vascular development and physiology. *Development* **134**, 2709-2718.

- Guilmeau, S., Flandez, M., Bancroft, L., Sellers, R. S., Tear, B., Stanley, P. and Augenlicht, L. H. (2008). Intestinal deletion of Pofut1 in the mouse inactivates notch signaling and causes enterocolitis. *Gastroenterology* **135**, 849-860.
- Guseh, J. S., Bores, S. A., Stanger, B. Z., Zhou, Q., Anderson, W. J., Melton, D. A. and Rajagopal, J. (2009). Notch signaling promotes airway mucous metaplasia and inhibits alveolar development. *Development* **136**, 1751-1759.
- Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K. and Honjo, T. (2002). Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int. Immunol.* **14**, 637-645.
- Harfe, B. D., Scherz, P. J., Nissim, S., Tian, H., McMahon, A. P. and Tabin, C. J. (2004). Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* **118**, 517-528.
- Harris, K. S., Zhang, Z., McManus, M. T., Harfe, B. D. and Sun, X. (2006). Dicer function is essential for lung epithelium morphogenesis. *Proc. Natl. Acad. Sci. USA* **103**, 2208-2213.
- Hayes, J. M., Kim, S. K., Abitua, P. B., Park, T. J., Herrington, E. R., Kitayama, A., Grow, M. W., Ueno, N. and Wallingford, J. B. (2007). Identification of novel ciliogenesis factors using a new *in vivo* model for mucociliary epithelial development. *Dev. Biol.* **312**, 115-130.
- Ishii, Y., Rex, M., Scotting, P. J. and Yasugi, S. (1998). Region-specific expression of chicken Sox2 in the developing gut and lung epithelium: regulation by epithelial-mesenchymal interactions. *Dev. Dyn.* **213**, 464-475.
- Ito, T., Udana, N., Yazawa, T., Okudela, K., Hayashi, H., Sudo, T., Guillemot, F., Kageyama, R. and Kitamura, H. (2000). Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium. *Development* **127**, 3913-3921.
- Johnston, C. J., Finkelstein, J. N., Oberdörster, G., Reynolds, S. D. and Stripp, B. R. (1999). Clara cell secretory protein-deficient mice differ from wild-type mice in inflammatory chemokine expression to oxygen and ozone, but not to endotoxin. *Exp. Lung Res.* **25**, 7-21.
- Kiernan, A. E., Xu, J. and Gridley, T. (2006). The Notch ligand JAG1 is required for sensory progenitor development in the mammalian inner. *PLoS Genet.* **2**, e4.
- Kong, Y., Glickman, J., Subramaniam, M., Shahsafaei, A., Allamneni, K. P., Aster, J. C., Sklar, J. and Sunday, M. E. (2004). Functional diversity of notch family genes in fetal lung development. *Am. J. Physiol. Lung Cell Mol. Physiol.* **286**, 1075-1083.
- Kurotani, R., Tomita, T., Yang, Q., Carlson, B. A., Chen, C. and Kimura, S. (2008). Role of secretoglobin 3A2 in lung development. *Am. J. Respir. Crit. Care Med.* **178**, 389-398.
- Linnoila, R. I. (2006). Functional facets of the pulmonary neuroendocrine system. *Lab. Invest.* **86**, 425-444.
- Liu, Y., Pathak, N., Kramer-Zucker, A. and Drummond, I. A. (2007). Notch signaling controls the differentiation of transporting epithelia and multiciliated cells in the zebrafish pronephros. *Development* **134**, 1111-1122.
- Lü, J., Qian, J., Izvolsky, K. I. and Cardoso, W. V. (2004). Global analysis of genes differentially expressed in branching and non-branching regions of the mouse embryonic lung. *Dev. Biol.* **273**, 418-435.
- Maeda, Y., Davé, V. and Whitsett, J. A. (2007). Transcriptional control of lung morphogenesis. *Physiol. Rev.* **87**, 219-244.
- McDowell, E. M., Newkirk, C. and Coleman, B. (1985). Development of hamster tracheal epithelium: I. A quantitative morphologic study in the fetus. *Anat. Rec.* **213**, 429-447.
- Murtaugh, L. C., Stanger, B. Z., Kwan, K. M. and Melton, D. A. (2003). Notch signaling controls multiple steps of pancreatic differentiation. *Proc. Natl. Acad. Sci. USA* **100**, 14920-14925.
- Okajima, T. and Matsuda, T. (2006). Roles of O-fucosyltransferase 1 and O-linked fucose in notch receptor function. *Methods Enzymol.* **417**, 111-126.
- Okamura, Y. and Saga, Y. (2008). Notch signaling is required for the maintenance of enteric neural crest progenitors. *Development* **135**, 3555-3565.
- Otani, E. M., Newkirk, C. and McDowell, E. M. (1986). Development of hamster tracheal epithelium: IV. Cell proliferation and cytodifferentiation in the neonate. *Anat. Rec.* **214**, 183-192.
- Park, K. S., Wells, J. M., Zorn, A. M., Wert, S. E., Laubach, V. E., Fernandez, L. G. and Whitsett, J. A. (2006). Transdifferentiation of ciliated cells during repair of the respiratory epithelium. *Am. J. Respir. Cell Mol. Biol.* **34**, 151-115.
- Post, L. C., Ternet, M. and Hogan, B. L. (2000). Notch/Delta expression in the developing mouse lung. *Mech. Dev.* **98**, 95-98.
- Que, J., Okubo, T., Goldenring, J. R., Nam, K. T., Kurotani, R., Morrissey, E. E., Taranova, O., Pevny, L. H. and Hogan, B. L. (2007). Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development* **134**, 2521-2531.
- Radtke, F. and Raj, K. (2003). The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat. Rev. Cancer* **3**, 756-767.
- Ramirez, M. I., Millien, G., Hinds, A., Cao, Y., Seldin, D. C. and Williams, M. C. (2003). T1alpha, a lung type I cell differentiation gene, is required for normal lung cell proliferation and alveolus formation at birth. *Dev. Biol.* **256**, 61-72.
- Rawlins, E. L. and Hogan, B. L. (2006). Epithelial stem cells of the lung: privileged few or opportunities for many? *Development* **133**, 2455-2465.
- Rawlins, E. L., Ostrowski, L. E., Randell, S. H. and Hogan, B. L. (2007). Lung development and repair: contribution of the ciliated lineage. *Proc. Natl. Acad. Sci. USA* **104**, 410-417.
- Reynolds, S. D., Reynolds, P. R., Pryhuber, G. S., Finder, J. D. and Stripp, B. R. (2002). Secretoglobins SCGB3A1 and SCGB3A2 define secretory cell subsets in mouse and human airways. *Am. J. Respir. Crit. Care Med.* **166**, 1498-1509.
- Rogers, D. F. (2003). The airway goblet cell. *Int. J. Biochem. Cell Biol.* **35**, 1-6.
- Sasamura, T., Ishikawa, H. O., Sasaki, N., Higashi, S., Kanai, M., Nakao, S., Ayukawa, T., Aigaki, T., Noda, K., Miyoshi, E. et al. (2007). The O-fucosyltransferase O-fut1 is an extracellular component that is essential for the constitutive endocytic trafficking of Notch in Drosophila. *Development* **134**, 1347-1356.
- Shan, L., Aster, J. C., Sklar, J. and Sunday, M. E. (2007). Notch-1 regulates pulmonary neuroendocrine cell differentiation in cell lines and in transgenic mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* **292**, 500-509.
- Shi, S. and Stanley, P. (2003). Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. *Proc. Natl. Acad. Sci. USA* **100**, 5234-5239.
- Shi, S., Stahl, M., Lu, L. and Stanley, P. (2005). Canonical Notch signaling is dispensable for early cell fate specifications in mammals. *Mol. Cell. Biol.* **25**, 9503-9508.
- Stripp, B. R., Maxson, K., Mera, R. and Singh, G. (1995). Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. *Am. J. Physiol.* **269**, 791-799.
- Stripp, B. R., Reynolds, S. D., Boe, I. M., Lund, J., Power, J. H., Coppens, J. T., Wong, V., Reynolds, P. R. and Plopper, C. G. (2002). Clara cell secretory protein deficiency alters clara cell secretory apparatus and the protein composition of airway lining fluid. *Am. J. Respir. Cell Mol. Biol.* **27**, 170-178.
- Stubbs, J. L., Davidson, L., Keller, R. and Kintner, C. (2006). Radial intercalation of ciliated cells during Xenopus skin development. *Development* **133**, 2507-2515.
- Tilley, A. E., Harvey, B. G., Heguy, A., Hackett, N. R., Wang, R., O'Connor, T. P. and Crystal, R. G. (2009). Downregulation of the notch pathway in human airway epithelium in association with smoking and COPD. *Am. J. Respir. Crit. Care Med.* **179**, 457-466.
- Toskala, E., Smiley-Jewell, S. M., Wong, V. J., King, D. and Plopper, C. G. (2005). Temporal and spatial distribution of ciliogenesis in the tracheobronchial airways of mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* **283**, 454-459.
- Tsao, P. N., Chen, F., Izvolsky, K. I., Walker, J., Kukuruzinska, M. A., Lu, J. and Cardoso, W. V. (2008). Gamma-secretase activation of notch signaling regulates the balance of proximal and distal fates in progenitor cells of the developing lung. *J. Biol. Chem.* **283**, 29532-29544.
- van Es, J. H., van Gijn, M. E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D. J., Radtke, F. et al. (2005). Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435**, 959-963.
- van Tuyl, M., Groenman, F., Kuliszewski, M., Ridsdale, R., Wang, J., Tibboel, D. and Post, M. (2005). Overexpression of lunatic fringe does not affect epithelial cell differentiation in the developing mouse lung. *Am. J. Physiol. Lung Cell Mol. Physiol.* **288**, 672-682.
- Warburton, D., Perin, L., Defilippo, R., Bellusci, S., Shi, W. and Driscoll, B. (2008). Stem/progenitor cells in lung development, injury repair, and regeneration. *Proc. Am. Thorac. Soc.* **15**, 703-706.
- Weibel, E. R. (1984). Lung cell biology. In *The Pathway for Oxygen: Structure and Function in the Mammalian Respiratory System*, pp. 231-233. Cambridge, MA: Harvard University Press.
- Weinmaster, G. and Kopan, R. (2006). A garden of Notch-ly delight. *Development* **133**, 3277-3282.
- Wettstein, D. A., Turner, D. L. and Kintner, C. (1997). The Xenopus homolog of Drosophila Suppressor of Hairless mediates Notch signaling during primary neurogenesis. *Development* **124**, 693-702.
- Zemke, A. C., Snyder, J. C., Brockway, B. L., Drake, J. A., Reynolds, S. D., Kaminski, N. and Stripp, B. R. (2009). Molecular staging of epithelial maturation using secretory cell-specific genes as markers. *Am. J. Respir. Cell Mol. Biol.* **40**, 340-348.