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Notch signaling prevents mucous metaplasia in mouse conducting airways during postnatal development

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SUMMARY

Goblet cell metaplasia and mucus overproduction contribute to the pathogenesis of chronic lung diseases, including asthma and chronic obstructive pulmonary disease (COPD). Notch signaling regulates cell fate decisions and is crucial in controlling goblet cell differentiation in the gut epithelium. Little is known, however, about how endogenous Notch signaling influences the goblet cell differentiation program that takes place in the postnatal lung. Using a combination of genetic and in vitro approaches here we provide evidence of a novel role for Notch in restricting goblet cell differentiation in the airway epithelium during the postnatal period. Conditional inactivation of the essential Notch pathway component *Pofut1* (protein O-fucosyltransferase1) in *Tgfb3-Cre*-expressing mice resulted in an aberrant postnatal airway phenotype characterized by marked goblet cell metaplasia, decreased Clara cell number and increase in ciliated cells. The presence of the same phenotype in mice in which the Notch transcriptional effector *Rbpjk* was deleted indicated the involvement of the canonical Notch pathway. Lineage study in vivo suggested that goblet cells originated from a subpopulation of Clara cells largely present in proximal airways in which Notch was disrupted. The phenotype was confirmed by a panel of goblet cell markers, showed no changes in cell proliferation or altered expression of proinflammatory cytokines and was associated with significant downregulation of the bHLH transcriptional repressor *Hes5*. Luciferase reporter analysis suggested that Notch directly repressed *MUC5AC* transcription in lung epithelial cells. The data suggested that during postnatal life Notch is required to prevent Clara cells from differentiating into goblet cells.

KEY WORDS: Notch, Airway, Mucus, Goblet cell metaplasia, Mouse

INTRODUCTION

The airway epithelium is in contact with the external environment, serving as a major defense barrier against inhaled toxic substances and pathogens. Proper differentiation of the airway epithelium into ciliated, neuroendocrine (NE), secretory Clara and goblet cells is crucial to maintain lung homeostasis and prevent disease (Knight and Holgate, 2003; Puchelle et al., 2006). Mucin-producing goblet cells are found in the epithelium of a variety of tissues, including the respiratory, digestive and reproductive tract, where they have multiple functions, including hydration and clearance of particulates and pathogens (Davis and Dickey, 2008; Gipson and Argueso, 2003; van Es et al., 2005). A number of studies show that interleukins, such as Il-4 and Il-13, and transcription factors, such as Foxa2 and Spdef (SAM pointed domain-containing ets transcription factor), influence goblet cell differentiation (Chen et al., 2009; Grunig et al., 1998; Jain-Vora et al., 1997; Park et al., 2007; Wan et al., 2004; Wills-Karp et al., 1998). In addition, the Notch pathway has been identified as a major regulator of goblet cell fate, both in development and in disease (Crosnier et al., 2005; Guseh et al., 2009; Kang et al., 2009; Okamoto et al., 2009; Shinoda et al., 2010; Tilley et al., 2009; van Es et al., 2005). In the gastrointestinal tract, disruption of Notch signaling by γ-secretase inhibitors results in increased number of goblet cells (Milano et al., 2004; Ridgway et al., 2006; Searfoss et al., 2003; Wong et al., 2004). Likewise, genetic inactivation of the Notch pathway leads to a massive conversion of proliferative crypt cells into postmitotic goblet cells (Crosnier et al., 2005; van Es et al., 2005). Conversely, constitutive Notch activation impairs goblet cell differentiation in the intestine (Fre et al., 2005; Stanger et al., 2005).

Respiratory conditions, such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis are typically associated with goblet cell metaplasia, which is characterized by overabundance of goblet cells and mucus hypersecretion (Jackson, 2001; Rogers, 2003; Rose and Voynow, 2006). Goblet cell metaplasia has been recently reported in a transgenic mouse model in which Notch signaling is constitutively activated in distal lung progenitors (Guseh et al., 2009). Previous studies in which important Notch pathway components have been deleted in the early developing lung epithelium using a *Shh-Cre* driver revealed a crucial role for Notch in formation of secretory Clara cells (Morimoto et al., 2010; Tsao et al., 2009). However, information on the goblet cell program was somewhat limited, as in murine airways differentiation of these cells has been reported to occur mostly during postnatal life (Pack et al., 1980) and nearly all the mutants died at birth. Thus, whether and how endogenous Notch signaling influences goblet cell differentiation postnatally remained an open question.

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In the present work, we addressed this issue by using a *Tgfb3-Cre* deleter mouse line and mice carrying floxed alleles of the *Pofut1* gene, which encodes an O-fucosyltransferase essential for Notch-ligand binding (Shi and Stanley, 2003). A preliminary analysis of lungs from a *Tgfb3-Cre*;*ROSA26-lacZ*(*R26R*) reporter mice suggested that the *Tgfb3-Cre* line targeted the developing airway epithelium in a mosaic fashion and at relatively late stages. Conditional inactivation of Notch using this approach resulted in an overall less severe phenotype than that reported previously (Morimoto et al., 2010; Tsao et al., 2009) and allowed survival to adulthood. Analysis of these mutants revealed a postnatal lung phenotype characterized by goblet cell metaplasia in areas of reduced number of Clara cells, without major changes in cell proliferation. The effect appears to have resulted from derepression of a Notch-mediated mechanism that restricts mucin gene expression in a subpopulation of Clara cells. Our findings reveal a novel role for Notch in restricting goblet cell differentiation in the airway epithelium during the postnatal period. Together the data suggest that Notch is crucial in maintaining lung homeostasis and preventing mucus hypersecretion, a major feature of COPD.

MATERIALS AND METHODS

Mouse strains

Tgfb3-Cre mice were generated as previously described (Yang et al., 2008). Floxed *Pofut1* (*Pofut1*$^{F/F}$) and *Rbpjk*$^{F/F}$ mice were kindly provided by Pamela Stanley (Shi et al., 2005) and Tasuku Honjo (Han et al., 2002), respectively. Both *Tgfb3-Cre* and *Rbpjk*$^{F/F}$ mice were on a C57BL/6 background, and *Pofut1*$^{F/F}$ mice were maintained on a mixed 129/C57BL/6 background. Rosa26 reporter mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA).

G-Red mice were generated using a transgenic vector consisting of the cytomegalovirus (CMV) enhancer, the chicken β-actin promoter, a loxP cassette containing eGFP cDNA and SV40 polyA, and the downstream DsRedT1 cDNA with SV40 polyA sequences (Lin et al., 2011). *Tgfb3-Cre* mice were mated to *Pofut1*$^{F/F}$ mice to generate *Tgfb3-Cre*;*Pofut1*$^{+/F}$ offspring, which were then crossed to *Pofut1*$^{F/F}$ to create *Tgfb3-Cre*;*Pofut1*$^{F/F}$ (*Pofut1*^{cTb3}) conditional knockout mice. For the *Rbpjk* model, the same approach was taken to generate conditional deletion of *Rbpjk* as above. *Pofut1*$^{F/F}$ and G-Red mice were crossed to obtain mice homozygous for the floxed *Pofut1* allele and G-Red transgene (*Pofut1*$^{F/F}$;G-Red$^{+/wt}$), which were mated to *Tgfb3-Cre*;*Pofut1*$^{+/F}$ to generate *Pofut1*$^{F/F}$;G-Red$^{+/WT}$;*Tgfb3*$^{Cre/+}$ conditional mutant reporter mice, respectively. Genotyping was performed on tail biopsies by PCR. The primers for genotyping the floxed *Pofut1* allele, *Cre* and G-Red have been described previously (Lin et al., 2011; Tsao et al., 2009; Yang et al., 2008). All mice were maintained on mixed genetic backgrounds.

Lung tissues of *Hes5* null mice were kindly provided by Dr Ryoichiro Kageyama (Cau et al., 2000). All protocols were approved by the Animal Care and Use Committee of the National Taiwan University Hospital and National Health Research Institutes.

X-gal staining

To detect β-galactosidase expression, lungs were inflated with 4% PFA for 30 minutes at 4°C and then processed for frozen sectioning. Sections were incubated in 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/ml X-gal solution for 4-8 hours at 37°C. Sections were counterstained with Eosin.

Immunohistochemistry

Lungs were inflated with 4% PFA for 1 hour to overnight at 4°C and embedded in OCT or paraffin. Periodic acid Schiff (PAS) and Alcian Blue (AB) staining and immunohistochemical staining for Ki67, Foxj1, β-tubulin, T1alpha, and proSP-C followed previously described methods (Tsao et al., 2009). The other primary antibodies used were: rabbit anti-Ttf1 (1:100, Leica); rabbit anti-Ccsp (1:1000, Upstate); goat anti-Ccsp (1:100, Santa Cruz); mouse anti-Mucin5ac (1:100, Abcam); rabbit anti-FOXA2 (1:400, Abcam); rabbit anti-HNF-3γ (Foxa3) (1:100, Santa Cruz); rabbit anti-GFP (1:500, Invitrogen); rabbit anti-Hes5 (1:200, Chemicon) (Batts et al., 2009; El-Hashash et al., 2011; Li et al., 2009; Wang et al., 2010). Immunofluorescence was performed using secondary antibodies conjugated to Alexa Fluor 488 or 596 (1:200, Invitrogen) and analyzed using a Zeiss confocal laser-scanning microscope (LSM510 META) as previously described (Tsao et al., 2008).

Morphometric analysis

Digital images of airways were acquired with a Leica DM750 LED microscope and imported into MetaMorph imaging software version 7.1 (Universal Imaging Corp.). At PN30, large (hilar airways with associated pulmonary artery and vein), medium and small (bronchioles down to terminal bronchioles) airways were analyzed (Park et al., 2007; Tsao et al., 2009). AB staining was quantified by tracing the area of epithelial AB positive staining in airways. The area measurements of AB staining were normalized to total longitudinal length along the basement membrane of epithelial surveyed to give a density value and presented as μm^2/mm. The percentage of labeled epithelial cells immunostained for Ccsp (Scgb1a1 – Mouse Genome Informatics) and Foxj1 was performed as previous described (Tsao et al., 2009). For our analysis of *Pofut1*$^{F/F}$;G-Red$^{+/WT}$;*Tgfb3*$^{Cre/+}$ conditional mutant reporter mice, we performed Ccsp-eGFP double staining in mutant lungs. The proportion of Ccsp+ cells within eGFP– cell population was calculated as the number of Ccsp+/eGFP– cells divided by the total number of eGFP– cells. The same was done to determine the proportion of Ccsp+ cells in the eGFP+ cell population. We then determined the proportion of eGFP+ or eGFP– cells in either Ccsp+ or Muc5ac+ cell populations using a similar method. A total of five sections was analyzed per animal (*n*=3) at 200 times magnification.

Real-time PCR

Total RNA from lung tissues or cell lines was isolated using the Trizol method. For reverse transcription, 2 μg of total RNA were transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time RT-PCR was performed in a DNA Engine Opticon 2 (Bio-Rad, CA, USA) using iQ SYBR Green supermix (Bio-Rad, CA, USA). Briefly, 100 ng of the reversed transcribed cDNA were used for each PCR reaction with 250 nM of forward and reverse primers. The primer sequences for real-time PCR were presented in supplementary Table S1. The threshold cycle (C_T) values were obtained for the reactions, reflecting the quantity of the product in the sample. The relative concentration of RNA for each gene to GAPDH mRNA was determined using the equation: $2^{-\Delta CT}$, where ΔCT=(CTmRNA – CTGAPDHmRNA).

Bronchoalveolar lavage and cell counts

Bronchoalveolar lavage (BAL) was performed as previous described (Hsieh et al., 2008). Cell pellets were resuspended in PBS. Cell numbers were counted with a hemocytometer, and inflammatory cell differentials were determined on cytospin smears (Tsao et al., 2007).

Luciferase activity and transfection in vitro

The *MUC5AC* promoter-luciferase (*MUC5AC*-Luc) construct consisted of a 3.7 kb segment of the 5′ flanking region of the human *MUC5AC* gene (Li et al., 1998) and was prepared as previous described (Wang et al., 2007). LA4 cells, a murine adenocarcinoma cell line that expresses *Muc5ac* endogenously, were transfected with 500 ng of *MUC5AC*-Luc and 10 ng *renilla* luciferase control vector (Promega, Madison, WI) by the MicroPorator (Digital Bio Technology, Seoul, Korea). The transfected cells were treated with DAPT {N-[N-(3, 5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester} (10 μM, Sigma) or DMSO (control) for 48 hours. Alternatively, LA4 cells were co-transfected with *MUC5AC*-Luc and 1.0 μg of constitutive active Notch1 intracelluar domain (caN1) or dominant negative (dnN1) constructs to constitutively activate or inhibit Notch1 signaling, respectively (Small et al., 2001), or *Hes5* cDNA (Hojo et al., 2000) for 48 hours. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), in a BD monolight 3010 luminometer (BD Biosciences, San Diego, CA) in accordance with the manufacturer's instructions and normalized to renilla

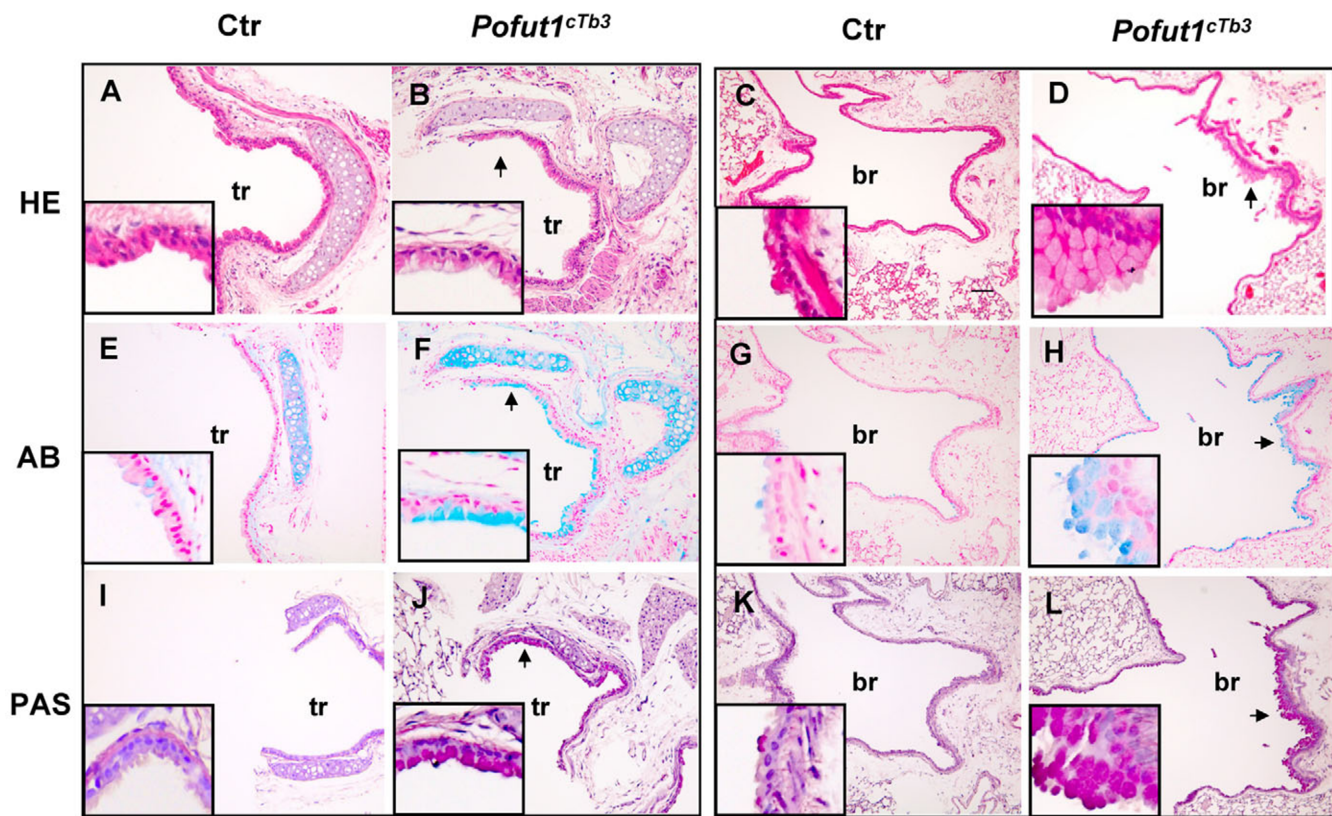

Fig. 1. Goblet cell metaplasia in *Pofut1^{cTb3}* mutant lungs at PN30. Tracheal (tr) (**A**,**B**,**E**,**F**,**I**,**J**) and bronchial (br) (**C**,**D**,**G**,**H**,**K**,**L**) epithelia stained with Hematoxilin and Eosin (HE), AB and PAS. Abundant AB or PAS staining (arrow) in cells with goblet cell morphology in *Pofut1^{cTb3}* mutant lungs (F,H,J,L). Scale bar: 100 μm.

activity. To compare gene expression in LA4 cells with a primary respiratory tract epithelial cell, we isolated adult mouse tracheal epithelium as previous described and processed for quantitative RT-PCR (qRT-PCR) analysis (You et al., 2002).

Statistics

Data were expressed as mean±s.e.m. Statistical analysis was performed using Student's *t*-test; differences were considered significant at $P<0.05$.

RESULTS

Inactivation of Pofut1 in Tgfb3-expressing sites in the lung

Previous analysis of disruption of *Pofut1* in the lung using *Pofut1$^{F/F}$* and *Shh-Cre* deleter mouse mutants (Harfe et al., 2004) showed that loss of Notch signaling in lung epithelial progenitors resulted in dramatic changes in the balance of differentiated cell types in the airway epithelium (Tsao et al., 2009). This model, however, did not allow inferring about how Notch influences postnatal differentiation of the lung, because of postnatal lethality. Whether lethality resulted from the deletion of Notch in all Shh-expressing cells in the lung or other organs has not been determined. We argued that using a different Cre-deleter mouse line that did not target all epithelial precursors at once and allowed survival could provide information about postnatal functions of Notch in the lung. Previous studies have shown that Tgfb3 is expressed in the developing airway epithelium and that *Tgfb3* regulatory sequences could drive *Cre* expression to the lung in transgenic mice (Coker et al., 1996; Pelton et al., 1990; Yang et al., 2008). In a preliminary analysis of *Tgfb3-Cre;ROSA26-lacZ*(*R26R*) reporter mice, we observed *lacZ* reporter expression in a mosaic-like pattern and at a relatively later stage in the developing airway epithelium, compared with *Shh-Cre;ROSA26-lacZ* (see Fig. S1A-C in the supplementary material). As represented in embryonic day 14.5 (E14.5) lungs, signals could be seen in only few epithelial cells of some airways, although it was strongly detected in the cartilage primordia, smooth muscle layer and pleura (see Fig. S1D-F in the supplementary material). Increasing labeling was observed postnatally, so that by postnatal day 30 (PN30, adult) most of the airway epithelium expressed *LacZ* (see Fig. S1G,H in the supplementary material). The data suggested that the *Tgfb3-Cre* deleter could target efficiently the developing airway epithelium, particularly postnatally at later stages. Thus, to investigate postnatal functions of Notch in the lung we used mice carrying a *Tgfb3-Cre* allele and a *Pofut1$^{F/F}$* mouse line.

Male mice heterozygous for the *Tgfb3-Cre* allele and the floxed *Pofut1* allele (*Pofut1$^{F/+}$*;*Tgfb3$^{Cre/+}$*) were crossed with female mice homozygous for the floxed *Pofut1* allele (*Pofut1$^{F/F}$*) (see Fig. S1I in the supplementary material). Genotyping of litters derived from this breeding scheme after birth (n>500) revealed *Pofut1$^{F/F}$;Tgfb3$^{Cre/+}$* (conditional deletion of both *Pofut1* alleles; termed *Pofut1^{cTb3}* onwards), *Pofut1$^{F/+}$*;*Tgfb3*$^{Cre/+}$ (conditional deletion of a single *Pofut1* allele), *Pofut1$^{F/F}$* and *Pofut1$^{F/+}$* (no deletion of *Pofut1*, hereby termed 'control') offspring at an expected Mendelian distribution. The *Pofut1^{cTb3}* mice were viable, although they showed progressive hair loss after 3 weeks

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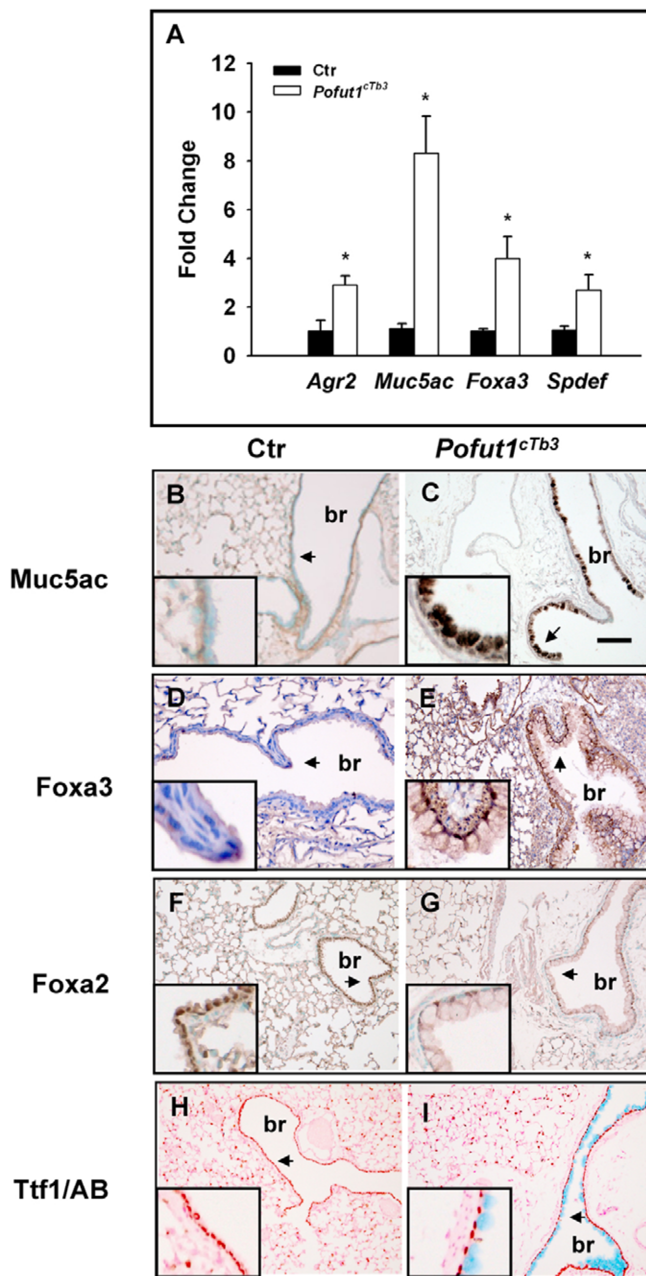

Fig. 2. Increased production of goblet cell markers and decreased Foxa2 expression in *Pofut1*^{cTb3} mutant airways. (**A**) Real-time PCR of control and *Pofut1*^{cTb3} mutant lungs at PN30 showing *Agr2*, *Muc5ac*, *Foxa3* and *Spdef* upregulated in mutants (*n*=3 or 4 per group). Data are mean+s.e.m. **P*<0.05 (**B-I**) Immunohistochemistry showing increased Muc5ac (B,C) and Foxa3 (D,E) signals and decreased Foxa2 (F,G) in goblet cells of *Pofut1*^{cTb3} mutant airways. (H,I), unchanged Ttf1 expression in AB-positive cells. Scale bar: 100 μm. br, bronchus.

postpartum. A detailed characterization of the skin and hair phenotype of the *Pofut1*^{cTb3} mutants has been reported recently (Lin et al., 2011).

Disruption of *Pofut1* and Notch signaling in *Pofut1*^{cTb3} mice was confirmed by real-time PCR analysis of *Pofut1* and known Notch target genes on whole-lung homogenates, as described in a subsequent section (Fig. 7B).

Notch restricts goblet cell differentiation in the airway epithelium postnatally

We analyzed the lungs of *Pofut1*^{cTb3} at PN30 and found no obvious differences in gross morphology compared with control lungs. Distal lung differentiation, including formation of alveolar sacs and type I and type II cells, was not affected by *Pofut1* deletion, as suggested by immunohistochemical assessment of markers, such as Ttf1 (thyroid transcription factor 1), T1alpha (podoplanin, Pdpn) and surfactant-associated protein C (Sftpc, SP-C) (see Fig. S2 in the supplementary material). Surprisingly, we found that the airway epithelium of mutant trachea and lungs was lined by a large number of goblet-like cells (Fig. 1A-D). We performed AB and PAS staining to identify acidic and neutral mucins characteristic of goblet cells in the airways of these animals. Staining was rarely observed in the trachea and bronchi of controls but was found in a large number of cells in *Pofut1*^{cTb3} mutants (Fig. 1E-L).

Real-time PCR for genes known to be associated with goblet cell differentiation, such as *Muc5ac*, *Spdef*, anterior gradient 2 (*Agr2*) and *Foxa3* showed marked upregulation in lungs from *Pofut1*^{cTb3} mutant compared with controls (Fig. 2A) (Chen et al., 2009; Gregorieff et al., 2009; Noah et al., 2010; Park et al., 2007). Immunohistochemistry confirmed the strong expression of these gene products in goblet cells and revealed the extensive mucous metaplasia in airways of mutants (Fig. 2B-E). Consistently with this, Foxa2, which is normally present in the airway epithelium and required to inhibit goblet cell differentiation (Wan et al., 2004), was absent in the goblet cells of mutants (Fig. 2F,G). The presence of Ttf1 in the goblet cells of mutants suggested that the respiratory cell identity was maintained in the airway epithelium and that transdifferentiation to an intestinal-like epithelium was unlikely (Fig. 2H,I).

To investigate the onset of the goblet cell metaplasia, we extended our analysis of *Pofut1*^{cTb3} lungs to different post- and prenatal stages, as a recent study suggests an early appearance of this cell type (Roy et al., 2011). Analyses of E14.5 and E18.5 showed no AB staining in airways of either controls or mutants (*n*=3 per group), suggesting that the goblet cell phenotype in *Pofut1*^{cTb3} mice did not develop before birth (not shown). We also had no evidence of this abnormality within the immediate days of postnatal life, as confirmed by morphometry and by similar levels of *Muc5ac*, *Foxa3* and *Spdef* expression in controls and mutants at P4, by real-time PCR (Fig. 3A and see Fig. S3 in the supplementary material). However, goblet cell metaplasia was found consistently at PN8 (not shown), PN17 and PN30 (Fig. 3).

Interestingly, the aberrant formation of goblet cells followed a proximodistal (PD) pattern of distribution with increasing age, even though *Tgfb3-Cre;ROSA26-lacZ*(*R26R*) signals could be seen already throughout the airway epithelium (see below and Fig. S1G-H in the supplementary material). Initially, the abundant AB-positive goblet cells of mutants were restricted almost exclusively to the trachea, later being found in both trachea and bronchi (large airways) at PN17. By PN30, morphometric analysis confirmed the aberrant goblet cell differentiation in smaller bronchioles, which normally lack goblet cells (Fig. 3).

To provide additional evidence that the *Pofut1*^{cTb3} phenotype was a function of disrupted Notch signaling, we used the same *Tgfb3-Cre* mouse deleter approach to inactivate expression of the crucial Notch canonical transcriptional effector gene *Rbpjk* (*Rbpj* – Mouse Genome Informatics) (previously known as *Rbpsuh*) in the airway epithelium. We found that *Rbpjk*$^{F/F}$;*Tgfb3*$^{Cre/+}$ (*Rbpjk*^{cTb3}) mutant mice at postnatal day 7 (PN7) recapitulated the

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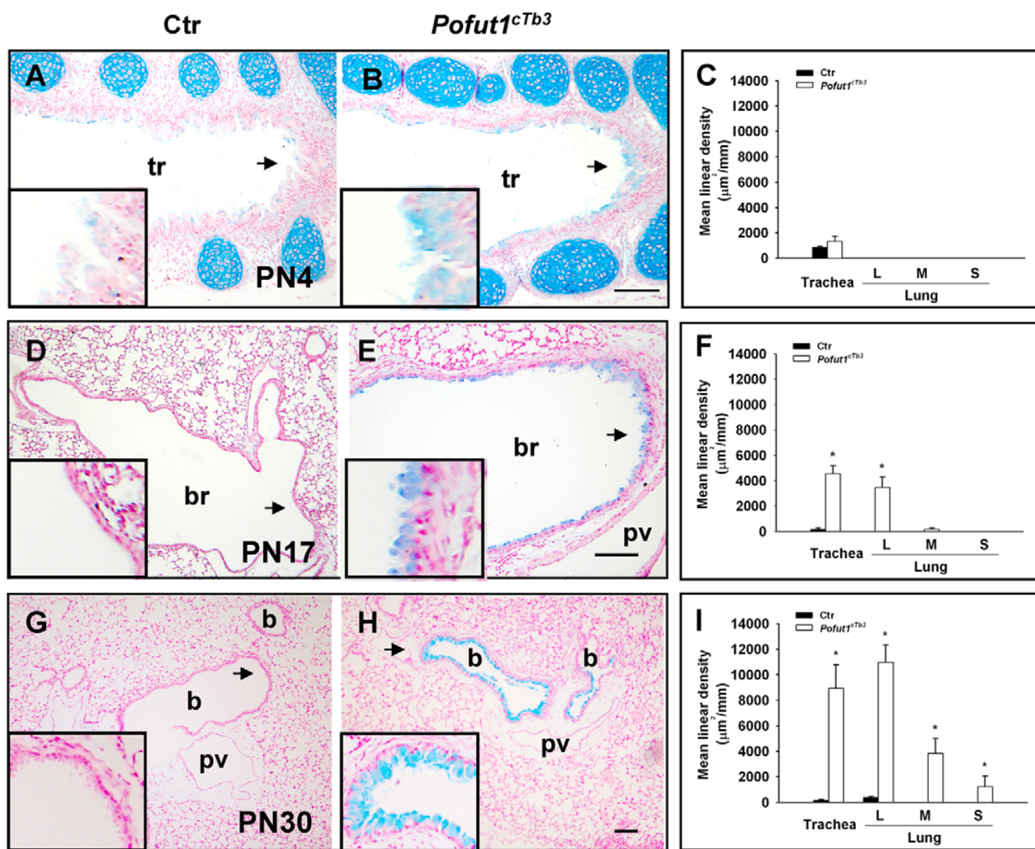

Fig. 3. Developmental progression of goblet cell metaplasia in *Pofut1*^{cTb3}airways. (**A-C**) At PN4, AB-positive goblet cells seen in trachea (tr) only but no difference in number between control and mutants. Data are mean+s.e.m. (**D-F**) At P17 increased number of goblet cells (mean linear density of AB staining, μm²/mm+s.e.m.) in trachea and large (L) airways of *Pofut1*^{cTb3} mutants. (**G-I**) At P30, increased AB-positive goblet cells extended from large (L), medium (M) and small (S) airways of *Pofut1*^{cTb3} compared with controls. *n*=3 per group; **P*<0.05 versus control. Data are mean+s.e.m. Scale bar: 100 μm. b, bronchiole; br, bronchus; pv, pulmonary vessel.

goblet cell metaplasia phenotype of the *Pofut1*^{cTb3} mouse (see Fig. S4 in the supplementary material). We concluded that Notch signaling postnatally restricts goblet cell differentiation in airways.

Pofut1^{cTb3} mutants show an imbalance in differentiated cellular phenotypes in airways

To start investigating the origin of the *Pofut1*^{cTb3} lung defect, we asked whether the phenotype could be a function of aberrant proliferation of goblet cells or the transformation of another resident airway epithelial cell into a goblet cell. Thus, first we assessed cell proliferation by Ki67 immunohistochemistry in lung sections from control and mutant mice at PN30. Sections were then counterstained with AB (Fig. 4A,B) and the number of putative proliferating goblet cells in proximal airways was quantified in both groups and compared. This analysis showed that, in spite of the increase in number of AB-labeled cells in mutants, Ki67 labeling in these cells relative to total goblet cells in the airway epithelium was nearly the same (1.9±0.3% in mutants vs 2.3±0.4 in controls, *P*=0.42). This suggested that increased proliferation of goblet cells was unlikely to play a role in the phenotype of mutants. We then determined the impact of *Tgfb3-Cre* mediated deletion of *Pofut1* in the relative abundance of other airway epithelial cell types. Real-time PCR analysis showed that PN30 mutant lungs had significant downregulation of the Clara cell secretory protein (*Ccsp*; *Scgb1a1* – Mouse Genome Informatics) gene marker and upregulation of the ciliated marker gene *Foxj1*, relative to controls (Fig. 4C). Immunohistochemistry followed by quantitation of Foxj1 and β-tubulin staining showed increased number of ciliated cells in the airways of *Pofut1*^{cTb3} mutant compared with controls (Fig. 4D-H). By contrast, the number of Ccsp-positive Clara cells was significantly reduced throughout the entire airway epithelium, including sites where goblet cells were abundant (Fig. 4I-M). The effect of *Tgfb3-Cre* deletion of *Pofut1* in airway epithelial differentiation was consistent with previous findings of the Notch requirement to balance secretory and ciliated cells (Morimoto et al., 2010; Tsao et al., 2009). The relatively unchanged levels of *Ccsp* and *Foxj1* mRNA between control and mutants at PN4 further supported the late onset of the *Pofut1*^{cTb3} phenotype postnatally (see Fig. S3 in the supplementary material).

Goblet cells originate from a subpopulation of Clara cells in proximal airways of Pofut1^{cTb3} mutant lungs

To further investigate the origin of the goblet cell metaplasia we performed lineage-tracing analysis of the cells undergoing *Tgfb3-Cre*-mediated deletion of *Pofut1*. We used a double fluorescent reporter mouse line (herein, G-Red reporter mice), which harbors a transgene comprised of a ubiquitous active chicken actin promoter that drives transcription of *eGFP* gene (Lin et al., 2011). Cre-mediated recombination induces expression of the second reporter, *DsRed*, by deletion of *loxP*-flanked *eGFP*-Stop cassette. Thus, in *Tgfb3*$^{Cre/+}$*;G-Red* double transgenic mice, cells that have undergone Cre-mediated excision could be distinguished by the absence of eGFP or the expression of DsRed (Fig. 5A). We analyzed *Pofut1*$^{F/F}$;G-Red$^{+/WT}$;*Tgfb3*$^{Cre/+}$ at PN30 by double immunofluorescence staining using anti-Muc5ac (visualized in red) and anti-eGFP (in green) antibodies. Fig. 5B shows extensive areas of recombination in proximal epithelium (eGFP negative) interspersed with only few positive for eGFP. Numerous Muc5ac-positive goblet cells lined the proximal airway epithelium of *Pofut1*$^{F/F}$;G-Red$^{+/WT}$;*Tgfb3*$^{Cre/+}$ mice but almost no overlap between eGFP and Muc5ac staining was found (Fig. 5B-D).

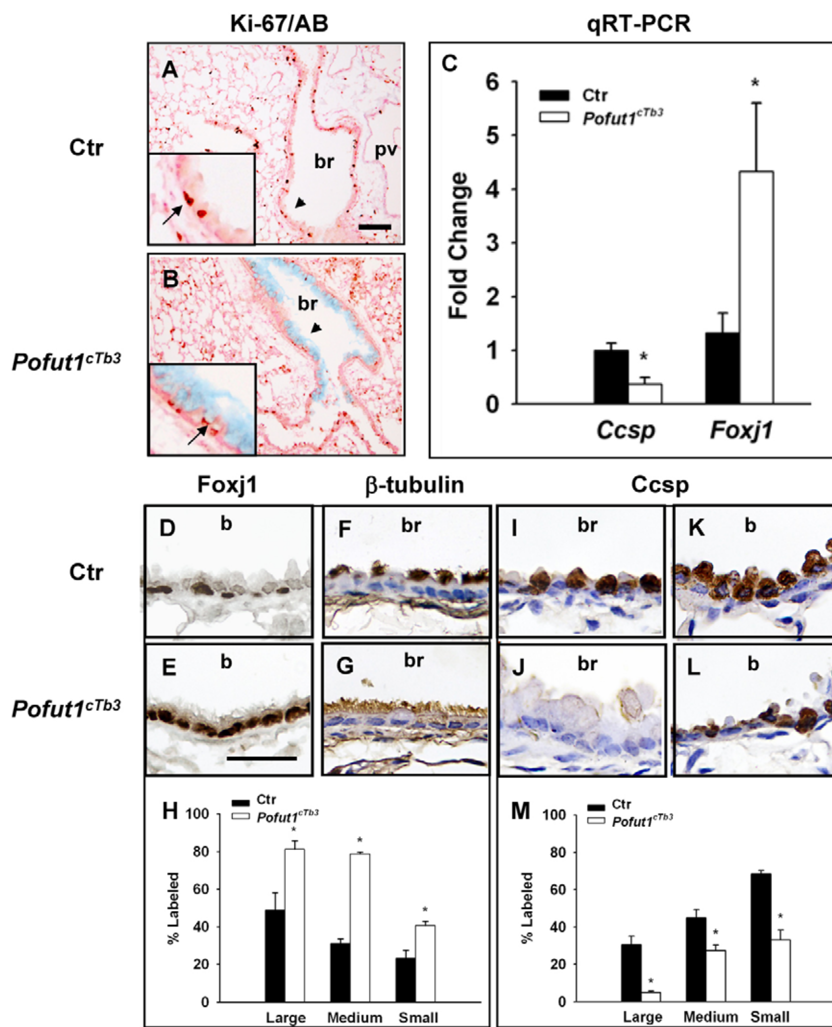

Fig. 4. Disrupted balance of Clara and ciliated cells adult *Pofut1^{cTb3}* mutant airways at PN30. (**A**,**B**) The proportion of Ki-67 (arrow) and AB double-positive cells was similar in control and *Pofut1^{cTb3}* mutants. Scale bar: 100 µm. (**C**) Real-time PCR of control and *Pofut1^{cTb3}* mutant lungs showing Ccsp downregulated and Foxj1 upregulated in *Pofut1^{cTb3}* mutants. Data are mean+s.e.m. **P*<0.05 (**D-M**) Increased number of Foxj1 and β-tubulin-expressing cells and decreased number of Ccsp immunostained cells in large, medium and small airways of *Pofut1^{cTb3}* mutants compared with control. (H,M) Quantitative analysis of Foxj1 (H) and Ccsp (M) labeling in these lungs. Data are mean+s.e.m. **P*<0.05 Scale bar: 50 µm. *n*=3 in each group. b, bronchiole; br, bronchus; pv, pulmonary vessel.

Morphometric analysis showed that nearly all Muc5ac+ cells (95.8%) were eGFP- (the remaining 4.2% non-targeted likely to be normally occurring goblet cells) (Fig. 5E). The data suggested that the majority of Muc5ac-positive goblet cells were derived or differentiated from *Tgfb3-Cre*-labeled cells in which *Pofut1* expression was disrupted. However, analysis of the relative proportion of targeted versus non-targeted cells in the Ccsp+ population by Ccsp-eGFP double staining revealed that most of these cells (89.2% of all Ccsp+) underwent Cre-recombination and thus Notch inactivation, but still remained Clara cells (Fig. 5E). This suggests that Notch is a crucial component of a mechanism that restricts the goblet cell program in a relatively small but important subpopulation of Clara cells postnatally. This subpopulation is likely to be functionally distinct, as suggested by previous studies in an antigen-challenged mouse model (Evans et al., 2004).

Loss of Notch signaling does not induce expression of proinflammatory cytokines

An increased number of goblet cells is often a manifestation of pulmonary inflammation, which is typically accompanied by increased expression of Th2-cytokines, such as *Il-4* and *Il-13* (Grunig et al., 1998; Jain-Vora et al., 1997; Temann et al., 1997; Wills-Karp et al., 1998). Here we tested whether the goblet cell metaplasia in *Pofut1^{cTb3}* mutants was associated with an underlying inflammatory process in the lung, altering levels of Th2 or proinflammatory cytokines. Histological sections did not reveal noticeable inflammatory infiltrates in mutants. Thus, we performed qRT-PCR of adult lung homogenates from control and *Pofut1c^{Tb3}* mutants (*n*=4, per group) looking for changes in expression of genes coding for selected Th2 cytokines. Fig. 6A shows no significant differences in *Il-4*, *Il-6* or *Il-13* mRNA expression between groups, suggesting that goblet cell differentiation occurred without obvious induction of these proinflammatory mediators. These results were further corroborated by the analysis of the total and differential cell counts in the BAL, which did not show differences between controls and *Pofut1^{cTb3}* mutants (data not shown).

Notch regulates *Muc5ac* gene expression in lung epithelial cell line

The *Pofut1^{cTb3}* phenotype strongly suggested that Notch signaling could regulate one or more aspects of the program of goblet cell differentiation, including mucin gene expression. To test whether Notch directly influenced transcription of a gene typically associated with this program, we assessed activity of a *MUC5AC*-luciferase reporter construct in transient transfection assays in LA4 cells, a murine lung adenocarcinoma cell line (Huang et al., 2009; Wang et al., 2009; Wang et al., 2007; Ye et al., 2011). PCR analysis showed that L4 cells express endogenous *Notch1*, *Hes5*, *Muc5ac*

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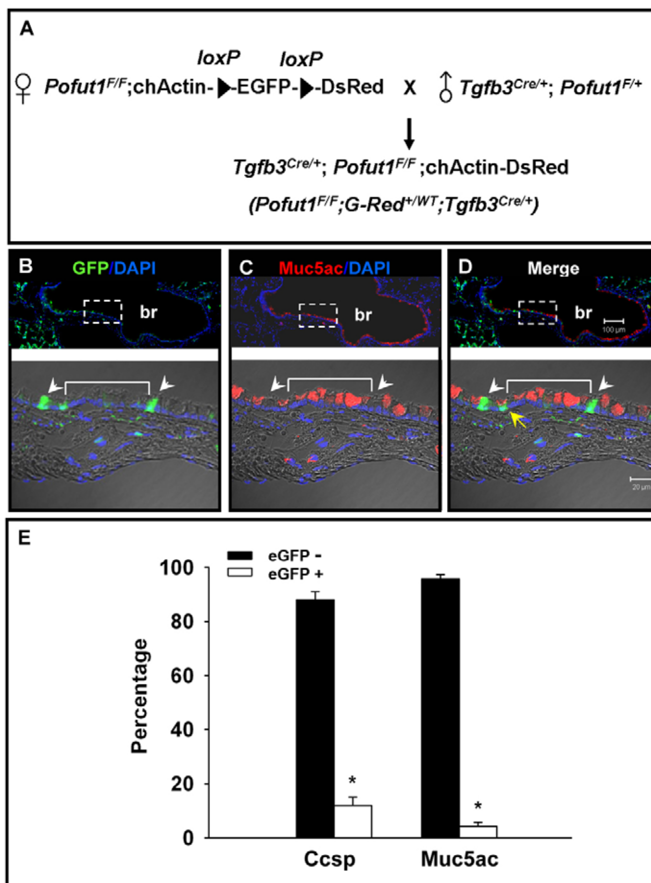

Fig. 5. Notch signaling-deficient Clara cells tend to differentiate into goblet cells in postnatal airways. (**A**) Generation of *Pofut1*$^{F/F}$;G-Red$^{+/WT}$;*Tgfb3*$^{Cre/+}$ mouse model. (**B-D**) Immunofluorescence of *Pofut1*$^{F/F}$;G-Red$^{+/WT}$;*Tgfb3*$^{Cre/+}$ lungs stained with anti-GFP (marking cells without Cre activity, green) and anti-Muc5ac (goblet cell marker, red), showing that the majority of goblet cells are eGFP negative (white bracket in B-D), thus, derived from Tgfb3-Cre-labeled cells deficient in Notch signaling. Rare eGFP/Muc5ac double-positive goblet cells can be seen (D, yellow arrow). (**E**) Morphometric analysis of PN30 *Pofut1*$^{F/F}$;G-Red$^{+/WT}$;*Tgfb3*$^{Cre/+}$ lungs (mean+s.e.m., $*P\leq0.05$). The majority of Ccsp-positive Clara cells and Muc5ac-positive goblet cells were eGFP negative.

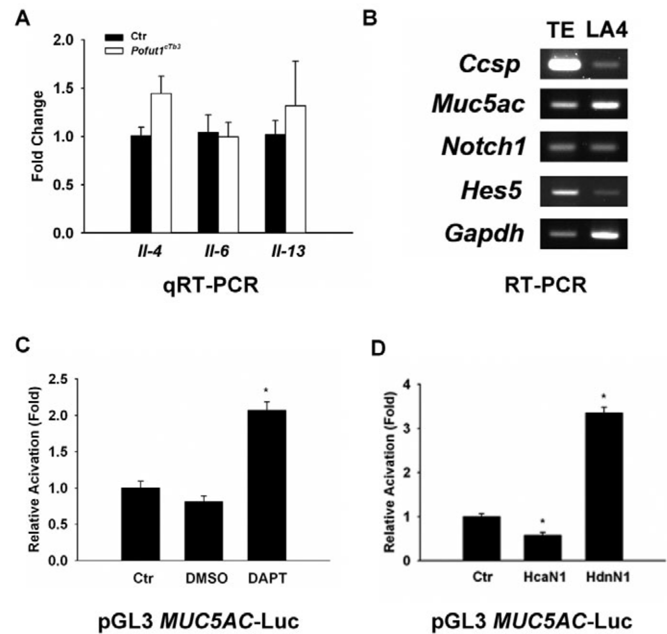

Fig. 6. *Notch1* inhibits *MUC5AC* transcription in *Pofut1*^{cTb3} mutants. (**A**) Real-time PCR: no difference in *Il-4, Il-13* (Th2 cytokines) and *Il-6* (proinflammatory cytokine) expression between control and *Pofut1*^{cTb3} mutant lungs at PN30. (**B**) Semi-quantitative RT-PCR comparing gene expression in isolated primary mouse tracheal epithelium and LA4 cells. (**C**,**D**) *MUC5AC*-Luc reporter assay in LA4 cells: DAPT significantly activates *MUC5AC* promoter; constitutively activating *Notch1* (HcaN1) inhibits *MUC5AC*-Luc transcription; transfection of a Notch dominant-negative (dnN1) construct reproduces the DAPT effect. Values are mean+s.e.m.,$*P<0.05$.

and *Ccsp* genes typically found in freshly isolated primary airway epithelial cells (Fig. 6B). Thus, although not primary, LA4 cells provide a suitable model to study Notch activity in a cell committed to a Clara-like secretory lineage.

We cultured LA4 cells for 48 hours in medium containing DMSO (control) or DAPT (10 μM) to inhibit gamma-secretase activity and thus prevent endogenous Notch activation (Tsao et al., 2008). Luciferase analysis showed that the gamma-secretase inhibitor DAPT significantly increased the activity of the *MUC5AC* promoter (Fig. 6C). To further support this observation, we modulated Notch signaling in LA4 cells using a *Notch1* constitutively active (caN1) construct or a dominant-negative (dnN1) construct (Small et al., 2001) in similar experiments. *MUC5AC*-Luc or pGL3 were co-transfected with caN1 or dnN1 constructs and luciferase activity was assessed at 48 hours. Consistent with the observations with DAPT treatment, we found a marked increase in *MUC5AC* promoter activity by downregulation of Notch signaling in dnN1-transfected cells. Conversely, Notch gain of function resulted in inhibited *MUC5AC*-luciferase expression in caN1-transfected cells (Fig. 6D).

Goblet cell metaplasia is associated with disruption of Notch signaling predominantly by Hes5

To gain insights into the Notch components and downstream targets potentially involved in the regulation of goblet cell fate in the airways, we compared levels of expression of key Notch-related genes in lung homogenates of adult *Pofut1*^{cTb3} and control mice. Real-time PCR showed a marked increase in *Jag1* expression and decreased *Notch1* expression (Fig. 7A), consistent with the major imbalance in the number of Jag1-expressing cells compared with Notch-expressing cells we previously reported in a *ShhCre-Pofut1* mouse (Tsao et al., 2009). With the exception of *Dll4* mRNA, which was also downregulated, expression of the other ligands and receptors was not significantly changed. We found no changes in expression of the Notch targets *Hes1 and Hey1* and significant but only marginal changes in *Hey2* mRNAs. By contrast, levels of *Hes5* mRNA were nearly fivefold reduced in mutants, paralleling the changes in *Pofut1* mRNA (Fig. 7B). To determine whether local changes in Hes5 protein expression correlated with the aberrant airway epithelial phenotype of mutants, we performed double Hes5-Ccsp immunostaining at PN30. This allowed us to infer cell type-selective expression of Hes5 in the airway and showed that in control lungs Hes5 signals were

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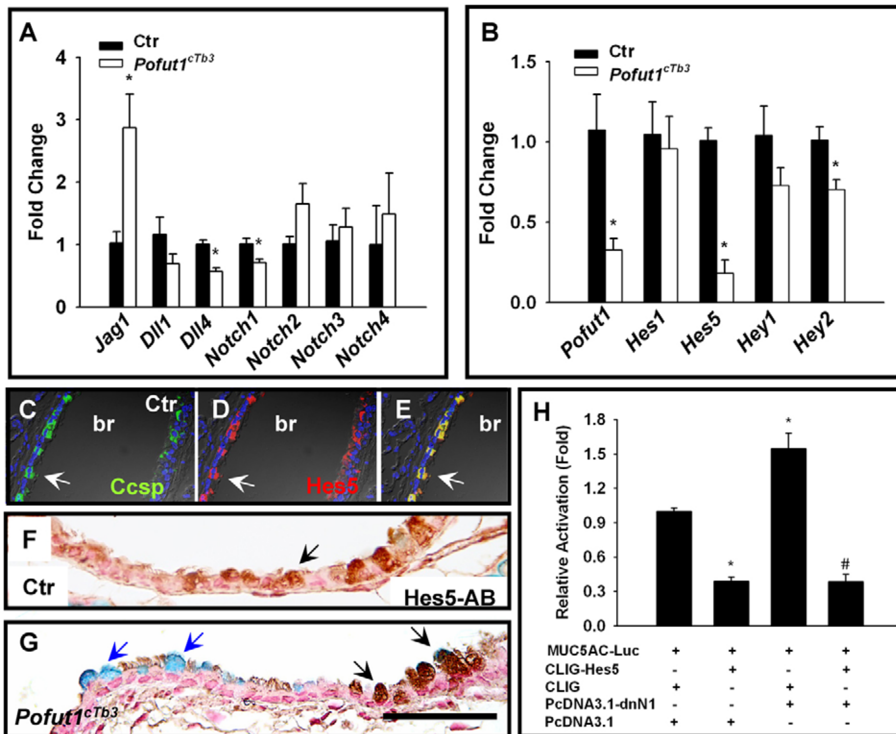

Fig. 7. Hes5 as a mediator of the Notch effects in mucin gene expression. (**A**,**B**) Real-time PCR of Notch pathway components in control and *Pofut1*^{cTb3} lungs at PN30: significant upregulation of *Jag1*, downregulation of *Dll4* and *Notch1* in mutants; marked downregulation of *Pofut1* and *Hes5* and, to a lesser extent, Hey2 in mutants (*n*=3 or 4 per group). **P*<0.05 versus control. (**C-E**) Double-immunostaining for Hes5 (red) and Ccsp (green) in PN30 control airways showing colocalization. (**F**,**G**) Hes5-AB double staining of controls shows Hes5 but no AB-labeled cells (F); in *Pofut1*^{cTb3} mutants (G) AB-positive goblet cells are present but negative for Hes5. (**H**) *MUC5AC-Luciferase* promoter activity in LA4 cells: *MUC5AC* co-transfected with *Hes5* cDNA rescues the negative effect of dnN1 on *MUC5AC* expression. Values are mean+s.e.m., **P*<0.05 versus control. #*P*<0.05 versus dnN1 transfection only.

restricted to Clara cells (Fig. 7C-E). Consistent with our RT-PCR findings, Hes5 staining was significantly reduced in the airway epithelium of *Pofut1*^{cTb3} mutants (Fig. 7F,G). Hes5 labeling was absent in goblet cells and only few Hes5-positive Clara cells could be detected (Fig. 7F).

Hes5 as a candidate mediator of the Notch effects in restricting mucin gene expression

Based on the observations above we hypothesized that Hes5-mediated Notch signaling could play a role in the regulation of mucin gene expression. To explore this possibility, first we tested the ability of Hes5 to directly regulate the transcriptional activity of *MUC5AC*. Thus we co-transfected a *Hes5* full-length cDNA construct [CLIG-*Hes5* (Hojo et al., 2000)] and the same *MUC5AC*-Luc construct previously used in LA4 epithelial cells and assessed luciferase activity after 48 hours. This resulted in a significant decrease in *MUC5AC*-Luc expression compared with controls in which no *Hes5* cDNA was transfected. As expected, disrupting Notch signaling with a dominant-negative *Notch1* construct (PcDNA3.1-dnN1) increased *MUC5AC* transcriptional activity (Fig. 7G, and Fig. 6D above). Remarkably, expression of a full-length *Hes5* cDNA prevented the increase in *MUC5AC* activity induced by PcDNA3.1-dnN1 in LA4 cells (Fig. 7G). This suggested that *Hes5* could play an important role in regulating the transcriptional activity of a mucin gene that is highly expressed in the airways of *Pofut1*^{cTb3} mutants and is typically found in goblet cell metaplasia.

To further investigate this issue, we analyzed lung differentiation in *Hes5* null mice. *Hes5* null mutants are fertile and have no obvious developmental defects; however, the lung phenotype has not been previously described (Cau et al., 2000). Analysis of E18.5 and PN30 lungs showed no significant differences in the profile of differentiation of *Hes5* mutants compared with controls, including number or timing of Clara or goblet cell differentiation as seen by expression of key markers (see Fig. S5 in the supplementary material and data not shown). We concluded that although *Hes5* is likely to restrict goblet cell differentiation by inhibiting transcription of mucin genes, this effect may be shared in vivo with other basic helix-loop-helix (bHLH) factors downstream targets of Notch. Alternatively, an additional stimulus is required to trigger a goblet cell metaplasia response in these mice.

DISCUSSION

Here we provide novel evidence that disruption of Notch signaling in the murine airway epithelium leads to goblet cell metaplasia during postnatal life. This phenotype is consistent with observations reported in the intestine in which Notch plays a prominent role in controlling goblet cell differentiation (Crosnier et al., 2005; Milano et al., 2004; Ridgway et al., 2006; Searfoss et al., 2003; van Es et al., 2005; Wong et al., 2004).

Previous reports using a *Shh-Cre* genetic approach to disrupt *Pofut1 or Rbpjk* genes early in development show that Notch deficiency prenatally has minimal effect in goblet cell differentiation in the airway epithelium. Instead, it has a dramatic effect in preventing formation of Clara cells (Morimoto et al., 2010; Tsao et al., 2009). Goblet cells constitute a very small population of the normal murine adult airway, although there is recent evidence of underestimation, largely owing to sensitivity of the standard labeling techniques (Roy et al., 2011). These cells can be greatly expanded upon exposure to environmental agents, such as allergens or cigarette smoke. Although the origin of the goblet cells is still little understood, there is increasing evidence that they arise from Clara cells, basal cells or an undifferentiated progenitor cell (Alessandrini et al., 2010; Chen et al., 2009; Evans et al., 2004; Hayashi et al., 2004; Kouznetsova et al., 2007; Morrisey and Hogan, 2010; Reader et al., 2003; Roy et al., 2011; Turner and Jones, 2009) A previous study shows that antigen challenge in ovalbumin-sensitized adult mouse elicits mucin production in Clara cells in the intrapulmonary airways (Evans et al., 2004). Another study shows that in the same model the increase in number of

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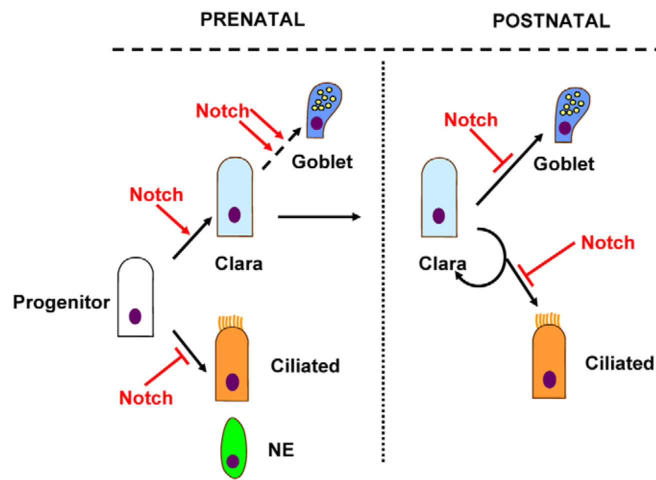

Fig. 8. Proposed model for the role of Notch in airway epithelium pre- and postnatally. Different thresholds of Notch activation influences cell fate in airway progenitors prenatally. In undifferentiated airway progenitors, no Notch fosters the ciliated and NE cell fate, whereas endogenous (physiologic) or high (supraphysiologic) levels of Notch signaling leads to differentiation in Clara cell or goblet cell fates, respectively. Once Clara cell fate is established, Notch signaling is required to restrict a goblet cell differentiation program in a subpopulation of Clara cells during the postnatal period. Notch could be also maintaining the balance of ciliated and secretory cells in adult airways.

goblet cells in the proximal airways is accompanied by a decrease in Clara cell number and a small decrease in ciliated cell number (Reader et al., 2003). These studies suggest that goblet cells derive from resident Ccsp-expressing secretory cells and that Clara cells serve as progenitors to goblet cells (Chen et al., 2009). Our findings from the *Pofut1*$^{F/F}$;G-Red$^{+/WT}$;*Tgfb3*$^{Cre/+}$ mouse model and the association of excessive goblet cells with decreased Clara cell abundance without significant changes in proliferation in *Pofut1*^{cTb3} mutants suggested that the goblet cell metaplasia in *Pofut1*^{cTb3} mutants probably resulted from conversion of Clara into goblet cells. Interestingly, the aberrant formation of goblet cells followed a PD pattern of distribution with increasing age in spite of the presence of *Tgfb3-Cre* activity in both proximal and distal regions. Moreover, our lineage study confirmed that a large number of the *Pofut1*^{cTb3} targeted cells remained Ccsp+ even in the adult PN30 lung. The observations above suggested intrinsic regional differences in susceptibility to Notch deletion in developing airways. This is in agreement with studies showing that the ability to induce a goblet cell metaplasia program in allergen-induced asthma is regionally limited to a population of Clara cells in large and medium-sized airways (Evans et al., 2004). This further supports the idea of a significant diversity of Clara cells in airways.

What could be the possible mechanism for goblet cell metaplasia in *Pofut1*^{cTb3} mutants? Disruption of Notch signaling in these mutants was consistently associated with enhanced expression of genes regulating goblet cell differentiation, including *Spdef, Foxa3* and *Agr2*. Moreover, we showed that Notch directly downregulates *Muc5ac* transcription. Thus, our data are consistent with the idea that Notch represses the goblet cell program in a subpopulation of Clara cells by preventing expression of goblet cell-associated genes. Our finding of the altered balance in ciliated versus Clara cells in *Pofut1*^{cTb3} lungs is in agreement with the lateral inhibition model proposed in previous reports (Morimoto et al., 2010; Tsao et al., 2009). This model, however, is unlikely to explain how Notch prevents goblet cell differentiation. Rather, we propose that during the postnatal period, Notch signaling may be modulated by intrinsic (part of the normal developmental program of goblet cells) or extrinsic (environmental insults: disease) stimuli to control formation of goblet cells from a subpopulation of Clara cells. Presumably this could be achieved by fine-tuning levels of expression of ligands, receptors or by modulating Notch activity, thus altering the secretory cell phenotype.

Paradoxically, increased goblet cell differentiation was observed in both our Notch loss-of-function model (postnatal) and the gain-of-function model (prenatal) reported in Guseh et al. (Guseh et al., 2009). Although we could not precisely determine the molecular basis for these differences, the observation is consistent with the known opposing role of Notch in controlling cell fate even within the same organ, depending on developmental timing and the cell population it acts upon. Indeed, Notch activation has been reported to increase goblet cell number by driving differentiation of postmitotic cells into mature goblet cells in the intestine; however, it can also inhibit the intestine goblet cell program if targeted to crypt cells, being highly context-dependent (Crosnier et al., 2005; Fre et al., 2005; Milano et al., 2004; Ridgway et al., 2006; Searfoss et al., 2003; Stanger et al., 2005; van Es et al., 2005; Wong et al., 2004).

Our model predicts that airway epithelial cell fate is strongly influenced by different thresholds of Notch activation. Prenatally, in undifferentiated airway progenitors, the absence of Notch fosters the ciliated and NE cell fate, whereas endogenous (physiologic) or high (supraphysiologic) levels of Notch signaling results in differentiation towards the Clara cell or goblet cell fates, respectively. However, once Clara cell fate is established, Notch signaling is required to restrict a goblet cell differentiation program in the Clara cells during the postnatal period (Fig. 8). Although in the adult lung there is evidence of Notch activation in regenerating Clara cells post-naphthalene injury, a definitive conclusion about the role of Notch on Clara cell homeostasis in the absence of injury has been confounded by the low turnover rates of the normal adult lung (Morimoto et al., 2010).

What triggered goblet cell metaplasia postnatally in *Pofut1*^{cTb3} mutants remains unclear. We propose that environmental stimuli, such as subliminal oxidative stress or exposure of the Notch-deficient Clara cells to a non pathogen-free environment could have been the triggering event. Although not obvious in our mouse strain, a transient increase in goblet cell number has been reported even in control postnatal mice (Roy et al., 2011). This further supports the requirement of a signal such as Notch to control the goblet cell program postnatally.

Interestingly, no changes in expression of known proinflammatory cytokine genes were detected, supporting the idea that Notch signaling regulates goblet cell differentiation in airway epithelial cells independent of active inflammatory events. This is in agreement with a previous study showing that Notch regulation of mucin gene expression is not affected by disruption of Stat6, a crucial effector of IL-mediated responses (Guseh et al., 2009).

The Notch-mediated control of goblet cell fate described here is likely to be relevant in the context of human disease. Failure of this mechanism may be part of the aberrant responses of the airway epithelium that lead to mucus hypersecretion in conditions, such as asthma and COPD. In support of this, a study from Tilley et al. (Tilley et al., 2009) shows that expression of Notch pathway components is downregulated in airways from human smokers and

smokers with COPD, compared with control subjects. Interestingly, in the COPD group, HES5 and HEY2 were the most significantly downregulated of the Notch targets, with no change in HES1, consistent with our findings in the *Pofut1*^{cTb3} model. Future experiments will further examine the potential cooperative contribution of Hes and Hey genes in regulating the goblet cell program in vivo under normal or exposure to environmental injurants.

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Competing interests statement
The authors declare no competing financial interests.

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DEVELOPMENT

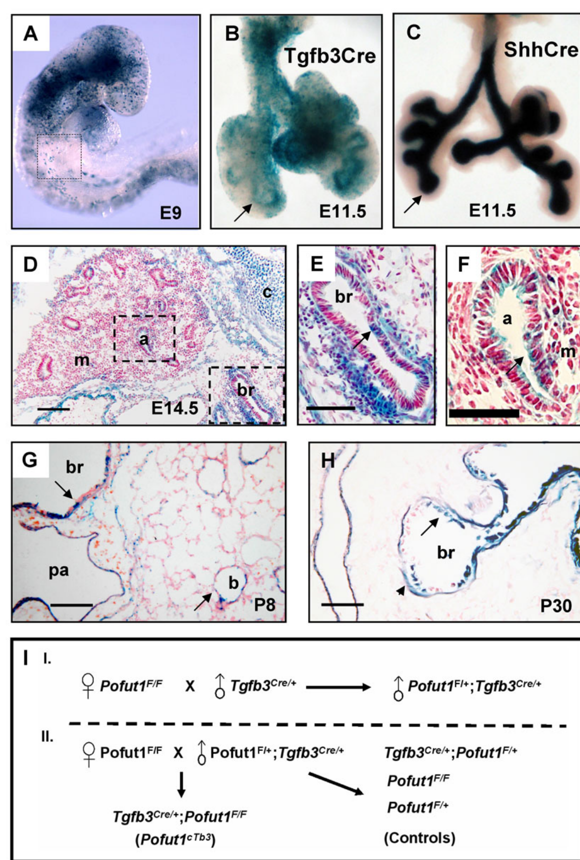

Fig. S1. *Cre* activity in *Tgfb3-Cre;R26R* mice and conditional deletion of *Pofut1*. (A) Whole-mount x-gal staining at E9 revealed x-gal staining in the head, heart and tail regions, but not in the foregut at the prospective lung region (square). **(B,C)** Whole-mount x-gal staining of E11.5 lung. Scattered positive staining in both mesenchyme and epithelium (arrow) of *Tgfb3-Cre;R26R* lungs **(B)**, in contrast to the very high and homogenous *lacZ* epithelial expression (arrow) in *Shh-Cre;R26R* lungs **(C)**. **(D-F)** Histological sections shows that at E14.5 only a few cells are labeled in the epithelium (arrow) of *Tgfb3-Cre;R26R* lungs, while signals are stronger in cartilage (c) and peribronchial smooth muscle layer (m). **(G)** In PN8 lungs, lacZ expression was still in scattered airway epithelium (arrow) and endothelium. **(H)** In adult PN30 lung, *lacZ* activity was detected in most airway epithelial cells (arrow) and smooth muscle (arrowhead) of airways and pulmonary vessels. Scale bar: 100 µm. **(I)** Strategy to create *Pofut1*^{cTb3} mutants. a, airway; br, bronchus; b, bronchiole; c, cartilage; m, lung mesenchyme; pa, pulmonary artery; pv, pulmonary vein.

Development • Supplementary information

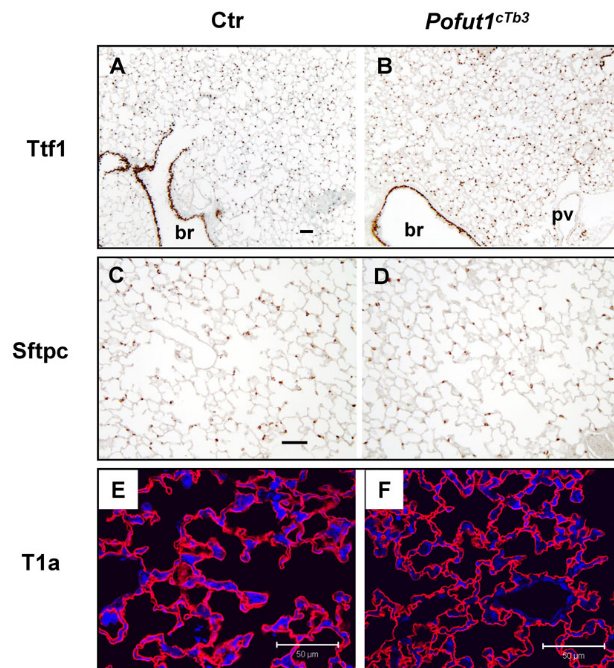

Fig. S2. Distal lung differentiation is intact in *Pofut1^{cTb3}* mutants at PN30.
Immunohistochemical staining for *Ttf1* labeling the distal epithelium **(A,B)**, *Sftpc* labeling the type **II** pneumocytes **(C,D)** and *T1alpha* (*T1a*) labeling the type **I** pneumocytes **(E,F)**. A similar pattern of staining is found in controls and *Pofut1^{cTb3}* mutant lungs. br, bronchus; pv, pulmonary vein.

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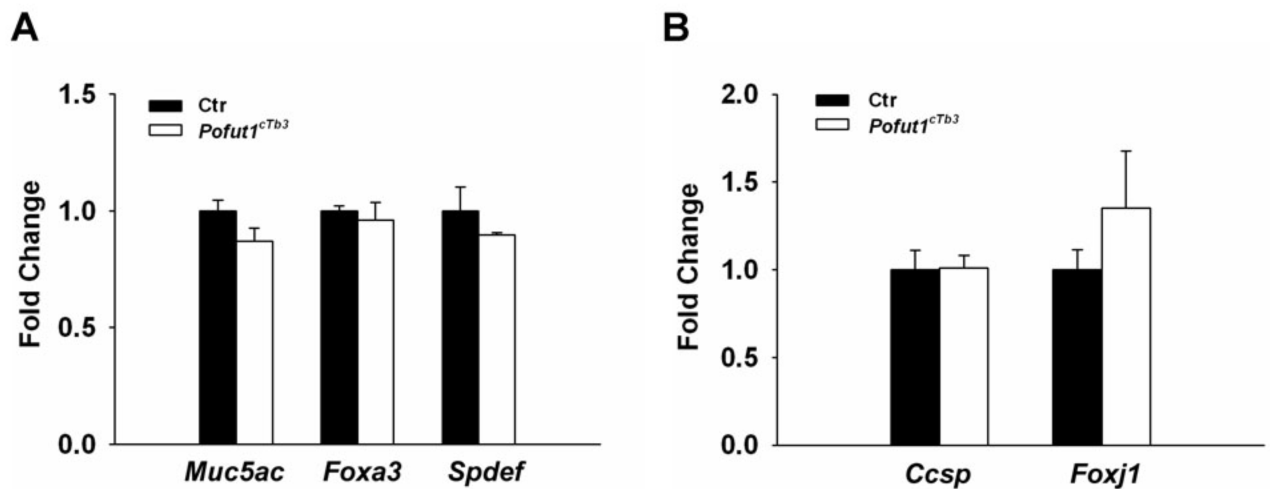

Fig. S3. Expression of mucin-related genes *Ccsp* and *Foxj1* in PN4 lungs. Real-time PCR showing that *Muc5ac*, *Foxa3*, *Spdef* **(A)**, *Ccsp* and *Foxj1* **(B)** expression is similar in lungs of control (*n*=5) and *Pofut1*^{cTb3} mutants (*n*=3) at P4.

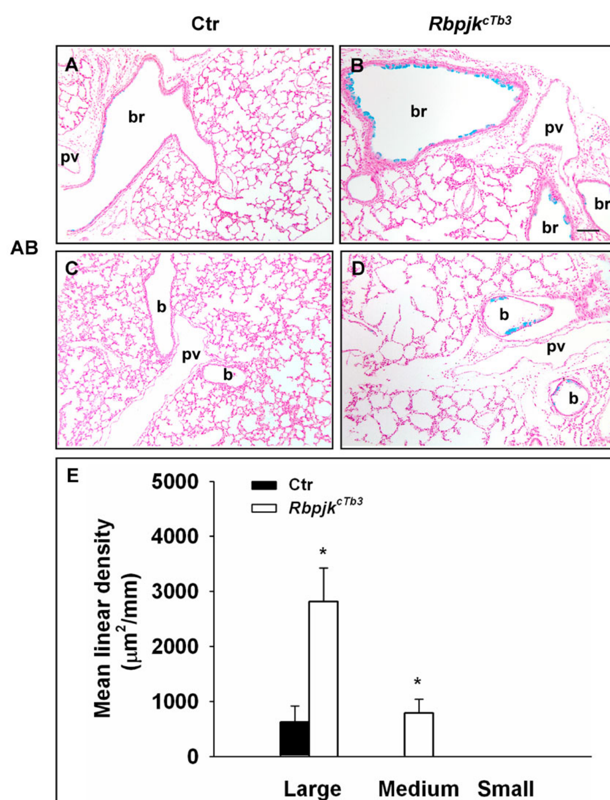

Fig. S4. Rbpjk deletion results in goblet cell dysplasia in airways. PN7 lungs of *Rbpjk*^{cTb3} mutants were analyzed by Alcian Blue staining. Only rare Alcian Blue-positive goblet cells were present in large airways of control mice (**A**), but not in mid-size airways (**C**). Similar to *Pofut1*^{cTb3} mutants, numerous goblet cells lined large (**B**) and medium (**D**) airways of *Rbpjk*^{cTb3} mutants. (**E**) Alcian Blue staining area was significantly increased in *Rbpjk*^{cTb3} mutant airways (*n*=3 in each group). br, bronchus; b, bronchiole; pv, pulmonary vessel.

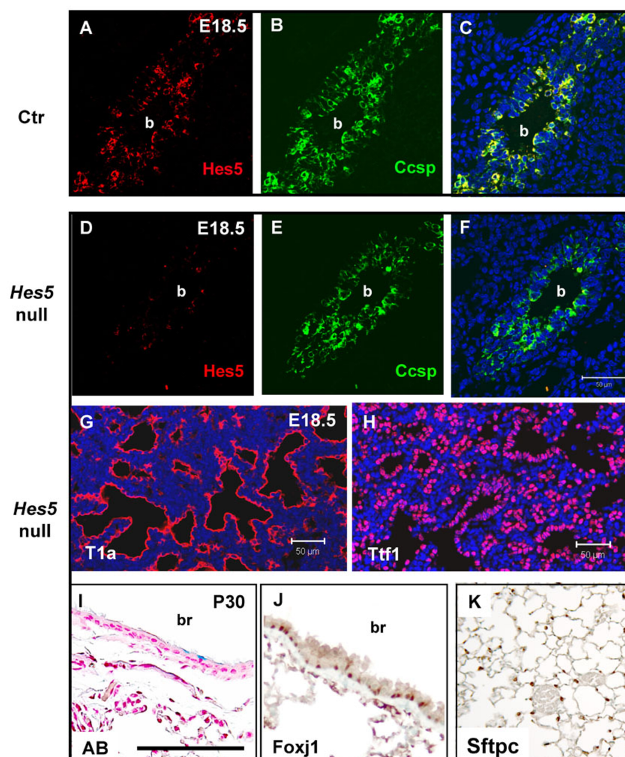

Fig. S5. *Hes5*-null mouse has normal Clara cell differentiation. (A-F) Co-immunostaining of Ccsp and Hes5 was performed in E18.5 control and *Hes5*-null lungs. Hes5 signals were detected in airway epithelium and co-localized with Ccsp in control lungs (A-C). No Hes5 expression by immunostaining is seen in *Hes5* null lung, which validates the specificity of the antibody used in our study (D); Ccsp expression was intact in *Hes5*-null airway epithelium (E-F). **(G-K)** The expression pattern of *T1a* (G), *Ttf1* (H), AB (I), *Foxj1* (J) and *Sftpc* (K) in *Hes5* mutant lungs was similar to that of control lungs (see Fig. 1). Scale bar: 50 ¼m. br, bronchus; b, bronchiole.

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Table S1. Real-time PCR primer sequences (5' to 3')

Gene	Forward primer	Reverse primer
Jag1	ACCACCTGCGAAGTGATTGAC	GAGATATACCGCACCCCTTCAG
Dll1	CCCATCCGATTCCCCTTCG	GGTTTTCTGTTGCGAGGTCATC
Dll4	CAGTTGCCCTTCAATTTCACCT	AGCCTTGGATGATGATTTGGC
Notch1	CCGTGGCTCCATTGTCTACCT	CATCGGTGGCACTCTGGAA
Notch2	CCAAGCGGAAGCAAGCAT	GGCGCTTGTGATTGCTAGAGT
Notch3	TGCCAGAGTTCAGTGGTGG	CACAGGCAAATCGGCCATC
Notch4	GGTTTGCCAGCTCCTATTGG	CAGCCAGCATCAAAGGTGTAGT
Hes1	CCAGCCAGTGTCAACACGA	AATGCCGGGAGCTATCTTTCT
Hes5	AGTCCCAAGGAGAAAAACCGA	GCTGTGTTTCAGGTAGCTGAC
Hey1	GCGCGGACGAGAATGGAAA	TCAGGTGATCCACAGTCATCTG
Hey2	AAGCGCCCTTGTGAGGAAAC	GGTAGTTGTCGGTGAATTGGAC
Pofut1	GGGTCACCTTCATGTACAAGTGAGTG	ACCCACAGGCTGTGCAGTCTTTG
Ccsp	ATGAAGATCGCCATCACAATCAC	GGATGCCACATAACCAGACTC T
Foxj1	CCCTGACGACGTGGACTATG	GCCGACAGAGTGATCTTGGT
Muc5ac	CTGTGACATTATCCCATAAGCCC	AAGGGGTATAGCTGGCCTGA
Spdef	AAGGCAGCATCAGGAGCAATG	CTGTCAATGACGGGACACTG
Agr2	GGAGCCAAAAAGGACCCAAAG	CTGTTGCTTGTCTTGGATCTGT
Foxa3	CCCACGCCAAACCACCATATT	CCCGGTAGTACGGGAAGAGG
Gapdh	CTTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGAG