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Different assemblies of Notch receptors coordinate the distribution of the major bronchial Clara, ciliated and neuroendocrine cells

Mitsuru Morimoto[1,2,*,‡], Ryuichi Nishinakamura[3], Yumiko Saga[1,2] and Raphael Kopan[4]

SUMMARY

In the developing lung, it is thought that the terminal buds of elongating airways contain a population of multipotent epithelial progenitors. As the bronchial tree extends, descendants of these cells give rise to lineage-restricted progenitors in the conducting airways via Notch signaling, which is involved in the establishment of epithelial Clara, ciliated and pulmonary neuroendocrine (NE) cell populations. However, the precise molecular details of this selection process are still emerging. Our stepwise removal of the three Notch receptors from the developing lung epithelium reveals that, whereas Notch2 mediates the Clara/ciliated cell fate decision with negligible contributions from Notch1 and Notch3, all three Notch receptors contribute in an additive manner to regulate the abundance of NE cells and the size of the presumptive pulmonary neuroepithelial body (pNEB) as a result of mutual interactions between NE cells and the Notch-dependent, SSEA-1$^+$, CC10$^-$ cell population surrounding the pNEB (SPNC cells). Ectopic expression of the Notch1 or Notch2 intracellular domain was sufficient to induce SSEA-1$^+$ cells and to suppress pNEB formation without expending Clara cells. We provide evidence that the additive functions of Notch receptors, together with other signaling pathways, maintains the expression of Hes1, a key regulator of NE cell fate, and that maintenance of Hes1 expression in epithelial cells is key to the regulation of pNEB size. These results suggest that two different assemblies of Notch receptors coordinate the numbers and distribution of the major epithelial cell types in the conducting airway during lung organogenesis.

KEY WORDS: Lung, Notch signaling, Neuroendocrine, Mouse

INTRODUCTION

Lung development relies on reciprocal mesenchymal-epithelial interactions orchestrated by temporal and spatial expression waves of multiple secreted factors and their downstream effectors (Morrisey and Hogan, 2010). Airway branching morphogenesis takes place at E11.5-16.5 in the mouse (the pseudoglandular stage). The terminal buds contain a population of multipotent epithelial progenitors during this period, which give rise to lineage-restricted descendants that produce at least seven major cell types in the 'stalk' region (Perl et al., 2002b; Rawlins et al., 2009). Thus, the early stalks form the proximal airway, then the distal airways and finally the alveoli (Cardoso and Lü, 2006). The Clara, ciliated and pulmonary neuroendocrine (NE) cells are common throughout the conducting airways, but their ratios vary along the proximodistal axis. In the murine tracheal epithelium, the basal cell generates goblet cells, a few Clara cells and many ciliated cells (Hong et al., 2004; Rock et al., 2009). In the distal bronchioles, Clara cells are more abundant than ciliated cells; a few NE cell clusters (called neuroepithelial bodies, or NEBs) per unit area are also present. The distal-most alveoli are lined with a single thin layer of flat type I cells and cuboidal type II cells (Morrisey and Hogan, 2010).

The Notch signaling pathway plays an important role in the developing respiratory system. Notch genes encode single-transmembrane receptors that mediate short-range communication between cells in all animal species (Kopan and Ilagan, 2009). When Notch binds to its ligand expressed on adjacent cells, a negative control region unfolds that permits the shedding of its extracellular domain by ADAM proteases. A subsequent cleavage within the transmembrane domain executed by the enzyme γ-secretase (Mumm and Kopan, 2000; Kopan and Ilagan, 2009) releases the Notch intracellular domain (NICD). NICD translocates to the nucleus, where it associates with Rbpj/Cbf-1, a DNA-binding protein. The NICD/Rbpj complex recruits the adaptor mastermind and assembles a transcription activation complex on target promoters (Lubman et al., 2004). In mammals, four Notch homologs (*Notch1* to *Notch4*) and at least five ligands (*Jag1*, *Jag2*, *Dll1*, *Dll3* and *Dll4*) mediate these signaling events. There are many auxiliary factors that modulate Notch signaling. One of these, protein *O*-fucosyltransferase 1 (Pofut1), conjugates *O*-fucose to specific extracellular EGF repeats, modifications that are essential for productive Notch-ligand interactions in mammals (Okamura and Saga, 2008; Stahl et al., 2008). Although Pofut1 may have additional substrates, *Pofut1*-null mice display Notch signaling defects that are similar to those associated with the loss of γ-secretase or Rbpj (Shi and Stanley, 2003; Okamura and Saga, 2008).

Conditional removal of Pofut1 or Rbpj from lung bud epithelia promoted ciliated cell expansion at the expense of Clara cells (Tsao et al., 2009; Morimoto et al., 2010). By contrast, constitutive

[1]Division of Mammalian Development, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan. [2]Department of Genetics, The Graduate University for Advanced Studies, Mishima, Shizuoka 411-8540, Japan. [3]Department of Kidney Development, Institute of Molecular Embryology and Genetics (IMEG), Kumamoto University, Kumamoto 860-061, Japan. [4]Department of Developmental Biology and Division of Dermatology, Washington University School of Medicine, Box 8103, 660 South Euclid Avenue, St Louis, MO 63110-1095, USA.

*Present address: Lung Development and Regeneration, RIKEN RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan

‡Author for correspondence (mmorimoto@cdb.riken.jp)

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expression of the Notch1 intracellular domain (N1ICD) in lung epithelial cells promoted mucous metaplasia and remarkably decreased the number of ciliated cells (Guseh et al., 2009). Jag1 expression is detected exclusively in ciliated cells (Tsao et al., 2009), and immunohistochemical analysis identified endogenous N1ICD in their neighbors (Morimoto et al., 2010). In vivo fate-mapping analysis of cells experiencing Notch1 activation provided evidence that such cells generate Clara cells, but not ciliated cells or NE cells (Morimoto et al., 2010). These observations suggest that the primary function of Notch1 in lung epithelial cells is to promote the Clara cell fate, either by suppressing the default ciliated cell fate via Notch1-mediated lateral inhibition or by inducing Clara cells directly. In the adult lung, Notch1 is also activated in Clara cells that are regenerating after naphthalene-induced injury (Morimoto et al., 2010). Regeneration of the proximal airways from tracheal stem cells relies on Notch activity to enable the cells to exit the stem cell niche and differentiate into Clara and goblet cells (Rock et al., 2011).

In the absence of *Hes1*, hyperplasia of presumptive NEBs (pNEBs) occurs in embryonic lung at the expense of Clara cells (Ito et al., 2000; Shan et al., 2007). This phenotype was also seen following conditional deletion of *Pofut1* from lung epithelium (Tsao et al., 2009). Because Hes1 is a target of Notch signaling in several biological systems, this led to the conclusion that epithelial Notch signaling was involved in NE cell fate selection. However, *Rbpj*-deficient mice on a mixed background retained *Hes1* expression and displayed only a mild reduction in NE cell numbers, resembling *Hes1* heterozygotes. This may indicate de-repression of Hes1 in *Rbpj*-null epithelia, involvement of Rbpj-independent Notch signaling in the regulation of pNEB size, or regulation of Hes1 by additional pathways.

To clarify the role of specific Notch receptors in establishing the cellular identities in the lung epithelium, we generated an allelic series for Notch1, Notch2 and Notch3 to eliminate Notch signaling from the lung epithelium while retaining Rbpj-mediated repression. Quantitative and histological analyses identified Notch2 as the primary receptor involved in Clara/ciliated cell fate selection, despite the detection of N1ICD in presumptive Clara cells (Morimoto et al., 2010). By contrast, all three Notch receptors were involved in regulating NE fate selection in an additive manner. The triple Notch knockout lung epithelium displays pNEB hyperplasia that resembles the *Hes1*-null phenotype. Additional analyses revealed that the Dll1-expressing NE cells are capped by SSEA-1^{+}, peri-pNEB, N1ICD^{+}, CC10^{-} (SPNC) cells. This novel epithelial cell population is involved in the regulation of NE cell number during pNEB formation. Thus, the numbers and distributions of the three major lung epithelial cell types are established by two distinct Notch-dependent mechanisms: Notch2 signaling controls Clara cell number, and three Notch receptors in SPNC cells activate Hes1 to control pNEBs.

MATERIALS AND METHODS

Mouse strains

We crossed *SHH-Cre; N1$^{flox/+}$, N2$^{flox/+}$* or *SHH-Cre; N1$^{flox/+}$, N2$^{flox/+}$, N3$^{-/+}$* males to *N1$^{flox/flox}$, N2$^{flox/flox}$* or *N1$^{flox/flox}$, N2$^{flox/flox}$, N3$^{-/-}$* females to generate a Notch allelic series (see Fig. 1A) (Krebs et al., 2003; Harfe et al., 2004; Yang et al., 2004; McCright et al., 2006). *SPC-rtTA, TetO-Cre; Rosa-GFP-N1ICD* and *Ascl1* knockout embryos were provided as previously described (Guillemot et al., 1993; Perl et al., 2002a; Guseh et al., 2009). All mice were maintained on the C57BL/6 × CD1 mixed background, with some transgenic lines also contributing FVB or 129 chromosome(s). Animal procedures were performed according to NIH guidelines. The mice were maintained in the animal facility under the Washington University animal care regulations or according to the Animal Care and Use Committee of the NIG.

Immunohistochemistry

Fetal lungs were dissected and fixed in 4% paraformaldehyde for 1 hour to overnight at 4°C, embedded in paraffin and sectioned at 6-7 μm. Sections were rehydrated and treated with 0.3% hydrogen peroxide in methanol for 10 minutes before staining. The antibodies and conditions used for individual immunohistochemical analyses are described in supplementary material Table S1. We define only the main bronchus as the bronchus and the terminal bronchiole as the bronchiole in this paper.

RNA isolation, cDNA synthesis and qRT-PCR analysis

E18.5 embryonic lung mRNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using the SuperScript III first-strand cDNA synthesis kit (Invitrogen). Quantitative RT-PCR was performed on a TaKaRa Thermal Cycler Dice Real Time System Single machine, using Power SYBR Green for Foxj1, CC10, CGRP and Gapdh. Amplification and analysis were performed according to the manufacturer's instructions. All reactions were normalized to Gapdh. Results were plotted as relative expression compared with control, where control was scaled to 1. The PCR primer pairs used in this paper were as follows: CC10-F, CACCAAAGCCTCCAACCTCTAC; CC10-R, GGGATGCCACATAACCAGACTC; Foxj1-F, ATCGTC-GTGCACATCTCGAAG; Foxj1-R, AGCAGAAGTTGTCCGTGATC; CGRP-F, GAGGGCTCTAGCTTGGACAG; CGRP-R, AAGGTGTG-AAACTTGTTGAGGT; Gapdh-F, AATGTGTCCGTCGTGGATCTGA; Gapdh-R, GATGCCTGCTTCACCACCTTCT.

RESULTS

Notch2 mediates Clara/ciliated cell selection

We and other groups have previously reported that deletion of Rbpj or Pofut1 in developing lung epithelium leads to expansion of ciliated cells at the expense of the Clara cell population, and we showed direct evidence for Notch1 receptor activation in Clara cells (Tsao et al., 2009; Morimoto et al., 2010). Given that all four mammalian Notch proteins bind to Rbpj with similar affinity (Kopan and Ilagan, 2009), we attempted to identify the contributions of each Notch receptor during Clara/ciliated cell fate selection (Guseh et al., 2009; Tsao et al., 2009; Morimoto et al., 2010). We used SHH-Cre to genetically remove conditional *Notch1* and *Notch2* alleles from the developing endodermal epithelium in wild-type or *Notch3*-null animals, creating an allelic series culminating in a triple knockout of all receptors (Shh-NTKO; Fig. 1A). Loss of Notch1 and Notch2 was confirmed by antibody staining (supplementary material Fig. S1A-D). We correlated the magnitude of ciliated cell expansion with the number of remaining Notch alleles (Fig. 1B,C) by measuring the levels of *CC10* (a Clara cell marker; *Scgb1a1* – Mouse Genome Informatics) and *Foxj1* (a ciliated cell marker) mRNAs by qRT-PCR (Fig. 1). We observed that *CC10* expression was decreased by 50% when one allele of *Notch1* and one allele of *Notch2* remained, which suggests a dose-dependent additive function for Notch1 and Notch2 in maintaining the Clara cell fate throughout lung epithelial development (Fig. 1B). However, whereas mice retaining only one allele of *Notch2* (*Shh-Cre; N1$^{f/f}$; N2$^{+/f}$*) retained 40.4% of *CC10* expression, mice retaining one allele of *Notch1* (*Shh-Cre; N1$^{+/f}$; N2$^{f/f}$*) lost *CC10* expression (1.29% of wild-type levels). These data suggest that despite the presence of activated Notch1 (N1ICD) in the nucleus of lung epithelial cells (Morimoto et al., 2010), Notch2 makes a dominant contribution in Clara cell fate determination. *CC10* expression was reduced further in the *Shh-Cre; N1$^{f/f}$; N2$^{f/f}$* double knockout (Shh-NDKO), and in the *Shh-Cre; N1$^{f/f}$; N2$^{f/f}$; N3$^{-/-}$* triple knockout (Shh-NTKO). These observations suggested that Notch1 and Notch3 make a modest contribution to Clara fate selection.

To examine the expansion of the ciliated cell fate, we quantified the *Foxj1* mRNA levels in the same samples. We saw a

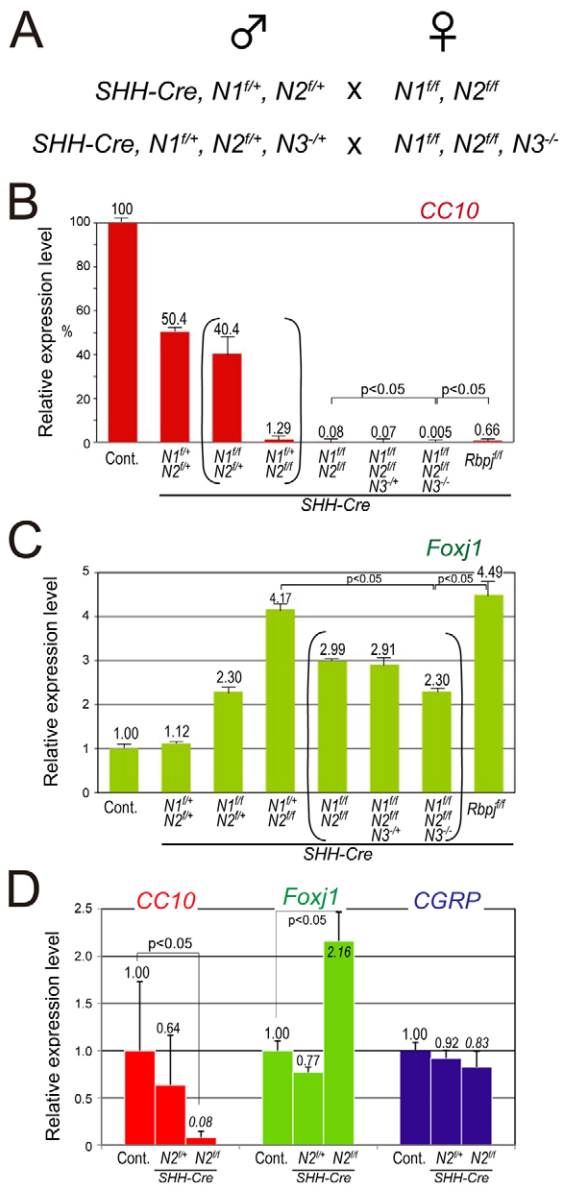

Fig. 1. Endodermal epithelium-specific stepwise removal of Notch paralogs revealed the dominant contribution of Notch2 in Clara/ciliated cell fate decision. (**A**) Two mating strategies were employed to achieve endodermal epithelium-specific knockout of Notch prologs. Male genotypes are noted on the left of the cross. (B-D) For each genotype, total RNA was extracted from one or two whole lungs collected at E18.5. The relative gene expression levels of *CC10* (B) and *Foxj1* (C) in the lungs were measured by quantitative RT-PCR. (**B**) *CC10* expression is progressively diminished by reduction in the Notch signal dose. Brackets highlight a comparison between *SHH-Cre; N1*$^{flox/flox}$, *N2*$^{flox/+}$ and *SHH-Cre; N1*$^{flox/+}$, *N2*$^{flox/flox}$ mice that unveiled a unique contribution of Notch2 in Clara cell fate determination. (**C**) Accordingly, *Foxj1* expression is upregulated in all mutants, reflecting the expansion of ciliated cells (see Fig. 2A). Although elevation of *Foxj1* expression correlates with a reduction of Clara cells, *SHH-Cre; N1*$^{flox/flox}$; *N2*$^{flox/flox}$, *SHH-Cre; N1*$^{flox/flox}$; *N2*$^{flox/flox}$; *N3*$^{-/+}$ and *SHH-Cre; N1*$^{flox/flox}$, *N2*$^{flox/flox}$; *N3*$^{-/-}$ lungs show lower levels of *Foxj1* expression relative to Shh-RKO lungs (brackets). (**D**) Analysis of *SHH-Cre; N2*$^{flox/flox}$ lung also demonstrated the reduction of *CC10* and the elevation of *Foxj1* expression in *Notch2*-null lung epithelium, while the *Cgrp* level was unaltered. Error bars from technical triplicates indicate s.d. *P* values are shown above the bars.

complementary expansion of *Foxj1* expression in *Shh-Cre; N1*$^{f/f}$; *N2*$^{+/f}$ and *Shh-Cre; N1*$^{+/f}$; *N2*$^{f/f}$ cells: the former showed a twofold (230%) expansion in *Foxj1* expression, while the latter, as well as *Shh-Cre; Rbpj*$^{f/f}$ (Shh-RKO), displayed a greater than fourfold increase in the expression of *Foxj1* (Fig. 1C). Accordingly, expanded ciliated cell populations were observed in the developing lung epithelium of both genotypes (Fig. 2A). Individual conditional deletion of Notch1 or Notch2 confirmed the dominant contribution of Notch2 to Clara/ciliated cell selection (Fig. 1D; supplementary material Fig. S2): *Shh-Cre; N2*$^{f/f}$ reproduced the phenotypes of ciliated cell expansion with a great reduction in Clara cell numbers, whereas *Shh-Cre; N1*$^{f/f}$ was indistinguishable from wild type. This unique role of Notch2 in Clara cell fate may be due to the greater abundance of Notch2 (supplementary material Fig. S1A,C), to activation of Notch2-specific targets or to an enhanced activation profile of Notch2 in response to Jag1 (Hicks et al., 2000).

All Notch receptors contribute to the regulation of pNEB abundance and size

We expected a similar expansion in *Foxj1* in Shh-RKO, Shh-NDKO and Shh-NTKO. However, we were surprised to see that *Foxj1* expression in Shh-NDKO and Shh-NTKO was much lower than in Shh-RKO, resembling instead *Shh-Cre; N1*$^{f/f}$; *N2*$^{+/f}$ (230%) (Fig. 1C, brackets). To address the cellular basis of this observation, we assessed the Clara/ciliated cell distribution using immunohistochemistry (Fig. 2A). Foxj1-expressing ciliated cells were distributed in a salt-and-pepper pattern in the distal conducting airways, where Notch dose correlated with the magnitude of ciliated cell expansion at the expense of CC10-postive cells (Fig. 2A, upper panels). The distal conducting airways of *Shh-Cre; N1*$^{+/f}$; *N2*$^{f/f}$, Shh-NDKO, Shh-NTKO and Shh-RKO mice were indistinguishable, containing mostly ciliated cells. However, in bronchi, an expanded Foxj1^{-}, CC10^{-} DAPI^{+} cell population was present in Shh-NDKO and Shh-NTKO mice (Fig. 2A, lower panel, asterisks). Because their cell morphology suggested that they were pNEBs, we examined the distribution of pNEBs in Shh-NTKO and Shh-RKO mice by immunohistochemistry for the NE cell marker CGRP (Calca – Mouse Genome Informatics). Although pNEB abundance in Shh-RKO mice remained similar to wild type and their size was only minimally affected (Fig. 2B) (Morimoto et al., 2010), Shh-NTKO bronchi contained an increased number of pNEBs with some displaying large increases in NE numbers (Fig. 2B, white asterisk). To quantify the abundance of pNEBs in bronchi, we counted pNEBs on histological sections and compared pNEB abundance among genotypes using the Kolmogorov-Smirnov test (K-S test) (Fig. 2C; Table 1). Interestingly, only Shh-NTKO lungs showed a significant increase in the abundance of pNEBs (P<0.05). In addition, we counted the number of NE cells in each pNEB and used the K-S test to determine whether pNEB size was altered in different genotypes (Fig. 2D). A significant increase in NE cell numbers per pNEB was detected in Shh-NTKO (P<0.001) and in Shh-NDKO (P<0.017), indicating that pNEB size reflects the abundance of NE cells in a pNEB. qRT-PCR for *Cgrp* mRNA further revealed that whenever *Foxj1* levels were lower than expected, a corresponding increase in *Cgrp* levels (by up to 6.86-fold in Shh-NTKO lungs) was detected (Fig. 2E). These data indicate that all three Notch receptors contribute in an additive manner to negatively regulate the pNEB size (Fig. 2C-E). Furthermore, Notch1, Notch2 or Notch3 single knockouts did not affect *Cgrp* expression, suggesting that the contribution of any single Notch receptor is redundant in the regulation of pNEB size (Fig. 1D; supplementary material Fig. S2B, Fig. S3).

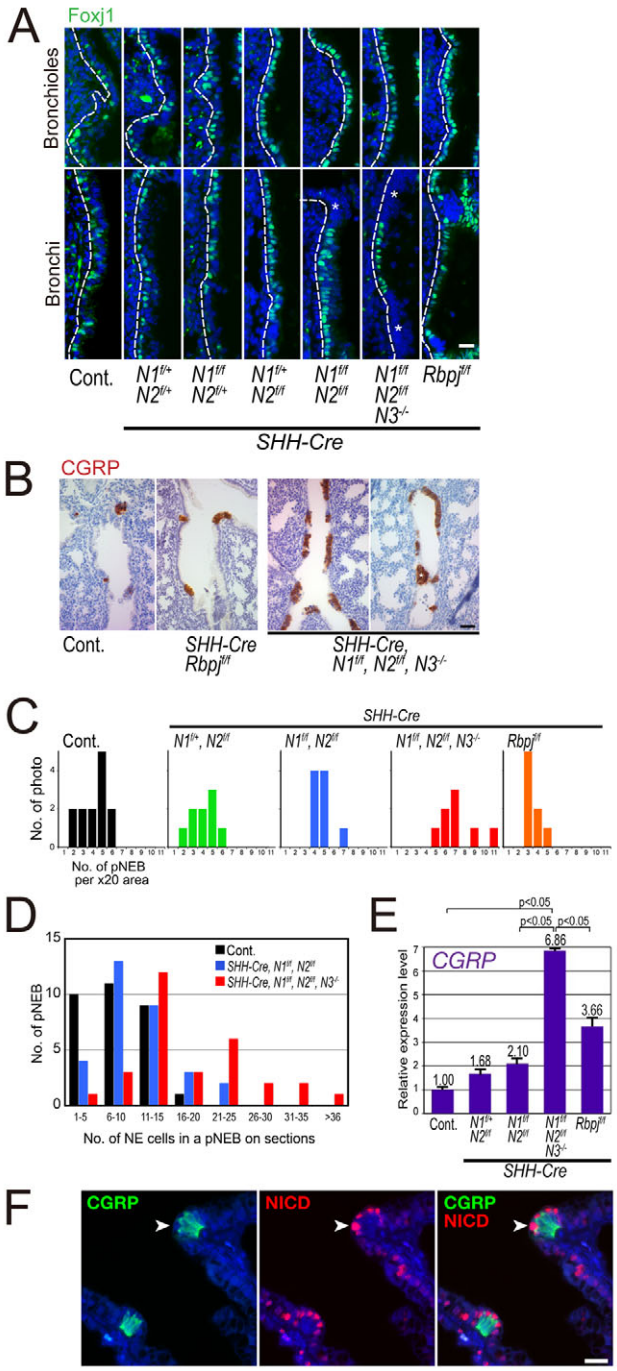

Fig. 2. Loss of Notch receptors affects NE cell numbers. For each genotype, two or three whole lungs were collected at E18.5 and at least six paraffin wax-embedded sections were stained for each individual sample. (**A**) Immunofluorescence of E18.5 bronchioles (upper panels) and bronchus (lower panels) stained with anti-Foxj1 (green) revealed that the ciliated cell expansion progressed as the Notch dose decreased. In addition, foci of Foxj1^{-} cells are observed in the Notch double knockout and triple knockout epithelia (asterisks). Dotted lines demarcate the mesenchyme (left) and the epithelium (right). (**B**) Immunohistochemistry for CGRP in E18.5 lungs shows a significant increase and enlargement of pNEBs (brown) in the Notch triple knockout but a moderate increase in Rbpj knockout epithelium. (**C**) Frequency distribution of pNEB abundance. Eight or nine photographs of proximal airways in 12 sections from two or three mice were taken at ×20 magnification for individual genotypes: control (black), *SHH-Cre; N1$^{flox/+}$; N2$^{flox/flox}$* (green), Notch double knockout (blue), triple knockout (red) and Rbpj knockout (orange) embryos. CGRP-positive clusters were determined as pNEBs on the monitor. Two to 11 pNEBs were observed in each photograph. (**D**) Frequency distribution of the numbers of NE cells per pNEB. The numbers of NE cells in a pNEB were counted in 30 pNEBs randomly selected from anti-CGRP-stained sections, for which at least two biological lung samples were collected from E18.5 control (black), Notch double knockout (blue) and triple knockout (red) embryos. (**E**) Quantification of *Cgrp* expression in E18.5 lungs estimates the magnitude of pNEB enlargement in each genotype. Total RNA was extracted from one to three whole lungs collected at E18.5 for each genotype. In comparison with the control, the Notch triple knockout displayed the greatest expansion, and Rbpj loss elevated the expression more than the loss of any two Notch receptors. (**F**) Double immunofluorescence of E18.5 bronchus with anti-CGRP (green) and anti-N1ICD (red) determined Notch activation in peri-pNEB cells (arrowheads). Scale bars: 10 μm in A,F; 20 μm in B. Error bars from technical triplicates and multiple samples indicate s.d. *P* values are shown above the bars.

To examine which cells receive the Notch signal, we used the anti-N1ICD V1744 antibody (which detects γ-secretase-cleaved Notch1) to identify signal-receiving cells in the vicinity of the pNEB (Xu et al., 2010) (supplementary material Fig. S4). Double staining for N1ICD and CGRP at E18.5 clearly visualized Notch1 activation in cells adjacent to Dll1-expressing NE cells, but never in the NE cells themselves (Fig. 2F, arrowheads). Our previous study in N1IP::CRE reporter mice, which permit the mapping of cell lineages experiencing Notch1 activation in vivo (Vooijs et al., 2007), illustrated that cells experiencing Notch1 activation never become NE cells (Morimoto et al., 2010). These observations suggest that Notch activation did not occur in NE precursors, but rather NE cells could activate Notch signaling in their neighbors. Notch activity is probably necessary for the proper regulation of pNEB size during lung development.

Notch signaling is indispensable to the SPNC cell population and to the regulation of pNEB size

These peri-pNEB cells containing N1ICD may be epithelial progenitor cells for non-NE cell types, including Clara and ciliated cells. We examined the distribution of the progenitor/stem cell marker SSEA-1/CD15 (Yanagisawa, 2011) to see whether peri-pNEB cells express this marker. At E15.5, SSEA-1-positive epithelial cells are abundant in the proximal airways (Xing et al., 2010). At E18.5, the distribution of SSEA-1-positive cells in the proximal airway became restricted in the vicinity of pNEBs (Fig. 3A,B, white arrowheads). Confocal imaging for SSEA-1 and CGRP confirmed the restricted expression pattern of this marker (supplementary material Fig. S5A). SSEA-1-positive cells were negative for CC10, distinguishing them from Clara cells (Fig. 3B, white and gray arrowheads). By contrast, SSEA-1-positive, CC10-positive cells were still abundant in the distal bronchioles at E18.5 (Fig. 3C, white arrowhead). Therefore, the SSEA-1 epitope distinguishes peri-pNEB cells at E18.5 as a distinct, CC10^{-} cell population.

To further characterize the peri-pNEB cells, we analyzed serial sections for the presence of N1ICD (Fig. 3D,E, white arrowhead and supplementary material Fig. S5B), and for Notch2 and Notch3 (supplementary material Fig. S1E,F). Co-staining for Notch and SSEA-1 differentiated these cells from Clara progenitors

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Table 1. *P* values determined by the Kolmogorov-Smirnov test for pNEB abundance in the Notch allelic mutant series

	Control	*SHH-Cre, N1$^{fl/+}$, N2$^{f/f}$*	*SHH-Cre, N1$^{f/f}$,N2$^{f/f}$*	*SHH-Cre, N1$^{f/f}$,N2$^{f/f}$,N3$^{-/-}$*
Control				
SHH-Cre, N1$^{fl/+}$, N2$^{f/f}$	1.0000			
SHH-Cre, N1$^{f/f}$,N2$^{f/f}$	0.3364	0.6994		
SHH-Cre, N1$^{f/f}$,N2$^{f/f}$,N3$^{-/-}$	0.0464*	0.0143*	0.0143*	
SHH-Cre, Rbpj$^{f/f}$	0.9850	0.7804	0.0731	0.0044*

*Significant difference in frequency distribution between two genotypes (P<0.05).

elsewhere. As we have reported previously (Morimoto et al., 2010), Clara (Fig. 3E, gray arrowhead), mesenchymal vascular smooth muscle and endothelial cell populations (Fig. 3E, asterisk) are all positive for N1ICD during lung development. Interestingly, although N1ICD detection requires tyramide amplification and is not quantitative, the signal intensity of N1ICD in peri-pNEB cells appeared faster and appeared more intense than in Clara cells, perhaps reflecting increased levels of Notch1 activation in these cells (Fig. 3E, gray arrowhead). We named these SSEA-1$^+$ peri-pNEB N1ICD$^+$ CC10$^-$ cells, 'SPNC' cells.

NE cell establishment in the proximal airway requires Hes1, Pofut1 or Rbpj (Ito et al., 2000; Tsao et al., 2009). This requirement prompted several authors to hypothesize that Dll1-expressing NE cells activate Notch signaling in their multipotent neighbors, preventing them from assuming the NE cell fate (Ito et al., 2000; Shan et al., 2007). If true, SPNC and NE cells may form an equivalence group. To clarify the relationship between SPNC cells and pNEBs, we examined the distributions of SPNC cells and NE cells in E18.5 Achaete-scute complex homolog 1 (*Ascl1/Mash1*)-deficient lungs (Guillemot et al., 1993; Borges et al., 1997) (Fig. 4). *Ascl1* is a basic-HLH type transcription factor that is indispensable in NE cells, and a presumed transcriptional activator of Dll1 that is repressed by Hes1 (Ito et al., 2000; Shan et al., 2007). Loss of Ascl1 results in loss of NE cells (Guillemot et al., 1993; Borges et al., 1997). Immunohistochemistry on multiple sections found instead that only a few SSEA-1$^+$ cells remained in the *Ascl1*-null bronchus (Fig. 4D). This is not because of the dependence of the SSEA1 markers on Ascl1, because bronchiolar SSEA-1$^+$ CC10$^+$ cell populations were unaltered in the *Ascl1* knockout (Fig. 4C). These data suggest that the existence of SPNC cells, but not of the SSEA-1 antigen, depends on *Ascl1*-expressing NE cells in the developing bronchus.

To investigate whether Notch activation is needed for SPNC cell maintenance, we examined the distribution of SPNC cells in *Shh-Cre; N2$^{f/f}$ and Shh-Cre; N1$^{+/f}$; N2$^{f/f}$* lungs. These mutants have

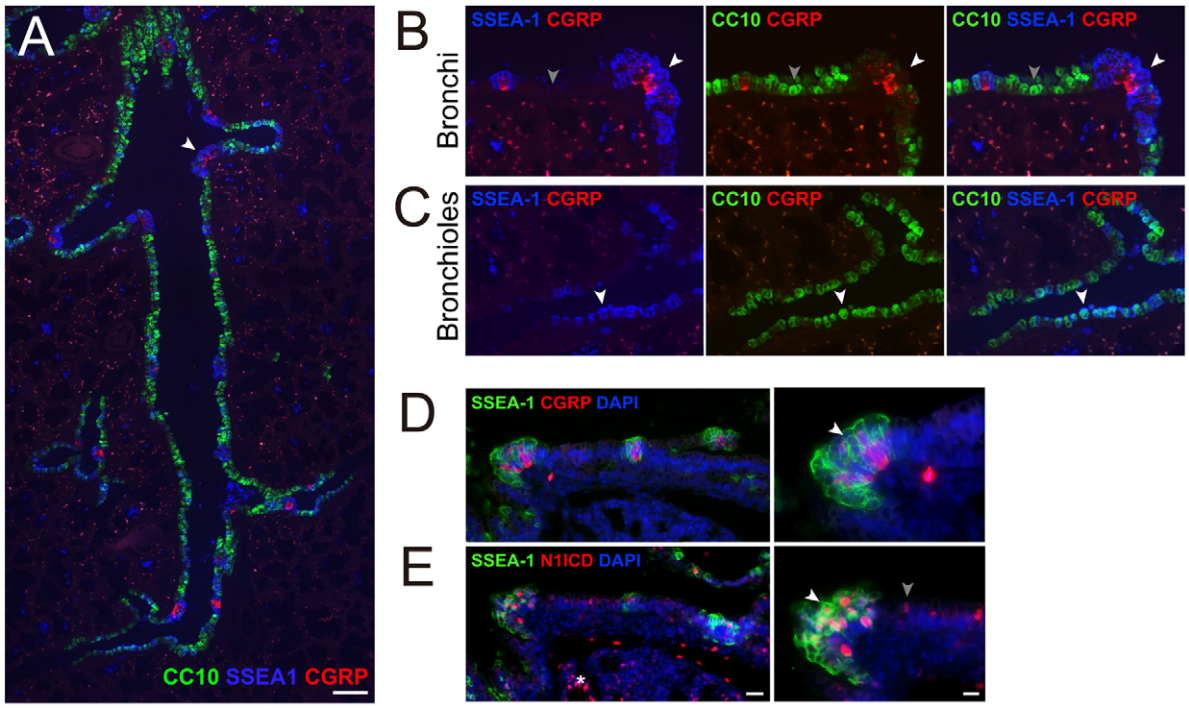

Fig. 3. Peri-pNEB, Notch signal-receiving cells in the bronchus visualized by SSEA-1 immunoreactivity. (**A-C**) Immunofluorescence staining of E18.5 lung with anti-CC10 (green), anti-CGRP (red) and anti-SSEA-1 (blue) shows localizations of Clara, NE and SSEA-1$^+$ cells. At the bronchus, SSEA-1$^+$ cells are restricted to the peri-pNEB area (white arrowheads in A and B). These are SSEA-1$^+$ CGRP$^-$ CC10$^-$ cells. The SSEA-1$^+$/pNEB clusters are interspaced between Clara cells (gray arrowheads in B). At the bronchioles, SSEA-1$^+$ cells that are often CC10$^+$ are broadly distributed (white arrowheads in C). (**D**,**E**) Immunofluorescence staining with anti-SSEA-1 (green) and anti-CGRP (red) (D), or anti-SSEA-1 (green) and anti-N1ICD (red) (E) illustrates the SSEA-1 immunoreactivity of peri-pNEB, Notch signal-receiving cells at the E18.5 bronchus (white arrowheads). A gray arrowhead identifies N1ICD in a Clara cell. Some N1ICD-positive vascular smooth muscle and endothelial cells are observed in mesenchyme (asterisk). These distribution patterns were confirmed in at least eight sections from three biological samples. Scale bars: 40 μm in A; 20 μm in B,C, D (left), E (left); 10 μm in D (right), E (right).

DEVELOPMENT

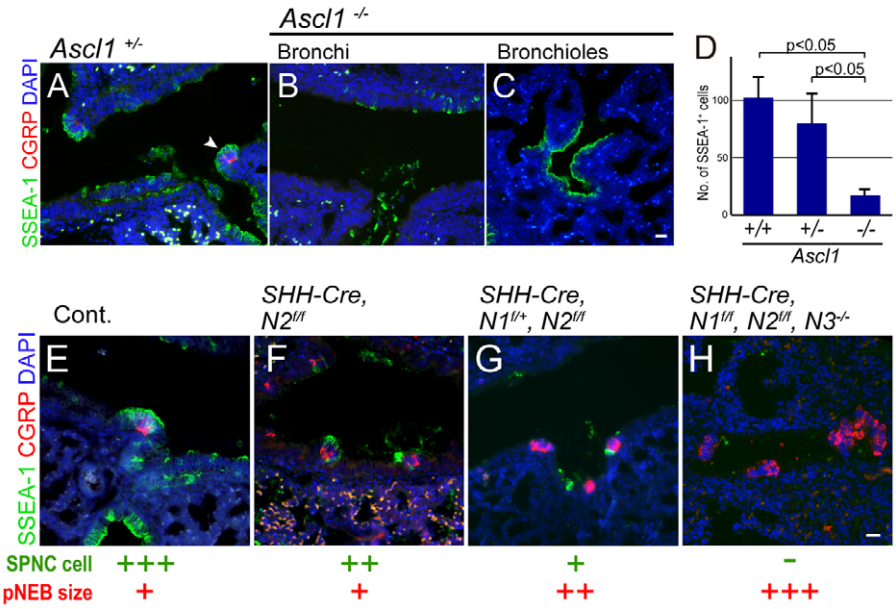

Fig. 4. Notch signals coordinate the NE/SPNC cell populations. (**A-C**) Immunofluorescence staining of SSEA-1 (green) and CGRP (red) in E18.5 *Ascl1* heterozygous (A) or null mutant (B,C) lungs. Wild-type and *Ascl1*$^{+/-}$ lungs contain pNEBs and SPNC cells (white arrowhead), whereas NE cells are not detected in *Ascl1*-null mutants. We also see a dramatic decrease in SSEA-1^{+} cells at the bronchus in *Ascl1*-null mutants, suggesting that the existence of this marker depends on a signal from Ascl1-dependent NE cells. SSEA-1-positive cells remain in *Ascl1*-deficient bronchioles, suggesting that the presence of SSEA1 antigen in this cell population is independent of Ascl1 and pNEBs. (**D**) Quantitative analysis of SSEA-1^{+} epithelial cells in the bronchi of E18.5 wild-type, *Ascl1* heterozygous and null mutant mice. At least 10 photos of the proximal airways at ×20 magnification from three biological samples for individual genotype were examined for this analysis. In the mutant, a significant decrease in the number of SSEA-1^{+} cells is observed. Error bars indicate s.d. *P* values are shown above the bars. (**E-H**) Immunofluorescence staining for SSEA-1 (green) and CGRP (red) in wild-type (E), *SHH-Cre;N2*$^{flox/flox}$ (F), *SHH-Cre;N1*$^{flox/+}$*;N2*$^{flox/flox}$ (G) and *SHH-Cre;N1*$^{flox/flox;}$*N2*$^{flox/flox}$*;N3*$^{-/-}$ (H) lungs. The relative expansion of the SPNC population (green) or pNEBs (red) is represented below. Scale bars: 10 μm in A-C,E; 20 μm in F-H.

abnormal Clara/ciliated cell fate allocation but maintain normal pNEB distribution with no or only a moderate increase in NE gene products (Fig. 1D; Fig. 2C,E). We observed fewer SPNC cells in lungs with these genotypes (Fig. 4F,G). By contrast, loss of SPNC cells and a dramatic increase in NE cells was seen when all three receptors were deleted (Shh-NTKO; Fig. 4H). Thus, SPNC cells require Notch signals and appear to be dependent on NE cells, presumably via the Notch ligand Dll1 expressed by NE cells. SPNC cell numbers show an inverse relationship with NE cell numbers, which is consistent with an additive function for all Notch receptors in NE cell regulation (Fig. 2).

Forced Notch activation increased the SPNC cell-like population but did not increase Clara cell numbers

We further attempted to express a constitutively active Notch1 (N1ICD) in developing lung epithelial cells using *SPC-rtTA, TetO-Cre; Rosa-GFP-N1ICD* tri-transgenic mice (Perl et al., 2002a; Murtaugh et al., 2003) in which GFP-N1ICD is expressed following a doxycycline pulse. Although DOX/rtTA toxicity to Type II lung epithelial cells caused lethality at birth (Morimoto and Kopan, 2009) and the lung epithelial structure was altered by N1ICD expression (Guseh et al., 2009), the bronchial region of the triple transgenic lung was still distinguishable (supplementary material Fig. S6). SSEA-1^{+} cells were now detected throughout the bronchial epithelium at E18.5 (Fig. 5A), implying that Notch1 activation is sufficient to expand a SPNC cell-like population. Ciliated cells and pNEBs became infrequent and their size was reduced (Fig. 5B,C). Ciliated cells that remained were GFP negative (Fig. 5D) and (Guseh et al., 2009), most probably reflecting incomplete targeting with the Dox-induction system. If Notch signaling was sufficient to promote Clara cell fate acquisition, the tri-transgenic mice would contain more Clara cells. However, N1ICD expression slightly decreased the frequency of Clara cells (compare Fig. 5E with Fig. 3B and Fig. 5F). This indicates that persistent Notch signaling blocks differentiation of both ciliated and NE cells, and may even interfere with Clara cell differentiation while maintaining the SPNC cell-like population. The observation of SSEA-1+, CC10+ cells suggests that some SPNC cells may differentiate into Clara cells in the presence of N1ICD (Fig. 5E).

To determine whether the Notch2 intracellular domain could promote Clara cell expansion, we examined the lung epithelium of E18.5 *Shh-Cre; Rosa-N2ICD* mice (Fujimura et al., 2010). Although constitutive Notch signaling altered the overall morphology of the lung and guts, more severely than in the tri-transgenic mice, many parts of the lung epithelium showed extensive SSEA-1 immunoreactivity (Fig. 5G). Again, whereas some SSEA-1^{+} cells also expressed CC10, we did not see an expansion in Clara cells. These data indicate that Notch signaling allows other factors to specify the Clara cell fate, most likely by blocking ciliated cell fate, and further indicates that the importance of Notch2 may be derived by its abundant expression in Clara cells and the selective affinity between the extracellular domains of Notch2 and Jag1 (see Discussion).

DISCUSSION

Ito et al. (Ito et al., 2000) reported that the Notch-Hes1 pathway regulates NE cell selection during lung epithelial development, but

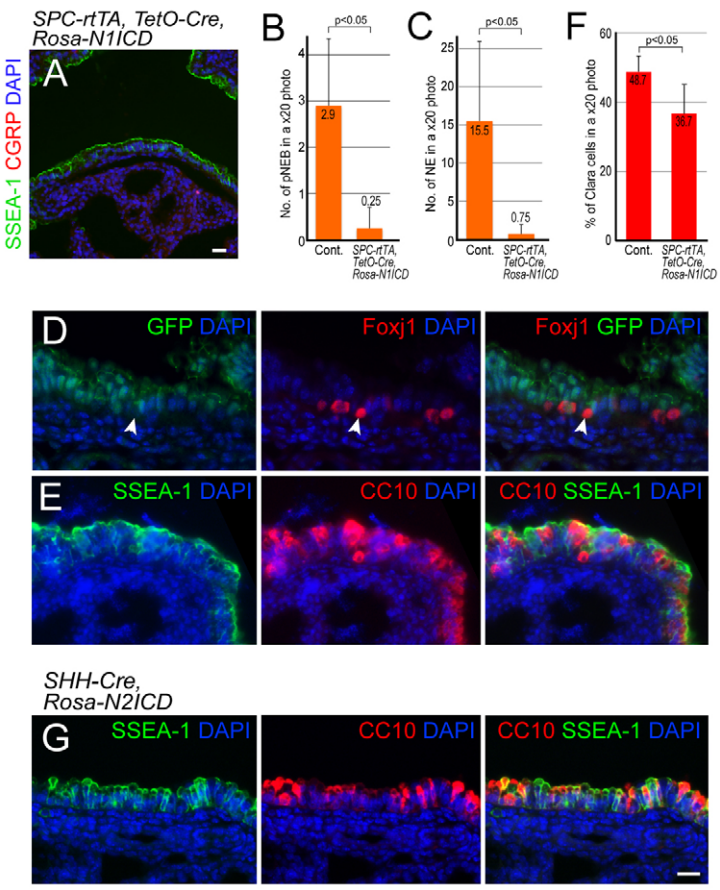

Fig. 5. Artificial Notch1 or Notch2 activation promotes the SPNC cell-like population but not Clara cells. (**A**) Immunofluorescence staining for SSEA-1 (green) and CGRP (red) in *SPC-rtTA;TetO-Cre;Rosa-GFP-N1ICD* lungs. (**B**,**C**,**F**) The numbers of pNEBs (B), NE (C) and Clara cells (F) were quantitatively evaluated in wild-type or triple-transgenic lung. Ten or more photographs of proximal airways in nine sections from three mice were taken at ×20 magnification. The numbers of pNEBs and NE cells were determined by counting cells on the PC display. *P* values are shown above the bars. (**D**) Anti-Foxj1 (red) and anti-GFP (green) identified a few ciliated cells in E18.5 *SPC-rtTA;TetO-Cre;Rosa-GFP-N1ICD* mice that were most likely due to incomplete recombination induced by Dox feeding. (**E**) Immunofluorescence staining for anti-CC10 (red) and anti-SSEA1 (green) determined that forced Notch1 activation does not result in Clara cell hyper-expansion. (**G**) Immunofluorescence staining for SSEA-1 (green) and CC10 (red) in *SHH-Cre;Rosa-N2ICD* mice. Notch2 activation also failed to promote Clara cells. Scale bars: 20 μm in A; 10 μm in D,E,G.

did not establish the cell type requiring Hes1. It has been shown that transgenic expression of N1ICD under the control of the CGRP promoter can inhibit Ascl1 activity and thus NE cell differentiation (Shan et al., 2007). Subsequent investigations revealed that Notch signaling regulates the Clara/ciliated cell fate (Guseh et al., 2009; Tsao et al., 2009; Morimoto et al., 2010) but created a controversy regarding the role of Notch in NE cell fate selection (Tsao et al., 2009; Morimoto et al., 2010). In the present study, we analyzed a series of Notch loss-of-function alleles, which disclosed interesting differences between the NE and Clara/ciliated cells fate decisions. Evidence for Notch1 activation during Clara/ciliated cell fate selection is provided by the accumulation of γ-secretase-generated nuclear N1ICD polypeptides and by linage tracing (Morimoto et al., 2010), yet we show conclusively that the contribution from Notch1 is minimal. We find that Notch2 regulates the Clara/ciliated cell fate decision, perhaps reflecting differences in abundance amplified by the response of Fringe-modified Notch1 and Notch2 to Jag1 (Hicks et al., 2000). Fringe inhibits Notch1 activation by Jag1, whereas activation of Notch2 is not inhibited by Fringe. If this interpretation is correct, we predict that in L-fringe deficient mice, Notch1 will be able to rescue Clara cells in Notch2-deficient lung epithelia. Because *Hes1* null lungs show only a 50% reduction in Clara cell numbers (Ito et al., 2000), and because Clara cells disappear in Notch2-null epithelia and Shh-RKO mice, we conclude that Notch2-mediated signaling acts through genes other than *Hes1* in Clara cell fate determination (Fig. 6F,G). Hes5 is not a candidate Clara cell regulator because *Hes5*-null mutants have no obvious developmental defects and show no significant differences in the profile of epithelial differentiation or the timing of Clara cell differentiation relative to controls (Tsao et al., 2011).

By contrast, the size and abundance of pNEBs appear to be regulated by an additive activity of all three Notch receptors in SPNC cells, most likely triggered by Dll1. Shh-NTKO mice have multiple large pNEBs that scarcely express Hes1 (Fig. 5D,E). By contrast, *Shh-Cre; N1$^{+/f}$; N2$^{f/f}$* and Shh-RKO mice do not show a significant reduction in Hes1 protein (*P*=0.17 and 0.24 in Student's *t*-test) or pNEB size (Fig. 5B,C,E). Combined with the observation that NE cell frequency is moderately increased in the Hes1 heterozygote (Ito et al., 2000), we concluded that pNEB size is determined by the dose of Hes1 in SPNC cells. Although *Hes1* is a well-characterized Notch target gene in some cells, it can be regulated by other pathways (Yoshiura et al., 2007; Nakayama et al., 2008). Thus, Rbpj ablation could lead to de-repression of Hes1 by the Rbpj co-repressor complex. Alternatively, the regulation of *Hes1* by *Alk5* (Xing et al., 2010) suggests that Notch proteins and TGFβ signaling co-regulate *Hes1* during NE cell selection in an RBPj-independent manner. The observation that Smad4 can interact with NICD on selected promoters (Li et al., 2011) may explain how Rbpj-independent (non-canonical) Notch signaling induces Hes1 expression in Shh-RKO.

What is the mechanism for controlling the abundance and size of pNEBs? A classical Notch-mediated lateral inhibition model has been proposed to explain the Hes1 knockout phenotype (Ito et al., 2000). In this model, a pNEB induction field is formed, which is defined by low-level expression of *Ascl1*, *Dll1* and Notch receptors. Ascl1 is presumed to drive expression of *Dll1*, which would activate Notch receptors on adjacent cells. Notch in turn activates *Hes1*, which inhibits *Ascl1* to prevent the cell from assuming the NE cell fate. We have previously shown that Notch is activated around primitive ciliated cells, consistent with the expected activation pattern

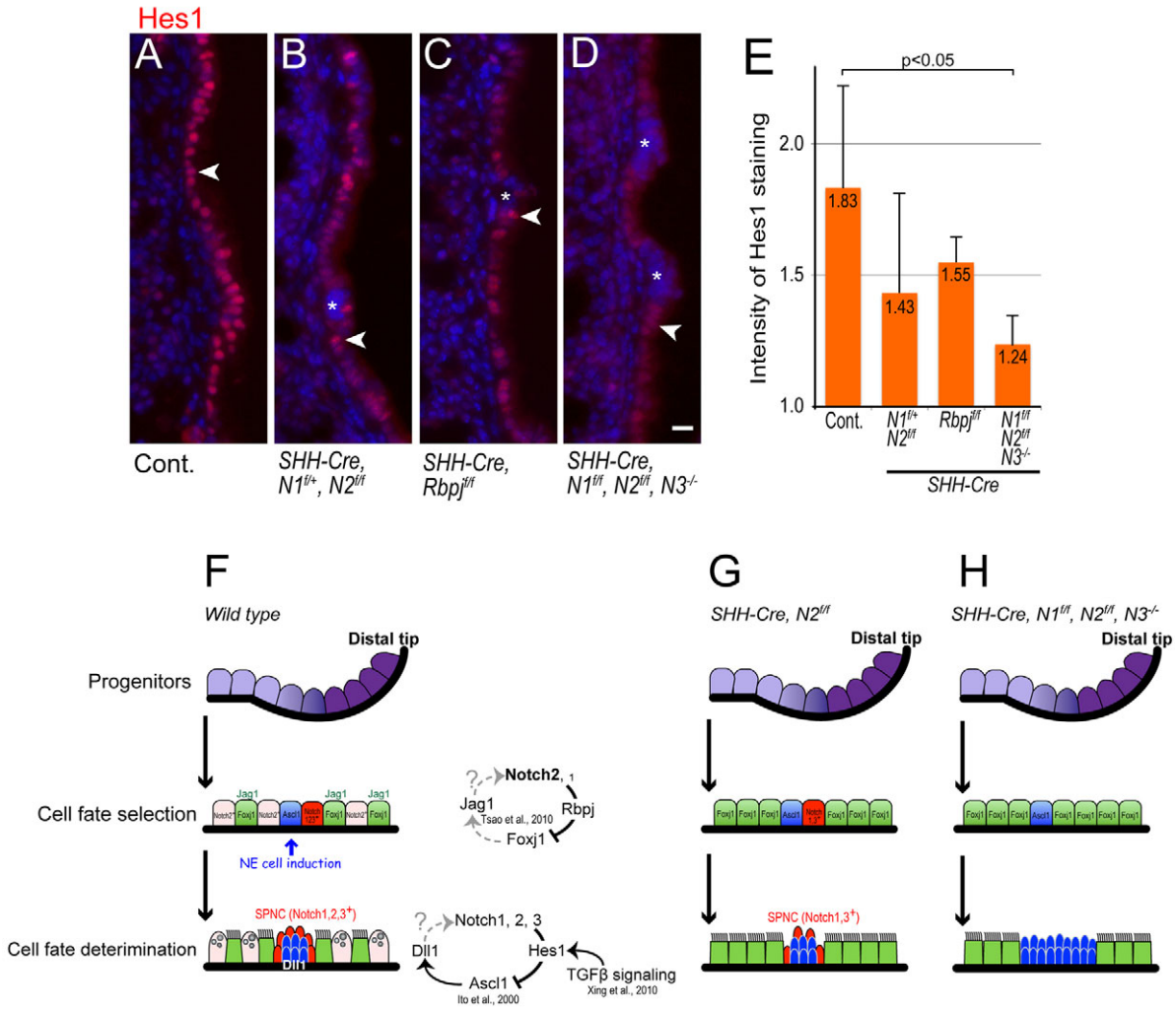

Fig. 6. Hes1 expression correlates with the severity of the pNEB defect in the Notch allelic series. (**A-D**) Hes1 protein levels (white arrowheads) in E18.5 wild-type (A), *SHH-Cre;N1*$^{flox/+}$*;N2*$^{flox/flox}$ (B), *SHH-Cre;Rbpj*$^{flox/flox}$ (C) and *SHH-Cre;N1*$^{flox/flox}$*;N2*$^{flox/flox}$*;N3*$^{-/-}$ (D) mice were assessed by immunofluorescence. Scale bar: 10 μm. Asterisks indicate NE cells. (**E**) Mean Hes1 intensity within the epithelium was measured using ImageJ. Normalization was performed by dividing the mean by the intensity from Hes1-negative NE cells within the same picture. At least four pictures from two to three biological samples were measured for each genotype. Error bars indicate s.d. *P* values are shown above the bars. (**F-H**) The proposed regulatoy mechanisms involved in Notch-dependent cell fate determination during bronchial epithelial development, which integrate our findings with reports from other groups. In the pseudoglandular stage, epithelial progenitors (purple) located at the distal tip produce descendants that differentiate into Clara (pink), ciliated (green), SPNC (red) and NE (blue) cells. See the text for details.

of Notch-mediated lateral inhibition (Morimoto et al., 2010). We examined the temporal sequence of pNEB formation relative to NICD accumulation at an early pNEB developmental stage. Ascl1 protein and the N1ICD epitope were detected at E14.5, at the onset of lung epithelial differentiation. If SPNC and NE cells are selected from within an equivalence group of bi-potential progenitors, Notch activation would be expected to occur in the cells surrounding each NE cell. Endothelial and vascular smooth muscle cells, which were used as controls for the staining reaction, contained N1ICD throughout lung development (supplementary material Fig. S7, asterisk). By contrast, N1ICD was detected only next to NE cell clusters (pNEB) at E14.5 (supplementary material Fig. S7, gray arrowhead), but not next to individual NE cells (supplementary material Fig. S7, white arrowhead) located more distally. Although we have not ruled out the possibility that Notch inhibits NE cell differentiation via lateral inhibition and thus determines the size of the NE induction field (Fig. 6F), the data prompted us to hypothesize that Dll1-Notch signaling might be not involved in repressing the neighbors of a nascent NE cell, but rather is deployed after NE cell differentiation, most likely to specify SPNC cells. Supporting this interpretation is the observation that selective ablation of the variant Clara cells surrounding the adult NEB caused hyperplasia (Hong et al., 2001). In this model, SPNC cells regulate the proliferation rates of adjacent NE cells to control the size of pNEB. The molecular mechanism underlying pNEB size regulation by Notch signaling will be clarified in future studies.

The adult lineage derived from SPNC cells is of interest. However, because SSEA-1 is a carbohydrate antigen on the cell surface, we cannot label the SPNC cells in vivo with a genetic tool. Anti-SSEA-1 immunoreactive cells disappear from peri-pNEB epithelial cells in the adult (data not shown). It is suggested that an equivalent population of SPNC cells expresses Uroplakin3A (*Upk3a*), and lineage labeling of Upk3A-expressing cells during development shows that these cells contribute to Clara and ciliated

lineages in the adult lung (Guha et al., 2012). This observation is consistent with our previous report that many Clara and a few ciliated cells are labeled in N1IP::CRE reporter mice (Morimoto et al., 2010). We confirmed that SPNC cells express Upk3A (data not shown). Therefore, SPNC cells become Clara and/or ciliated cells after the pNEB size has been determined.

In conclusion, this report demonstrates that although Notch signaling is used reiteratively to organize the major cell types within a tissue, each step uses different receptors. Selection is either robust (pNEB regulation, where three Notch genes act with some redundancy in SPNC cells to negatively regulate NE cells) or sensitive to perturbations (Clara cells, where it relays only the Notch2 receptor signal). We speculate that these differences may explain the constant spacing and size of pNEBs, and the diverse patterns of Clara/ciliated cells throughout the conducting airway.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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