

# The loss of *Hoxa5* function promotes Notch-dependent goblet cell metaplasia in lung airways

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## Summary

*Hox* genes encode transcription factors controlling complex developmental processes in various organs. Little is known, however, about how HOX proteins control cell fate. Herein, we demonstrate that the goblet cell metaplasia observed in lung airways from *Hoxa5*<sup>-/-</sup> mice originates from the transdifferentiation of Clara cells. Reduced CC10 expression in *Hoxa5*<sup>-/-</sup> embryos indicates that altered cell specification occurs prior to birth. The loss of *Hoxa5* function does not preclude airway repair after naphthalene exposure, but the regenerated epithelium presents goblet cell metaplasia and less CC10-positive cells, demonstrating the essential role of *Hoxa5* for correct differentiation. Goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice is a FOXA2-independent process. However, it is associated with increased Notch signaling activity. Consistent with these findings, expression levels of activated NOTCH1 and the effector gene HEY2 are

enhanced in patients with chronic obstructive pulmonary disease. In vivo administration of a  $\gamma$ -secretase inhibitor attenuates goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice, highlighting the contribution of Notch signaling to the phenotype and suggesting a potential therapeutic strategy to inhibit goblet cell differentiation and mucus overproduction in airway diseases. In summary, the loss of *Hoxa5* function in lung mesenchyme impacts on epithelial cell fate by modulating Notch signaling.

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Key words: *Hox* genes, Goblet cells, Notch pathway

## Introduction

During development, the undifferentiated endodermal epithelium of the lung primordium will give rise to specialized cell types along the anterior-posterior axis of the respiratory tree. Trachea and bronchi are lined by a pseudostratified epithelium consisting of basal, ciliated and secretory cells, the latter including Clara cells and few goblet cells. Non-cartilaginous airways of the bronchial tree are covered by a simple columnar epithelium mainly composed of ciliated and Clara cells. In the distal lung, type 1 and type 2 cells constitute the alveolar epithelium. All these cells fulfill critical functions, including mucociliary clearance, gas exchange and maintenance of surface tension (Morrisey and Hogan, 2010). In response to chronic insults, differentiation of the bronchial tree epithelium is altered with an increased number of goblet cells. Mucus normally produced by goblet cells is critical for host defense, but in excess it represents a major cause of airway obstruction. Goblet cell metaplasia and mucus hypersecretion contribute to the morbidity and mortality of chronic pulmonary disorders like chronic obstructive pulmonary disease (COPD) (Fahy and Dickey, 2010).

Cell fate determination of the respiratory epithelium is under the concerted action of multiple molecules (Morrisey and Hogan, 2010; Whitsett et al., 2011). Depending on the pathology, the goblet cell population may exhibit a distinct mucin content and

express a specific array of transcription factors (Plantier et al., 2011). Several intertwining molecular networks are involved in goblet cell differentiation. Classical knock-out experiments and tissue-specific conditional mutations in mice have contributed to our understanding of the mechanisms involved in the acquisition of respiratory cell fate. For instance, the mutation of *Foxa2*, a gene encoding a winged helix/forkhead box transcription factor, in the respiratory epithelial cells induces pulmonary eosinophilic inflammation and goblet cell metaplasia (Wan et al., 2004; Chen et al., 2010). Overexpression of the SAM-pointed domain-containing Ets-like factor *Spdef* gene in Clara cells triggers goblet cell differentiation and decreased *Foxa2* expression (Park et al., 2007; Chen et al., 2009). Expression of an activated form of  $\beta$ -catenin, a central member of the Wnt canonical pathway, in respiratory epithelial cells causes goblet cell hyperplasia and reduced *Foxa2* expression (Mucenski et al., 2005). The Notch pathway has also emerged as a key player in airway epithelial cell fate. The mosaic mutations in airway epithelium of *Pofut-1*, an O-fucosyltransferase essential for Notch-ligand binding, and *Rbpjk*, a transcriptional effector, lead to goblet cell metaplasia with reduced FOXA2 expression (Tsao et al., 2011). In contrast, overexpression of the active intracellular domain of the mouse NOTCH1 receptor (NICD) in lung epithelium promotes goblet cell metaplasia without change in FOXA2 expression.

Altogether, these data demonstrate the prevalent role of *Foxa2* and the importance of thresholds in Notch pathway activation in the determination of airway epithelial cell fate (Guseh et al., 2009; Rock et al., 2011).

Lung branching morphogenesis and epithelial cell fate determination require reciprocal interactions between the contiguous epithelium and the lung mesenchyme (Alescio and Cassini, 1962; Shannon et al., 1998). Despite accumulated evidence showing that mesenchyme can instruct epithelial differentiation, the nature of the mesenchymal factors involved still remains elusive. *Hox* genes encode transcription factors specifying regional identity along the body axes and regulating morphogenesis during animal development (Pourquié, 2009). In human and mouse, 39 *Hox* genes are characteristically organized in four clusters and classified in 13 paralog groups. Several *Hox* genes, predominantly from paralog groups 2 to 6, are expressed in a distinct spatio-temporal fashion during lung ontogeny (Bogue et al., 1994; Mollard and Dziadek, 1997). Their expression is mainly restricted to lung mesenchyme. Except for *Hoxa5*, the lack of overt lung phenotype in single mutants suggests that, taken individually, these *Hox* genes do not play a predominant role in lung ontogeny. Functional redundancy by other *Hox* genes may mask anomalies (Rossel and Capecchi, 1999).

The *Hoxa5* mutation results in panoply of phenotypes indicative of the broad range of *Hoxa5* actions throughout life (Jeannotte et al., 1993; Aubin et al., 2002; Garin et al., 2006; Gendronneau et al., 2012). Most *Hoxa5*<sup>-/-</sup> mice die at birth from respiratory distress due to tracheal and lung dysmorphogenesis (Aubin et al., 1997). Surviving mutants display lung airspace enlargement and goblet cell metaplasia (Mandeville et al., 2006). *Hoxa5* expression is confined to the mesenchyme of the entire respiratory tract suggesting that it provides regional cues to the contiguous epithelia and participates to cell fate determination (Aubin et al., 1997; Coulombe et al., 2010). Herein, we have focused on the cellular and molecular mechanisms underlying goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice. The loss of *Hoxa5* function induces Clara to goblet cell transdifferentiation, a FOXA2-independent process accompanied by an increased activity of Notch signaling. Overall, our data indicate that mesenchyme-expressed *Hoxa5* participates to epithelial secretory cell fate by controlling the Notch pathway.

## Materials and Methods

### Mice, genotyping and tissue collection

The *Hoxa5* mutant mouse line was maintained in the MF1-129/Sv-C57BL/6 background or in the 129/Sv inbred background (Jeannotte et al., 1993). Transgenic mice carrying the *TCF/Lef-lacZ* reporter gene, provided by Dr. Dufort, were used to study the impact of the *Hoxa5* mutation on Wnt canonical signaling (Mohamed et al., 2004). Age of the embryos was estimated by considering the morning of the day of the vaginal plug as embryonic (E) day 0.5. Experimental specimens were genotyped by Southern blot analysis.

Lungs from control and mutant embryos were collected at E18.5. For adult specimens, lungs were dissected, instilled with 4% cold paraformaldehyde (PFA) and paraffin-embedded (Jaffe et al., 1990). For RNA extraction, lungs were snap-frozen in N<sub>2</sub>. Experiments were performed according to the guidelines of the Canadian Council on Animal Care and approved by the institutional animal care committee.

### Human specimens

Human lung samples from anonymous patients were provided by the "Banque de Tissus du Réseau en Santé Respiratoire du FRSQ" from l'Institut universitaire de cardiologie et de pneumologie de Québec (IUCPQ, Canada). Specimens were characterized on the basis of clinical history, physical examination, pulmonary function tests and chest X-ray. The study was approved by IUCPQ ethics

committee and written informed consent was obtained from patients before entry into the study.

### Naphthalene injury model

Wild-type (wt) and *Hoxa5*<sup>-/-</sup> mice of 8 weeks received one intraperitoneal injection of 275 mg/kg of naphthalene (Sigma-Aldrich) or the equivalent volume of corn oil as vehicle (Zemke et al., 2009). At day 1 (wt, n=6; *Hoxa5*<sup>-/-</sup>, n=6), day 3 (wt, n=7; *Hoxa5*<sup>-/-</sup>, n=8), day 14 (wt, n=10; *Hoxa5*<sup>-/-</sup>, n=9), day 42 (wt, n=8; *Hoxa5*<sup>-/-</sup>, n=10), and day 84 (wt, n=5; *Hoxa5*<sup>-/-</sup>, n=6) after naphthalene injection, mice were euthanized and their lungs were collected. Vehicle-injected mice were sacrificed at day 3 after injection (wt, n=8; *Hoxa5*<sup>-/-</sup>, n=9).

### Treatment with the $\gamma$ -secretase inhibitor GSI L685,458

Control and *Hoxa5*<sup>-/-</sup> mice were anesthetized before receiving intranasally at postnatal day (D) 30 and D37, 50  $\mu$ l of 0.3 mg/kg of GSI L685,458 (Sigma-Aldrich; wt, n=10; *Hoxa5*<sup>-/-</sup>, n=11) or 50  $\mu$ l of 1.5% DMSO as vehicle (wt, n=11; *Hoxa5*<sup>-/-</sup>, n=11). Mice were sacrificed 3 days (D40) after the second instillation of GSI L685,458 (Kang et al., 2009).

### Quantitative RT-PCR experiments

Total RNA was isolated from lungs of E18.5 embryos, D30 and D60 mice with TRIzol reagent (Invitrogen). cDNA was synthesized with the Superscript II Reverse Transcriptase (Invitrogen) using 2  $\mu$ g of total RNA and random primers. Quantitative PCR (qPCR) was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) and a thermal cycler ABI PRISM 7000. The *Rpl19* gene was used as control. Primer sequences are listed in supplementary material Table S1.

### Immunohistochemistry (IHC) and immunofluorescence (IF) analyses

Paraffin-embedded lungs were serially sectioned at 4  $\mu$ m for e18.5 and 6  $\mu$ m for postnatal specimens. Immunostaining experiments were performed as described (Gendronneau et al., 2012). Slides were counterstained with either Alcian blue (for detection of acid mucus-producing cells) and nuclear Fast Red or Periodic Acid-Schiff staining and Hematoxylin.

Primary and secondary antibodies used are listed in supplementary material Table S2. For IF studies, nuclei were visualized by DAPI staining. To identify goblet cells by fluorescence, the periodic acid fluorescent Schiff (PAF) procedure was applied (Evans et al., 2004). Images were captured with an FV1000 confocal system or with a Leica DMR microscope coupled to a QImaging camera.

### Proliferation and apoptosis analyses

To assess the proliferation rate, mice were injected intraperitoneally with 250  $\mu$ g/g BrdU (Sigma-Aldrich) in phosphate buffer (PBS). Lungs were collected 4 hours later, fixed in 4% PFA overnight and paraffin-embedded for IHC. Proliferation index was calculated as the number of BrdU-positive bronchial epithelial cells divided by the number of bronchial epithelial cells. A mean of 400 airway epithelial cells per specimen were counted.

Apoptotic cells were detected by the terminal transferase (TdT) DNA end labeling method (Giroux and Charron, 1998).

### $\beta$ -galactosidase staining

Lungs were fixed in ice-cold 4% PFA, 0.2% glutaraldehyde in PBS for 1 hour. Samples were transferred into ice-cold 30% sucrose in PBS overnight and then in Tissue-Tek O.C.T. (Sakura Finetek). 10  $\mu$ m cryostat sections were processed for X-gal staining and counterstained with nuclear Fast Red as described (Trainor et al., 1999).

### Statistical analysis

To study the effect of the genotype on the number of MAC3-positive cells, a two-way ANOVA with repeated measures was applied. Student's *t* test was performed to compare the number of BrdU-positive cells and the different cell types after naphthalene and GSI L685,458 treatments. When appropriate, Student's *t* test or the nonparametric Wilcoxon test was performed for gene expression analyses. Statistical analyses were done using the SAS 9.2 statistical software (SAS Institute, Cary, NC). A significance level inferior to 5% (*p*<0.05) was considered statistically significant.

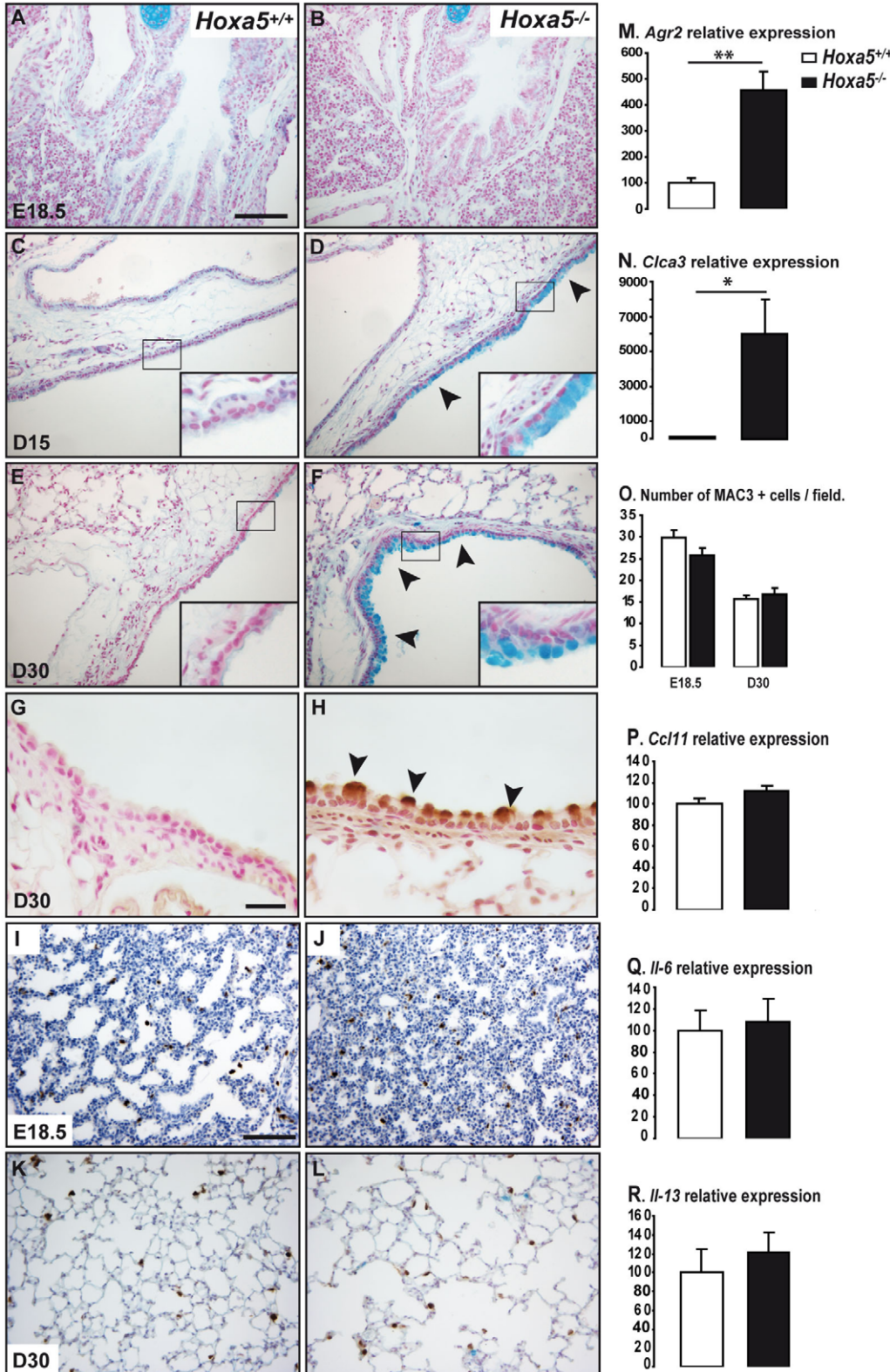
## Results

### Goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice is not linked to an inflammatory response

We previously reported goblet cell hyperplasia and mucus hypersecretion in lungs from *Hoxa5*<sup>-/-</sup> mice, a phenotype associated with an inflammatory response (Mandeville et al.,

2006). We further characterized the goblet cell phenotype using alcian blue (AB) staining of lungs from wt and *Hoxa5*<sup>-/-</sup> specimens at E18.5, D15 and D30 (Fig. 1A–F). At E18.5, goblet cells were barely found in trachea and primary bronchi of wt and *Hoxa5*<sup>-/-</sup> embryos. In D15 control specimens, goblet cells

remained scattered along the bronchial epithelium, whereas numerous AB-positive cells were observed in the bronchi of *Hoxa5*<sup>-/-</sup> mice. Goblet cell hyperplasia worsened with age as shown by the intense AB staining in D30 *Hoxa5*<sup>-/-</sup> samples. Goblet cells were mainly located along intrapulmonary



**Fig. 1. Goblet cell hyperplasia in *Hoxa5*<sup>-/-</sup> mice.** (A–F) Detection of goblet cells by AB staining in lungs from wt and *Hoxa5*<sup>-/-</sup> E18.5 embryos, and D15 and D30 mice. Goblet cells were scarce in wt specimens (A,C,E), but numerous in bronchi from D15 and D30 *Hoxa5*<sup>-/-</sup> mice (arrowheads; D,F). MUC5AC-positive cells were solely detected in *Hoxa5*<sup>-/-</sup> specimens (arrowheads; G,H). (I–L) Detection of macrophages by MAC3 immunostaining in lungs from wt and *Hoxa5*<sup>-/-</sup> E18.5 embryos and D30 mice. No major difference was observed. Values are expressed as mean ± SD (O; n=5–7 animals/group). (M,N) qPCR analysis for *Agr2* and *Clca3* expression in lungs from wt and *Hoxa5*<sup>-/-</sup> D30 mice revealed respectively a 4.5- and 60-fold increase in mutants. (P–R) qPCR analysis for *Ccl11*, *Il-6* and *Il-13* expression in lungs from wt and *Hoxa5*<sup>-/-</sup> D30 mice did not show difference. Values are expressed as mean ± SEM (n=5–8 animals/group). \**p*<0.05; \*\**p*<0.01. Scale bars, 100 μm (A–F, I–L), 25 μm (G,H). (C–F) Insets correspond to a 7.5× magnification of the boxed areas.



conducting airways, extending from the primary bronchi to the bronchioli where basal cells were sparsely found (data not shown). No MUC5AC immunostaining was detected along the respiratory tract of wt animals whereas MUC5AC-positive cells were abundant in *Hoxa5*<sup>-/-</sup> mice (Fig. 1G,H). In lungs from D30 *Hoxa5*<sup>-/-</sup> animals, qPCR analysis revealed a significant increase in expression levels for the anterior gradient 2 (*Agr2*) and the calcium-activated chloride channel family member 3 (*Clca3*) genes, both involved in the regulation of goblet cell differentiation (Fig. 1M,N) (Nakanishi et al., 2001; Chen et al., 2009).

Goblet cell hyperplasia often reflects pulmonary inflammation. We evaluated the inflammatory status of our *Hoxa5* mutant mouse colony by assessing macrophage recruitment to the lung by IHC with the MAC3 macrophage marker. In contrast to our previous observations, there was no significant difference in the number of MAC3-positive cells between wt and *Hoxa5*<sup>-/-</sup> specimens at E18.5 and D30 (Fig. 1I–L,O) (Mandeville et al., 2006). Moreover, qPCR analysis did not reveal significant changes in expression for the T helper (Th) type 2 cytokines, *Il-6* and *Il-13*, and the chemokine *Ccl11* genes between wt and mutants, indicating that goblet cell hyperplasia occurred without induction of proinflammatory mediators (Fig. 1P–R) (Zhu et al., 1999; Dent et al., 2004). Thus, inflammation did not contribute to goblet cell hyperplasia in *Hoxa5*<sup>-/-</sup> mice. The airway phenotype observed likely resulted from the impact of the *Hoxa5* mutation on the respiratory epithelium.

#### The *Hoxa5* mutation induces a developmental defect causing Clara to goblet cell transdifferentiation

To define the origin of goblet cells in the airway epithelium of *Hoxa5*<sup>-/-</sup> mice, we examined cellular proliferation and apoptosis in proximal airways from D30 wt and *Hoxa5*<sup>-/-</sup> specimens. Goblet cell hyperplasia in mutants occurred without evidence of increased proliferation of goblet cells or augmented apoptosis of other cell types, as assessed by BrdU (Fig. 2A–C) and TUNEL labeling (data not shown), respectively.

Models developed to mimic pathological conditions have shown that Clara, ciliated and basal cells can display plasticity and contribute to mucous cell hyperplasia (Evans et al., 2004; Tyner et al., 2006; Chen et al., 2009; Turner et al., 2011; Rock et al., 2011). To assess the integrity of the airway epithelium of *Hoxa5*<sup>-/-</sup> mice, the relative abundance of these cell types was examined in lungs from D30 and D60 wt and mutant mice (Fig. 2; data not shown). In areas of mucous cell hyperplasia, no major change in the number and distribution of basal cells was observed when using the transcription factor p63 as marker (Fig. 2D–F). A comparable observation was made for ciliated cells detected by FOXJ1, a forkhead transcription factor required for the assembly of the ciliary apparatus (Chen et al., 1998). In *Hoxa5*<sup>-/-</sup> specimens, ciliated cells appeared intermingled with goblet cells raising questions about the localization of Clara cells (Fig. 2G–I). Immunostaining with the Clara cell marker CC10 (encoded by the *Scgb1a1* gene) showed the widespread distribution of Clara cells along the proximal airways of wt mice with a strong cytoplasm labeling. A weaker CC10 immunostaining was detected in *Hoxa5*<sup>-/-</sup> specimens. Moreover, AB-positive cells displayed CC10 co-staining, which was restricted to the apex of the cells (Fig. 2J–M). qPCR analysis confirmed the decreased *Scgb1a1* expression in lungs from D60 *Hoxa5*<sup>-/-</sup> mice (Fig. 2N). The co-localization of AB staining

with CC10 labeling suggested that mucus-producing cells might derive from Clara cells.

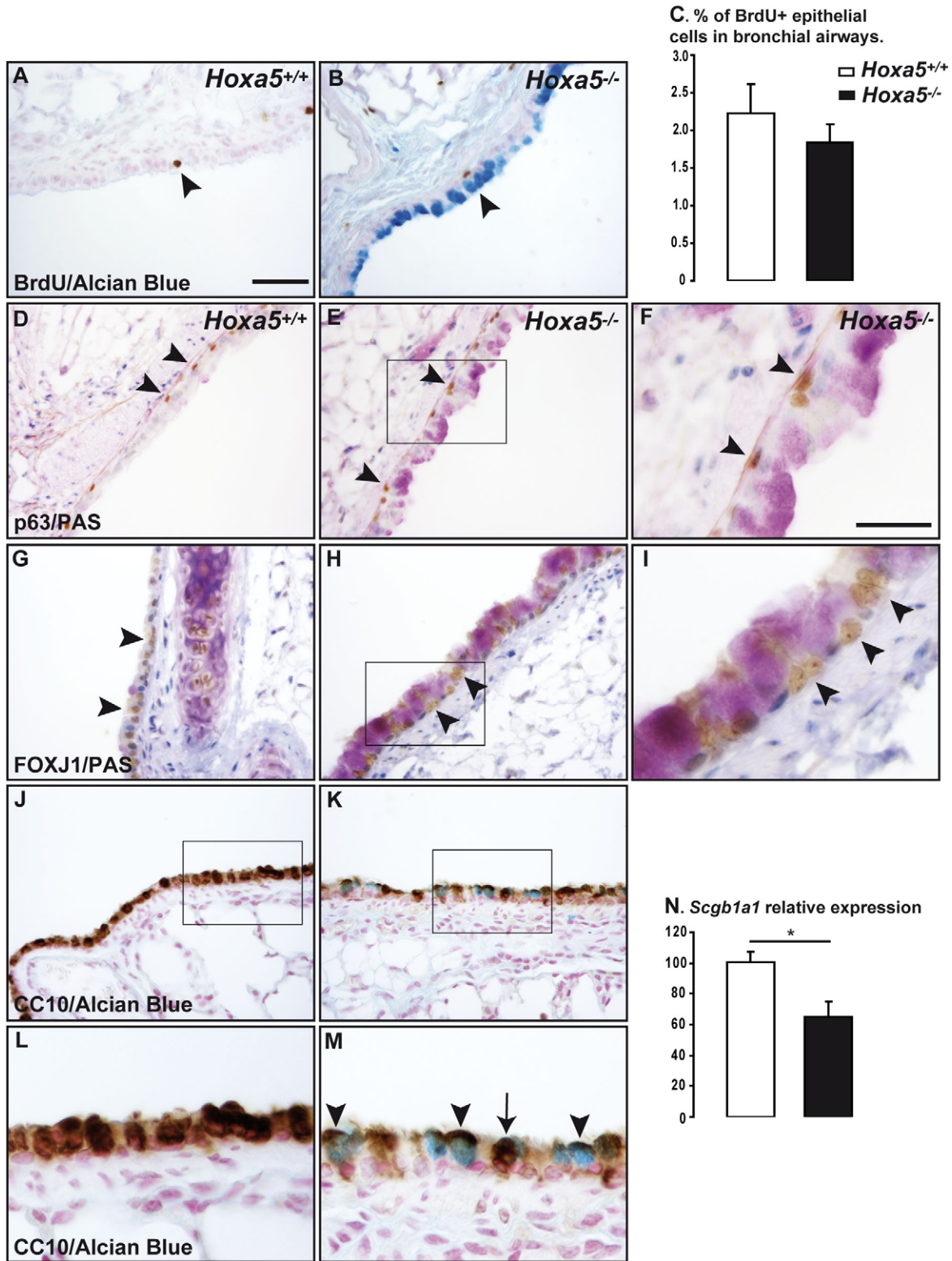
To corroborate the dual identity of goblet cells in *Hoxa5*<sup>-/-</sup> specimens, IF co-staining experiments were performed on D60 wt and *Hoxa5*<sup>-/-</sup> mice using CC10 antibody with periodic acid fluorescent Schiff (PAF), the latter revealing intracellular mucins, or with an AGR2 antibody (Fig. 3). Airway epithelium of wt mice exhibited CC10 labeling of Clara cells' cytoplasm and weak or no PAF and AGR2 staining (Fig. 3A–C,G–I). In *Hoxa5*<sup>-/-</sup> mice, PAF- and AGR2-positive cells were abundant and most of them co-localized with CC10-positive cells (Fig. 3D–F,J–L). Again, CC10 staining was confined to the apex of the mucus cells even in the few goblet cells detected in wt specimens (Fig. 3C,F,L). Thus in absence of *Hoxa5* function, goblet cell metaplasia occurs via Clara to goblet cell transdifferentiation.

To determine if the transdifferentiation of Clara cells results from impaired cell specification during development, we examined the respiratory epithelial cell types in E18.5 wt and *Hoxa5*<sup>-/-</sup> embryos. No major difference in the proportion and the proximo-distal distribution of p63-positive basal cells and  $\beta$ IV-tubulin-positive ciliated cells was observed (Fig. 4A–D). Consistent with the AB staining, no PAF-positive cells were detected in E18.5 wt and *Hoxa5*<sup>-/-</sup> specimens (data not shown). However, a reduced CC10 signal was seen in *Hoxa5*<sup>-/-</sup> lungs (Fig. 4E,F). qPCR analysis of *Scgb1a1* expression also showed decreased levels in E18.5 *Hoxa5*<sup>-/-</sup> lungs ( $p=0.06$ ) (Fig. 4I). To define if airway epithelial cells were stalled in a progenitor state in *Hoxa5*<sup>-/-</sup> embryos, the Stage-Specific Embryonic Antigen-1 (SSEA1) antibody, a marker of mouse embryonic stem cells, was used (Xing et al., 2010). No difference in SSEA1 staining intensity and distribution was observed between wt and *Hoxa5*<sup>-/-</sup> specimens (Fig. 4G,H). Thus, the goblet cell metaplasia observed in lungs from *Hoxa5*<sup>-/-</sup> mice results from Clara cell transdifferentiation, an event that initiates prior to birth.

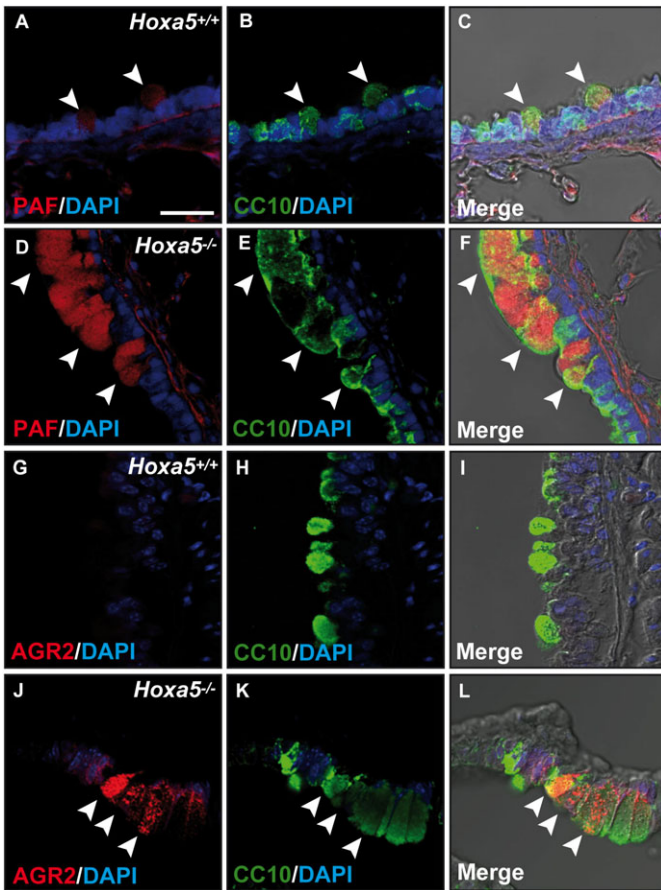
#### A role for *Hoxa5* in lung repair

Accumulation of evidences supports the notion that lung developmental processes also govern regeneration of lung tissue in pathological conditions. To assess the capacity of lung epithelium from *Hoxa5*<sup>-/-</sup> mice in restoring its integrity, a naphthalene-induced lung injury approach was tested in wt and *Hoxa5*<sup>-/-</sup> mice (Van Winkle et al., 1995). Clara cells expressing the cytochrome P-450 monooxygenase CYP2F2 are the primary targets for the cytotoxicant naphthalene, CYP2F2 being essential for the bioactivation and toxicity of naphthalene in lung. Naphthalene-resistant variant Clara cells show multipotent differentiation and the ability to regenerate the destroyed airway epithelium (Hong et al., 2001). Lungs from wt and *Hoxa5*<sup>-/-</sup> treated animals were analyzed after 1, 3, 14, 42 and 84 days. Epithelial regeneration was monitored by CC10 IHC combined to AB staining. As expected, injection of vehicle did not produce apparent damage in wt and *Hoxa5*<sup>-/-</sup> mice three days after injection. In *Hoxa5*<sup>-/-</sup> mice, AB staining was found significantly higher while CC10 signal was weaker (Fig. 5A,B). On days one and three following naphthalene injection, airway epithelial injuries appeared similar in wt and *Hoxa5*<sup>-/-</sup> mice (Fig. 5C–F). A majority of Clara cells were sloughed from the basement membrane and the bronchi were lined by a simple squamous epithelium composed of FOXJ1-positive cells





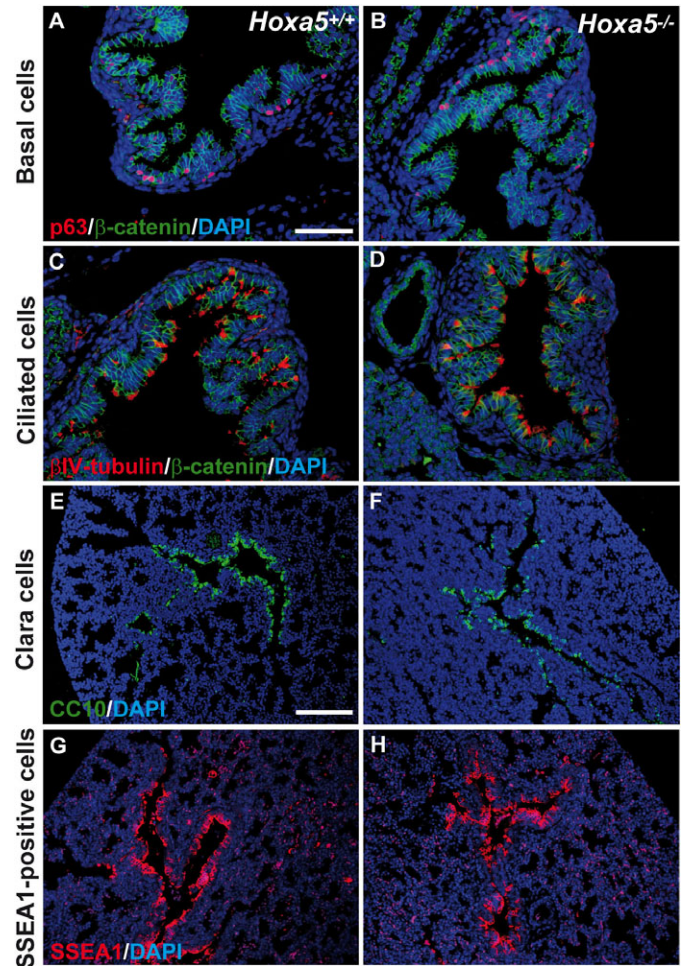
**Fig. 2. Characterization of the respiratory epithelium of *Hoxa5*<sup>-/-</sup> mice.** (A–C) BrdU-labeled airway cells (arrowheads) from wt and *Hoxa5*<sup>-/-</sup> D30 mice. BrdU labeling index was similar for both genotypes. Values are expressed as mean ± SD (n=4–5 animals/group). (D–I) Correct specification of basal and ciliated cells as shown respectively by immunostaining with p63 (arrowheads; D–F) and FOXJ1 (arrowheads; G–I) in lungs from wt and *Hoxa5*<sup>-/-</sup> D30 mice. (J–M) Decreased CC10-staining in *Hoxa5*<sup>-/-</sup> D30 mice. AB-stained goblet cells displayed weak but positive CC10 immunolabeling in mutants (arrowheads, the arrow points to a Clara cell; M). (N) qPCR analysis of *Scgb1a1* expression in lungs from wt and *Hoxa5*<sup>-/-</sup> D60 mice revealed decreased expression in mutants. Values are expressed as mean ± SEM (n=6–8 animals/group). \**p*<0.05. Scale bars, 50 μm (A,B,D,E,G,H,J,K), 25 μm (F,I,L,M).



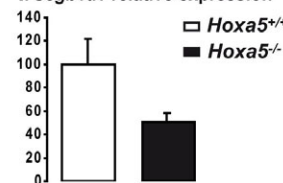
**Fig. 3. Clara to goblet cell transdifferentiation in *Hoxa5*<sup>-/-</sup> airway epithelium.** (A–L) Proximal airway sections from wt and *Hoxa5*<sup>-/-</sup> D60 mice. Clara cells were detected by CC10 immunolabeling (green) and goblet cells by PAF staining or AGR2 immunolabeling (red). Nuclei were counterstained with DAPI. Co-localization of CC10 and PAF or AGR2 within *Hoxa5*<sup>-/-</sup> airway epithelial cells is shown by the yellow color (arrowheads; C,F,I,L). Scale bar, 25  $\mu$ m.

(Table 1; data not shown). Goblet cells in the airways of *Hoxa5*<sup>-/-</sup> mice were totally exfoliated indicating that in addition to CC10, they might also express CYP2F2 (Li et al., 2011). Starting on day 14 onwards, CC10-positive cells were detected with an irregular distribution along the airways (Fig. 5G–L). Similar to our observations with untreated mice, *Hoxa5*<sup>-/-</sup> mice exhibited a weak CC10 immunoreactivity when compared to controls (Fig. 3, Fig. 5G–L). On day 84 after naphthalene injection, goblet cells reappeared along the airway epithelium of *Hoxa5*<sup>-/-</sup> mice (Fig. 5K,L). CD3 immunostaining revealed areas invaded by lymphocytes in lungs from wt and *Hoxa5*<sup>-/-</sup> mice, suggesting that inflammation is not a predominant causal factor for goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> specimens (supplementary material Fig. S1A–C).

To define how the different cell types recovered from the naphthalene-induced injury, cell quantification was performed (Table 1). At days 14, 42, and 84 after injury, the airway epithelium of naphthalene-treated *Hoxa5*<sup>-/-</sup> mice contained a significant lower number of Clara cells than wt specimens, which corroborated the histological data. No difference in the number of FOXJ1-positive ciliated cells was observed between wt and *Hoxa5*<sup>-/-</sup> mice, whereas the number of AB-positive goblet cells



**I. *Scgb1a1* relative expression**



**Fig. 4. Characterization of airway epithelium in *Hoxa5*<sup>-/-</sup> embryos.** Correct specification of basal (A,B) and ciliated cells (C,D), shown respectively by immunostaining with p63 (red) and  $\beta$ IV-tubulin (red) in lungs from wt and *Hoxa5*<sup>-/-</sup> E18.5 embryos. (E,F) CC10 immunolabeling (green) showed a reduced number of Clara cells in *Hoxa5*<sup>-/-</sup> specimens. (G,H) SSEA1 immunostaining (red) was similar for both genotypes. (I) qPCR analysis for *Scgb1a1* expression in lungs from E18.5 wt and *Hoxa5*<sup>-/-</sup> embryos indicated decreased expression in mutants. Values are expressed as mean  $\pm$  SEM (n=7 animals/group). Scale bars, 50  $\mu$ m (A–D), 100  $\mu$ m (E–H).

was significantly higher in *Hoxa5*<sup>-/-</sup> mice at day 84 after naphthalene injection. qPCR analysis of *Scgb1a1*, *Cyp2f2*, *Agr2* and *Foxj1* expression levels was also performed to appreciate lung airway re-epithelialisation (Fig. 5M–O; data not shown). As expected, *Hoxa5*<sup>-/-</sup> mice injected with vehicle displayed reduced *Scgb1a1* and *Cyp2f2* expression and increased *Agr2* transcript levels when compared to wt. At day 3 after injury, *Scgb1a1*, *Cyp2f2* and *Agr2* expression was barely detectable in both wt and mutant lungs. At days 14 and 42 after injury, airway epithelium recovery was characterized by a tendency to



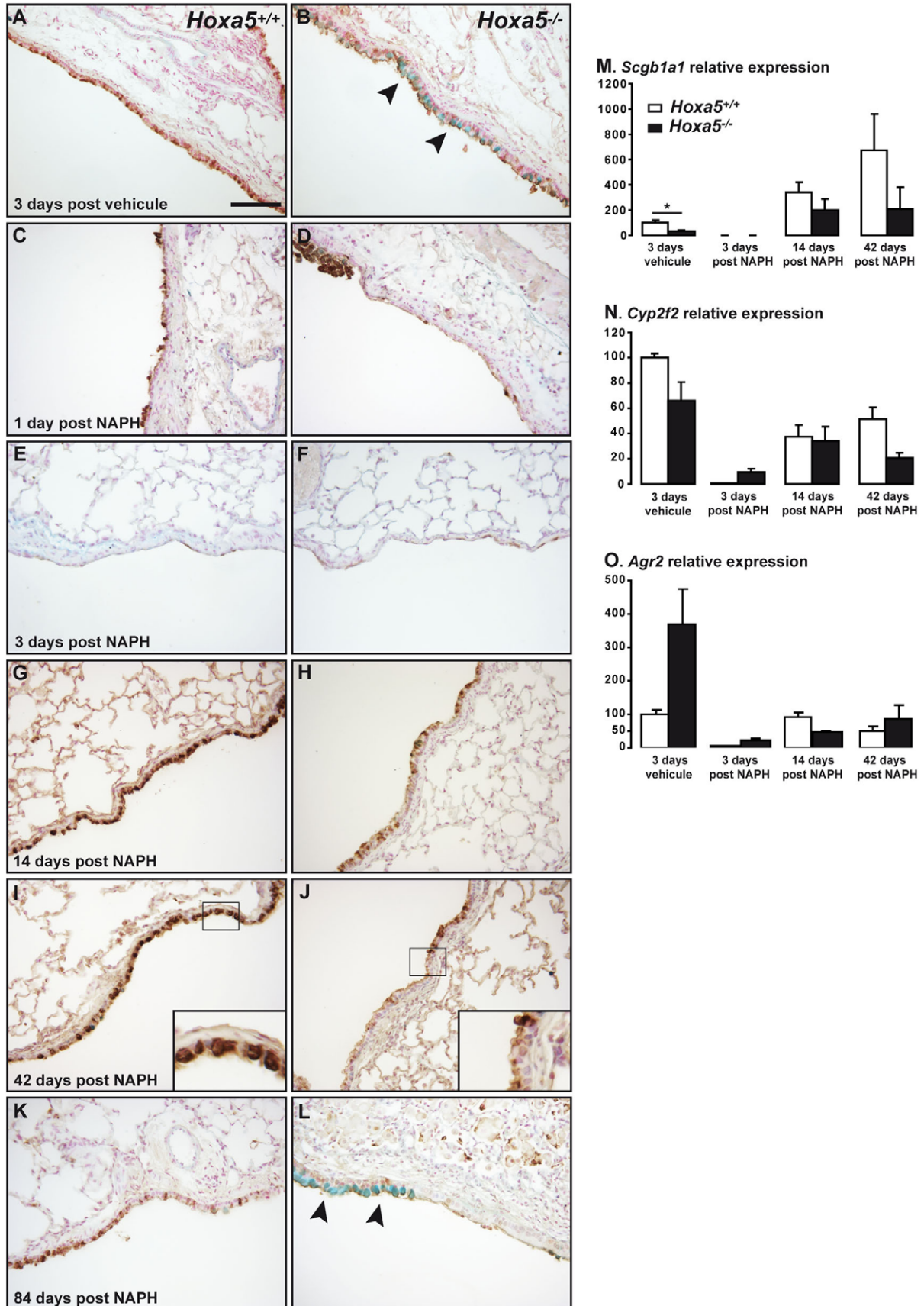


Fig. 5. See next page for legend.



**Table 1. Percentage of Clara, ciliated, and goblet cells in bronchial airways after naphthalene-induced injury.**

	3 days post vehicle		3 days post NAPH		14 days post NAPH		42 days post NAPH		84 days post NAPH	
	wt n=4	<i>Hoxa5</i> <sup>-/-</sup> n=5	wt n=4	<i>Hoxa5</i> <sup>-/-</sup> n=3	wt n=7	<i>Hoxa5</i> <sup>-/-</sup> n=6	wt n=5	<i>Hoxa5</i> <sup>-/-</sup> n=7	wt n=5	<i>Hoxa5</i> <sup>-/-</sup> n=6
<b>CC10+ cells</b>	43.4±5.5	24.5±2.6**	n.d.	n.d.	32.3±6.2	20.8±4**	31.2±6.5	19.1±3.9*	30.1±5.5	18.9±2.3**
<b>FOXJ1+ cells</b>	43.7±1.2	43.4±1.6	91.6±4.4	89.6±6.8	51.5±6.7	50.8±3.7	45.6±3.3	43.4±2	47.9±4.6	46.1±1.3
<b>Alcian blue+ cells</b>	3.3±4.8	14.9±9.3*	n.d.	n.d.	6.8±6.4	6.7±4.5	5.4±2.7	7.4±5.6	6±4.6	19.5±9.0*

Values are expressed as % of the total number of cells. A minimum of 1000 cells were counted per specimen.

Data are presented as mean ± SD.

\*p<0.05,

\*\*p<0.01

n.d.: not determined

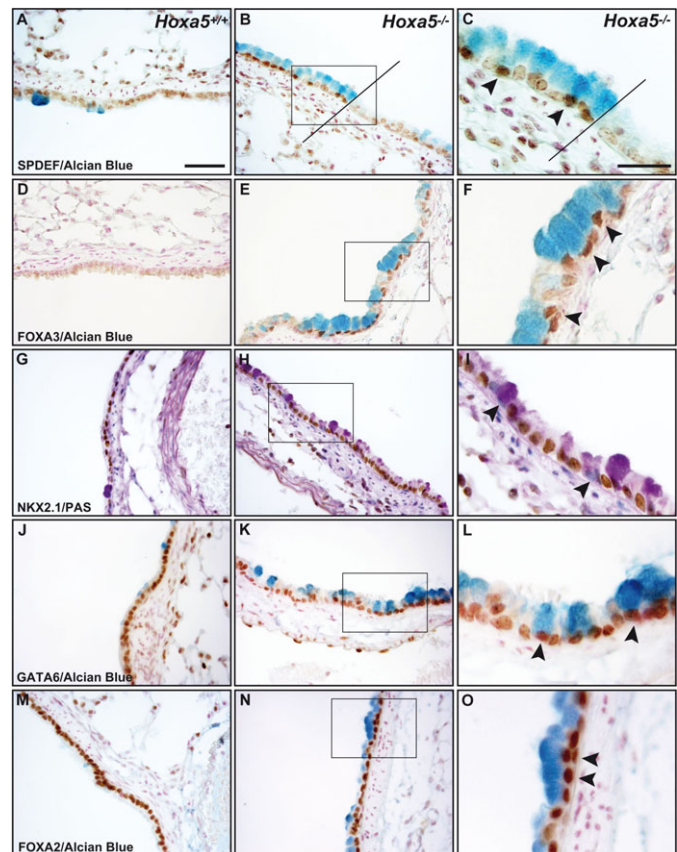
decreased expression of *Scgb1a1* and *Cyp2f2* in *Hoxa5*<sup>-/-</sup> lungs. No significant difference was observed for *Foxj1* expression from day 3 to day 42 after injury, supporting the IHC data (Table 1; data not shown). Similar *Agr2* expression levels were observed in wt and *Hoxa5*<sup>-/-</sup> lungs up to day 42 after naphthalene injection, consistent with the lack of detectable goblet cells at this time point and suggesting that abnormal Clara cell differentiation precedes goblet cell metaplasia (Fig. 5O).

Thus, the lack of *Hoxa5* function did not prevent lung airway re-epithelialisation after naphthalene injection. However, the cellularity of the regenerated epithelium in naphthalene-treated *Hoxa5*<sup>-/-</sup> mice remained similar to that from untreated mutant animals indicating that mesenchymal *Hoxa5* expression is necessary for correct lung airway re-epithelialisation and differentiation.

#### Impact of the loss of *Hoxa5* function on regulators involved in lung epithelial cell differentiation and specification

To determine the mechanisms by which *Hoxa5* controls goblet cell differentiation, we analyzed the expression of transcription factors known to govern lung epithelial cell differentiation in airways from D30 wt and *Hoxa5*<sup>-/-</sup> mice (Fig. 6). SPDEF expression was markedly increased at sites of goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice (Fig. 6A–C). A similar observation was made for the forkhead box transcription factor FOXA3, a nuclear marker of goblet cells (Fig. 6D–F) (Park et al., 2009). In contrast, no difference was detected for NKX2.1 expression between wt and mutant specimens. As reported, NKX2.1 was expressed in basal and secretory cells, but not in goblet cells (Fig. 6G–I) (Whitsett et al., 2011; Plantier et al., 2011). We also looked at GATA6 expression as GATA6 and NKX2.1 act synergistically in regulating pulmonary epithelial differentiation (Zhang et al., 2007). GATA6 expression was detected in most cells, including goblet cells, along the airway epithelium with no major difference between wt and *Hoxa5*<sup>-/-</sup> mice (Fig. 6J–L).

The loss of FOXA2 expression is associated with goblet cell metaplasia (Wan et al., 2004; Chen et al., 2009). Surprisingly, we did not observe any impact of the *Hoxa5* mutation on FOXA2 staining even if we repeated the experiments with two distinct



**Fig. 6. Expression of transcriptional regulators of airway epithelial cell differentiation in *Hoxa5*<sup>-/-</sup> mice.** Expression of SPDEF (A–C), FOXA3 (D–F), NKX2.1 (G–I), GATA6 (J–L), and FOXA2 (M–O) was assessed in bronchial tissues from wt and *Hoxa5*<sup>-/-</sup> D30 mice. SPDEF and FOXA3 immunostaining was markedly increased at sites of goblet cell metaplasia. No NKX2.1 expression was detected in goblet cells from both genotypes. GATA6 and FOXA2 were expressed along the airway epithelium with no difference between wt and *Hoxa5*<sup>-/-</sup> lungs. Arrowheads indicate goblet cells. Scale bars, 50 μm (A,B,D,E,G,H,I,K,M,N), 25 μm (C,F,I,L,O).

**Fig. 5. Impact of naphthalene-induced injury in *Hoxa5*<sup>-/-</sup> mice.** CC10 IHC combined to AB staining was performed on lungs from vehicle-treated (A,B) and naphthalene-treated wt and *Hoxa5*<sup>-/-</sup> mice (C–L). At days 1 and 3 after naphthalene injection, Clara and goblet cells were shed into the airway lumen in wt and *Hoxa5*<sup>-/-</sup> mice. On days 14, 42, and 84 after injury, CC10 expression was restored in wt mice, while CC10 staining was less intense in *Hoxa5*<sup>-/-</sup> mice. Goblet cells were detected in *Hoxa5*<sup>-/-</sup> specimens 84 days after injury (arrowheads; L). (M–O) qPCR analysis for *Scgb1a1*, *Cyp2f2*, and *Agr2* expression in lungs from wt and *Hoxa5*<sup>-/-</sup> mice treated or not with naphthalene. Values are expressed as mean ± SEM (n=3–4 animals/group). \*p<0.05. Scale bar, 100 μm. (I,J) Insets correspond to a 7.5× magnification of the boxed areas.

antibodies (Fig. 6M–O; data not shown). FOXA2 staining was detected along the airway epithelium in *Hoxa5*<sup>-/-</sup> specimens, even in regions of goblet cell metaplasia. We also looked at FOXA2 expression in naphthalene-treated wt and *Hoxa5*<sup>-/-</sup> mice on day 84 after injection (supplementary material Fig. S1D–F). FOXA2 staining was similar for both genotypes and goblet cell metaplasia occurred in *Hoxa5*<sup>-/-</sup> specimens without reduced FOXA2 expression. Thus, the molecular mechanisms responsible for goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice are FOXA2-independent.

$\beta$ -catenin, a key regulated effector of the Wnt canonical pathway, plays a critical role in the differentiation of respiratory epithelial cells (Mucenski et al., 2005). We examined the expression of members from the Wnt signaling pathways (canonical and non-canonical) involved in lung organogenesis by qPCR in trachea/primary bronchi and lung from D30 wt and *Hoxa5*<sup>-/-</sup> mice (Li et al., 2002; Mandel et al., 2008; Goss et al., 2009; Foronjy et al., 2010; Xu et al., 2011). *Wnt5a* and *Wnt2* expression was significantly augmented in *Hoxa5*<sup>-/-</sup> lungs when compared to controls, whereas a significant decrease in *Wnt4* expression was observed in mutant lung specimens (supplementary material Fig. S2A–C). No change in expression levels for the *sFrp1* gene encoding a Wnt signaling inhibitor was observed in mutant specimens but a tendency to diminished expression of the Wnt inhibitory factor 1, *Wif1*, gene was detected in *Hoxa5*<sup>-/-</sup> trachea and primary bronchi specimens (supplementary material Fig. S2D,E). To assess the impact of the *Hoxa5* mutation on the Wnt canonical pathway in lung epithelium, we used the *TCF/Lef-lacZ* transgenic reporter mouse line as a functional read-out of the activity of the cascade (Mohamed et al., 2004). As reported, *lacZ* expression was mainly confined to the epithelium and the staining was faint along the airways and in the pulmonary epithelium of E18.5 and D30 *Hoxa5*<sup>+/+</sup> *TCF/Lef-lacZ*<sup>+</sup> specimens (Dean et al., 2005). In *Hoxa5*<sup>-/-</sup> *TCF/Lef-lacZ*<sup>+</sup> specimens, *lacZ* expression was more pronounced in intensity and in the number of positive cells suggesting that *Hoxa5* may influence the activity of the Wnt/ $\beta$ -catenin cascade in lung epithelium (supplementary material Fig. S2F–K). However, we could not decisively establish that the X-gal staining colocalized with regions of goblet cell metaplasia, inferring that increased Wnt canonical signaling did not directly contribute to goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> specimens.

#### *Hoxa5* action in lung epithelial differentiation and Notch signaling

As NIICD overexpression in mouse lung epithelium results in goblet cell metaplasia without change in FOXA2 expression, we hypothesized that Notch signaling may be involved in transducing mesenchymal *Hoxa5* function, and triggering goblet cell metaplasia in airway epithelium from *Hoxa5*<sup>-/-</sup> animals (Guseh et al., 2009). Expression analysis of members of the Notch pathway was performed by qPCR in trachea/primary bronchi and lung from D30 wt and *Hoxa5*<sup>-/-</sup> mice (Fig. 7A–F). No change in expression was observed for the ligand *Jag1* and for the transcriptional effectors *Hes1* and *Hey1*. However, significant increased expression of the ligand *Jag2* and the target gene *Hey2* was detected in *Hoxa5*<sup>-/-</sup> lungs, while significant increased expression of the transcriptional regulator *Rbpjk* was observed in the trachea/primary bronchi from mutant specimens. We evaluated the functional status of the Notch signaling pathway in *Hoxa5*<sup>-/-</sup> airways by IHC experiments for NIICD and HEY2.

Expression of both proteins was markedly increased at sites of goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> specimens (Fig. 7G–L). A strong NIICD staining was also associated with regions of goblet cells in naphthalene-treated *Hoxa5*<sup>-/-</sup> mice on day 84 after injection (supplementary material Fig. S1G–I). Thus, specific activation of the Notch pathway in airway epithelium may underlie the goblet cell phenotype in *Hoxa5*<sup>-/-</sup> mice with HEY2 as a potential effector of goblet cell metaplasia.

#### Increased expression of activated-NOTCH1 and HEY2 in human pulmonary disease

To test whether increased Notch signaling is a hallmark of goblet cell hyperplasia associated with human pulmonary diseases, we performed NIICD and HEY2 IHC experiments on bronchial tissues from COPD patients. They revealed a robust expression of NIICD and HEY2 in areas of goblet cell metaplasia along the airway epithelium and in submucosal glands from COPD patients when compared to controls (Fig. 7M–R).

#### Attenuation of goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice treated with a $\gamma$ -secretase inhibitor

To directly assess the potential role of Notch signaling in the goblet cell phenotype of *Hoxa5*<sup>-/-</sup> mice, we used the cell permeable  $\gamma$ -secretase inhibitor (GSI) L685,458, which blocks Notch signaling by inhibiting the cleavage of the transmembrane domain of the receptor Notch by the  $\gamma$ -secretase and the liberation of active NICD (Kang et al., 2009). Controls and *Hoxa5*<sup>-/-</sup> mice were intranasally instilled with GSI L685,458 and sacrificed three days after treatment (Fig. 8A). Co-staining experiments with AB and CC10 revealed that the proportion of AB-positive cells was significantly diminished in airways of GSI-treated *Hoxa5*<sup>-/-</sup> mice when compared to vehicle-treated *Hoxa5*<sup>-/-</sup> mice (33% vs 13%) (Fig. 8B–H). No effect of the inhibitor was observed in wt mice. *Agr2* and *Clca3* expression levels were both reduced in lungs from GSI-treated *Hoxa5*<sup>-/-</sup> mice, while *Scgb1a1* expression was significantly restored in GSI-treated mutant mice (Fig. 8I–K). Thus, goblet cell metaplasia was attenuated in *Hoxa5*<sup>-/-</sup> mice by the Notch inhibitor GSI L685,458.

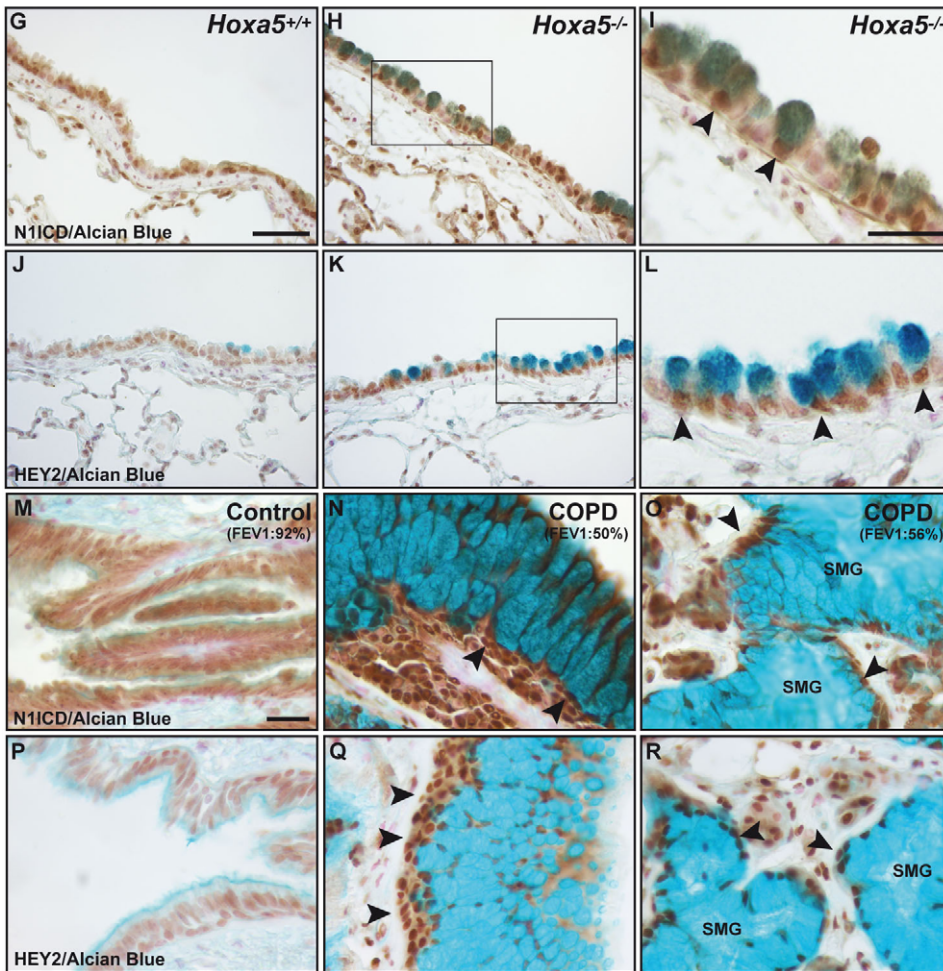
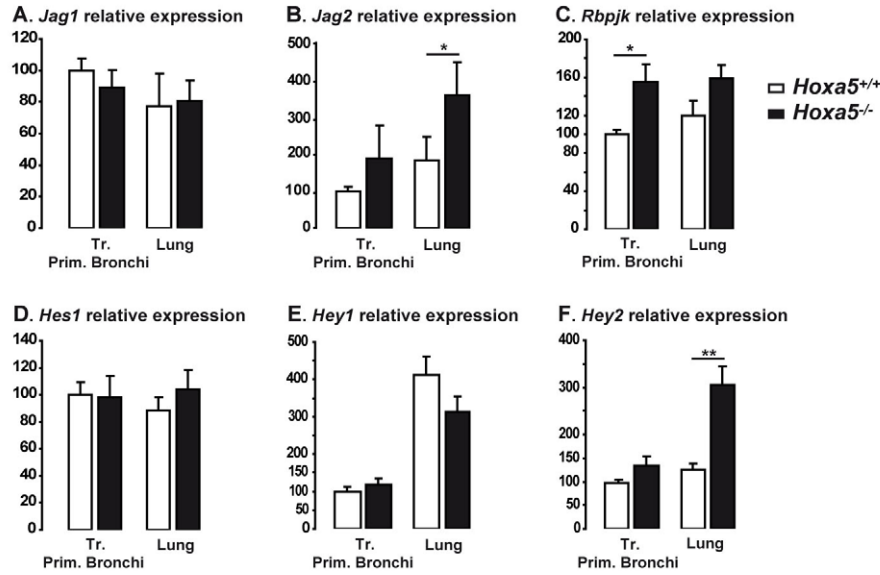
To validate the impact of the inhibitor on the Notch pathway, NIICD immunostaining was performed on airway sections. The intensity of NIICD labeling was diminished in GSI-treated compared to vehicle-treated *Hoxa5*<sup>-/-</sup> mice (Fig. 9A–F). Decreased Notch signaling activity was further confirmed by the down-regulation of the target gene *Hey2* in GSI-treated *Hoxa5*<sup>-/-</sup> mice as revealed by IHC and qPCR (Fig. 9G–M). Altogether, the data support the notion that Notch activation mediates at least in part goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice.

## Discussion

### The origin of goblet cells in *Hoxa5*<sup>-/-</sup> mice

We have previously shown that *Hoxa5* is critical for lung formation (Aubin et al., 1997). The present study enlightens the role of *Hoxa5* in airway epithelial cell fate. The loss of *Hoxa5* function in airway mesenchyme hampers normal Clara cell differentiation leading to their transdifferentiation into goblet cells, an event occurring without change in epithelial proliferation. Despite evidences that ciliated cells could display plasticity contributing to mucous cell metaplasia (Tyner et al., 2006; Turner et al., 2011), our data are concurring with several studies showing that mucus-producing cells derive preferentially





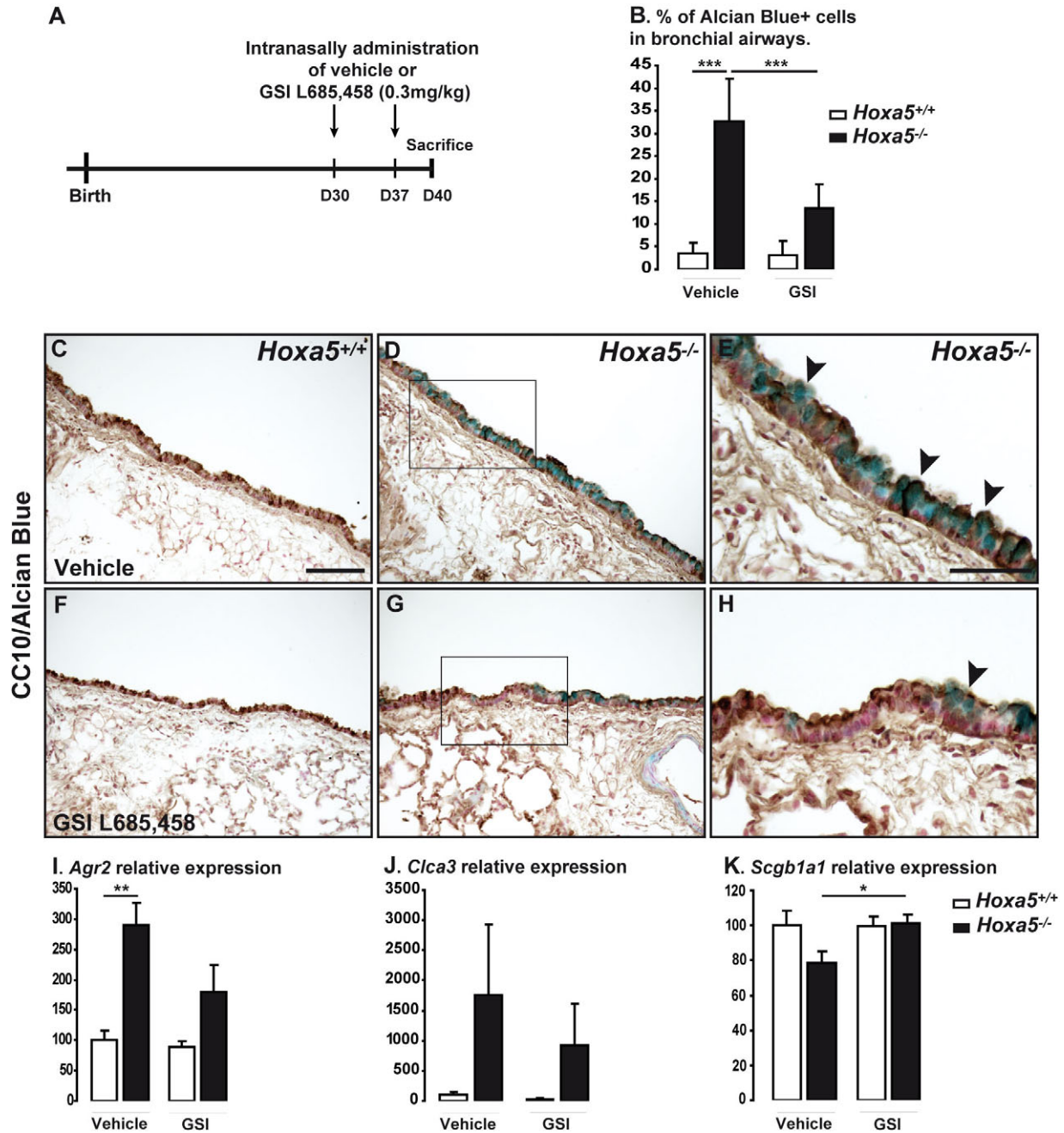
**Fig. 7. Increased Notch signaling in airways from *Hoxa5*<sup>-/-</sup> mice and COPD patients.** (A–F) qPCR analysis for *Jag1*, *Jag2*, *Rbpjk*, *Hes1*, *Hey1*, and *Hey2* expression in trachea/primary bronchi and lungs from wt and *Hoxa5*<sup>-/-</sup> D30 mice. Values are expressed as mean ± SEM (n=7–8 animals/group). \**p*<0.05, \*\**p*<0.01. (G–L) Immunostaining for N1ICD (G–I) and HEY2 (J–L) was performed in lungs from wt and *Hoxa5*<sup>-/-</sup> D30 mice. A salt-and-pepper N1ICD staining was observed in airway epithelium of controls. Goblet cells in *Hoxa5*<sup>-/-</sup> lungs displayed a strong N1ICD staining. HEY2 staining was weak along the bronchial epithelium of controls, but intense in goblet cells lining the airways of *Hoxa5*<sup>-/-</sup> mice. (M–R) Expression of N1ICD (M–O) and HEY2 (P–R) in lungs from controls and COPD patients. A strong N1ICD and HEY2 expression was detected in areas of goblet cell metaplasia along the airway epithelium and in submucosal glands (SMG) from COPD patients. Arrowheads indicate positive cells. FEV1: forced expiratory volume in 1 second. Scale bars, 50 μm (G,H,I,K), 25 μm (J,L–R).

from Clara cells (Evans et al., 2004; Park et al., 2007; Chen et al., 2009; Tsao et al., 2011). Considering that Clara cells were identified as epithelial progenitor cells in intrapulmonary airways and that Clara and goblet cells share similar machinery for protein secretion at their surface, the transdifferentiation of Clara

cells into goblet cells in *Hoxa5*<sup>-/-</sup> mice is a logical outcome (Rawlins et al., 2009).

Studies of the mouse trachea established that goblet cell metaplasia might also originate from basal cells (Rock et al., 2011). In *Hoxa5*<sup>-/-</sup> mice, no goblet cell metaplasia was detected



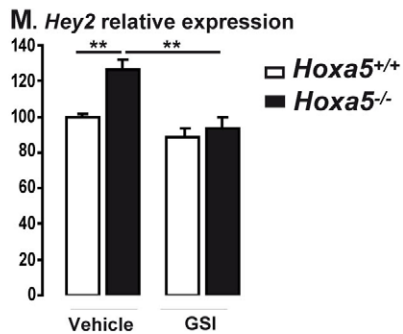
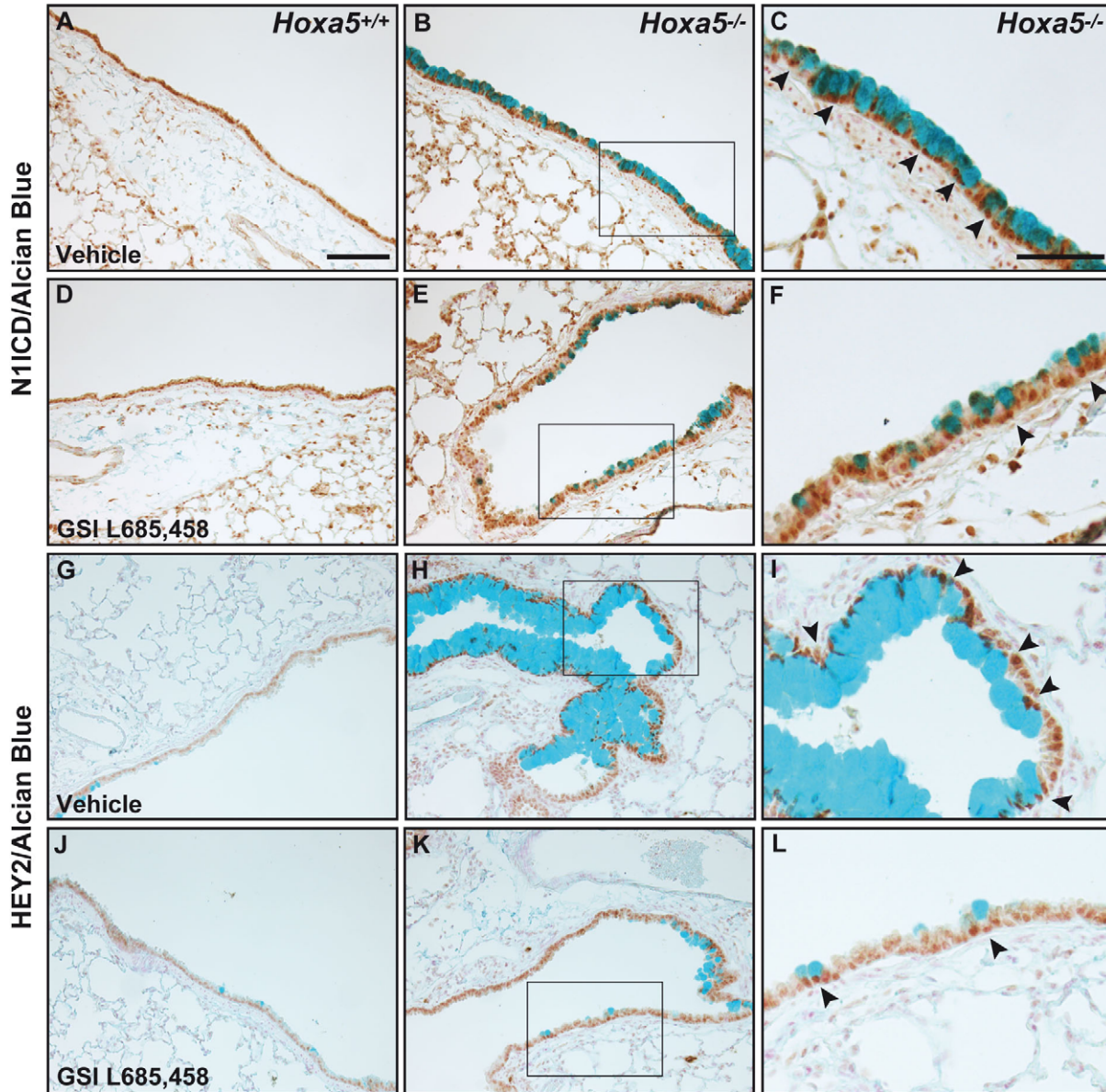


**Fig. 8. Attenuated goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice after GSI treatment.** (A) Diagram of the experimental protocol. (B) *Hoxa5*<sup>-/-</sup> mice displayed less goblet cells after GSI treatment when compared to vehicle-treated mutants. Percentage of goblet cells was calculated as the number of AB-positive bronchial epithelial cells relative to the total number of bronchial epithelial cells. Values are expressed as mean ± SD (n=5–6 animals/group). (C–H) CC10 IHC combined to AB staining in wt and *Hoxa5*<sup>-/-</sup> mice after vehicle (C–E) or GSI (F–H) treatments. Arrowheads indicate goblet cells. (I–K) qPCR analysis for *Agr2*, *Clca3*, and *Scgb1a1* expression in lungs from wt and *Hoxa5*<sup>-/-</sup> mice treated with vehicle or GSI. Values are expressed as mean ± SEM (n=5–6 animals/group). \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001. Scale bars, 100 μm (C,D,F,G), 50 μm (E,H).

in the trachea where basal cells are the most abundant (data not shown). While all these data reveal how the airway epithelial cells are remarkably plastic and capable of extensive remodeling, our results did not provide evidence for a role of ciliated or basal cells in goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice.

Goblet cell metaplasia is closely associated with pulmonary inflammation (Whitsett et al., 2011). Reduced *Foxa2* expression is also coupled to goblet cell metaplasia and inflammation (Wan et al., 2004; Chen et al., 2010). In *Hoxa5*<sup>-/-</sup> mice, goblet cell

metaplasia is neither accompanied by inflammation, nor by a change in FOXA2 expression. We previously reported that recruitment of inflammatory cells characterized the lung phenotype of *Hoxa5*<sup>-/-</sup> mice, a result not reproduced in the current analysis (Mandeville et al., 2006). A likely explanation for the disparity between the inflammation statuses of the mice is the improvement of the mouse husbandry conditions over the years, which positively impacted on the health of the animals. Interestingly, inflammatory cells were detected in wt and



**Fig. 9. Decreased Notch signaling activity in *Hoxa5*<sup>-/-</sup> mice after GSI treatment.** Immunostaining for N1ICD (A–F) and HEY2 (G–L) was performed in lungs from wt and *Hoxa5*<sup>-/-</sup> mice treated with vehicle (A–C,G–I) or GSI (D–F,J–L). Reduced N1ICD and HEY2 staining was observed along the epithelium of GSI-treated *Hoxa5*<sup>-/-</sup> mice. Arrowheads indicate goblet cells. (M) qPCR analysis of *Hey2* expression in lungs from wt and *Hoxa5*<sup>-/-</sup> mice treated with vehicle or GSI. Values are expressed as mean ± SEM (n=5 animals/group). \*\**p*<0.01. Scale bars, 100 μm (A,B,D,E,G,H,J,K), 50 μm (C,F,I,L).

*Hoxa5*<sup>-/-</sup> lung specimens on day 84 after naphthalene injection, most likely resulting from the stress due to the naphthalene-induced injury. Although inflammation was observed for both genotypes, goblet cell metaplasia was solely seen in mutants with no effect on FOXA2 expression (supplementary material Fig. S1A–E). Altogether, our data indicate that in normal and regenerative conditions, the loss of *Hoxa5* function causes goblet cell metaplasia that develops independently of inflammation and *Foxa2*-driven mechanisms.

Goblet cell metaplasia was detectable after birth in *Hoxa5*<sup>-/-</sup> specimens, but abnormal Clara cell differentiation was already observed prior to birth as revealed by the reduced CC10 expression levels in E18.5 *Hoxa5*<sup>-/-</sup> embryos. SSEA1 staining was similar between wt and E18.5 *Hoxa5*<sup>-/-</sup> embryos. According to the widespread SSEA1 expression along nascent airways epithelium during embryogenesis, it was proposed that SSEA1-positive cells might serve as progenitors for the differentiated airway epithelial cells, including Clara cells (Xing et al., 2010). The reduced CC10 staining observed in E18.5 *Hoxa5*<sup>-/-</sup> specimens may thus reflect abnormal terminal differentiation, contributing to the impaired Clara cell identity observed in the postnatal period, rather than impaired cell fate specification. This is further supported by our observations that except for Clara and goblet cells, all airway cell types analyzed were not affected by the *Hoxa5* mutation indicating that *Hoxa5* does not act on multipotent progenitors but may specifically instruct the secretory cell lineage.

The characterization of the lung airway epithelium of *Hoxa5*<sup>-/-</sup> mice following naphthalene-induced injury revealed cellular and molecular similarities with the abnormal epithelial cell differentiation phenotype seen in *Hoxa5*<sup>-/-</sup> mice during normal development. This supports the concept that lung morphogenesis, repair, and regeneration are tightly coordinated by conserved transcriptional networks (Whitsett et al., 2011). Evidences accumulated herein also demonstrate that the mesenchymal expression of *Hoxa5* is essential for the correct cell fate of the secretory cell lineage in lung airway epithelium during development and regeneration.

#### Molecular mechanisms and goblet cell differentiation

The SPDEF, FOXA2 and NKX2.1 transcription factors participate in molecular networks governing goblet cell differentiation (Wan et al., 2004; Chen et al., 2009; Maeda et al., 2011). In *Hoxa5*<sup>-/-</sup> mice, SPDEF expression was augmented at sites of goblet cell metaplasia whereas the NKX2.1 protein, normally expressed in Clara cells, was not detected in goblet cells. These results indicate that in the *Hoxa5*<sup>-/-</sup> environment, SPDEF and NKX2.1 may contribute to the regulatory pathway leading to the transdifferentiation process. As well, *Hoxa5* may indirectly act on *Spdef* and *Nkx2.1* gene expression in airway epithelium to determine the secretory cell fate. However FOXA2 expression, reported to be repressed by SPDEF in a mouse model in which *Spdef* expression is specifically targeted to Clara cells, remained unchanged in goblet cells from *Hoxa5*<sup>-/-</sup> specimens indicating the involvement of alternative transcriptional networks (Park et al., 2007).

Our study on the possible participation of Wnt signaling in goblet cell metaplasia in *Hoxa5*<sup>-/-</sup> mice demonstrated that the expression of Wnt ligands in the lung was altered by the *Hoxa5* mutation. Activation of the canonical Wnt pathway, when assessed with a Wnt responsive reporter mouse line, appeared

also more pronounced in lungs from *Hoxa5*<sup>-/-</sup> specimens. However, *lacZ* expression did not convincingly coincide with areas of goblet cell metaplasia, reducing the potential of the Wnt canonical pathway to be a major player in goblet cell fate in *Hoxa5* mutants. This is supported by the lack of change in FOXA2 expression in *Hoxa5* mutants. Indeed, mice expressing an activated form of  $\beta$ -catenin in airway epithelial cells develop goblet cell metaplasia associated with reduced FOXA2 expression (Mucenski et al., 2005). Nevertheless, the variations in expression of several members of the Wnt pathway raise the possibility that Wnt signaling may be involved in other aspects of the *Hoxa5* lung phenotype.

Several studies have highlighted the key role of Notch signaling in controlling the delicate balance between ciliated and secretory cell fates (Tsao et al., 2009; Morimoto et al., 2010). NIICD misexpression in lung epithelium resulted in goblet cell metaplasia without change in FOXA2 expression, a situation reminiscent to what we observed in *Hoxa5* mutants (Guseh et al., 2009). Moreover, administration of a Notch inhibitor reduces goblet cell metaplasia in an ovalbumin-induced asthma model (Kang et al., 2009). Apparent discrepancies between the data were reported, as selective inhibition of the Notch pathway can also result in airway goblet cell metaplasia (Tsao et al., 2011). However, all these results suggest that different thresholds of Notch activation may influence airway epithelial cell fate depending on the developmental timing and the cell population it acts upon. In *Hoxa5*<sup>-/-</sup> mice and in patients suffering from COPD, activation of the Notch signaling pathway in airway goblet cells occurred. The contribution of the Notch pathway to goblet cell metaplasia was further confirmed in *Hoxa5*<sup>-/-</sup> mice by partially rescuing the phenotype via administration of a GSI. Although promising, these data did not allow concluding whether the decreased number of goblet cells in airways from GSI-treated *Hoxa5*<sup>-/-</sup> mice results in the hampering or the reversion of the phenotype.

In *Hoxa5*<sup>-/-</sup> mice, goblet cell metaplasia was associated with increased expression of *Rbpjk*, *Jag2* and *Hey2*. Whereas the conditional removal of *Rbpjk* in lung endoderm promotes ciliated cell expansion at the expense of Clara cells, the augmented *Rbpjk* expression levels in *Hoxa5*<sup>-/-</sup> specimens support the notion that quantitative regulation of Notch signaling is crucial for airway epithelial cell fate (Tsao et al., 2009). The ligand *Jag2* is expressed in lung mesenchyme and epithelium but its role in lung development still remains vague since *Jag2*<sup>-/-</sup> mice die at birth from cleft palate (Jiang et al., 1998; Kong et al., 2004).

We described for the first time *Hey2* expression in airway goblet cells. *Hey2* transcripts were previously detected in the distal epithelium of primary lung buds and *Hey2* downregulation was reported in lung buds from GSI-treated explants, a result in agreement with our data (Tsao et al., 2008). These observations indicate that *Hey2* is a putative mediator of the Notch pathway in airway epithelium differentiation. However, the exact role of *Hey2* in goblet cell differentiation remains an issue.

Collectively, our observations expand the *in vivo* data reported so far and confirm that modulation of Notch signaling represents a potential therapeutic approach to restrain goblet cell differentiation and mucus hyperproduction in airways from patients with chronic lung diseases. As misregulated *Hoxa5* gene expression is associated with emphysema, the alveolar component of COPD, one can hypothesize that *Hoxa5* downregulation may confer susceptibility to develop COPD by



impairing alveolar formation and homeostasis and by promoting goblet cell metaplasia (Golpon et al., 2001; Mandeville et al., 2006; Boucherat et al., 2007). Even though the nature of the factors influenced by the loss of *Hoxa5* function in lung mesenchyme and impacting on Notch signaling in the airway epithelium remains to be defined, our findings unveil *Hoxa5* as a micromanager controlling not only organ formation and axial patterning but also details of cell morphogenesis and function.

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### Competing Interests

The authors declare that there are no competing interests.

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