WNT/RYK signaling restricts goblet cell differentiation during lung development and repair

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Decided by Edward E. Morrisey, University of Pennsylvania, Philadelphia, PA, and accepted by Editorial Board Member Roeland Nusse November 1, 2019


Goblet cell metaplasia and mucus hypersecretion are observed in many pulmonary diseases, including asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis. However, the regulation of goblet cell differentiation remains unclear. Here, we identify a regulator of this process in an N-ethyl-N-nitrosourea (ENU) screen for modulators of postnatal lung development; RYK mutant mice exhibit lung inflammation, goblet cell hyperplasia, and mucus hypersecretion. RYK functions as a WNT coreceptor, and, in the developing lung, we observed high RYK expression in airway epithelial cells and moderate expression in mesenchymal cells as well as in alveolar epithelial cells. From transcriptomic analyses and follow-up studies, we found decreased WNT/β-catenin signaling activity in the mutant lung epithelium. Epithelial-specific RYK deletion causes goblet cell hyperplasia and mucus hypersecretion but not inflammation, while club cell-specific RYK deletion in adult stages leads to goblet cell hyperplasia and mucus hypersecretion during regeneration. We also found that the airway epithelium of COPD patients often displays goblet cell metaplastic foci, as well as reduced RYK expression. Altogether, our findings reveal that RYK plays important roles in maintaining the balance between airway epithelial cell populations during development and repair, and that defects in RYK expression or function may contribute to the pathogenesis of human lung diseases.

Significance

Goblet cell metaplasia and mucus hypersecretion are observed in many pulmonary diseases. However, the regulation of goblet cell differentiation remains unclear. We identified an ENU-induced mutation in the mouse RYK gene which causes goblet cell hyperplasia in the lung. From transcriptomic analyses and follow-up studies, we found decreased WNT/β-catenin signaling activity in the airway epithelium of mutant lungs. Epithelial-specific RYK deletion causes goblet cell hyperplasia and mucus hypersecretion during lung development and repair. We also found that the airway epithelium of COPD patients often displays goblet cell metaplastic foci, as well as reduced RYK expression, suggesting a conserved role for RYK in the regulation of goblet cell fate in both development and disease.
and in craniofacial morphogenesis (28, 29). Recent studies reveal that RYK participates in mammary epithelial growth and branching morphogenesis (30) as well as in cardiac development (31). However, the potential role of RYK in lung development and homeostasis has not been studied.

The conducting airways of the lung are lined by a simple columnar epithelium containing multiciliated cells, basal cells, neuroendocrine cells, and secretory cells (32). The mucus-producing goblet cells are specialized secretory cells found throughout the mucosal epithelia in the gastrointestinal tract, airway, and ocular surface, and they are required for lubrication and barrier function against external pathogens and debris (33). Several growth factors, inflammatory cytokines, and transcription factors have been implicated in goblet cell differentiation and mucin production in the airway (6, 7). For instance, overexpression of Notch1 intracellular domain in airway epithelial cells leads to increased airway mucous production (34). Airway epithelial cell-specific Foxp1/4 knockout mice exhibit ectopic activation of the goblet cell lineage program (35). SPDEF regulates a transcriptional network required for airway goblet cell differentiation, and its overexpression is sufficient to cause goblet cell metaplasia in lung (36, 37). Overexpression of Foxa3 in club cells causes goblet cell metaplasia and pulmonary inflammation (38). However, the roles of WNT/RYK signaling in airway goblet cell differentiation remain poorly understood.

In the present study, we reveal a role for RYK in the differentiation of airway epithelial cells during lung development and regeneration. Notably, we find that Ryk deficiency leads to decreased activity of WNT/β-catenin signaling in the lung epithelium as well as increased expression of genes associated with goblet cell hyperplasia in the lung epithelium in vitro and in vivo. Consistent with Ryk expression, epithelial cell-specific Ryk deletion leads to goblet cell hyperplasia and mucus hypersecretion during postnatal lung development and epithelial cell regeneration. Our results bring mechanistic insight into the regulation of lung epithelial cell differentiation, and how this program goes awry in disease, leading to goblet cell hyperplasia and mucus hypersecretion.

Results
An N-ethyl-N-nitrosourea Mutant Displays Growth Retardation and Goblet Cell Hyperplasia. To identify regulators of early postnatal lung development in mouse, we performed N-ethyl-N-nitrosourea (ENU) mutagenesis (39, 40). From screening the F3 progeny of 170 G1 mice, we identified 10 families displaying recessive lung inflammation phenotypes. In one of these families, mutant pups exhibited growth retardation and low body weight (Fig. 1 A and B) around postnatal day 1 (P1) and subsequently died around P14 (Fig. 1C). The mutant lungs were hypoplastic (Fig. 1D) and characterized by severe infiltration of inflammatory cells into airway lumens and alveolar spaces, as well as alveolar simplification (Fig. 1 E and F). Since viscous material was observed in airway lumens and alveolar spaces, we tested whether secretory cells, especially goblet cells, were affected in the mutant animals. Alcian blue staining and immunostainings revealed a massively increased expression of genes associated with goblet cell hyperplasia in the lung epithelium in vitro and in vivo. Consistent with Ryk expression, epithelial cell-specific Ryk deletion leads to goblet cell hyperplasia and mucus hypersecretion during postnatal lung development and epithelial cell regeneration. Our results bring mechanistic insight into the regulation of lung epithelial cell differentiation, and how this program goes awry in disease, leading to goblet cell hyperplasia and mucus hypersecretion.

An ENU-induced mutant exhibits goblet cell hyperplasia in the lung. (A) General appearance of P7 wild-type (n = 30) and mutant (n = 20) mice. (B) Body weight progression at postnatal stages of wild-type (n = 12) and mutant (n = 12) mice. (C) Survival rate of wild-type and mutant mice during postnatal development. Genotyping of 48 pups at P0, 52 pups at P7, 36 pups at P10, 25 pups at P14, and 20 pups at P30. (D) Hematoxylin and Eosin (H&E) staining of P7 wild-type (n = 20) and mutant (n = 12) lung sections. (E) High magnification of H&E-stained P7 wild-type (n = 20) and mutant (n = 12) lung sections. (F) Immunostaining for E-cadherin (E-Cad) and Isolectin B4 (IsoB4) in P10 wild-type (n = 12) and mutant (n = 10) lung sections. (G) Alcian blue/nuclear fast red staining in P10 wild-type (n = 12) and mutant (n = 10) lung sections. (H) Immunostaining for MUC5AC in P10 wild-type (n = 12) and mutant (n = 10) lung sections. A high-magnification image of the area in the dashed boxes is shown on the right. (I) Immunostaining for AGR2 and FOXJ1 in P7 wild-type (n = 12) and mutant (n = 10) lung sections. (J) Immunostaining for MUC5AC and KRT5 in P7 wild-type (n = 12) and mutant (n = 10) lung sections. (K) Immunostaining for MUC5AC and SCGB1A1 in P7 wild-type (n = 12) and mutant (n = 10) lung sections. Arrowheads point to MUC5AC/SCGB1A1 double-positive/coexpressing cells. Error bars are means ± SEM. (Scale bars: D, 2 mm; E, 50 μm; F [Top] and H [Left], 500 μm; F [Bottom], G, H [Right], and I–K, 20 μm.)
increased number of mucus-containing secretory cells in the airway epithelium of mutant bronchi and bronchioles at P10 (Fig. 1G). While MUC5AC-positive goblet cells are rarely detected in the airway epithelium of wild-type animals at P10, the number of goblet cells was dramatically increased in mutant airway epithelium (Fig. 1H). In mutant lungs, goblet cells were found mainly along the distal tracheal tube and intrapulmonary conducting airway, extending from the primary bronchi to the bronchioles. Since goblet cells differentiate after birth (32), we determined the stage at which an increased goblet cell number was first observed in the mutants. Wheat germ agglutinin (WGA) lectin-positive mucin secreting cells (41) were increased in number at around P4 in mutant lungs (SI Appendix, Fig. S1A), correlating with the first wave of goblet cell differentiation (32).

In order to investigate the origin of the supernumerary goblet cells in the mutant airway epithelium, we first examined cellular proliferation and apoptosis in P7 wild-type and mutant lungs. Apoptotic cell death in the mutant airway epithelium was not significantly altered compared to wild type, even though it was increased in the mutant mesenchyme (SI Appendix, Fig. S1B). In contrast, cellular proliferation was significantly increased in the airway epithelium of mutant lungs (SI Appendix, Fig. S1C). Additionally, while the proliferation of basal (SI Appendix, Fig. S1D), ciliated (SI Appendix, Fig. S1E), and goblet cells (SI Appendix, Fig. S1F) was unaltered in mutant lungs, the number of proliferating club cells was significantly increased in mutant lungs compared to wild type (SI Appendix, Figs. S1 G and H).

Next, we performed immunostaining for goblet cell markers (AGR2 and MUC5AC) in parallel with several airway epithelial cell markers, including FOXJ1 for ciliated cells, KRT5 for basal cells, and SCGB1A1 for club cells. In the mutant airway epithelium of wild-type animals at P10, the number of SCGB1A1-positive club cells was dramatically increased in mutant airway epithelium (Fig. S2A), suggesting that, in the mutant animals as in wild type, goblet cells derive from club cells.

A Stop-Loss Mutation in Ryk Is the Causal Variant in the Mutant.

To identify the mutation causing the described phenotypes, we carried out whole-exome sequencing comparing wild-type and mutant genomes and found a stop-loss (SL) mutation (c.A1776G;p.X595W) in the receptor-like tyrosine kinase (Ryk) gene, which results in a 45-amino acid extension of the carboxy (C) terminus (SI Appendix, Figs. S2 A and D). The mutant allele could be distinguished by high-resolution melt analysis and Sanger sequencing (SI Appendix, Fig. S2 B and C). Since the C-terminal PDZ-binding motif in Ryk acts as a ligand for DVL (26), we tested whether mutant Ryk could physically interact with DVL1 by immunoprecipitating the Ryk protein complex in HEK293T cells. While wild-type Ryk stably interacts with DVL1, mutant Ryk showed impaired binding to DVL1 (SI Appendix, Fig. S2E). This result indicates that the C-terminal Ryk protein extension introduced by the SL mutation prevents the physical interaction between Ryk and the PDZ domain of DVL1. To quantify the expression levels of Ryk messenger RNA (mRNA) and protein in mutant lungs, we performed RT-qPCR, Western blotting, and immunostaining. Expression of Ryk mRNA and protein was significantly decreased in RykSL/SL (hereafter RykSL/SL) lungs compared to wild type (Fig. 2C). We also found significantly up-regulated genes in RykSL/SL lungs, including Muc5b, Ii1r2, and Iltora (Fig. 2D). Among the down-regulated genes, Mecn, is known as a downstream target of Wnt/b-catenin signaling, and it is expressed in airway and alveolar epithelial cells (42, 43). We tested Mecn expression in the airway epithelium by in situ hybridization and Western blotting. While Mecn mRNA expression was strongly detected in the airway epithelium of P1 wild-type animals, its expression was dramatically reduced in RykSL/SL lungs (Fig. 2E). In addition, Mecn expression was also reduced in RykSL/SL lungs compared to wild-type littermates (Fig. 2F). These data indicate that Wnt/b-catenin signaling is down-regulated in Ryk-deficient lungs.

As Ryk is known to modulate both b-catenin–dependent and -independent signaling (18, 19), we first examined whether Ryk participates in Wnt/b-catenin signaling, by crossing RykSL/SL mutant mice with the TOPGAL reporter line. While b-catenin–dependent transcription was active in the airway epithelium of wild-type lungs, it was dramatically decreased in RykSL/SL airway epithelium (Fig. 2G). Next, we used a second Wnt/b-catenin reporter (TOPFLASH) and observed that, in HEK293 cells, Wnt-3A and Ryk led to increased Wnt/b-catenin reporter activation compared to basal levels (Fig. 2H). When Ryk-transfected cells were treated with WNT-3A, luciferase activity was further increased (Fig. 2I). However, treatment with WNT-5A, a b-catenin–independent WNT ligand, did not induce reporter activation. Simultaneous treatment with WNT-5A and Ryk induced the reporter at the same level as...
treatment with 

Fig. 2. RYK acts as a positive regulator of WNT/β-catenin signaling in the lung. (A) Differentially expressed genes (DEGs) between P1 Ryk<sup>−/−</sup> and Ryk<sup>SL/SL</sup> lungs from RNA-Seq analysis. Fold change (log2) of DEGs (50 genes) in mutant compared to wild-type lungs. (B) Gene Ontology classification of DEGs between P1 Ryk<sup>−/−</sup> and Ryk<sup>SL/SL</sup> lungs. (C) The qPCR validation of down-regulated genes (WNT signaling-related genes and Fgf9) in P1 Ryk<sup>−/−</sup> and Ryk<sup>SL/SL</sup> lungs. (D) The qPCR validation of up-regulated genes (Muc5b, Il1r2, and Ifira) in P1 Ryk<sup>−/−</sup> and Ryk<sup>SL/SL</sup> lungs. (E) Mycn expression in P1 Ryk<sup>−/−</sup> (n = 6) and Ryk<sup>SL/SL</sup> (n = 4) lung sections by in situ hybridization. A high-magnification image of the area in the dashed boxes is shown on the Right. (F) Representative Western blot (from 3 individual sets of lung lysates) for MYCN in P1 Ryk<sup>−/−</sup> and Ryk<sup>SL/SL</sup> lungs. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (G) X-gal staining of P1 Ryk<sup>−/−;</sup>TOPGAL (n = 6) and Ryk<sup>SL/SL;</sup>TOPGAL (n = 6) lung sections. A high-magnification image of the area in the dashed boxes is shown on the Right. (H) TOPFLASH-based WNT/β-catenin reporter assay upon stimulation with WNT3A or WNT5A or cotransfection of Ryk. (I) Expression levels of Ryk, AXIN2, and MYCN in control small interfering RNA (siRNA) and Ryk siRNA-treated HEK293T cells by RT-qPCR. (J) WNT/β-catenin reporter assay upon Ryk overexpression or Ryk siRNA knockdown after transfection of the TOPFLASH reporter. Error bars are means ± SEM; ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, 2-tailed Student’s t test. Ct values are listed in SI Appendix, Table S2. (Scale bars: E [Left], 30 μm; E [Right], 10 μm; G [Left], 500 μm; G [Right], 100 μm.)
RykSL/SL lungs (Fig. 3D), indicating that Ryk negatively regulates Muc5b mRNA and protein expression in airway epithelial cells.

To test whether WNT/β-catenin signaling and Ryk regulate Muc5b expression transcriptionally, we isolated a 2-kilobase mouse Muc5b promoter fragment and generated a pMuc5b-luciferase reporter construct (Fig. 3E). This reporter exhibited high basal activity in AS49 human lung epithelial cells (Fig. 3E). However, while mouse and human Ryk overexpression in AS49 cells led to a reduction in Muc5b promoter activity, significantly increased luciferase activity was observed when Ryk was knocked down (Fig. 3E). Moreover, WNT activation by WNT-3A inhibited the basal transcriptional activity from the Muc5b promoter (Fig. 3F). When WNT-3A was used in combination with Ryk, they acted synergistically to reduce luciferase activity (Fig. 3F). We also found that Mycn represses Muc5b promoter activity (Fig. 3F). However, when Mycn was cotransfected with Ryk, they showed no obvious synergistic effects on Muc5b promoter activity (Fig. 3F), indicating that WNT/Ryk signaling represses Muc5b expression through MYCN in AS49 lung epithelial cells.

Since Ryk deficiency leads to goblet cell hyperplasia in the lung, we next examined the expression of goblet cell hyperplasia-associated genes in vitro and in vivo. First, we used C22 cells, an immortalized mouse club cell line. When Ryk was knocked down in C22 cells, the expression of goblet cell hyperplasia-associated genes, Muc5ac, Muc5b, and Agr2, was dramatically increased, as was that of Foxa3, although to a lesser extent (Fig. 3G). The expression of Spdef, a regulator of goblet cell differentiation (36, 37), appeared to be unaltered in Ryk knockout cells (Fig. 3G). However, the expression of these genes was not altered when we overexpressed Ryk, even though Spdef was down-regulated (Fig. 3G). The expression of Scgb1a1, a club cell marker, was also down-regulated in Ryk knockout C22 cells (Fig. 3G). Next, we asked whether the hyperplasia signature was also present in RykSL/SL lungs at P4, the stage when goblet cell hyperplasia is first detectable. Similar to our Ryk knockdown results, the expression of Muc5ac, Muc5b, and Agr2 was dramatically increased in P4 RykSL/SL lungs compared to wild-type littermates (Fig. 3H). The expression of Foxa3 was also significantly increased (Fig. 3H). In contrast, Spdef and Scgb1a1 expression was not changed in RykSL/SL lungs (Fig. 3H). MUC5AC, AGR2, and FOXA3 expression was also increased in the airway epithelium of P4 RykSL/SL lungs compared to wild-type littermates (Fig. 3I), indicating that Ryk inhibits the expression of goblet cell hyperplasia-associated genes both in vitro and in vivo. Altogether, the up-regulation of goblet cell markers together with the down-regulation of club cell markers suggests that Ryk inhibits club to goblet cell differentiation.

Epithelial-specific Ryk Deletion Leads to Goblet Cell Hyperplasia. To investigate its cell type-specific functions during mouse lung development, we deleted Ryk in a panepithelial manner using an Shh-Cre line and in club cells using a CCSP-iCre line. When we deleted Ryk in epithelial cells using Shh-Cre and Ryklox mice, Ryk expression was dramatically decreased in epithelial Shh-RykKO lungs compared to wild-type littermates (Fig. 4A). Unlike RykKO (23) and RykSL/SL mice, Shh-RykKO mice showed no obvious growth retardation, lung hypoplasia, or lung inflammation (Fig. 4 B–D and SI Appendix, Fig. S6 A and B). Instead, Shh-RykKO mice exhibited a dramatically increased number of mucus-secreting cells in the airway epithelium as well as mucous hypersecretion into the airway lumen (Fig. 4E). In addition, the number of MUC5AC-positive goblet cells was massively increased in the bronchi and bronchioles of Shh-RykKO lungs compared to wild-type littermates (Fig. 4F). However, Shh-RykKO mice exhibited no obvious defects in the differentiation of alveolar epithelial cells (SI Appendix, Fig. S6C). Similar to what we observed in RykSL/SL mice, the number of MUC5B-positive cells and MUC5B expression were greatly increased in Shh-RykKO lungs compared to wild-type littermates (SI Appendix, Fig. S6 D and E). Whereas the number of ciliated cells did not appear to be altered.
in Shh-Ryk^{−/−} lungs compared to wild-type littermates (Fig. 4 G and H), the number of AGR2-positive goblet cells was increased, and that of club cells was significantly decreased (Fig. 4 G and H). In the airway epithelium of Shh-Ryk^{−/−} mice, basal (SI Appendix, Fig. S6F) and ciliated (SI Appendix, Fig. S6G) cell markers did not overlap with goblet cell markers; however, many goblet cells expressed club cell markers (SI Appendix, Fig. S6G), suggesting that the goblet cells derive from club cells and not from basal or ciliated cells. Additionally, while the proliferation of ciliated cells appeared to be unaltered, the number of proliferating goblet and club cells was significantly increased in Shh-Ryk^{−/−} lungs compared to wild-type littermates (SI Appendix, Fig. S6 I and J). To test the expression of airway epithelial cell markers in Shh-Ryk^{−/−} lungs, we performed RT-qPCR. Expression of the goblet cell genes Foxa3, Agr2, Muc5ac, and Muc5b was significantly up-regulated in Shh-Ryk^{−/−} lungs compared to wild type, while that of Scgb1a1 was down-regulated (Fig. 4I). However, expression of Foxj1, Krt5, and Spdef did not appear to be significantly altered in Shh-Ryk^{−/−} lungs (Fig. 4I). Overall, these data indicate that RYK inhibits goblet cell differentiation in the airway epithelium.

Next, to delete Ryk in club cells, we crossed CCSP-Cre-YFP mice with Ryk^{lox/lox} mice (hereafter CCSP-Ryk^{−/−}). Ryk expression was reduced to undetectable levels in YFP-positive club cells compared to wild-type littermates (SI Appendix, Fig. S6K). As development progressed, the number of goblet cells gradually increased in the CCSP-Ryk^{−/−} airway epithelium compared to that in wild-type littermates (Fig. 4J), indicating that Ryk deficiency in club cells leads to goblet cell hyperplasia at postnatal stages. However, when Ryk was deleted in ciliated cells by using Foxj1-CreER^{T2}, we found no obvious lung phenotypes (SI Appendix, Fig. S7 A–D). Taken together, these data suggest that RYK has a cell-autonomous function in club cells to inhibit goblet cell differentiation and hyperplasia.

**Ryk Deficiency Causes Goblet Cell Hyperplasia during Lung Regeneration.** As Ryk is also strongly expressed in the lung epithelium at adult stages (SI Appendix, Fig. S4 G and H), we hypothesized that it might function during adult lung homeostasis and repair. To test this hypothesis, we first administered naphthalene to wild-type adult mice and collected lung samples at 0, 2, 7, 14, and 21 dpi after injury (dpi). In mouse, club cells are more susceptible to naphthalene-induced airway injury than other epithelial cell types, and they are considered fully regenerated only at around 21 dpi (47, 48). We examined the expression of Ryk, Muc5b, and Mucn during wild-type lung repair. The expression of Muc5b was decreased at 2 and 5 dpi but reached peak expression at 14 dpi, after which time uninjured expression levels were restored at 21 dpi (Fig. 5A). In contrast, the expression of Ryk and Mucn gradually increased over time after injury (Fig. 5A). These data suggest that Ryk is active upon airway epithelial cell injury.

To test whether Ryk haploinsufficiency can interfere with airway epithelial cell differentiation after injury, we administered naphthalene to wild-type and Ryk^{SL/+} mice. At 21 dpi, wild-type lungs exhibited no significant differences in the epithelial cell populations compared to uninjured wild-type lungs (Fig. 5B). However, at 21 dpi, Ryk^{SL/+} mice exhibited dramatically increased numbers of goblet cells and a reduced number of club cells, with no obvious differences in basal or ciliated cell populations (Fig. 5B). This indicates that Ryk is required for the maintenance of club cell functions.

Furthermore, we tested whether Ryk deficiency in club cells led to defects in airway epithelial cell differentiation after injury by administering naphthalene to CCSP-Ryk^{−/−} mice. At 21 dpi,
CCSP-RykKO mice exhibited mucus hypersecretion and increased numbers of goblet cells in the airway epithelium compared to wild-type littermates (Fig. 5C). The expression of MUCSC and MUCSB and the number of goblet cells were dramatically increased in lungs with club cell-specific Ryk deletion compared to wild type (Fig. 5 D and F). At 21 dpi, while basal cells (KRT5+) and ciliated cells (FOXJ1+) were not changed in number, the number of club cells (SCGB1A1+) was decreased in CCSP-RykKO airway epithelium (Fig. 5 E and F). In addition, during airway epithelial cell regeneration, basal cells and ciliated cells did not express MUCSC and AGR2, respectively (Fig. 5 G and H). However, the expression of club cell markers, including CYP2F2, SCGB1A1, and SCGB3A1, was weakly detected in most goblet cells (AGR2+ and MUCSC+)(Fig. SI and SI Appendix, Fig. S7 E and F), suggesting that goblet cells may derive from club cells during airway epithelial cell regeneration. Moreover, in CCSP-RykKO lungs, NKX2-1 and SOX17 expression was not changed compared to wild type (SI Appendix, Fig. S5G), indicating that Ryk does not participate in respiratory versus digestive fate determination during regeneration. Overall, these results suggest that Ryk functions to maintain the balance among airway epithelial cell populations during lung regeneration.

**Reduction of Ryk Expression in COPD Patients’ Airways.** To determine whether Ryk is expressed in human lungs, we examined control samples from embryonic and adult stages. Similar to our observations in mouse, Ryk is specifically detected in the airway epithelium at early embryonic stages (Fig. 6A). At adult stages, Ryk is strongly expressed in airway and alveolar epithelial cells (Fig. 6B), and weakly expressed in immune, endothelial, and smooth muscle cells (Fig. 6C).

Goblet cell metaplasia including hyperplasia is one of the characteristic features of asthma and COPD including emphysema and chronic bronchitis (1, 2). WNT/β-catenin signaling activity is decreased in COPD, and its reactivation can attenuate pathological features of COPD patient-derived cells and murine models (49, 50). In order to test whether Ryk expression is altered in COPD patient lungs, we performed immunostaining. In samples from healthy donors, Ryk was strongly detected in small airway epithelial cells and distal alveolar epithelial cells (Fig. 6D and SI Appendix, Fig. S8A). In contrast, while Ryk was still strongly detected in alveolar epithelial cells, its expression in the airway epithelium was significantly reduced in samples from COPD patients exhibiting goblet cell metaplasia (Fig. 6D and SI Appendix, Fig. S8B). By immunostaining for Ryk and MUCSC in healthy donor and COPD patient samples, we observed reduced Ryk expression in MUCSC-positive cells (Fig. 6E and SI Appendix, Fig. S8B), indicating that Ryk deficiency may lead to goblet cell metaplasia in the airway of COPD patients. Taken together, these data indicate that Ryk may play a protective role against goblet cell metaplasia in COPD.

**Discussion**

Here, by performing an ENU mutagenesis screen, we identified a mutation in the mouse Ryk gene and investigated its role during lung development and repair. Our Ryk−/−mutant mice exhibit goblet cell hyperplasia and mucus hypersecretion, as well as growth retardation and lung inflammation. Ryk expression in the lung is strongly detected in airway epithelial cells and alveoli. Consistent with this expression pattern, lung epithelial cell-specific Ryk deletion led to goblet cell hyperplasia and mucus hypersecretion. These and other data indicate that Ryk is a negative regulator of goblet cell differentiation by positively regulating WNT/β-catenin signaling during lung homeostasis and repair.

The WNT signaling pathway plays pivotal roles in cell fate determination, cell migration, and polarity in development, and it also controls stem cell self-renewal and tissue homeostasis during repair (51). Ryk is a pseudokinase which participates in β-catenin-dependent and β-catenin-independent signaling in a cell- and tissue-dependent manner (18, 19, 26–29). For example, Ryk potentiates WNT/β-catenin signaling when mammalian cells are treated with the canonical ligands WNT1 and WNT3a.
In zebrafish (21) and Xenopus (22), RYK can act as a receptor for Wnt5b and Wnt11, respectively, to activate PCP signaling. Similarly, in mammals, WNT5A/RYK regulates PCP signaling by stabilizing VANGL2, a core PCP pathway component (28). In the present study, using WNT reporters in vitro and in vivo, we found that RYK functions autonomously in the lung airway epithelium as a positive regulator of WNT/β-catenin signaling. RYK consists of a WNT-binding WIF domain at its N terminus, a transmembrane domain, a pseudokinase domain, and a PDZ-binding motif at its C terminus (18, 19). The SL mutation in the Ryk ENU allele leads to a 45-amino acid extension of its C terminus, a transmembrane domain, a pseudokinase domain, and a PDZ-binding motif at its C terminus (18, 19). The SL mutation in mouse cFlip generates a peptide extension that interacts with an E3 ligase, subsequently degraded via the ubiquitin proteasome system. A high-magnification image of the area in the dashed boxes is shown on the Bottom. Immunostaining for RYK and MUC5AC in lung sections from healthy donors and COPD patients. (Scale bars: A, B [Right], and C [Right], 50 μm; B [Left] and D [Top], 100 μm; C [Left], 200 μm; D [Bottom] and E, 30 μm.)

Airway epithelial cells represent the first line of defense against inhaled agents, including cigarette smoke, allergens, and microorganisms. Goblet cells secrete mucus as a physical barrier to protect the airway from dehydration, while trapping air particles which are swept away by mucociliary clearance (33). Under normal physiological conditions, the airway epithelium contains only a few goblet cells, and moderate secretion of mucus exerts a protective function. However, in response to acute or chronic airway injury, goblet cells undergo hyperplastic growth, followed by mucus hypersecretion, ultimately leading to mucus accumulation and airway obstruction, as well as infection (6, 7). Under these conditions, in vivo studies have shown that mouse club cells, but not ciliated cells, transdifferentiate into goblet cells (46, 54). These results are consistent with goblet cell hyperplasia originating from club cells in the Ryk-deficient epithelium, indicating that signaling through WNT/RYK, β-catenin, and MYCN plays an important role in the maintenance of club cell fate during lung development and repair. Goblet cell metaplasia is often observed in parallel with pulmonary inflammation, and inflammatory signals are proposed to drive the metaplastic transition (3, 6, 7). In contrast, Ryk-depleted airway epithelia exhibited goblet cell hyperplasia and mucus hypersecretion without inflammation, suggesting that inactivating mutations in Ryk are sufficient to drive goblet cell hyperplasia.

Acquisition of airway goblet cell fate is controlled by several signals, including inflammatory stimuli, growth factors, and transcription factors. Even though the ETS transcription factor SPDEF is a known master regulator of goblet cell differentiation and maturation (36, 37), SPDEF is not required for FOXA3-mediated goblet cell metaplasia (38), and its mRNA levels were not significantly altered in Ryk mutant lungs. In our in vitro and in vivo studies, loss of Ryk function caused increased expression of genes associated with goblet cell hyperplasia, including Agr2, Muc5ac, Muc5b, and Foxa3, but not Spdef. In addition, while SPDEF directly regulates Muc5ac expression, it does not affect Muc5b expression (55). In contrast, Ryk deficiency increased the expression of both Muc5ac and Muc5b, indicating that WNT/RYK signaling regulates mucin gene expression in the airway epithelium without affecting Spdef mRNA levels.

Goblet cell metaplasia/hyperplasia accompanied by mucus hypersecretion is a common feature of asthma, COPD, and pulmonary fibrosis (1, 2). In the lungs of COPD patients, WNT/β-catenin signaling has been reported to be down-regulated (56, 57), and smoking, a major risk factor in COPD, leads to reduced WNT/β-catenin activity in airway epithelia of COPD lungs. Moreover, activation of WNT/β-catenin signaling can attenuate pathological features of COPD patient-derived cells and murine models (49, 57, 58). By analyzing COPD/empysema patient samples, we found that RYK expression was down-regulated in goblet cell metaplastic foci expressing MUC5AC and MUC5B in the small airways.
11. M. Zhang, J. Shi, Y. Huang, L. Lai, Expression of canonical WNT/β-catenin signaling in the lung mesenchymal compartment results in lower cell proliferation rates, leading to mesenchymal deficiency, reduction of ECM protein expression in alveolar cells, and, as a consequence, emphysema and airway inflammation (50). Our results suggest that, in COPD patients, goblet cell metaplasia may be caused by defective epithelial cell proliferation or specification programs, rather than by inflammatory signals. In addition, we show that goblet cell fate is controlled by the WNT/RYK signaling pathway. Thus, we propose that therapeutic approaches for goblet cell metaplasia in COPD might exploit control of the WNT/β-catenin signaling pathway through RYK.
12. A. M. Goss et al., Wnt5b-Ryk pathway provides diagnostically and/or critical reading of the manuscript. Y.N. was supported by grants-in-aid from the Japan Society for the Promotion of Science (Grant 19K22728). This work was supported by funds from the Max Planck Society to Dr. K.R.S.

**Materials and Methods**

All animal care and experimental procedures in this study were approved by the local animal ethics committee at the Regierungspräsidium Darmstadt, Hessen, Germany. ENU-induced C57BL/6 males were obtained from Monica Justice, Baylor College of Medicine, Houston, TX. Tamoxifen (Sigma) was dissolved in corn oil (Sigma) at 50 mg/mL and injected intraperitoneally at the early and development stages and frequencies. For whole-exome sequencing, genomic DNA samples were extracted from 2 wild types and 2 mutants. For RNA sequencing (RNA-Seq), total RNA was isolated from P1 lungs of 2 Ryk(−/−) and 2 Ryk(+/−) mice. Whole-exome sequencing and RNA-Seq were performed as previously described (39).

The materials and methods used in this study are described in detail in **SI Appendix, SI Materials and Methods**.

**Data Availability.** The RNA-Seq dataset produced in this study is available in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131946 (accession no. GSE131946).

**ACKNOWLEDGMENTS.** We thank Saverio Belluscii for the Shh-Cre mice; Birgit Spinzagel, Isabelle Salwig, and Thomas Braun for the CMV-Cre, CCSP-Cre-YP, and Foxj1-CreERT2 mice; and Srikanth Karnati for the C22 mouse club cell line. We also thank Young-June Jin, Felix Gunawan, Konstantinos Gkatzis, Josephine Gollin, and Simon Perathoner for discussions and/or critical reading of the manuscript. Y.N. was supported by grants-in-aid from the Japan Society for the Promotion of Science (Grant 19K22728). This work was supported by funds from the Max Planck Society to Dr. K.R.S.