

Noncanonical WNT-5A signaling impairs endogenous lung repair in COPD

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Chronic obstructive pulmonary disease (COPD) is a leading cause of death worldwide. One main pathological feature of COPD is the loss of functional alveolar tissue without adequate repair (emphysema), yet the underlying mechanisms are poorly defined. Reduced WNT- β -catenin signaling is linked to impaired lung repair in COPD; however, the factors responsible for attenuating this pathway remain to be elucidated. Here, we identify a canonical to noncanonical WNT signaling shift contributing to COPD pathogenesis. We demonstrate enhanced expression of noncanonical WNT-5A in two experimental models of COPD and increased posttranslationally modified WNT-5A in human COPD tissue specimens. WNT-5A was increased in primary lung fibroblasts from COPD patients and induced by COPD-related stimuli, such as TGF- β , cigarette smoke (CS), and cellular senescence. Functionally, mature WNT-5A attenuated canonical WNT-driven alveolar epithelial cell wound healing and transdifferentiation in vitro. Lung-specific WNT-5A overexpression exacerbated airspace enlargement in elastase-induced emphysema in vivo. Accordingly, inhibition of WNT-5A in vivo attenuated lung tissue destruction, improved lung function, and restored expression of β -catenin-driven target genes and alveolar epithelial cell markers in the elastase, as well as in CS-induced models of COPD. We thus identify a novel essential mechanism involved in impaired mesenchymal-epithelial cross talk in COPD pathogenesis, which is amenable to therapy.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity and mortality in the world, resulting in a growing social and economic burden (Mathers and Loncar, 2006; Vestbo et al., 2013). It is anticipated that the prevalence and burden of COPD will further rise over the next decades, as a result of the aging population and the persistent exposure of individuals to risk factors associated with the disease (Mathers and Loncar, 2006). In accordance, aging has recently been highlighted as a significant risk factor for chronic lung diseases (Meiners et al., 2015). Long-term cigarette smoke (CS) exposure is a primary causative risk factor for COPD, although the disease can also develop in individuals who never smoked (Salvi and Barnes, 2009; Vestbo et al., 2013). COPD is characterized by progressive, irreversible air-

flow limitation and loss of functional parenchymal pulmonary tissue, called emphysema. Emphysema comprises alveolar airspace enlargement and impaired pulmonary regeneration; it has a poor prognosis and there are currently no effective medical treatments aside from lung transplantation. The molecular mechanisms underlying the development and progression of COPD/emphysema are not yet fully clarified. Recent studies from our laboratory and others have demonstrated that alterations in the WNT microenvironment potentially contribute to disease pathogenesis (Baarsma et al., 2011; Kneidinger et al., 2011; Wang et al., 2011; Heijink et al., 2013).

WNT ligands (19 in human) are evolutionarily conserved secreted glycoproteins that are indispensable for proper organ, especially lung, development (Morrisey et al., 2013; Kotton and Morrisey, 2014). Specific WNT ligands can either activate the β -catenin-dependent (canonical) or β -catenin-independent (noncanonical) pathways by acting on various

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Abbreviations used: ABC, active β -catenin; ATI, alveolar epithelial type I; CM, conditioned medium; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CSE, CS extract; DOX, doxycycline; FA, filtered air; GOLD, Global Initiative for Chronic Obstructive Lung Disease; IPF, idiopathic pulmonary fibrosis; pHLF, primary human lung fibroblast; PKC, protein kinase C; SFTPC, surfactant protein C; siRNA, small interference RNA.

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transmembrane receptors (Baarsma et al., 2013). In emphysematous COPD patients, nuclear expression of the transcriptional coactivator β -catenin, a surrogate marker for active canonical WNT signaling, is decreased in alveolar epithelial type II (ATII) cells (Kneidinger et al., 2011; Jiang et al., 2016). The cause of reduced canonical WNT- β -catenin signaling in the alveolar epithelium and, consequently, limited lung repair capacity in COPD patients remains to be elucidated.

The structural and cellular alterations observed in the lungs of individuals with COPD phenotypically resemble accelerated aging of the organ and WNT signal alterations have been shown to impact cellular aging mechanisms, such as senescence (Ito and Barnes, 2009; Muñoz-Espín et al., 2013; Scheraga and Thannickal, 2014; Meiners et al., 2015). Recent evidence indicates that noncanonical WNT signaling is able to inhibit canonical WNT signaling, resulting in decreased β -catenin stability and/or impaired downstream signaling (Mikels and Nusse, 2006; Nemeth et al., 2007). Nevertheless, this mechanism has not been linked to chronic lung disease pathology. In the current study, we hypothesize that a transition of canonical to noncanonical WNT signaling contributes to COPD development.

We report for the first time that WNT-5A expression, a ligand known to trigger noncanonical WNT signaling, is increased in experimental and human COPD. We provide evidence of WNT signaling being crucially involved in impaired cellular crosstalk in which fibroblast-derived WNT-5A negatively regulates canonical WNT- β -catenin signaling in alveolar epithelial cells in vitro and in vivo, thereby impairing the capacity of the lung for wound healing and regeneration.

RESULTS

Noncanonical WNT-5A is increased in murine models of COPD and contributes to emphysema development in vivo

We first examined the expression of the noncanonical WNT ligands in well-established mouse models of COPD. WNT-5A was the only noncanonical WNT ligand significantly increased in mice subjected to short-term (3 d) CS (Δ CT: *Wnt-4*, -1.93 ± 0.04 vs. -1.98 ± 0.11 ; $P > 0.05$; *Wnt-5A*, -2.20 ± 0.18 vs. -1.61 ± 0.15 ; $P < 0.01$; *Wnt-5B*, -4.50 ± 0.05 vs. -4.22 ± 0.19 ; $P > 0.05$; and *Wnt-11*, -2.45 ± 0.10 vs. -2.00 ± 0.16 ; $P > 0.05$; filtered air [FA] versus CS-exposed mice; $n = 4$). Increased WNT-5A protein expression, accompanied by reduced active β -catenin (ABC) expression, was observed in whole-lung homogenate of mice chronically exposed to CS (4 mo) in comparison to FA-exposed mice (fold change, 3.5 ± 0.7 ; Fig. 1 A). Accordingly, expression of the WNT/T- β -catenin target gene *Axin2* was significantly attenuated, whereas expression of the neutrophil chemoattractant *KC* (*Cxcl1*) was increased by CS exposure (Fig. 1 B). Similar results were obtained in our mouse model of elastase-induced emphysema with WNT-5A transcript and protein expression being increased (protein: 2.4 ± 0.3 -fold over vehicle control; Fig. 1, C and D). Elastase-induced emphysema development was also accompanied by a reduc-

tion in the canonical WNT target genes *Axin2* and *Naked1* (*Nkd1*) and by an up-regulation of elastin (*Eln*; Fig. 1 D), further corroborating previously published results (Kneidinger et al., 2011; Uhl et al., 2015).

Next, we analyzed whether WNT-5A contributes to the development of experimental emphysema in vivo. To this end, we used mice that conditionally overexpress WNT-5A specifically in the lung (SFTPC-rtTA TetO-WNT-5A mice). Pulmonary overexpression of WNT-5A was initiated by supplementation of in the drinking water with doxycycline (DOX) 1 wk before the induction of emphysema and was well tolerated by the mice in all the experimental groups (Fig. 1, E and F). Overexpression of the ligand aggravated the development of elastase-induced emphysema, as determined by an increase in tropoelastin expression (Fig. 1 F), histological assessment, and quantitative morphometry (Fig. 1, G and H). A significant increase in mean chord length was observed in emphysematous mice that overexpressed WNT-5A compared with respective control group (Fig. 1 H; 35.0 ± 3.0 versus $26.8 \pm 1.9 \mu\text{m}$, respectively; $P < 0.05$). Collectively, these data indicate a potential pathophysiological role of increased WNT-5A in the development and progression of emphysema.

Enhanced expression and posttranslationally modified WNT-5A in biosamples of individuals with COPD

To determine the potential clinical relevance of WNT-5A in human COPD, we investigated whether WNT-5A expression was altered in lung samples of COPD patients. Notably, WNT-5A transcript was enhanced in lung tissue specimens from individuals with COPD compared with tissue from individuals without COPD (donor; Fig. 2 A). Additionally, analysis of WNT-5A expression in induced sputum of COPD patients revealed that expression of the ligand is dependent on disease severity (i.e., Global Initiative for Chronic Obstructive Lung Disease [GOLD] status; Fig. 2 B; data derived from Gene Expression Omnibus [GEO] microarray available under accession no. GSE22148; Singh et al., 2011). The data from this large cohort (GSE22148) with 140 COPD patients (GOLD stage II, $n = 71$; stage III, $n = 59$; and stage IV, $n = 13$) revealed a weak ($r = 0.18$), but significant ($P < 0.05$), linear correlation between age of the patient and WNT-5A expression, independent of disease severity (Fig. 2 C).

Monomeric WNT ligands are predicted to have a molecular mass of ~ 40 kD; however, they are heavily subjected to posttranslational modifications, including palmitoylation and glycosylation (Willert and Nusse, 2012; Baarsma et al., 2013). In addition, (secreted) WNT ligands can form high molecular weight homomers/oligomers, which may influence the signaling capacity of ligands (Cha et al., 2008; MacDonald et al., 2014; Zhang et al., 2015). We first verified if the antibody that we used for detection of WNT-5A was also able to detect the ligand when oligomerized (Fig. S1). Notably, mature (~ 49 kD) and homomeric/oligomeric WNT-5A protein (~ 230 kD) was increased in lung tissue of individuals with COPD, whereas the expression of WNT-5A with a mo-

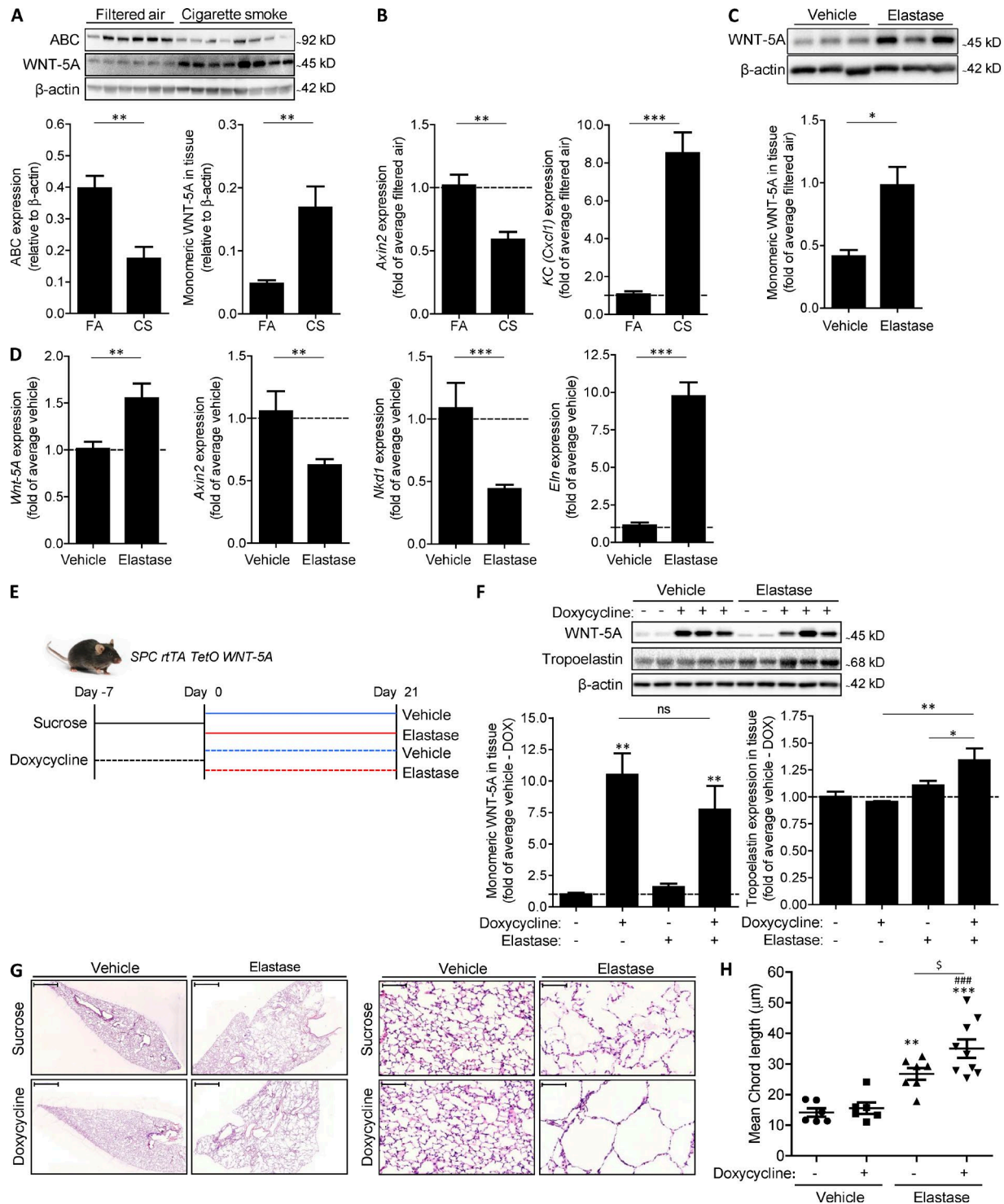


Figure 1. Noncanonical WNT-5A is increased in murine models of COPD and contributes to emphysema development in vivo. (A) Immunoblots and quantification of WNT-5A and ABC in whole-lung homogenate of mice exposed to FA ($n = 6$) or CS (CS; $n = 8$) for 4 mo. (B) Expression *Axin2* and *KC* (*CXCL1*) in whole-lung homogenate of mice exposed to FA ($n = 6$) or CS ($n = 8$) for 4 mo. (C) Immunoblots and quantification of WNT-5A expression in whole lung homogenate of mice 14 d after exposure to vehicle (PBS; $n = 3$) or elastase ($n = 3$). (D) Expression of *Wnt-5A*, *Axin2*, *Nkd1*, and *Eln* in whole-lung homogenate of mice 7 d after exposure to vehicle (PBS; $n = 6$) or elastase ($n = 6-12$). (A-D) *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, unpaired Student's *t* test. (E) Experimental setup to determine the impact of lung-specific WNT-5A overexpression on emphysema development. Animals had ad libitum access to drinking water containing 5% sucrose (solid line) or DOX (2 mg/ml) in 5% sucrose (dashed line). Animals were treated on day 0 with either elastase (PPE; 40

lecular weight of ~45 kD tended to be increased, although not significantly (Fig. 2 D). In accordance, gene expression of porcupine (*PORCN*), an O-acyltransferase that posttranslationally modifies WNT proteins, is increased in lung tissue of COPD patients (Fig. 2 E).

Pulmonary fibroblasts are a cellular source of noncanonical WNT-5A

We next aimed to identify the main cellular source of WNT-5A in COPD. WNT-5A expression was analyzed in 3D human lung tissue cultures (3D-LTCs), primary human ATII (phATII) cells, and primary human lung fibroblasts (phLF; Fig. 3 A). The highest basal transcript level of WNT-5A was detected in phLF compared with phATII and 3D-LTCs. WNT-5A protein could be detected in supernatants from phLF, but not in supernatants of ATII-like A549 cells (Fig. 3 B). Notably, RNA sequencing of phLF from individuals without (donor) and with COPD further revealed aberrant gene expression of specific WNT ligands, with increased noncanonical *WNT-5A* expression in COPD fibroblasts (Fig. 3 C; 2.4-fold of mean donor; $P < 0.001$; unpublished data). Moreover, *WNT-5A* expression was increased in senescent phLF of smokers, as well as from individuals with COPD compared with respective nonsenescent fibroblasts (Fig. 3 D). Next, we stimulated phLF with TGF- β and/or CS extract (CSE). TGF- β treatment of phLF resulted in increased expression of *WNT-5A*, whereas expression of another noncanonical WNT ligand, *WNT-4*, was unaffected (Fig. 3 E). Stimulation with TGF- β led to increased WNT-5A protein expression and secretion of the ligand (Fig. 3, F and G). Although CSE alone (up to 15%) only resulted in a slight increase in intracellular WNT-5A protein expression, CSE showed a synergistic effect on both TGF- β -induced WNT-5A protein expression and secretion in phLF (Fig. 3, F and G).

Given our initial finding that WNT-5A appears to be posttranslationally modified and oligomerized differently in human COPD samples, we next investigated the role of glycosylation on WNT-5A synthesis and secretion by phLF. The phLF were stimulated without or with TGF- β in the absence or presence of tunicamycin, a compound that prevents glycosylation of proteins. Tunicamycin largely decreased cellular expression and secretion of WNT-5A in cell lysates and supernatants from phLF, respectively (Fig. 3 H). These findings highlight the requirement of WNT-5A glycosylation to be produced and secreted by phLF, which is in line with a biochemical study investigating the relevance of posttranslational modifications of WNT-5A (Kurayoshi et al., 2007).

Fibroblast-derived WNT-5A inhibits canonical WNT- β -catenin signaling in alveolar epithelial cells

Next, we endeavored to elucidate the underlying mechanism by which WNT-5A impairs lung repair. Our previous work demonstrated that canonical WNT- β -catenin signaling is attenuated in ATII cells of emphysematous COPD patients (Kneidinger et al., 2011; Uhl et al., 2015). Given that canonical and noncanonical WNT signaling can reciprocally regulate each other's signaling activity, we investigated the direct effect of WNT-5A on WNT- β -catenin signaling in alveolar epithelial cells. Stimulation with WNT-3A resulted in increased phosphorylation of LRP6 (p-ser1490 LRP6) and accumulation of ABC in human (A549) and murine (MLE12) alveolar-like epithelial cells (Fig. 4, A and B). WNT-5A cotreatment resulted in a concentration-dependent attenuation of WNT-3A-induced LRP6 phosphorylation and ABC accumulation. DVL2 phosphorylation, a positive control for WNT signaling in general, was induced by either WNT-3A or WNT-5A, and this response was enhanced by stimulation with both ligands together (Fig. 4, A and B). Moreover, in murine alveolar epithelial cells (MLE12), WNT-3A enhanced cytosolic and/or nuclear localization of β -catenin (indicated by arrows), which was largely decreased by direct cotreatment with WNT-5A (Fig. 4 C). Accordingly, activation of β -catenin-dependent gene transcription (determined by TOP/FOP flash activity) by WNT-3A or pharmacological inhibition of GSK-3 β by either LiCl (10 mM) or SB216763 (5 μ M) was attenuated by WNT-5A in lung epithelial cells (Fig. 4, D–F). The intracellular effects of WNT-5A were at least in part mediated by protein kinase C (PKC), but not JNK1/2 or TAK1/NLK (percentage inhibition of WNT-3A signaling by WNT-5A in the absence or presence of the PKC inhibitor GF109203X was $69.2 \pm 3.3\%$ and $43.3 \pm 9.4\%$, respectively; $P < 0.05$; and not depicted). These findings indicate that WNT-5A impairs β -catenin activation and signaling not only at the receptor level (i.e., regulating LRP6 activation) but also downstream of canonical WNT receptors.

This effect was specific for WNT-5A, as WNT-4 was not able to inhibit β -catenin activity in alveolar epithelial cells (Fig. 4 G). In addition, the WNT-5A effect seems to be cell specific, as WNT-5A was unable to attenuate WNT-3A-induced activation of β -catenin-dependent gene transcription in fibroblasts (Fig. 4 H). Collectively, these results suggest that noncanonical WNT-5A is a specific negative regulator of WNT- β -catenin signaling in alveolar epithelial cells.

We further investigated the importance of N-glycosylation on the signaling properties of WNT-5A. Deglycosylated

U/kg body weight) or vehicle control (PBS). Data are derived from two independent animal experiments. (F) Analysis of WNT-5A and tropoelastin at day 21 in whole-lung homogenate of SFTPC rtTA TetO-WNT-5A mice exposed to DOX or 5% sucrose in the drinking water and treated with elastase or vehicle control ($n = 3-6$ mice/group). *, $P < 0.05$; **, $P < 0.01$, determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. (G) H&E-stained lung tissue sections. Bars: (left) 1 mm; (right) 100 μ m. (H) Mean chord length as determined by quantitative morphometry ($n = 6-9$ animals per group). **, $P < 0.01$; ***, $P < 0.001$, compared with vehicle treatment with sucrose; ###, $P < 0.001$ compared with vehicle treatment with DOX; §, $P < 0.05$, compared with elastase treatment with sucrose; determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test.

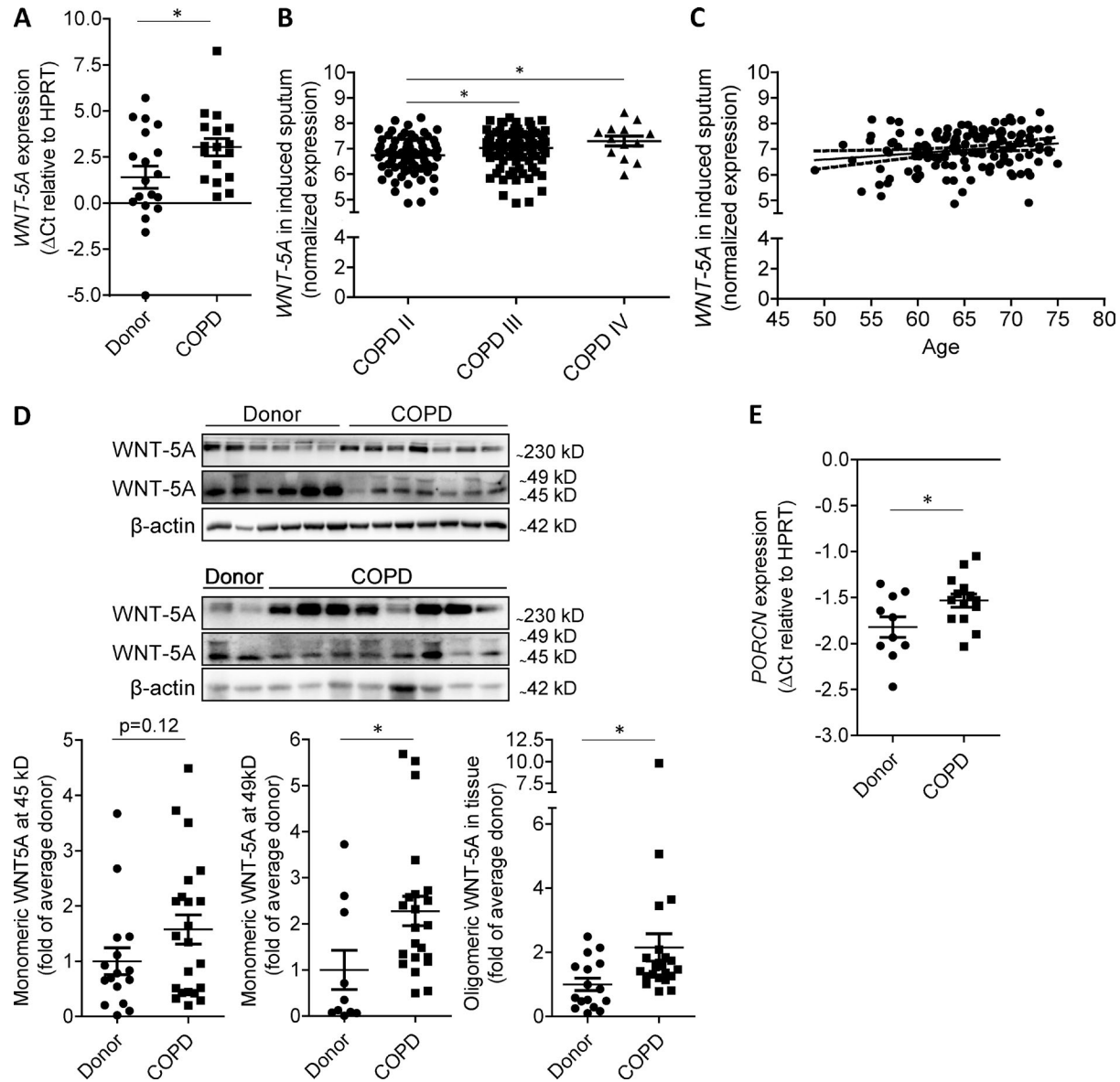


Figure 2. Enhanced expression and posttranslationally modified WNT-5A in biosamples of individuals with COPD. (A) *WNT-5A* in whole-lung homogenate of individuals without (Donor; $n = 19$) and with COPD ($n = 17$). *, $P < 0.05$, unpaired Student's t test with Welch's correction. (B) *WNT-5A* expression in induced sputum of individuals with COPD (GOLD stage II, $n = 71$; III, $n = 59$; IV, $n = 13$). *, $P < 0.05$, determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. (C) *WNT-5A* expression in induced sputum of individuals with COPD correlated to age of the individual (COPD stage II/III/IV; $n = 143$). Linear regression analysis; $r = 0.18$ and $P < 0.05$. Data presented in B and C are derived from microarray data available under GEO accession no. GSE22148 (Singh et al., 2011). (D) Immunoblots and quantification of *WNT-5A* protein expression in whole-lung homogenate of individuals without (Donor; $n = 16$) and with COPD ($n = 22$). *, $P < 0.05$, unpaired Student's t test with Welch's correction. (E) Expression of *porcupine* (*PORCN*) in whole-lung homogenate of individuals without (Donor; $n = 10$) and with COPD ($n = 14$). *, $P < 0.05$, unpaired Student's t test with Welch's correction.

WNT-5A was generated by treating *WNT-5A* conditioned medium (*WNT-5A* CM) with Peptide-*N*-Glycosidase F (PNGase F), which did not influence protein stability of the ligand (Fig. 5 A). Deglycosylated *WNT-5A* was not able to significantly attenuate canonical *WNT* signaling, whereas untreated *WNT-5A* CM attenuated *WNT-3A*-induced activation of β -catenin dependent gene transcription in A549 cells (Fig. 5 B, left). Furthermore, control CM treated with

PNGase F did not affect *WNT-3A* driven transcriptional activity of β -catenin (Fig. 5 B, right). These results demonstrate that glycosylation of the ligand is essential for the observed negative regulation of *WNT*- β -catenin signaling by *WNT-5A* in alveolar epithelial cells.

Next, we wondered if fibroblast-derived *WNT-5A* was sufficient to affect canonical *WNT*- β -catenin signaling in alveolar epithelial cells. We experimentally addressed this

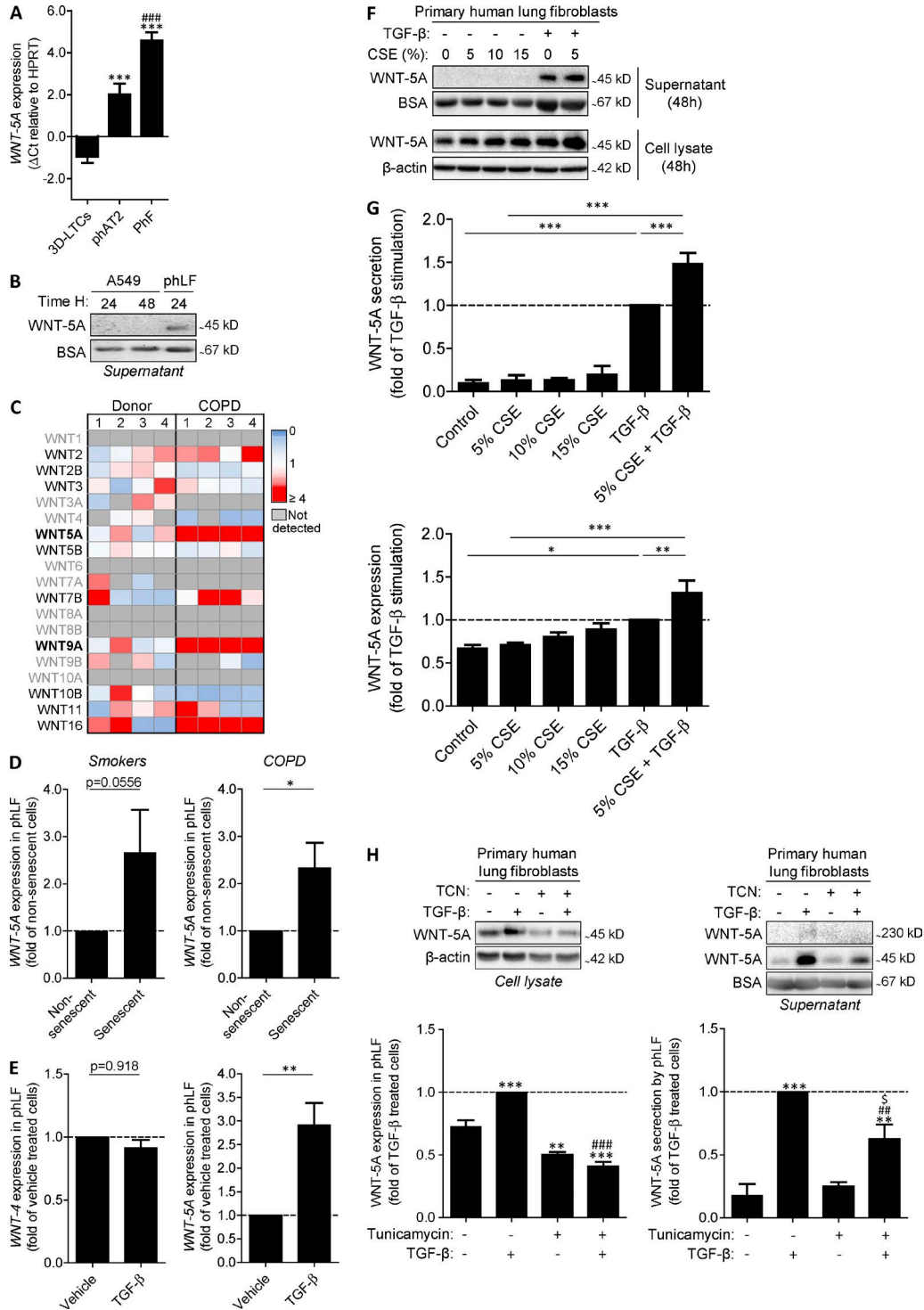


Figure 3. **WNT-5A is highly expressed and secreted by human lung fibroblasts, which are augmented by CSE and/or TGF-β.** (A) Comparison of *WNT-5A* expression in human 3D lung tissue cultures (3D-LTCs; $n = 3$), primary human alveolar epithelial cells ($n = 4$), and phLFs ($n = 5$). ***, $P < 0.001$, compared with 3D-LTCs; ###, $P < 0.001$, compared with phLF; determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. (B) Comparison of *WNT-5A* secretion by human alveolar epithelial cells (A549; 24 and 48 h) and phLF (24 h; $n = 4$ independent experiments). (C) RNA sequence analysis of *WNT* ligand expression in phLF of individuals without (Donor; $n = 4$) and with COPD ($n = 4$). Gray boxes indicate that *WNT* ligands could not be detected in the respective sample. Adjusted p -value < 0.05 (Benjamini-Hochberg multiple testing correction). (D) *WNT-5A* in nonsenescent and senescent phLF of smokers ($n = 7$) and individuals with COPD ($n = 8$). *, $P < 0.05$, paired Student's t test. (E) *WNT-4* and *WNT-5A* in phLF in the absence

question by treating alveolar epithelial cells with supernatants of fibroblasts in which WNT-5A was silenced by small interference RNA (siRNA). Treatment of MRC5 human lung fibroblasts with WNT-5A-specific siRNA attenuated both basal and TGF- β -induced expression of WNT-5A in cell lysates and supernatants (Fig. 5, C–E). WNT-5A at >200 kD was only detected in the supernatant fraction and not in cell lysate, further corroborating that WNT-5A homomers/oligomers are a secreted form of WNT-5A. Remarkably, supernatants of TGF- β -stimulated fibroblasts in which WNT-5A was knocked down caused a significant activation of β -catenin/TCF-dependent gene transcription in alveolar epithelial cells (Fig. 5 F), whereas supernatants of TGF- β -stimulated fibroblasts (scrambled siRNA with TGF- β) caused a slight, although not significant, decrease in β -catenin signaling in alveolar epithelial cells (Fig. 5 F). Collectively, these experiments demonstrate that WNT-5A secreted by lung fibroblasts is sufficient to negatively affect canonical WNT signaling in alveolar epithelial cells.

WNT-5A impairs WNT- β -catenin-mediated alveolar epithelial cell repair

These results urged us to study the functional consequence of noncanonical WNT-5A on alveolar epithelial cell repair capacity. WNT-5A significantly delayed WNT-3A- β -catenin-mediated wound closure of murine lung epithelial cells (Fig. 6 A). We also used the well-characterized in vitro model in which primary mouse ATII cells trans-differentiate spontaneously to ATI-like cells over culture, a process highly dependent on autocrine WNT- β -catenin signaling (Fig. 6, B and C; Flozak et al., 2010; Mutze et al., 2015). WNT-5A CM treatment impaired β -catenin activation and attenuated ATII-to-ATI cell trans-differentiation over time of culture, as determined by protein expression of ABC and T1 α /podoplanin (ATI cell marker; Fig. 6 D). The inhibitory effect of WNT-5A on β -catenin signaling in the alveolar epithelium was once more confirmed by analyzing microarray data of primary mouse ATII cells treated with WNT-5A (unpublished data). Canonical WNT target genes, such as *Nkd1* and *Lgr5*, were significantly down-regulated by WNT-5A compared with control treatment (Fig. 6 E). Interestingly, WNT-5A also negatively affected the expression of *Nkx2.1* (TTF-1; pulmonary epithelial cell marker), whereas elastin (*Elm*), which is up-regulated in experimental emphysema (Fig. 1 D) and in patients with severe emphysema (Deslee et al., 2009), was among the highest up-regulated genes (Fig. 6 E).

Inhibition of WNT-5A signaling in vivo recuperates alveolar cell function and attenuates lung pathogenesis in murine models of COPD

To evaluate if targeting WNT-5A in vivo is beneficial in developing and/or established experimental COPD, we first used the model of elastase-induced emphysema and prophylactically treated mice with either neutralizing antibodies directed against WNT-5A (α WNT-5A) or respective IgG control antibodies (Fig. 7 A). Histological assessment of the lungs 7 d after elastase treatment revealed ameliorated lung pathology in animals that received α WNT-5A compared with mice treated with IgG control antibodies (Fig. 7, B–D). This was confirmed by quantitative morphometric analysis (mean chord length, 26.2 ± 2.3 versus 32.1 ± 1.6 μ m, respectively; $P = 0.06$) and by a significant increase in lung function (elastance, 24.3 ± 1.6 versus 20.4 ± 0.9 cmH₂O/ml, respectively; $P < 0.05$; Fig. 7 D). Furthermore, increased expression of ABC (Fig. 7 E), the canonical WNT target genes *Axin2* and *Nkd1*, and the alveolar epithelial cell markers *T1 α* (ATI) and *Sftpc* (ATII) were observed in mice treated with α WNT-5A compared with respective IgG-treated mice (Fig. 7 F).

A contributing factor to the development of COPD is the inflammatory response to CS. Therefore, we further investigated if WNT-5A is involved in CS-induced pulmonary inflammation in vivo (Fig. 7 G). CS increased the number of inflammatory cells, mainly neutrophils and macrophages, in the bronchoalveolar lavage fluid (BALF; Fig. 7 H). No increase in either eosinophils or lymphocytes was observed in response to CS (not depicted). Systemic application of BOX5, a synthetic peptide that acts as an antagonist of WNT-5A (Jenei et al., 2009), did not influence macrophage and neutrophil recruitment into the lung (Fig. 7 H). Nevertheless, CS decreased the expression of *Axin2* and the alveolar epithelial cell marker *Nkx2.1*, which was partially restored by WNT-5A antagonism (Fig. 7 I). Plotting of *Axin2* against *Nkx2.1* revealed a clear linear correlation ($r = 0.95$; $P < 0.0001$; Fig. 7 I). Moreover, both of these genes were significantly down-regulated in COPD patients (donor versus COPD: *AXIN2*, $P = 0.0026$; *NKX2.1*, $P = 0.0003$), and a similar linear correlation between *AXIN2* and *NKX2.1* was observed in human lung tissue (all individuals, $r = 0.46$; Donor, $r = 0.41$; COPD, $r = 0.45$; $P < 0.0001$ for all groups; Fig. 7 J). These findings further indicate a close link between WNT signal activity and alveolar epithelial cell function in vivo.

Finally, to address if WNT-5A is involved in the progression of emphysema and a potential therapeutic target for the treatment of emphysema, we exposed mice to elastase and

and presence of TGF- β (2 ng/ml; 24 h) in pHLF ($n = 5$ –8). **, $P < 0.01$, paired Student's *t* test. (F and G) Representative immunoblots and quantification of WNT-5A expression in supernatant (secretion) and cell lysate after stimulation with CSE (5–10–15% CSE) in the absence and presence of TGF- β (2 ng/ml) for 48 h ($n = 4$ independent experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. (H) WNT-5A in supernatant (secretion) and cell lysate after stimulation TGF- β (2 ng/ml) in the absence and presence of tunicamycin (TCN; 5 μ M) for 48 h ($n = 3$ –5 independent experiments). **, $P < 0.01$; ***, $P < 0.001$ compared with vehicle without TNC; ##, $P < 0.01$; ###, $P < 0.001$, compared with TGF- β without TNC; §, $P < 0.05$, compared with vehicle with TCN; determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test.

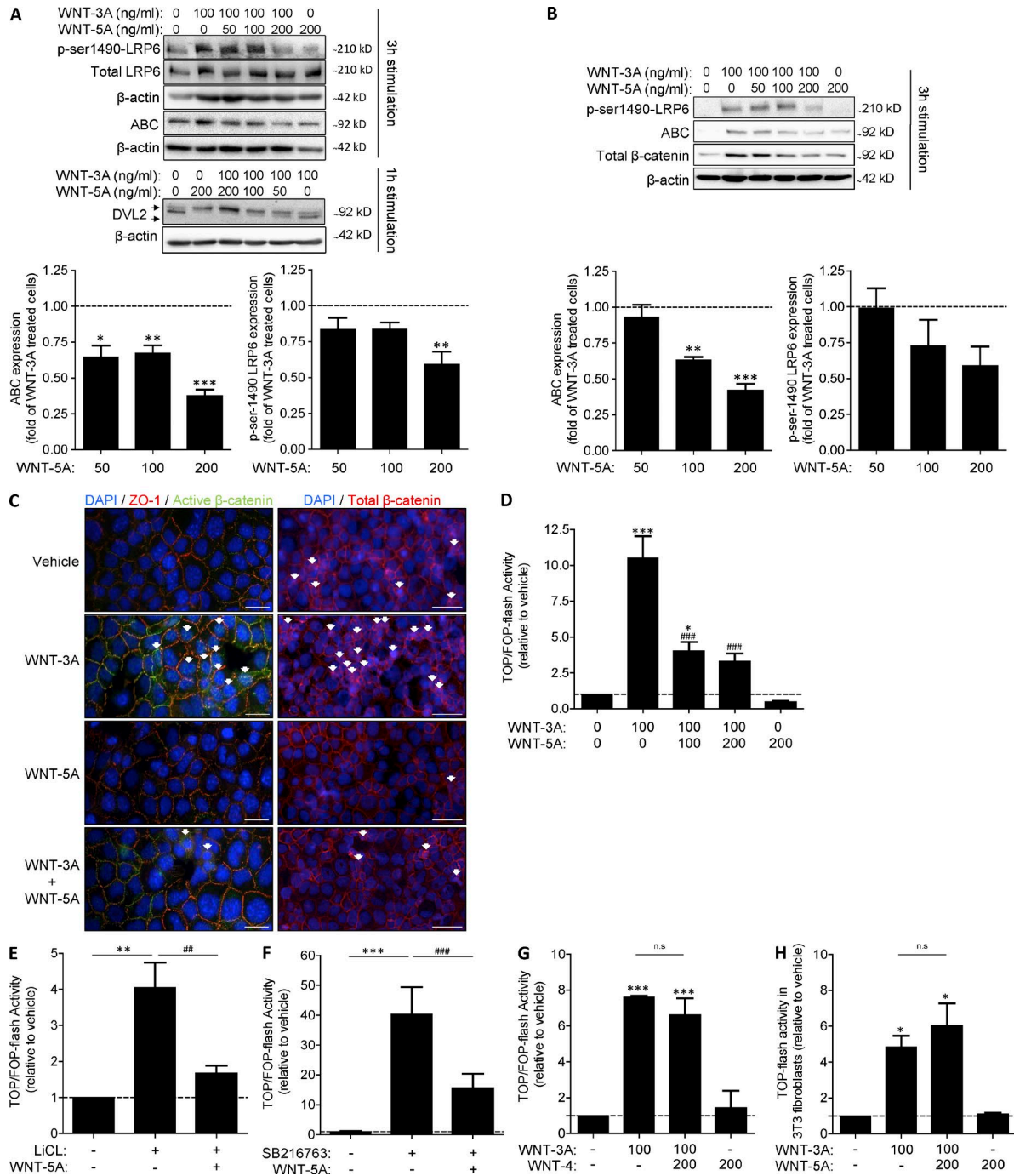


Figure 4. WNT-5A attenuates canonical WNT-β-catenin signaling in alveolar epithelial cells. (A and B) Immunoblots and quantification of LRP6 phosphorylation and ABC accumulation in the absence and presence of WNT-3A and/or WNT-5A in (A) human (A549) and (B) murine (MLE12) alveolar epithelial cells ($n = 3-5$ independent experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WNT-3A stimulation in the absence of WNT-5A; determined by one-way ANOVA followed by a Dunnett's multiple comparison test. (C) Representative immunofluorescence images of active and total β-catenin (left and right, respectively) in murine alveolar epithelial cells (MLE12) treated with WNT-3A, WNT-5A, or both for 3 h. Arrows indicate cytosolic and/or nuclear β-catenin expression. ZO-1 staining (left) was included to show tight junctions between cells. Bars: (left) 20; (right) 50 μm ($n = 3$). (D) β-Catenin-dependent gene transcription (TOP/FOP-flash assay) activated by WNT-3A in the absence and presence of WNT-5A in human alveolar epithelial cells ($n = 4-5$). *, $P < 0.05$; ***, $P < 0.001$ compared with vehicle; ###, $P < 0.001$, compared with WNT-3A; determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. (E and F) β-Catenin-dependent gene transcription activated by GSK-3β inhibitor (E) LiCl or (F) SB216763 in the absence and presence of WNT-5A (100 ng/ml) in human alveolar epithelial cells (A549). Data represents $n = 3-5$ independent experiments. **, $P < 0.01$; ***, $P < 0.001$, compared with vehicle control; #, $P < 0.01$; ###, $P < 0.001$, compared with respective GSK-3β alone; determined by one-way ANOVA, followed by

started treatment with IgG control antibodies or α WNT-5A upon established emphysema (6 d after exposure; Fig. 8 A). Consistent with the preventative approaches, mice with established emphysema that received α WNT-5A had significantly improved lung architecture, better lung function (elastance and compliance), and reduced mean chord length as compared with mice receiving IgG treatment (Fig. 8, B–D). Similarly to what was seen in the CS model, inhibition of WNT-5A did not influence inflammatory cell influx (Fig. 8 E), while resulting in an increased expression of canonical WNT target genes (*Axin2* and *Nkd1*) and the alveolar epithelial cell marker *Nkx2.1* (Fig. 8 F). Consistent with the CS model, a linear correlation between *Axin2* and *Nkx2.1* was also observed in the therapeutic model of elastase-induced emphysema (Fig. 8 G). Furthermore, these data were confirmed on the protein level with enhanced NKX2.1 ($P = 0.075$) expression in mice receiving α WNT-5A antibodies in comparison to mice receiving IgG antibody treatment (Fig. 8, H and I). These data indicate that inhibition of WNT-5A reduces the development and progression of emphysema by restoring canonical WNT signaling and alveolar epithelial cell function in vivo. Collectively, our data suggest that attenuated canonical WNT signaling and disturbed lung repair observed in COPD is in part a consequence of increased WNT-5A secretion by pulmonary fibroblasts (Fig. 9).

DISCUSSION

In the current study, we demonstrate that increased noncanonical WNT signaling activated by WNT-5A contributes to COPD pathogenesis. Increased expression of noncanonical WNT-5A was detected in two distinct animal models of emphysema/COPD. Moreover, elevated expression together with posttranslational modification and oligomerization of the ligand was observed in human COPD. WNT-5A gene expression correlated to the age of COPD patients. Accordingly, we found increased WNT-5A in primary COPD fibroblasts, as well as senescent fibroblasts. Functionally, WNT-5A impaired the activation of β -catenin, the main effector protein in canonical WNT signaling, in alveolar epithelial cells, thereby inhibiting wound healing and ATII-to-ATI cell trans-differentiation in vitro. Lung-specific overexpression of WNT-5A aggravated elastase-induced emphysema, and both preventive and therapeutic inhibition of WNT-5A restored canonical WNT signaling, resulting in decreased lung pathology in experimental emphysema/COPD. Collectively, WNT-5A is increased in COPD and negatively affects canonical WNT signal-driven alveolar epithelial cell function, thereby contributing to disease development and progression.

Canonical and noncanonical WNT signaling are both indispensable for proper lung development (Li et al., 2002; Goss et al., 2009). Mice carrying a targeted disruption of the WNT-5A locus show distinct abnormalities in distal lung morphogenesis, characterized by increased proliferation of epithelial and mesenchymal cells, as well as impaired lung maturation (Li et al., 2002). Inducible WNT-5A knockout mice do not currently exist and conventional, constitutive WNT-5A knockout mice (*WNT-5A*^{-/-}) die shortly after birth, apparently due to respiratory failure (Yamaguchi et al., 1999). To determine the relevance of WNT-5A in emphysema development in the adult mouse, we first performed gain-of-function experiments in which WNT-5A was overexpressed in vivo (SFTPC rtTA TetO WNT-5A mice). This mouse line is based on the incorporation of the 3.7-kb fragment of the human SFTPC promoter and is one of the most widely used transgenic mouse strains that targets the respiratory epithelium (Wert et al., 1993). In the adult lung, SFTPC promoter activity has been reported to target ATII cells and subsets of cuboidal bronchiolar cells (Rawlins and Perl, 2012). Lung-specific overexpression of WNT-5A during development has been demonstrated to negatively affect epithelial branching in vivo (Li et al., 2005). Although mesenchymal cells most likely represent the majority of WNT-5A-producing cells in the lung, the SFTPC rtTA TetO WNT-5A model allowed us to artificially enhance expression of the ligand into the extracellular space of the lung, similar to what is observed in COPD pathogenesis. WNT-5A overexpression resulted in significant aggravation of elastase-induced emphysema in vivo, and thus indicates that WNT-5A enhances the susceptibility of the lung to develop emphysema/COPD.

In human lung tissue, we detected several forms of WNT-5A with distinct molecular weights. WNT ligands are heavily subjected to posttranslational modifications, such as palmitoylation and glycosylation, and they can also agglomerate into multimeric/oligomerized complexes (~95–230 kD; Panáková et al., 2005; Kurayoshi et al., 2007; Baarsma et al., 2013). We observed an increase in oligomerized WNT-5A in the lung tissue of individuals with COPD. Similar findings have been observed in murine lung tissue; however, further in-depth studies are needed to corroborate these results. Fully matured, monomeric WNT-5A is equivalent to ~49 kD, and expression of WNT-5A with this molecular weight was almost exclusively detected in individuals with COPD. Analogous to COPD, increased expression of mature WNT-5A (~49 kD) is present in other chronic pathologies, such as melanoma and ovarian carcinoma (Da Forno et al., 2008; Barbolina et al., 2011). The clinical relevance of fully matured WNT-5A compared with partially, posttranslationally modi-

a Newman-Keuls multiple comparison test. (G) β -Catenin-dependent gene transcription activated by WNT-3A in the absence and presence of recombinant WNT-4 (200 ng/ml) in A549 cells ($n = 6$). ***, $P < 0.001$, compared with vehicle control; determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. (H) β -Catenin-dependent gene transcription activated by WNT-3A in the absence and presence of recombinant WNT-5A (200 ng/ml) in 3T3 fibroblasts ($n = 3$). *, $P < 0.05$, compared with vehicle control; determined by one-way ANOVA followed by a Newman-Keuls multiple comparison test.

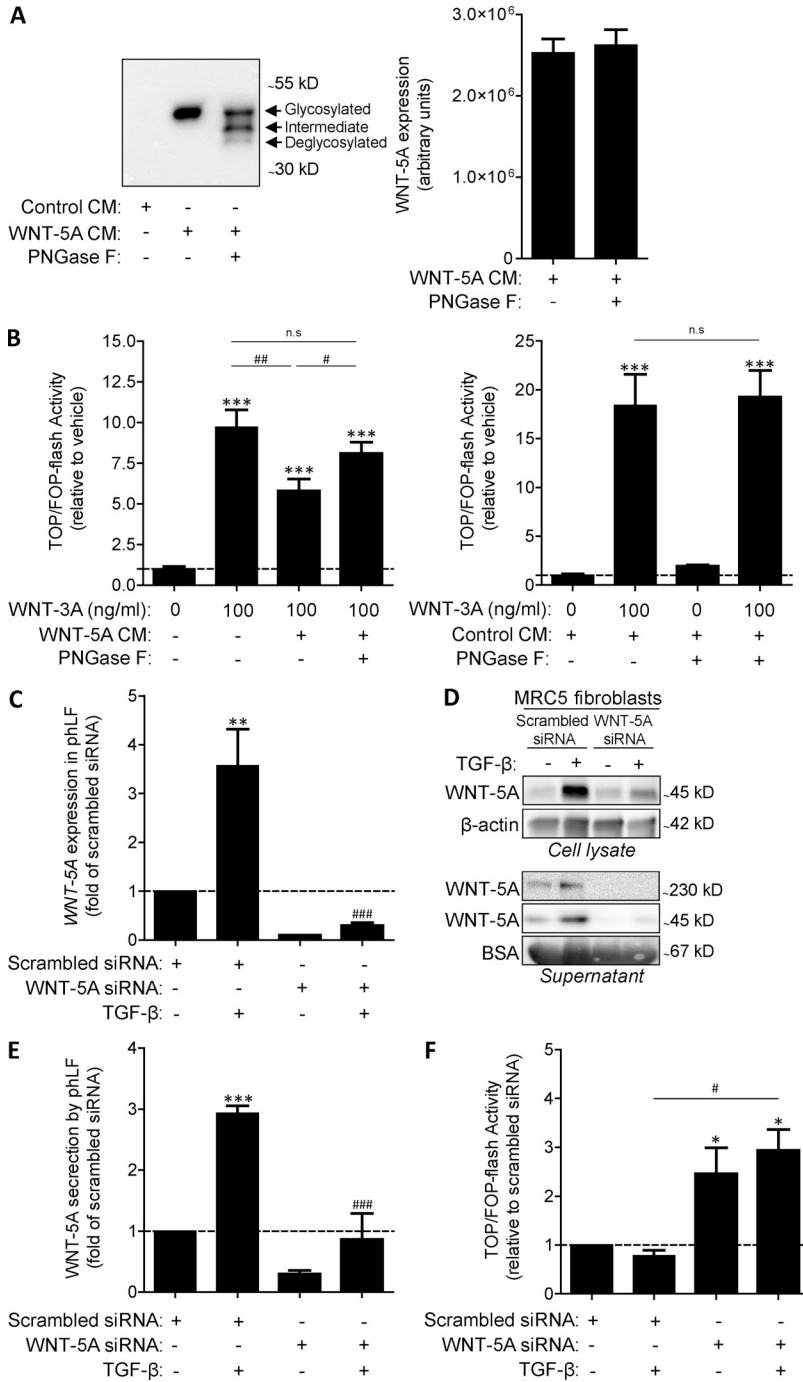


Figure 5. Glycosylated, fibroblast-derived WNT-5A is a negative regulator of canonical WNT-β-catenin signaling in alveolar epithelial cells. (A) Immunoblots and quantification of WNT-5A in L-cell-derived control CM or WNT-5A CM in the absence and presence of PNGase F for 1 h ($n = 3-6$ independent experiments). (B) β-Catenin-dependent gene transcription (TOP/FOP-flash assay) in A549 cells activated by WNT-3A in the absence and presence of glycosylated WNT-5A (WNT-5A CM), deglycosylated WNT-5A (WNT-5A CM + PNGase F), or control CM without and with PNGase F ($n = 3-6$ independent experiments). ***, $P < 0.001$, compared with vehicle control; #, $P < 0.05$; ##, $P < 0.01$, compared with WNT-3A with WNT-5A CM; determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. (C-E) Confirmation of WNT-5A silencing on (C) mRNA and (D and E) protein level in MRC5 fibroblast ($n = 3$) transfected with scrambled siRNA or WNT-5A siRNA and subsequently treated with TGF-β (2 ng/ml for 48 h). **, $P < 0.01$; ***, $P < 0.001$, compared with vehicle control; ###, $P < 0.001$, compared with scrambled siRNA with TGF-β; determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. (F) β-Catenin-dependent gene transcription in A549 cells after 24 h of stimulation with supernatants derived from MRC5 human lung fibroblasts treated with scrambled siRNA or WNT-5A siRNA with or without additional TGF-β (2 ng/ml) treatment ($n = 3$). *, $P < 0.05$, compared with scrambled siRNA control; #, $P < 0.05$, compared with scrambled siRNA with TGF-β; determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test.

fied WNT-5A in these diseases has not yet been investigated. Here, we provide in vitro evidence that attenuation of canonical WNT signaling by WNT-5A was largely diminished when posttranslational modification (i.e., glycosylation and palmitoylation) within WNT-5A were removed, indicating that these posttranslational modifications are important for the secretion and function of WNT-5A.

Canonical and noncanonical WNT signaling can be independent signaling entities; however, over the last decade,

increasing evidence indicates that these pathways are strongly interconnected (Ishitani et al., 2003; Topol et al., 2003; Mikels and Nusse, 2006). Although inhibitory action of noncanonical WNT-5A on β-catenin signaling has been established previously, to the best of our knowledge, we are the first to demonstrate that this mechanism is central to a specific lung disease in human. Our study shows that WNT-5A attenuates canonical WNT-β-catenin signaling by reducing LRP6 activation and accumulation of transcriptionally ABC in alveolar

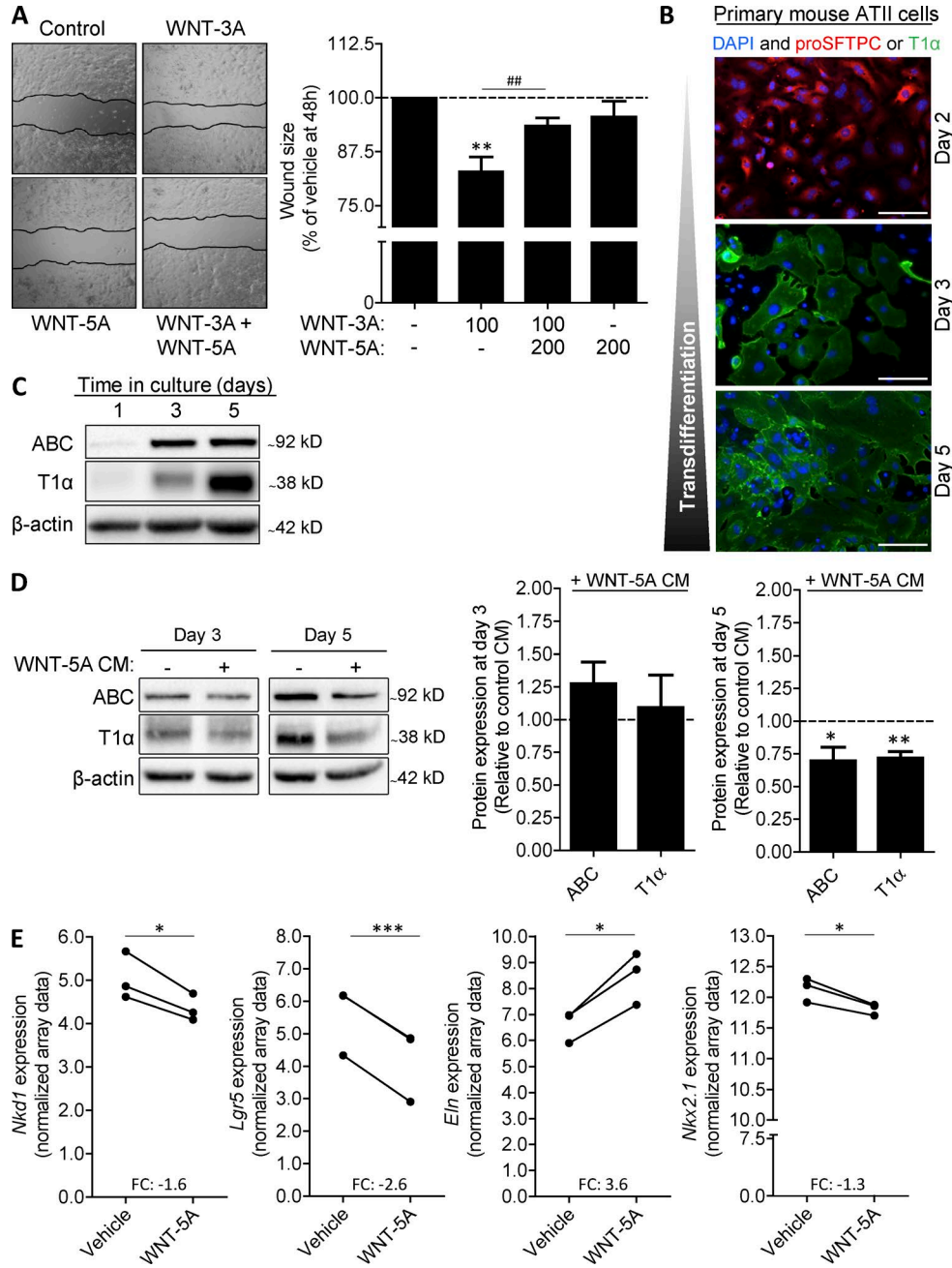


Figure 6. WNT-5A impairs canonical WNT signaling driven alveolar cell repair. (A) Representative images and quantification of wounds size of murine epithelial cells (MLE12) treated with WNT-3A, WNT-5A, or the combination (each WNT ligand: 100 ng/ml) for 48 h after wounding ($n = 4$ independent experiments). **, $P < 0.01$ compared with vehicle control; ###, $P < 0.01$, compared with WNT-3A stimulation; determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. (B) Representative immunofluorescence images of SPC (ATII cell marker) or T1α (ATI cell marker) in primary mouse ATII cells cultured up to 5 d. Bar, 100 μ m. (C) Immunoblots of ABC and T1α (ATI cell marker) protein expression in primary mouse ATII cells cultured up to 5 d. (D) Representative immunoblot and quantification of ABC and T1α in primary mouse ATII cells cultured for 3 or 5 d in the presence of control CM or WNT-5A CM (each diluted 1:1; $n = 5$). *, $P < 0.05$; **, $P < 0.01$, compared with control CM; determined by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. (E) Microarray data for *Nkd1*, *Lgr5*, *Eln*, and *Nkx2.1* of primary mouse ATII cells stimulated with WNT-5A (200 ng/ml; 6 h) or vehicle control ($n = 3$). *, $P < 0.05$; ***, $P < 0.001$, paired Student's *t* test.

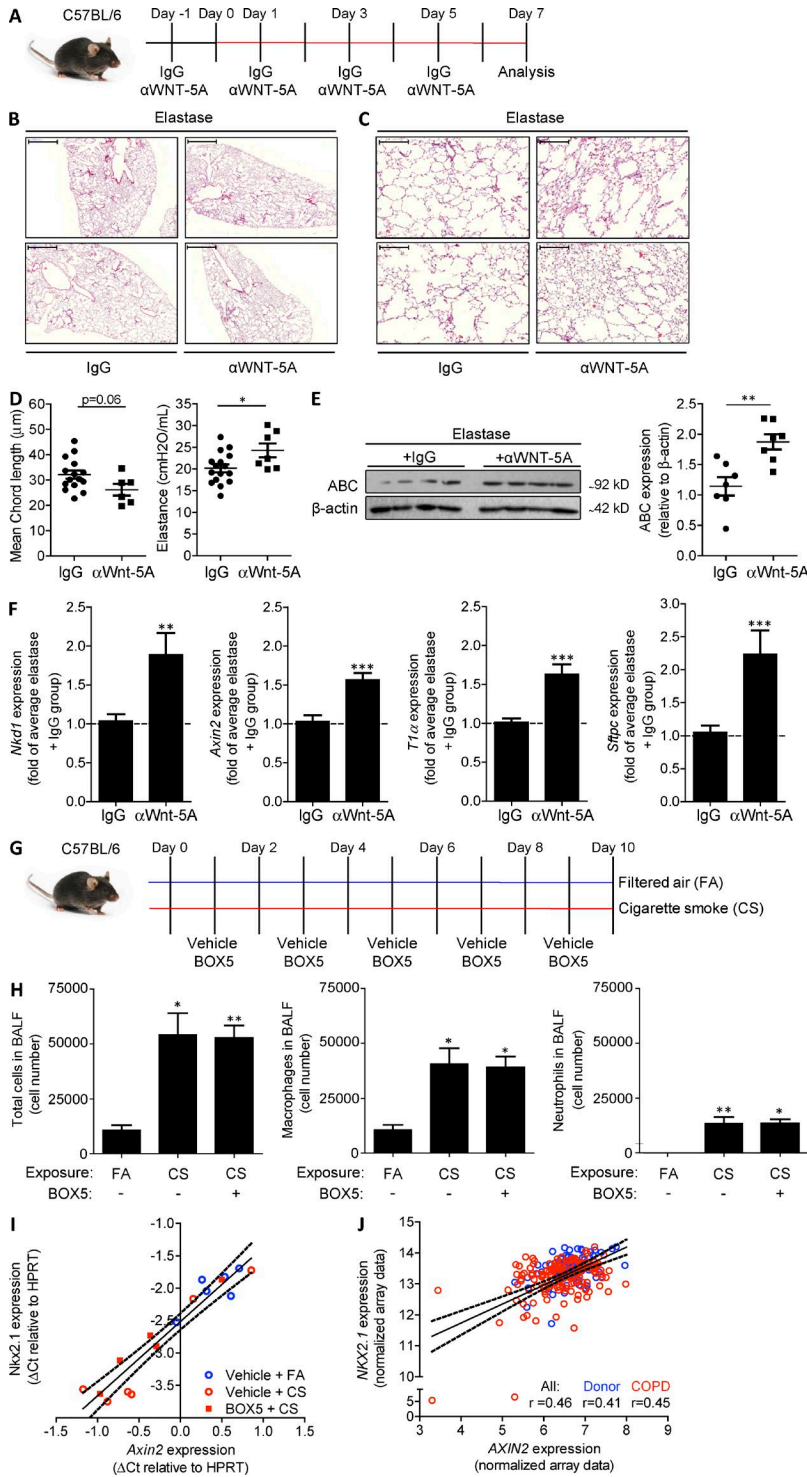


Figure 7. Prophylactic inhibition of WNT-5A signaling in vivo recovers alveolar cell function and attenuates lung pathogenesis in murine models of COPD. (A) Experimental setup to determine the effect of antibody-mediated inhibition of WNT-5A on emphysema development in vivo. Animals were treated on day 0 with elastase (PPE: 80 U/kg body weight) and received either αWNT-5A antibodies or respective IgG control antibodies as treatment. Data are derived from two independent animal experiments. (B and C) Representative images of H&E-stained lung tissue sections. Bars: (B) 500; (C) 100 μm. (D) Mean chord length and lung elastance measurements in mice receiving elastase and either αWNT-5A or IgG antibodies ($n = 6-16$ animals per group). *, $P < 0.05$, unpaired Student's t test. (E) Representative immunoblots and quantification of ABC in mice with elastase-induced emphysema and either IgG or αWNT-5A treatment ($n = 7$ for each group). **, $P < 0.01$, unpaired Student's t test. (F) Expression of *Nkd1*, *Axin2*, *Ttα* (podoplanin), and *Sftpc* in whole-lung homogenate of mice with elastase-induced emphysema and either IgG ($n = 12$) or αWNT-5A ($n = 6$) treatment. **, $P < 0.01$; ***, $P < 0.001$, unpaired Student's t test. (G) Experimental setup to determine the effect of WNT-5A blocking peptide BOX5 on CS-induced pulmonary inflammation and alterations in lung epithelial cell function. Data are derived from a single animal experiment. (H) Analysis of total cells, macrophages, and neutrophils in BALF of mice exposed for 10 d to FA (control) or CS in the absence or presence of BOX5 (40 μg per i.p. injection; $n = 3-8$ animals per group). *, $P < 0.05$; **, $P < 0.01$ compared with FA-exposed mice; determined by one-way ANOVA followed by a Newman-Keuls multiple comparison test. (I) Correlation between *Axin2* and *Nkx2.1* expression in whole-lung homogenate of mice exposed to FA or CS in the absence or presence of BOX5 ($n = 5-7$ animals per group). Linear regression analysis, $r = 0.95$ and $P < 0.0001$. (J) Correlation between *AXIN2* and *NKX2.1* expression in whole-lung homogenate of individuals without (Donor; $n = 91$) and with COPD ($n = 144$). Linear regression analysis: all individuals, $r = 0.46$; donor, $r = 0.41$; and COPD, $r = 0.45$; $P < 0.0001$ for all groups. Data derived from GSE47460-GPL14550.

epithelial cells. Moreover, WNT-5A attenuates β-catenin signaling activated by pharmacological inhibition of GSK-3β, indicating that the ligand additionally activates an intracellular signaling cascade that negatively influences β-catenin signaling. Alongside WNT-5A, WNT-4 is up-regulated in COPD (Durham et al., 2013; Heijink et al., 2013); however, this li-

gand did not affect canonical WNT-β-catenin signaling in alveolar epithelial cells. Recently, an in vitro study linked CS-related lung carcinogenesis to WNT-5A and PKC signaling (Whang et al., 2013). Analogously, we found that pharmacological inhibition of PKC partially prevented the action of WNT-5A on β-catenin-dependent gene transcription in

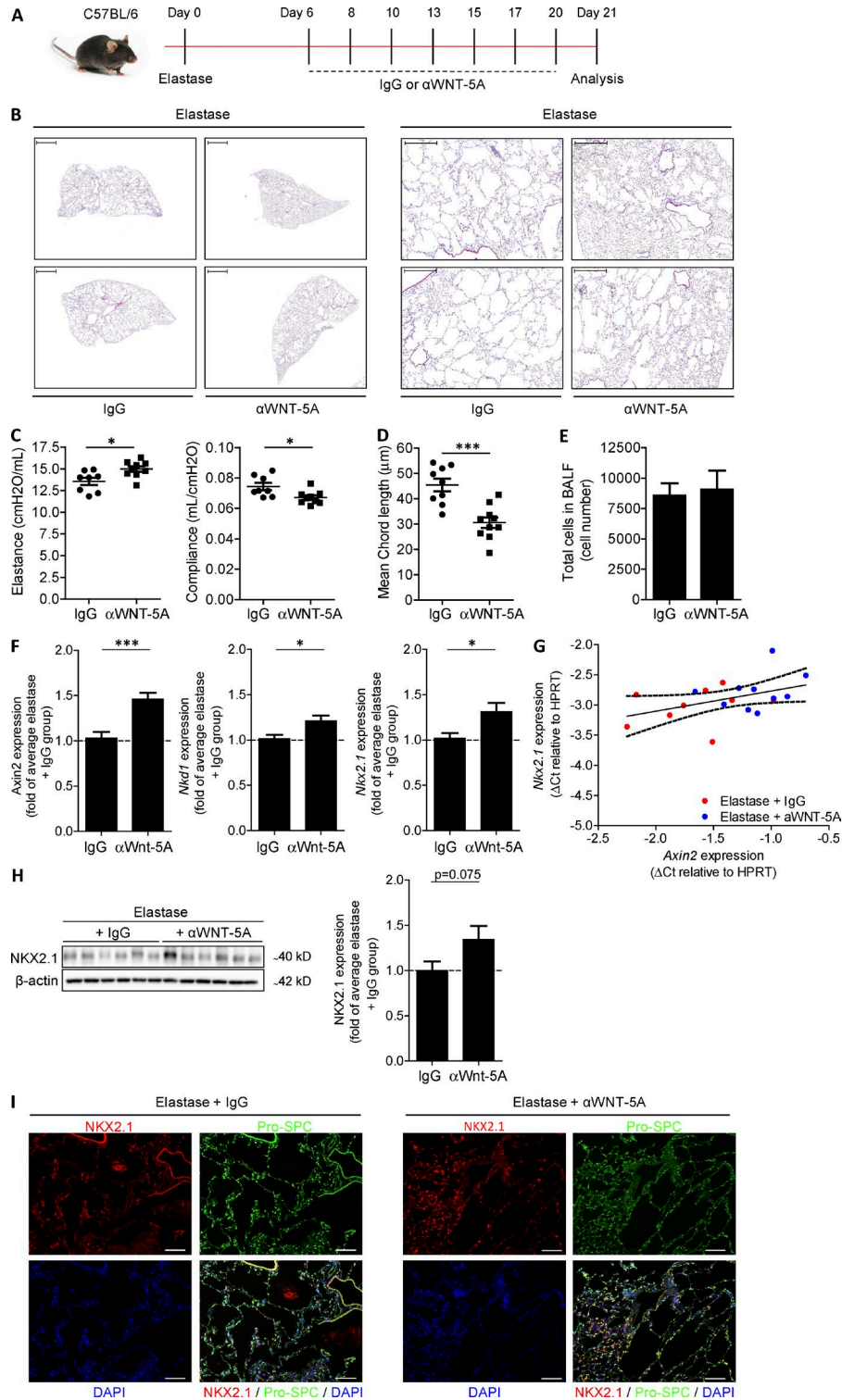


Figure 8. Therapeutic inhibition of WNT-5A signaling in vivo recovers alveolar cell function and attenuates lung pathogenesis in murine models of COPD. (A) Experimental setup to determine the effect of antibody-mediated inhibition of WNT-5A on established emphysema in vivo. Animals were instilled on day 0 with elastase (PPE: 40 U/kg body weight) and received either α WNT-5A antibodies or respective IgG control antibodies as treatment starting at day 6 after elastase. Data are derived from two independent animal experiments. (B) Representative images of H&E-stained lung tissue sections. Bars: (left) 75; (right) 25 μ m. (C and D) Lung function parameters (C) elastance and compliance and (D) mean chord length in mice receiving elastase and either α WNT-5A or IgG antibodies ($n = 8-10$ animals per group). *, $P < 0.05$; ***, $P < 0.001$, unpaired Student's t test. (E) Analysis of total cells in BALF

alveolar epithelial cells. Moreover, removal of posttranslational modifications within WNT-5A diminished the capacity of the ligand to down-regulate WNT-3A- β -catenin-mediated gene transcription, which is in agreement with biochemical studies investigating the relevance of posttranslational modifications of WNT ligands (Komekado et al., 2007; Kurayoshi et al., 2007). Our *in vivo* intervention studies, in which the biological/pathogenic effect of WNT-5A was investigated by treating mice subjected to elastase with WNT-5A-neutralizing antibodies or mice exposed to CS with the WNT-5A antagonist BOX5, show that inhibition of WNT-5A, independently of inflammation, increases canonical WNT signaling and alveolar cell function accompanied by improved lung function and structure. Collectively, these data strongly suggested that WNT-5A is a negative regulator of endogenous lung repair *in vivo* and is a novel and suitable molecular target for therapeutic intervention in emphysema/COPD.

We report that, in the lung, WNT-5A is most likely, but not exclusively, secreted by parenchymal fibroblasts and that secretion of the ligand is enhanced by TGF- β and/or soluble components of CS. In agreement, components and/or contaminants of CS, including nicotine and LPS, have been demonstrated to directly enhance WNT-5A expression in lung cells (Whang et al., 2013; Villar et al., 2014). In addition, WNT-5A is a direct target of miR-487b, a microRNA that is repressed by CS (Xi et al., 2013). Collectively, these data provide evidence that noncanonical WNT-5A represents a prime target of COPD-related exposures. These data are further corroborated by our finding that WNT-5A expression is enhanced in pHLFs from COPD patients. Furthermore, WNT-5A expression in nonepithelial cells increases with age in the human lung, as shown in a case report by immunofluorescence staining of the ligand in lung tissue obtained from a 73-yr old compared with a 21-yr old (Kovacs et al., 2014). Moreover, WNT-5A can suppress telomerase activity, and impaired telomerase activity increases the susceptibility to develop COPD/emphysema (Olson et al., 1998; Stanley et al., 2015). Consistent with these findings, we report that WNT-5A gene expression correlates to the age of the COPD patients and we observed enhanced WNT-5A expression *in vitro* when pHLFs of smokers or COPD patients become senescent. These data further indicate a potential age-associated shift to noncanonical WNT signaling contributing to COPD pathogenesis. Florian et al. (2013) demonstrated that noncanonical WNT-5A is able to negatively affect stem cell aging by inducing ageing-associated stem cell apolarity and

the occurrence of aging-like differentiation. Here, we investigated the effects of WNT-5A on ATII cells, which function as progenitor cells in the lung (Barkauskas et al., 2013). Our data underline the relevance of WNT- β -catenin signaling in repair processes by the alveolar epithelium and show that WNT-5A negatively affects these processes. Canonical WNT signaling enhanced wound closure (i.e., migration) by alveolar epithelial cells, which was largely prevented by cotreatment with WNT-5A. Moreover, ATII-to-ATI-like cell trans-differentiation, which is dependent on autocrine WNT- β -catenin signaling (Flozak et al., 2010; Mutze et al., 2015), was also impaired by WNT-5A. In addition, WNT-5A down-regulated the expression of canonical WNT target genes (*Nkd1* and *Axin2*), as well as the epithelial cell marker *Nkx2.1*, in primary mouse ATII cells. Notably, pulmonary *Nkx2.1* expression closely correlates to the expression of the canonical WNT target gene *Axin2* in individuals without and with COPD, which could be recapitulated *in vivo* in our murine models of COPD. The transcription factor NKX2.1 transactivates promoters of surfactant proteins (i.e., Sp-A, Sp-B, and Sp-C) and podoplanin (T1 α) in pulmonary epithelial cells, and is essential for lung morphogenesis (Ramirez et al., 1997; Minoo et al., 1999). Accordingly, inhibition of WNT-5A *in vivo* in experimental emphysema enhances NKX2.1 expression, accompanied by increases in *T1 α* and *Sftpc* and improved lung architecture. In addition, WNT-5A up-regulated *elastin* (*Eln*) expression in primary mouse ATII cells and recent studies demonstrated that *ELN* (human elastin) is among the highest up-regulated genes in COPD compared with controls (Deslee et al., 2009; Brandsma et al., 2016), which may be caused in part by increased WNT-5A expression in COPD, as our study indicates.

WNT signal alterations have also been implied in other chronic lung diseases, such as asthma and idiopathic pulmonary fibrosis (IPF; Selman et al., 2008; Kumawat et al., 2014). In IPF, increased canonical WNT- β -catenin signal activity in the alveolar epithelium has been reported (Chilosi et al., 2003; Königshoff et al., 2009). Currently, knowledge about the contribution of noncanonical signaling in IPF is limited; however, increased WNT-5A expression has been detected in IPF fibroblasts (Vuga et al., 2009). This raises the question, why WNT-5A is not able to achieve the herein described inhibition in canonical WNT- β -catenin signaling in the IPF lung. Potentially several factors, such as disease-specific genetic susceptibility, interplay between various other growth factors, and alterations in the extracellular ma-

of mice receiving elastase and either α WNT-5A or IgG antibodies ($n = 9$ – 10 animals per group). (F) Expression of *Axin2*, *Nkd1*, and *Nkx2.1* in whole-lung homogenate of mice with elastase-induced emphysema and either IgG or α WNT-5A treatment ($n = 13$ – 15 animals per group). *, $P < 0.05$; ***, $P < 0.001$, unpaired Student's *t* test. (G) Correlation between *Axin2* and *Nkx2.1* expression in whole lung homogenate of mice exposed to elastase and treated with either IgG or α WNT-5A ($n = 9$ – 10 animals per group). Linear regression analysis, $r = 0.44$ and $P = 0.0575$. (H) Immunoblots and quantification of NKX2.1 in whole-lung homogenate of mice receiving elastase and either α WNT-5A or IgG antibodies ($n = 9$ animals per group). $P = 0.075$, unpaired Student's *t* test. (I) Representative immunofluorescent images of NKX2.1 (red), pro-SPC (green), and nuclei (DAPI; blue) in lung tissue of mice exposed to elastase and treated with either IgG (left) or α WNT-5A (right). Bars, 20 μ m.

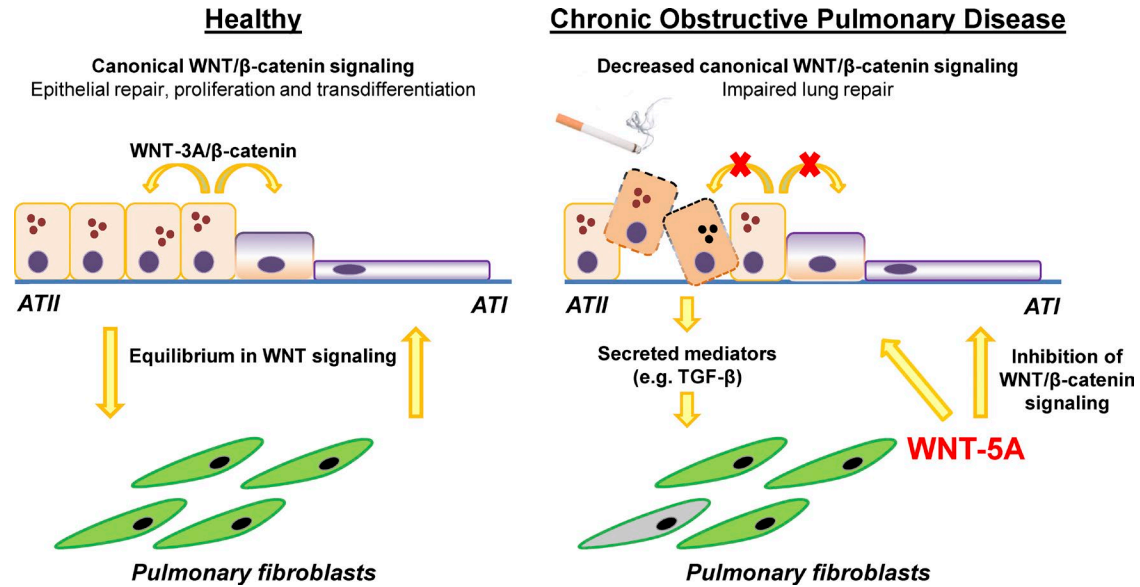


Figure 9. **Schematic representation showing the proposed mechanism of how WNT-5A contributes to COPD pathogenesis.** Healthy lung: canonical WNT- β -catenin signaling contributes to homeostasis of the alveolar epithelium by regulating cell proliferation, migration, and transdifferentiation. Diseased lung: CS-induced damage of the alveolar epithelium causing release of mediators (e.g., TGF- β) which act on pulmonary fibroblasts. Pulmonary fibroblasts produce noncanonical WNT-5A, which is enhanced by senescence (indicated in gray), TGF- β , and/or soluble components of CS. Noncanonical WNT-5A attenuates canonical WNT- β -catenin signaling in ATII cells, thereby disrupting alveolar homeostasis and resulting in impaired endogenous lung repair and emphysema.

trix, determine the outcome of WNT-5A signaling in the lung. Further studies addressing this question will provide important additional insight needed to fully understand disease-specific WNT signaling.

Collectively, our data demonstrate increased expression of mature noncanonical WNT-5A in experimental and human COPD. Functionally, WNT-5A impairs alveolar epithelial cell repair by attenuating canonical WNT- β -catenin signaling in vitro and in vivo. We thus identify a novel essential mechanism involved in impaired lung repair and COPD pathogenesis, which is amenable to therapy.

MATERIALS AND METHODS

Cell culture

Murine lung epithelial cells (MLE12; ATCC CRL-2110) were cultured in RPMI medium supplemented with 10% (vol/vol) FCS, 100 mg/liter streptomycin, and 100 U/ml penicillin. Human alveolar epithelial cells (A549; CCL-185; ATCC) were cultured in Dulbecco's Modified Eagle's medium/nutrient mixture F12 medium (DMEM/F12) supplemented with 10% (vol/vol) FCS and antibiotics. MRC5 human lung fibroblasts (ATCC CCL-171) were cultured in Ham's F12 medium to which 10% (vol/vol) FCS, antibiotics, and 2 mM L-glutamine were added. Before cell stimulation, cells were synchronized by culturing them for 24 h in corresponding plain medium supplemented with 0.1% (vol/vol) FCS and antibiotics. In case of MRC5 fibroblasts, HAM's F12 medium was additionally supplemented with 2 mM L-glutamine. Cell

stimulations were performed with fresh medium with identical composition as medium for cell synchronization.

phLFs

phLFs were isolated from lung tissue biopsies from the University Hospital Grosshadern of the LMU (Ludwig Maximilians University, Munich, Germany). Specimens from lung lobes or segmental lung resections were dissected into 1–2-mm² pieces and digested with 5 mg of collagenase I at 37°C for 2 h. Subsequently, digested samples were filtered through nylon filters with a pore size of 70 μ m. Filtrates, containing the lung fibroblasts, were centrifuged at 400 g at 4°C for 5 min. Cell pellets were resuspended and subsequently cultured in DMEM/F-12 medium supplemented with 20% (vol/vol) FCS and antibiotics. Before cell stimulation, cells were synchronized by culturing them for 24 h in DMEM/F12 medium supplemented with 0.1–0.5% (vol/vol) FCS and antibiotics. For cell stimulation, fresh medium with identical composition as medium for cell synchronization was used. Fibroblasts from COPD and smoker controls were isolated and cultured until they achieved replicative senescence, as previously described (Dagouassat et al., 2013), from lung tissue of patients undergoing lung surgery.

RNA sequencing of phLFs

Total RNA of phLFs derived from COPD explants or donor material (*n* of 4 per group) was sequenced using the Illumina system (HiSeq 2000). Sample preparation and sequencing was performed by GATC and Eurofins Genomic. Raw

data were analyzed by Genomatrix. Expression analysis was based on reads uniquely aligned to the human genome. Gene expression values were calculated for all loci available and provided as RPKM (reads per kilobase million per mapped reads) values. Genes differentially expressed between donor and COPD were determined by using edgeR 3.6.2 and defined by a minimum \log_2 fold change of ± 1 and a significance level of the adjusted p -value of 0.05 (Benjamini-Hochberg multiple testing correction).

Quantitative (q)RT-PCR

Total RNA was extracted using the QIAGEN RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. cDNA of all samples was generated by reverse transcription using SuperScriptII (Life Technologies). qRT-PCR was performed using SYBR Green and the LightCycler 480 System (both from Roche). Hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used as a reference gene in all qRT-PCR reactions. Relative transcript levels are expressed in ΔCt values ($\Delta Ct = Ct^{\text{reference}} - Ct^{\text{target}}$) or fold change ($2^{\Delta\Delta Ct}$). Primer sequences are presented in Table S1.

Canonical WNT- β -catenin activity assay

Transcriptional activity of canonical WNT signaling was determined with the M50 Super 8x TOPflash and M51 Super 8x FOPflash vectors, which contain a firefly luciferase gene under the control of 7 TCF/LEF binding sites (TOPflash) or mutated TCF/LEF binding sites (FOPflash). A549 cells were plated in 48-well plates at a density of 55×10^3 per well. The next day, cells were transfected with either 500 ng/well of M50 Super 8x TOPflash plasmid or the negative control M51 Super 8x FOPflash using Lipofectamine LTX with PLUS reagent (Life Technologies) in serum-free Opti-MEM medium (Life Technologies). After 6 h of transfection cells were stimulated with WNT ligands in the absence or presence of pharmacological inhibitors. Stimulations were performed in DMEM/F12 (A549) medium supplemented with 0.1% (vol/vol) FCS. Transcriptional activity of canonical WNT signaling in fibroblasts was analyzed in the Leading Light WNT reporter cell line (ENZ-61002-0001; Enzo Life Sciences). For each independent experiment, stimulations were performed in duplicates or more repetitions. After 24 h of stimulation the cells were lysed using Glo lysis buffer and luciferase activity was assayed using the Bright-Glo luciferase assay system (Promega). TOPflash activity was normalized to FOPflash activity and expressed relative to control conditions.

WNT-5A siRNA transfection

MRC5 fibroblasts (ATCC CCL-171) were grown to $\sim 90\%$ confluence in six-well cluster plates and transiently transfected with specific double-stranded siRNA targeted against the WNT-5A transcript (SC-41112; Santa Cruz Biotechnology, Inc.). Cells were transfected in serum-free Opti-MEM medium (Life Technologies) without any additional supplements using 200 pmol of siRNA in combination with Li-

pofectamine 2000 transfection reagent (Life Technologies). Control transfections were performed using 200 pmol nonsilencing control siRNA (SC-37007; Santa Cruz Biotechnology, Inc.). After 6 h of transfection, the medium was changed to Ham's F12 medium supplemented with 10% (vol/vol) FCS, 2 mM L-glutamine, 100 mg/l streptomycin, and 100 U/ml penicillin for 16 h. Cells were subsequently stimulated for the indicated time points with TGF- β (2 ng/ml) in Ham's F12 medium supplemented with 0.1% FCS, L-glutamine, and antibiotics. Supernatants were collected and cells were lysed using ice-cold radioimmunoprecipitation assay buffer (RIPA, pH 7.4; 20 mM Tris HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2.5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1% $\text{C}_{24}\text{H}_{39}\text{NaO}_4$, and 1% NP-40) supplemented with protease and phosphatase inhibitors (Roche). Supernatants and cell lysates were stored at -20°C until further use.

Preparation of WNT-CM

Mouse fibroblasts-like L-cells stably expressing WNT-3A or WNT-5A were used to obtain WNT-CM. Parental L-cells (control: ATCC CRL-2648), L-WNT-3A cells (ATCC CRL-2647), or L-WNT-5A cells (ATCC CRL-2814) were cultured in DMEM/F12 medium supplemented with 10% (vol/vol) FCS, 100 mg/l streptomycin, and 100 U/ml penicillin. WNT CM or control CM was prepared according to the ATCC guidelines. In short, confluent L-cell cultures were split 1:10 and cultured for 4 d in supplemented DMEM/F12 medium in 10-cm culture dishes. After 4 d, the medium was collected and the cells were cultured for another 3 d in fresh DMEM/F12 medium with supplements. The second batch of medium was collected after 3 d and mixed with medium of day 4 (ratio 1:1). The combined medium, which is referred to as CM, was filtered and stored at -20°C till further use. For cell stimulation, the conditioned media were prewarmed to 37°C and diluted in DMEM/F12 medium with 0.1% (vol/vol) FCS and antibiotics.

Deglycosylation of WNT-5A

Deglycosylated WNT-5A was generated by incubating WNT-5A CM with Peptide-N-Glycosidase F (PNGase F). This enzyme is a native glycoaminidase cleaving the link between asparagine and N-acetylglucosamines, thereby reversing N-glycosylation of proteins. Freshly prepared WNT-5A CM (500 μl) was incubated for 2 h at 37°C in the absence or presence of 1 μl PNGase F (New England Biolabs). Control CM was incubated at identical conditions. The conditioned media were directly used to determine their effect on β -catenin/TCF-dependent gene transcription. In short, A549 cells transfected with either M50 Super 8x TOPflash plasmid or the negative control M51 Super 8x FOPflash were stimulated with 100 μl /well of control CM, WNT-5A CM, or PNGase F-treated conditioned media added to 300 μl /well DMEM/F12 medium without or with recombinant WNT-3A (final volume, 400 μl /well; final concentration WNT3A, 100 ng/ml). For each independent experiment, 3 distinct batches of

WNT-5A CM without or with PNGase F were prepared, and stimulations were performed in duplicates. After 24 h of stimulation, the cells were lysed using Glo lysis buffer and luciferase activity was assayed using the Bright-Glo luciferase assay system (Promega). The degree of PNGase F–induced deglycosylation of WNT-5A was determined by immunoblotting. To examine the relevance of glycosylation in WNT-5A secretion, confluent pHLFs were stimulated without or with TGF β (2 ng/ml) in the absence or presence of tunicamycin (5 μ M) for 48 h. Tunicamycin prevents glycosylation of newly synthesized glycoproteins by inhibiting the formation of *N*-acetylglucosamine intermediates. After 48 h of stimulation the supernatants were collected and the cells were lysed in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors (Roche). Supernatants and cell lysates were stored at -20°C till further use.

Supernatant protein concentration

Supernatants were concentrated at a factor of 10 using Amicon Ultra-0.5 centrifugal filter devices according to the manufacturer's instructions (Merck). In short, 500 μ l of cell-free supernatant was loaded onto an Ultra 3K filter device and centrifuged at 14,000 *g* for 30 min. To recover the concentrated solute, the filter was placed upside down in a collection tube and centrifuged at 1,000 *g* for 2 min. The obtained ultrafiltrate (50 μ l) was stored at -20°C till further use.

Animals

Pathogen-free female C57BL/6 mice (8–10 wk old) were obtained from Charles River and housed in rooms maintained at constant temperature and humidity with a 12-h light cycle. Animals were allowed food and water ad libitum. In the model of CS-induced COPD, smoke was generated from 3R4F Research Cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY). Mice were exposed to mainstream smoke as described previously (John-Schuster et al., 2014). In the intervention study, mice received every other day an i.p. injection with PBS (vehicle; 100 μ l) or *t*-Boc-NH-Met-Asp-Gly-Cys-Glu-Leu-CO $_2$ H (BOX5: 40 μ g/mouse/injection) dissolved in PBS at least 1 h before exposure to CS. For the lung-specific WNT-5A overexpression experiments, hemizygous SFTPC-rtTA mice (The Jackson Laboratory) were interbred with TetO-WNT-5A mice (provided by R. Smits, Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam, the Netherlands; Bakker et al., 2012) obtaining double transgenic mice. Overexpression of WNT-5A was initiated 1 wk before the start of the experiment by supplying either DOX (2 mg/ml in 5% sucrose) or 5% sucrose solution in the drinking water, which was refreshed every other day till the end of the experiment. Emphysema was induced by dissolving pancreatic porcine elastase (40 or 80 U/kg body weight) in sterile phosphate-buffered saline, which was subsequently applied orotracheally. Control mice received 80 μ l sterile PBS. In the intervention experiments, the mice were orotracheally

treated with either α WNT-5A antibodies (R&D Systems; MAB645 and AF645) or respective control IgG antibodies (R&D Systems; MAB006 and AB108) dissolved in PBS (15 μ g/mouse/instillation) at the indicated time points.

Lung function measurement

Lung function measurement was performed as previously described (John-Schuster et al., 2014). Mice were anaesthetized with ketamine/xylazine and tracheostomized, and their pulmonary function was analyzed using the flexiVent system (Scireq). To obtain a mean lung volume similar to that of spontaneous breathing, mice were ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min. Lung mechanical properties were tested using the SnapShot and Primewave perturbations. Four readings per animal were taken.

Lung tissue processing and quantitative morphometry

The right lung lobe was shock-frozen in liquid nitrogen for protein and RNA isolation and the left lung lobe was fixed at a constant pressure (20 cm fluid column) by intratracheal instillation of PBS-buffered 6% PFA and embedded into paraffin for histological analysis of hematoxylin-eosin (H&E)-stained slides. Design-based stereology was used to analyze sections via an Olympus BX51 light microscope equipped with a computer-assisted stereological toolbox (newCAST; Visiopharm) on H&E-stained lung tissue slides, as previously described (John-Schuster et al., 2014). Air space enlargement was assessed by quantifying mean linear chord length (MLI) on 30 fields of view per lung.

Human lung tissues

Lung tissue was obtained from 20 COPD patients classified as GOLD IV undergoing lung transplant due to their underlying COPD (7 females, 13 males; mean age = 56 ± 5 yr) and 16 control subjects (transplant donors, 7 females and 9 males; mean age, 50 ± 11 yr). COPD samples were taken from the parenchyma with histological validation of emphysematous changes. Human 3D lung tissue cultures (3D-LTCs) were obtained and maintained as described previously (Uhl et al., 2015).

Immunoblotting

Cells or pulverized lung homogenates were lysed with radioimmunoprecipitation assay buffer (RIPA) supplemented with protease and phosphatase inhibitors (Roche). Protein concentrations were determined using BCA assay (Thermo Fisher Scientific). To each protein sample 4 \times Laemmli loading buffer (composition: 150 mM Tris HCl, 275 mM SDS, 400 mM dithiothreitol, 3.5% [wt/vol] glycerol, and 0.02% bromophenol blue) was added with the exception of samples used for the detection of WNT-5A to which nonreducing 4 \times Laemmli loading buffer (identical composition as 4 \times Laemmli loading buffer, but without reducing agent dithiothreitol) was added. A similar protocol was used for analysis of proteins in cell supernatants. To 20–30 μ l of ultrafiltrate, 10 μ l

of either nonreducing 4× Laemmli loading buffer (detection of WNT-5A) or reducing 4× Laemmli loading buffer was added. Equal amounts of protein (10–20 µg/lane) were subjected to electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes and analyzed for the proteins of interest using specific primary and HRP-conjugated secondary antibodies. By using enhanced chemiluminescence reagents (Thermo Fisher Scientific) bands were recorded with a ChemiDoc XRS+ system equipped with Image Lab 5.0 software (Bio-Rad Laboratories).

Scratch assay

Murine lung epithelial cells (MLE12; CRL-2110; ATCC) were grown in 24-well plates with RPMI medium supplemented with 10% (vol/vol) FCS and antibiotics until confluence. Cells were synchronized for 24 h with RPMI medium containing 0.1% FCS and antibiotics. Confluent monolayers of MLE12 cells were wounded by scraping a pipette tip across the monolayer. Cells were once washed with warm PBS (composition: 140.0 mM NaCl, 2.6 mM KCl, 1.4 mM KH₂PO₄, and 8.1 mM Na₂HPO₄·2H₂O, pH 7.4) and stimulated with vehicle control (0.1% BSA in PBS), WNT-3A (100 ng/ml), WNT-5A (100 ng/ml), or the combination of WNT-3A with WNT-5A (both 100 ng/ml). Cells were stimulated in quadruplicates for each independent experiment. Images of wounds were captured using a canon EOS 1000D DSLR mounted on an Axiovert 40C microscope with a 5× objective (Zeiss). Images were obtained at initial time of wounding and 48 h after wounding. The size of the wound was analyzed (three measurements per image) using ImageJ software and data are expressed relative to wound size of vehicle control after 48 h.

Normalization of microarray data

Raw .cel files from a previously published study were downloaded from GEO under accession no. GSE22148 (Singh et al., 2011), processed using R Software, and normalized using Robust Multi-Array Average expression measure (RMA) in the affy package from BioConductor. The mean of the WNT-5A probe set was calculated for each patient and grouped according to GOLD status using available clinical data. Deposited normalized microarray data from the Lung Genome Research Consortium for whole-lung tissue homogenates from normal and COPD cohort sets were used (GSE47460–GPL14550).

Alveolar epithelial cell isolation and culture

Primary ATII cells were isolated from mice, as previously described (Königshoff et al., 2009). In brief, lungs were lavaged twice with sterile PBS and tissues digested with dispase and minced. The suspension was sequentially filtered through 100-, 20-, and 10-µm nylon meshes and centrifuged at 200 g for 10 min. The pellet was resuspended in DMEM, and negative selection for lymphocytes/macrophages was performed by incubation on CD16/32- and CD45-coated Petri dishes

for 30 min at 37°C. Cell purity and viability were analyzed in freshly isolated ATII cells directly after isolation. Cell purity was routinely assessed by epithelial cell morphology and immunofluorescence analysis with panCK and SPC (both positive), as well as α-SMA and CD45 (both negative) of cytocentrifuge preparations of ATII cells.

Immunofluorescence staining

Primary mouse ATII cells were fixed with acetone/methanol (1:1), and blocked with 5% (w/vol) BSA (Sigma-Aldrich) for 30 min. Cells were subsequently incubated with the respective primary antibody at room temperature for 1 h in PBS containing 0.1% (w/vol) BSA, followed by incubation with the fluorescently labeled secondary antibody (goat anti-rabbit Alexa Fluor 555; Life Technologies). DAPI staining (Roche) was used to visualize cell nuclei. For staining of tissue sections, murine lung tissue was fixed in 4% formalin before paraffin embedding. Sections (4-µm) were prepared and mounted on slides, followed by deparaffinization and immunofluorescent staining according to a standard protocol. For deparaffinization, the paraffin-embedded sections were placed at 60°C for at least 30 min, incubated twice in xylene, and then consecutively transferred into 100% ethanol, 90% ethanol, 80% ethanol, and 70% ethanol at room temperature. For antigen retrieval, slides were immersed in citrate buffer, pH 6.0, and heated in a decloaking chamber (Biocare Medical) for 30 s at 125°C, followed by 10 s at 90°C. Primary antibody dilutions were prepared in antibody diluent (Zytomed Systems), added to each tissue section in a wet chamber and incubated overnight. Primary antibodies used were mouse monoclonal anti-TTF-1 (NKX2.1; dilution 1:50; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-prosurfactant protein C (Pro-SPC; dilution 1:200; Merck), polyclonal goat anti-podoplanin (T1α; dilution 1:500, R&D Systems), rabbit polyclonal anti-tight junction protein 1 (TJP1 or ZO-1; 1:50; Invitrogen), mouse monoclonal anti-β-catenin (1:200; BD), and mouse monoclonal anti-ABC (ABC clone 8E7; Merck). Secondary antibodies were Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 555 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG H+L, Alexa Fluor 555 goat anti-rabbit IgG (all Life Technologies; dilution 1:400), or FITC polyclonal goat anti-mouse (Dako). Nuclei were stained with DAPI (1:2,500; Sigma-Aldrich). The slides covered with Fluorescence Mounting Medium (Dako) and examined under an Axio Imager Microscope (Zeiss).

Antibodies and reagents

The rat anti-WNT-5A (MAB645) antibody, recombinant WNT-3A (mouse; 1324, human; 5036), recombinant WNT-5A (mouse/human: 645), and recombinant TGF-β (human; 240) were obtained from R&D Systems. The mouse anti-BSA (SC57504) antibody was obtained from Santa Cruz Biotechnology, Inc. The horseradish peroxidase (HRP)-conjugated sheep anti-mouse antibody (NA931V), HRP-conjugated donkey anti-rabbit antibody (NA934V), and

HRP-conjugated goat anti-rat antibody (NA935V) were purchased from GE Healthcare. Mouse anti-total β -catenin antibody was purchased from BD. Mouse anti-nonphosphorylated- β -catenin antibody (clone 8E7) was obtained from Merck. All other chemicals were of analytical grade.

Study approval

Human lung tissue. Lung tissue was obtained from 20 COPD patients classified as GOLD IV undergoing lung transplant as a result of their underlying COPD and 16 control subjects (donor). The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine and the Ludwig Maximilians University (LMU; #330-10). Informed consent was obtained in written form from each subject for the study protocol.

phLF isolation. phLFs were isolated from lung tissue biopsies from the University Hospital Grosshadern of the LMU (Ludwig Maximilian University of Munich). Participants provided written informed consent to participate in this study, in accordance with approval by the local ethics committee of the LMU (Project 333-10, 455-12).

Animal studies. All animal experiments were conducted under strict governmental and international guidelines and were approved by the local government for the administrative region of Upper Bavaria (Project 55.2-1-54-2532-129-14).

Statistical analysis

Data represent means \pm SEM, from n separate experiments. Statistical significance of differences was evaluated by either a Student's t test, Student's t test with Welch correction, or one-way ANOVA followed by a Newman-Keuls multiple comparison test or followed by a Dunnett's multiple comparison test where appropriate. Differences were considered to be statistically significant when $P < 0.05$.

Online supplement material

Fig. S1 shows that oligomerized WNT-5A can be detected in murine and human samples using human/mouse WNT-5A antibody MAB645. Table S1 lists primer sequences for determination of gene expression in human and murine lung tissue.

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