ORIGINAL ARTICLE

# A pro-inflammatory role for the Frizzled-8 receptor in chronic bronchitis

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## ABSTRACT

**Rationale** We have previously shown increased expression of the Frizzled-8 receptor of the Wingless/integrase-1 (WNT) signalling pathway in COPD. Here, we investigated if the Frizzled-8 receptor has a functional role in airway inflammation associated with chronic bronchitis.

**Methods** Acute cigarette-smoke-induced airway inflammation was studied in wild-type and Frizzled-8-deficient mice. Genetic association studies and lung expression quantitative trait loci (eQTL) analyses for Frizzled-8 were performed to evaluate polymorphisms in *FZD8* and their relationship to tissue expression in chronic bronchitis. Primary human lung fibroblasts and primary human airway epithelial cells were used for in vitro studies.

**Results** Cigarette-smoke-exposure induced airway inflammation in wild-type mice, which was prevented in Frizzled-8-deficient mice, suggesting a crucial role for Frizzled-8 in airway inflammation. Furthermore, we found a significant genetic association (p=0.009) between single nucleotide polymorphism (SNP) rs663700 in the *FZD8* region and chronic mucus hypersecretion, a characteristic of chronic bronchitis, in a large cohort of smoking individuals. We found SNP rs663700 to be a *cis*-eQTL regulating Frizzled-8 expression in lung tissue. Functional data link mesenchymal Frizzled-8 expression to inflammation as its expression in COPD-derived lung fibroblasts was regulated by pro-inflammatory cytokines in a genotype-dependent manner. Moreover, Frizzled-8 regulates inflammatory cytokine secretion from human lung fibroblasts, which in turn promoted MUC5AC expression by differentiated human airway epithelium.

**Conclusions** These findings indicate an important pro-inflammatory role for Frizzled-8 and suggest that its expression is related to chronic bronchitis. Furthermore, our findings indicate an unexpected role for fibroblasts in regulating airway inflammation in COPD.

### Key messages

**What is the key question?**

- What is the functional role for the Frizzled-8 receptor in airway inflammation in COPD?

**What is the bottom line?**

- We show that the Frizzled-8 receptor is associated with chronic bronchitis and is involved in cytokine secretion from human pulmonary fibroblasts as well as acute cigarette-smoke-induced inflammation in a mouse model.

**Why read on?**

- Our findings concerning the pro-inflammatory role for Frizzled-8 in airway inflammation in COPD provide a rationale for further exploration of the therapeutic potential of the Frizzled-8 receptor in COPD.

## INTRODUCTION

COPD is a complex lung disease, characterised by airflow limitation that is not fully reversible. Airflow limitation is usually progressive and associated with chronic inflammation, remodelling of the small airways and emphysema development in some patients.[1 2]

The heterogeneity of COPD is represented by different phenotypes within the patient population, chronic bronchitis being one of them. Chronic bronchitis is defined by chronic cough and idiopathic sputum production for at least 3 months per year for two consecutive years.[1] Inflammatory signals can cause mucus cell metaplasia, resulting in more mucus by both an increased production of mucus by goblet cells and decreased elimination from the airways.[3] This is defined in patients as chronic mucus hypersecretion (CMH). CMH is associated with bronchial inflammation, accelerated lung function decline,[4] increased mortality[5] and is a risk factor for COPD.[6]

COPD treatment is neither curative nor directed at different COPD phenotypes, including chronic bronchitis. At present, the pathophysiology and underlying mechanisms of chronic bronchitis are poorly understood. This hampers the development of targeted drugs. Therefore, a better understanding of the mechanisms behind chronic bronchitis is needed.

The Wingless/integrase-1 (WNT) signalling pathway plays an important role in lung development.[7] Recent studies indicate involvement of the WNT signalling pathway in remodelling[8 9] and inflammation[10 11] in the lung. WNT ligands bind

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to transmembrane Frizzled (FZD) receptors, thereby controlling cell differentiation, growth and polarity.[12] Our previous results showed that FZD8 expression is higher in pulmonary fibroblasts of patients with moderate (GOLD stage II) and severe (GOLD stage IV) COPD after stimulation with transforming growth factor (TGF)-β compared with control fibroblasts.[8] However, the functional role for FZD8 in COPD is as yet unknown. WNT-3A,[10] WNT-4[13] and WNT-5A[14] have recently been shown to function as pro-inflammatory stimuli in the lung. Importantly, we previously showed that WNT-5A is a ligand for FZD8 in airway smooth muscle cells,[9] reinforcing the possibility that the increased FZD8 expression in COPD fibroblasts may play a role in pro-inflammatory signalling in the lung.

Here, we aimed to investigate the link between FZD8 and airway inflammation in COPD. We studied the role of FZD8 in acute cigarette-smoke-induced airway inflammation in vivo using a mouse model and in vitro in human lung fibroblasts obtained from patients with COPD with and without CMH. Furthermore, we investigated the association between single nucleotide polymorphisms (SNPs) in the *FZD8* region and CMH, and their roles as *cis*-expression quantitative trait loci (eQTL), regulating FZD8 expression in lung tissue.

## METHODS

A more extensive description of methods and statistics can be found in the online supplementary file.

### Ethics statement

The study protocol followed national ethical and professional guidelines ('Code of conduct; Dutch federation of biomedical scientific societies'; http://www.federa.org) for all lung tissue and explant cell culture studies in Groningen. For the genetics study on CMH, approval by the local medical ethics committee and written informed consent from all patients were obtained (see online supplementary file). All animal experiments were performed according to the national guidelines and approved by the University of Groningen Animal Ethical Committee (committee approval number 5912B).

### Animal studies

Heterozygous, inbred, specified-pathogen-free breeding colonies FZD8$^{+/-}$ mice (C57BL/6;129P2-*FZD8*$^{tm1Dgen}$/J), showing no obvious phenotype, were obtained from The Jackson Laboratory (USA). After breeding, homozygous male FZD8$^{-/-}$ mice and wild-type$^{+/+}$ (WT) littermates were used for experiments as described in detail in the online supplementary file.

**Table 1** Patient characteristics, patients with GOLD stage IV COPD

| | GOLD stage IV COPD without CMH | GOLD stage IV COPD with CMH |
|---|---|---|
| Number of subjects | 8 | 8 |
| Age (range) | 58 (57–62) | 54 (48–61) |
| Sex | | |
| Male | 3 | 4 |
| Female | 5 | 4 |
| Pack-years (range) | 44.9 (30–72) | 31.3 (15–40) |
| Smoking status | Ex-smoker | Ex-smoker |
| % predicted FEV1 (range) | 19.2 (12.23–25.69) | 14.6 (13.12–20.12) |
| FEV1/FVC (range) | 0.30 (0.19–0.66) | 0.23 (0.16–0.29) |
| Bronchiectasis | None | None |

Age, pack-years, predicted FEV1 and FEV1/FVC are represented as mean (range).
CMH, chronic mucus hypersecretion.

### Genetic association

Genetic association studies for *FZD8* and CMH were performed in the NELSON cohort; a large cohort of smoking individuals with and without COPD which was originally set up to detect lung cancer.[15] After quality control, 717 heavy smokers with CMH (cases) and 1795 heavy smokers without CMH (controls) all with ≥20 pack-years were included for the analysis as previously published.[16] Six SNPs were tested using multivariate logistic regression analysis under an additive model, with adjustment for ex-smokers or current smokers and the two population sites (Groningen and Utrecht).

### eQTL analysis in lung tissue

The lung eQTL analysis was performed in a large dataset of lung tissue samples where both genotype and genome-wide gene expression data are available through a collaboration of three universities (Laval University, Quebec City, Canada; University of British Columbia, Vancouver, Canada; University of Groningen, Groningen, the Netherlands), and Rosetta/Merck Sharpe and Dohme as has been described previously, including patient characteristics.[17] After quality control, 1095 patients out of 1111 were included in the analysis. A *cis*-eQTL analysis was performed to determine the association between SNP rs663700 and the regulation of gene expression of genes within 100 kb.[17] In short, the association between genotype and expression of *cis*-eQTL genes was analysed using linear regression on imputed data, first in the three cohorts separately, followed by a meta-analysis.[18 19]

### Fibroblast and epithelial cell culture

Human airway and parenchymal lung fibroblasts from ex-smoking patients with GOLD stage IV COPD with and without CMH were isolated from transplanted lungs as has been described previously.[20] Presence of CMH was defined by patient records. Patient characteristics are shown in table 1 and showed no differences in age, gender, smoking status, lung function or medication use. At the same time, we determined that none of the patients used for the study in fibroblasts was diagnosed with bronchiectasis, excluding the possible influence on the data. MRC-5 human lung fibroblasts[21] were obtained from Sigma (St Louis, Missouri, USA). Specific small interfering (si) RNA was used to knock down FZD8. Human airway epithelial cells were isolated from donor lungs. Cells were cultured and stimulated to measure gene expression using mRNA isolation and real-time PCR analysis, to perform immunohistochemistry for goblet cell number or to measure cytokine release using ELISA and Milliplex as described in detail in the online supplementary file.

### Statistical analysis

Statistical analysis of in vitro and in vivo experiments was performed with the SigmaPlot software (Systat Software, San Jose, California, USA). All real-time PCR data were log-transformed before analysis. For comparison between two conditions, the Student's t test ($\alpha$=0.05, tested two-tailed) was used. Differences in FZD8 gene expression in fibroblasts between two genotypes were tested using a one-tailed Mann–Whitney test, based on the results of the eQTL analysis. To test differences between different patient groups, one-way analysis of variance (ANOVA) on ranks was used. For comparison between multiple conditions within the primary MRC-5 human lung fibroblast

experiments and within the different groups of mice, two-way ANOVA was used. Tests used for the analyses of the genetic studies are described in the appropriate sections. Where appropriate, ANOVA was followed by a post hoc analysis using the Student–Newman–Keuls multiple comparisons test. $p<0.05$ was considered significant.

## RESULTS

### FZD8 regulates cigarette-smoke-induced airway inflammation in mice

Inflammation in COPD follows a characteristic pattern of increased numbers of macrophages, lymphocytes and neutrophils.[2] To investigate a possible role for FZD8 in inflammation, we used $FZD8^{-/-}$ mice and studied acute cigarette-smoke-induced airway inflammation, as described previously.[22] $FZD8^{-/-}$ mice were born according to normal Mendelian ratio and survived after birth with no differences compared with WT mice. Baseline lung structure was not different for the $FZD8^{-/-}$ mice and WT mice in terms of gross morphological structure and expression of α-sm-actin around the airways and vessels (see online supplementary figures S1A–C). Baseline presence of inflammatory cells and pro-inflammatory cytokines was slightly higher in $FZD8^{-/-}$ mice compared with WT mice (figure 1A–G) and this was significant for neutrophil number ($p=0.004$) and CXCL5 ($p=0.005$) gene expression when using a Student's t test on the air-exposed animals only.

Four days of cigarette-smoke exposure induced an increase in the number of lymphocytes and neutrophils in the bronchial alveolar lavage fluid (BALF) of WT mice (figure 1A, B). No effect of cigarette smoke was found on total cells and macrophages (figure 1C, D). Notably, cigarette smoke did not increase the number of lymphocytes and neutrophils in $FZD8^{-/-}$ mice and neutrophil number in cigarette-smoke-exposed $FZD8^{-/-}$ mice was significantly lower compared with cigarette-smoke-exposed WT mice (figure 1B). Keratinocyte-derived cytokine (KC), CXCL2 and CXCL5 are chemotactic for neutrophils, and the gene expression of all three cytokines was increased upon cigarette-smoke exposure in WT mice (figure 1E–G). KC and CXCL2 tended to be reduced in $FZD8^{-/-}$ mice, whereas for CXCL5, the cigarette-smoke-induced increase was completely absent in $FZD8^{-/-}$ mice. Gene expression of FZD8 and its putative ligands WNT-5A and WNT-5B was also measured in whole lung homogenates and was not different between groups, although small, non-significant increases in cigarette-smoke-exposed WT mice were apparent for all three genes (see online supplementary figures S1D–F). MUC5AC gene expression significantly increased after cigarette-smoke exposure in WT mice but not in $FZD8^{-/-}$ mice (see online supplementary figure S1G). However, consistent with previous studies,[22 23] we did not find any cells positive for MUC5AC protein expression in lung tissue sections before and after cigarette-smoke exposure in WT and $FZD8^{-/-}$ mice. Together, these results suggest that FZD8 is involved in acute cigarette-smoke-induced airway inflammation in mice in vivo.

### A genetic association between FZD8 and CMH

In view of this pro-inflammatory role, we next studied the role of FZD8 in chronic bronchitis and found a genetic association between SNP rs663700 in the *FZD8* region and CMH ($p=0.009$; OR=0.8059; 0.684–0.9495) in a large cohort of smoking individuals with and without COPD, implying that *FZD8* may play a role in the development of CMH (figure 2A). Notably, we found that SNP rs663700 was associated with CMH already in the healthy smokers of the cohort ($n=1348$; $p=0.002$), indicating that the SNP links to CMH specifically and is independent of the presence of COPD. The protective C allele is associated with lower risk of having CMH and the susceptibility allele T with higher risk of having CMH. The five other SNPs tested in the *FZD8* region were less or not associated with CMH (see online supplementary table S3).

Next, we performed an eQTL analysis in a large dataset of lung tissue samples of non-smokers, current smokers and ex-smokers with and without COPD[17] to determine the association between SNP rs663700 and the lung mRNA expression levels of genes within 100 kb, as has been described previously.[17] The eQTL analysis showed that SNP rs663700 has a *cis*-eQTL effect on FZD8 ($p=4.58\times10^{-11}$) and CCNY (Cyclin Y; $p=4.72\times10^{-4}$) gene expression in lung tissue ($n=1095$). The strongest eQTL effect was on FZD8 where the protective C allele is associated with lower expression and the susceptibility allele T with higher expression of FZD8 (results from the meta-analysis across the three cohorts: $p=4.94\times10^{-10}$; Spearman $\rho=0.19$; figure 2B–E). The allele frequency within the study population was 65.8% for the homozygous CC, 30.3% for the heterozygous CT and 3.9% for the homozygous TT.

### FZD8 expression in lung fibroblasts is associated with CMH

We previously established that FZD8 is expressed in fibroblasts and that its expression is increased in COPD.[8] To link these previous findings with the current observation that FZD8 regulates inflammation and is involved in chronic bronchitis, we studied the pro-inflammatory role of FZD8 in human airway and parenchymal lung fibroblasts from ex-smoking patients with GOLD stage IV COPD with and without CMH. We found that interleukin (IL)-1β and epidermal growth factor (EGF) induced a strong increase in FZD8 gene expression in lung fibroblasts. Interestingly, this normalised increase in FZD8 gene expression was stronger in primary airway and parenchymal lung fibroblasts of patients with GOLD stage IV COPD with CMH than in those without CMH (figure 3A, C). The mean basal FZD8 gene expression did not differ with respect to CMH status. No differences in gene expression with respect to CHM status were observed for the effects of IL-1β or EGF on WNT-5A, WNT-5B, WNT-16, FZD2 and FZD6 gene expression in parenchymal lung fibroblasts (see online supplementary figures S2A, B), indicating a specific effect on FZD8. Although TGF-β did augment FZD8 gene expression, there was no significant difference between the two patient groups regarding CMH status, whereas tumour necrosis factor (TNF)-α and cigarette-smoke extract did not augment FZD8 gene expression (see online supplementary figure S2C). This indicates a specific effect for IL-1β and EGF. Interestingly, receptor expression of EGFR was higher in airway fibroblasts and pulmonary fibroblasts of patients with GOLD stage IV COPD with CMH than without CMH, as well as IL1R1 expression in airway fibroblasts of patients with GOLD stage IV COPD with CMH than without CMH (figure 3B, D), suggesting a link between receptor expression and functional FZD8 induction by IL-1β and EGF.

### FZD8 gene expression in lung fibroblasts is regulated by SNP rs663700

We further analysed the genetic basis of FZD8 gene expression in airway and parenchymal lung fibroblasts of patients with GOLD stage IV COPD with and without CMH. The fold induction of FZD8 gene expression after stimulation with IL-1β and EGF was found to be dependent on the genotype of the patients with respect to SNP rs663700. In line with the results of our lung eQTL analysis, airway fibroblasts of patients with GOLD

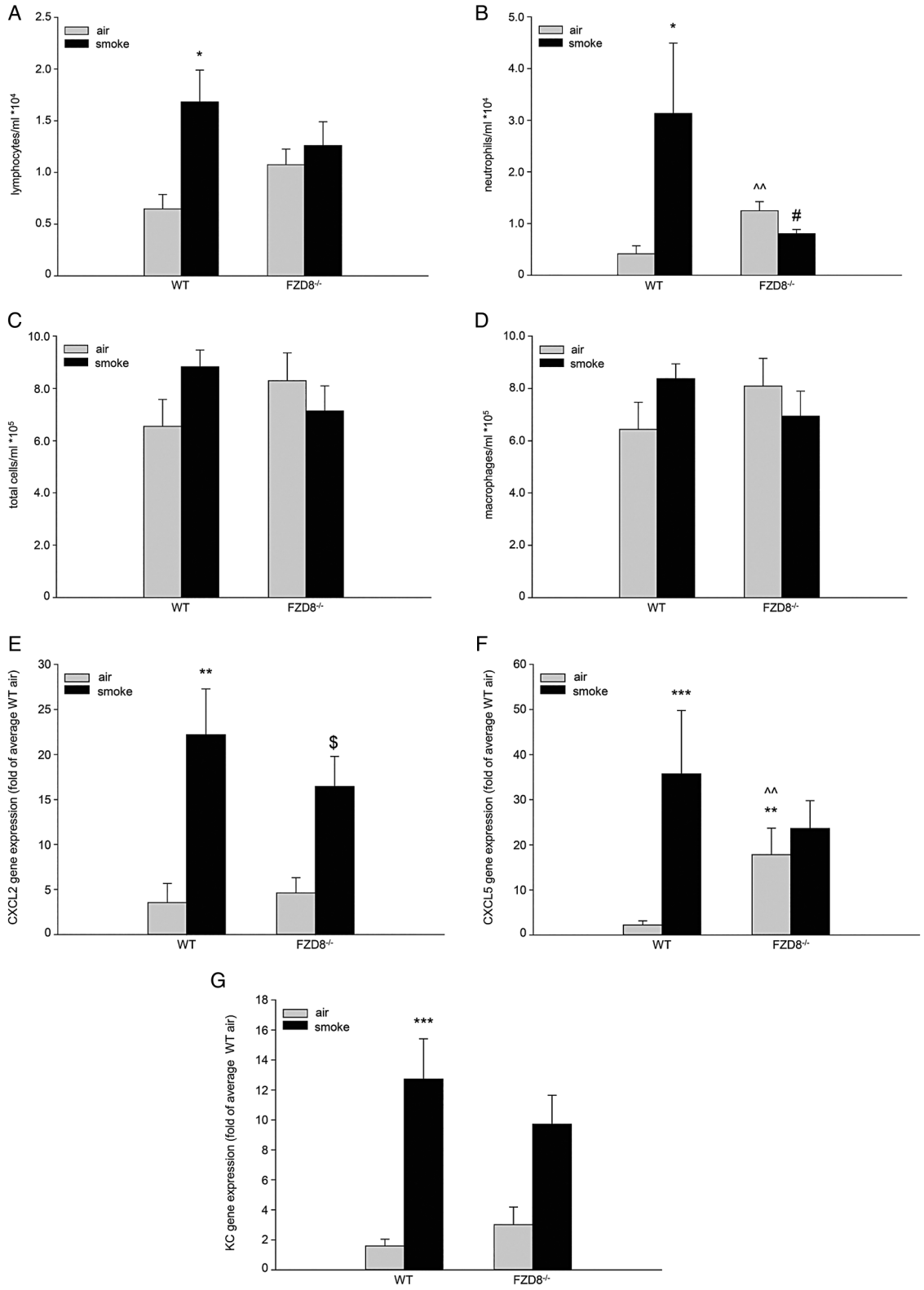


**Figure 1** Acute cigarette-smoke-induced airway inflammation is dependent on FZD8. Wild-type (WT) and FZD8$^{-/-}$ mice were exposed to cigarette smoke for 4 days. (A) Lymphocyte numbers in bronchial alveolar lavage fluid (BALF); p=0.068 for the interaction of smoke exposure and genotype (two-way ANOVA). (B) Neutrophil numbers in BALF; p=0.035 for the interaction of smoke exposure and genotype (two-way ANOVA). (C) Total cell number in BALF; p=0.079 for the interaction of smoke exposure and genotype (two-way ANOVA). (D) Macrophage numbers in BALF; p=0.105 for the interaction of smoke exposure and genotype (two-way ANOVA). (E) CXCL2 gene expression in whole lung homogenates; p=0.343 for the interaction of smoke exposure and genotype (two-way ANOVA). (F) CXCL5 gene expression in whole lung homogenates; p=0.021 for the interaction of smoke exposure and genotype (two-way ANOVA). (G) KC gene expression in whole lung homogenates. Data represent mean±SEM of eight mice per group. *p<0.05, **p<0.01, ***p<0.001 compared with air-exposed WT mice $^{\#}$p<0.05 compared with smoke-exposed WT mice $^{\$}$p<0.05 compared with air-exposed FZD8$^{-/-}$ mice (two-way ANOVA with Student–Newman–Keuls multiple comparisons test). ^^p<0.01 compared with air-exposed FZD8$^{-/-}$ mice (two-tailed Student's t test). ANOVA, analysis of variance; CXCL, chemokine ligand; KC, keratinocyte-derived cytokine; EGF, epidermal growth factor.

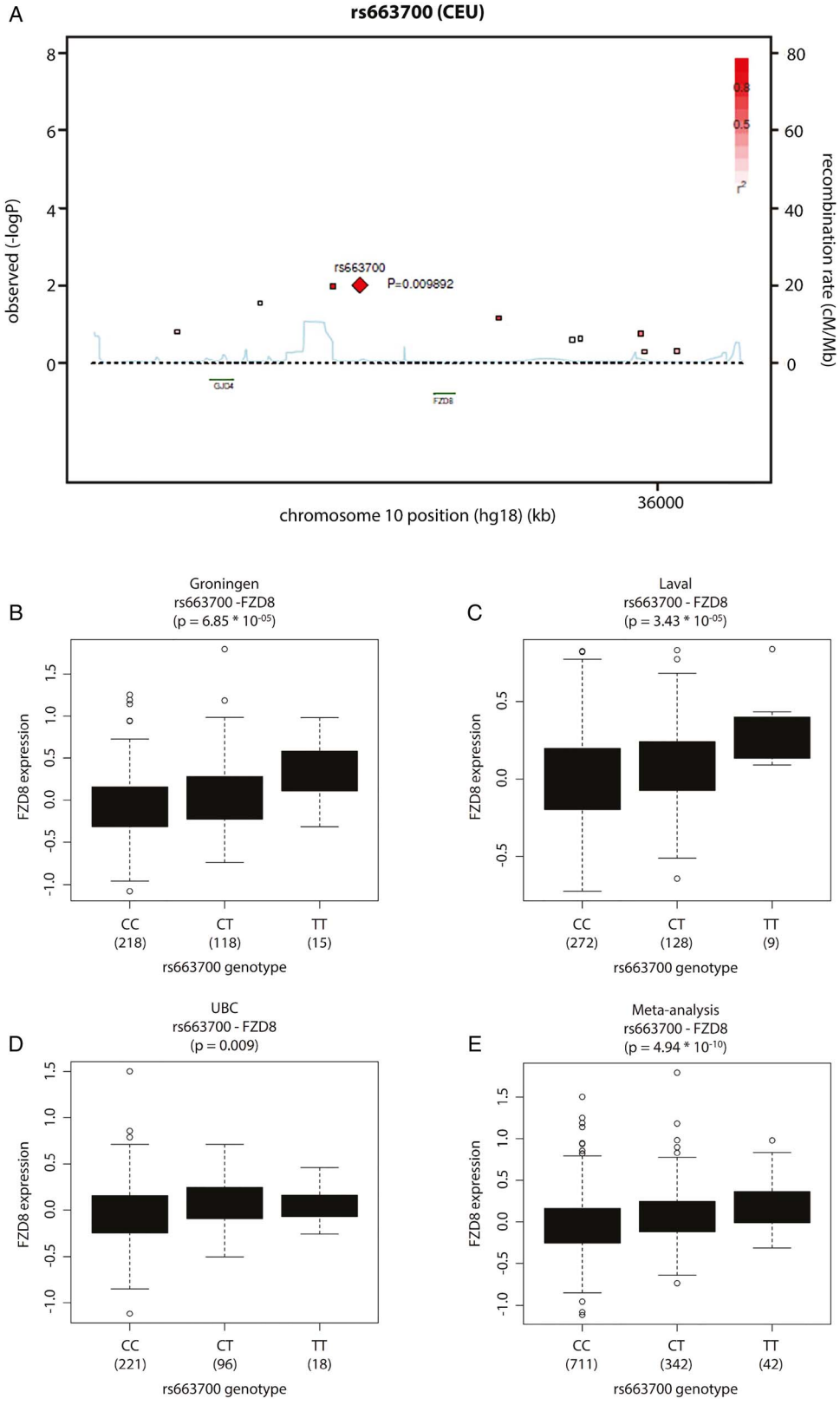


**Figure 2** Genetic association between FZD8 and chronic mucus hypersecretion (CMH). (A) Genetic association between single nucleotide polymorphism (SNP) rs663700 in the *FZD8* region and CMH within a cohort of heavy smokers with and without COPD (n=717 with CMH and n=1795 without CMH); p=0.009; OR=0.8059; 0.684–0.9495. *FZD8* and SNP rs663700 are located on chromosome 10. C is the wild-type (WT) allele and protective, and T is the variant, susceptibility allele of SNP rs663700. CEU stands for Caucasian population in the HapMap project. (B) Expression quantitative trait loci (eQTL) analysis results from the Groningen cohort (n=363). Boxplots show the relationship between gene expression and genotype; $p=6.85\times10^{-05}$. (C) eQTL analysis results from the Laval cohort (n=409). Boxplots show the relationship between gene expression and genotype; $p=3.43\times10^{-05}$. (D) eQTL analysis results from the UBC cohort (n=339). Boxplot show the relationship between gene expression and genotype UBC; p=0.009. (E) eQTL analysis results from the meta-analysis across the three cohorts. Boxplots show the relationship between gene expression and genotype; $p=4.94\times10^{-10}$.

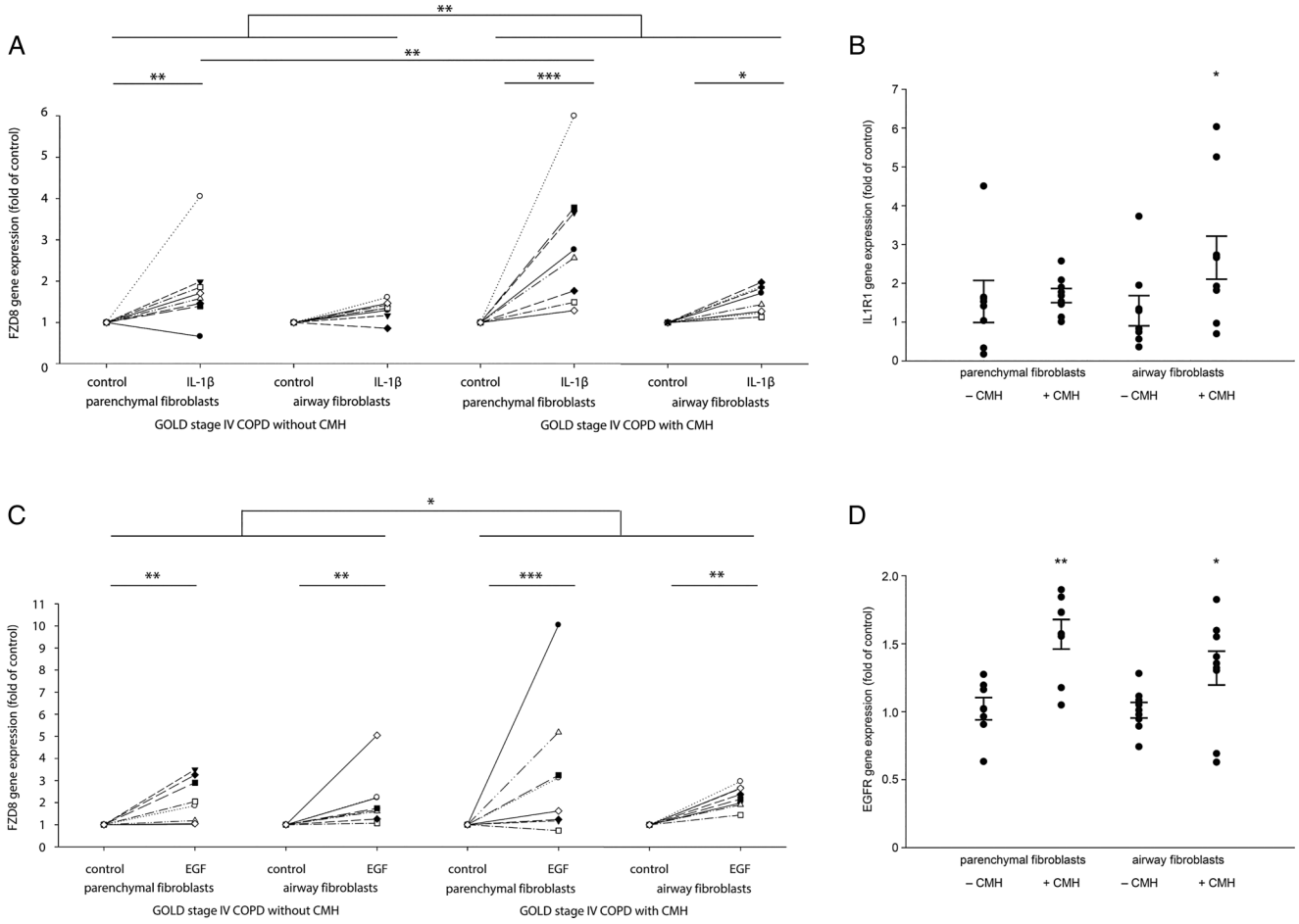


**Figure 3** Interleukin (IL)-1β- and epidermal growth factor (EGF)-induced FZD8 expression is associated with chronic mucus hypersecretion (CMH). Primary airway and parenchymal lung fibroblasts of patients with GOLD stage IV COPD with and without CMH were stimulated for 4 h with IL-1β (1 ng/mL) or EGF (10 ng/mL) to study FZD8 gene expression. Basal delta Cq-values did not differ between groups: parenchymal fibroblasts GOLD stage IV COPD: 16.29±0.61, airway fibroblasts GOLD stage IV COPD: 16.28±0.28, parenchymal fibroblasts GOLD stage IV COPD with CMH: 15.96 ±0.38, airway fibroblasts GOLD stage IV COPD with CMH: 15.99±0.51. (A) IL-1β-induced FZD8 gene expression. (B) Basal IL1R1 gene expression. (C) EGF-induced FZD8 gene expression. (D) Basal EGFR gene expression. Data represent induction of gene expression of eight patients per group. *p<0.05, **p<0.01 (one-way ANOVA with Student–Newman–Keuls multiple comparisons test). ANOVA, analysis of variance.

stage IV COPD with the CT variant showed a higher induction of FZD8 gene expression than did patients with the CC variant after IL-1β but not after EGF stimulation (figure 4A, B). In parenchymal lung fibroblasts, this effect was less pronounced (see online supplementary figure S3A, B). The low allele frequency (3.9%) of the TT variant precluded us from obtaining sufficient numbers of donors to analyse the impact of the TT variant on gene expression in these studies. Our results suggest that variants in FZD8 gene expression in lung fibroblasts play a role in patients with COPD who have CMH.

### FZD8 regulates IL-6 and CXCL8 secretion by lung fibroblasts

In view of our findings using the FZD8$^{-/-}$ mice, our next aim was to investigate in what way the changed expression in FZD8 gene expression in lung fibroblasts may affect pro-inflammatory responses. We investigated which cytokines are produced by primary MRC-5 human lung fibroblasts in response to IL-1β and EGF. Stimulation with IL-1β-induced granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage CSF (GM-CSF), IL-6, chemokine ligand (CXCL)8, interferon gamma-induced protein (IP)-10 and monocyte chemotactic protein (MCP)-1 secretion (figure 5A). Stimulation with EGF induced GM-CSF, IL-6 and CXCL8 secretion (figure 5B). We hypothesised that FZD8 may regulate the IL-1β-induced and EGF-induced release of IL-6 and CXCL8 from fibroblasts, as these cytokines are increased in COPD and contribute largely to the inflammation seen in COPD.[24] Moreover, in accordance with a previous study,[25] we observed that IL-6 and CXCL8 induce MUC5AC gene expression and CXCL8 induces PAS positive mucin protein expression indicative of goblet cell differentiation in differentiated primary human airway epithelial cells from healthy donors (figures 5C, D), further linking these cytokines to CMH. We were, however, not able to detect MUC5AC protein release. We found that IL-1β dose dependently increased both IL-6 and CXCL8 secretion from primary MRC-5 human lung fibroblasts (figure 6A, B), whereas EGF dose dependently increased CXCL8 secretion (figure 6C), but not IL-6 secretion (see online supplementary figure S4A). FZD8 knock-down by specific siRNA (knockdown efficiency 65% on average; see online supplementary figures S4B, C) significantly reduced IL-1β-induced IL-6 secretion (p=0.037 for the interaction of IL-1β stimulation and siRNA knock-down (two-way ANOVA) and CXCL8 secretion (p=0.008 for the interaction of IL-1β stimulation and siRNA knock-down (two-way ANOVA)) (figures 6A, B). EGF-induced CXCL8 secretion was also reduced by FZD8 knock-down (p<0.001 for the interaction of

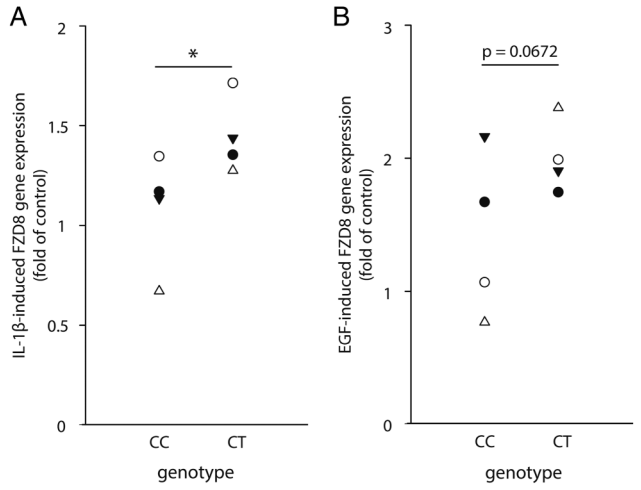


**Figure 4** Interleukin (IL)-1β- and epidermal growth factor (EGF)-induced increase of FZD8 gene expression in patients with GOLD stage IV COPD with and without chronic mucus hypersecretion (CMH) is regulated by single nucleotide polymorphism (SNP) rs663700. Relationship between the fold induction in FZD8 gene expression in airway fibroblasts of patients with GOLD stage IV COPD after stimulation with IL-1β or EGF and the FZD8 genotype of the patients, with regard to SNP rs663700. (A) IL-1β-induced FZD8 gene expression. (B) EGF-induced FZD8 gene expression. The experiment was performed for eight patients per group, four of which had a known genotype. Data represent four patients with CC and four patients with CT. *p<0.05 (one-tailed Mann–Whitney test).

EGF stimulation and siRNA knockdown (two-way ANOVA)) (figure 6C). These data show that FZD8 is involved in the IL-1β-induced secretion of IL-6 and CXCL8 as well as in the EGF-induced secretion of CXCL8. Furthermore, these data stress the importance of the fibroblast in the inflammatory response as seen in chronic bronchitis in COPD.

## DISCUSSION

This study shows for the first time that FZD8 plays a critical role in inflammatory processes involved in chronic bronchitis, including CMH, and reveals an unexpected role for the fibroblast in this process. We found that acute cigarette-smoke-induced airway inflammation in an in vivo mouse model is partly FZD8 dependent. In FZD8$^{-/-}$ mice, cigarette smoke did not induce neutrophilic inflammation, which was accompanied by the reduced expression of CXCL5 and to a lesser extent of CXCL2 and KC compared with cigarette-smoke-exposed WT mice. Furthermore, we show that there is an association between pro-inflammatory cytokine-induced FZD8 gene expression in airway and parenchymal lung fibroblasts of patients with GOLD stage IV COPD and the presence of CMH. This is explained in part by increased EGFR and IL1R1 receptor expression in fibroblasts of patients with GOLD stage IV COPD with CMH compared with patients without CMH and in part by a polymorphism in the *FZD8* region. SNP rs663700 in the *FZD8* region is associated with CMH in a cohort of smokers and additionally is a *cis*-eQTL in lung tissue, regulating FZD8 expression. Moreover, SNP rs663700 is associated with increased FZD8 gene expression in fibroblasts of patients with GOLD stage IV COPD with CMH. Upon stimulation with IL-1β and EGF, primary MRC-5 human lung fibroblasts secrete IL-6 and CXCL8, a process which is regulated by FZD8, and we show that both of these cytokines are able to initiate MUC5AC gene expression and CXCL8 induces goblet cells in differentiated primary human airway epithelial cells.

COPD is a heterogeneous disease characterised by different patient subpopulations.[26] We investigated the potential role for FZD8 in chronic bronchitis, and found a clear association between the cytokine-induced expression of FZD8 gene expression and CMH, a clinical expression which is a consequence of the pathologic process in chronic bronchitis. Of importance is to emphasise that the genetic association was independent of the presence of COPD, indicating that FZD8 likely plays a role in CMH in healthy smokers as well. CMH is associated with bronchial inflammation, accelerated lung function decline, COPD morbidity and increased mortality.[4–6] The association of the genetic variance in *FZD8* with CMH is the first evidence to support a genetic link between the WNT signalling pathway and a phenotype of COPD. Interestingly, we also found a specific role for FZD8 in the chronic bronchitis phenotype, reinforcing the contention that phenotypes of COPD may be characterised by specific pathophysiological mechanisms.

While the communication of both damaged and intact epithelium to the fibroblasts is widely recognised in the airways,[27] we now find indications that fibroblasts play an additional crucial role in inflammation and epithelial mucus production. Upon damage, the epithelium secretes inflammatory cytokines such as EGF, IL-1β and IL-1α. IL-1α has been shown to induce IL-6 and CXCL8 in fibroblasts.[28] Importantly, IL-6 and CXCL8 secretion from primary MRC-5 human lung fibroblasts are increased upon coculturing these cells with human bronchial epithelial cells. This process is mediated via IL-1α.[29] IL-1α and IL-1β are known to function via the same receptor, IL1R1. IL-1β and EGF stimulated fibroblasts to produce IL-6 and CXCL8 via FZD8 in our study. We propose that in this way, FZD8 contributes to the inflammation present in chronic bronchitis. Our data support this hypothesis, as neutrophil and lymphocyte recruitment in the BALF are reduced in FZD8$^{-/-}$ mice as well as mRNA expression of the neutrophil attractants CXCL5 and KC in whole lung homogenates, whereas IL-6-induced and CXCL8-induced mucus production in differentiated primary human airway epithelial cells. Both cytokines are secreted by human lung fibroblasts upon stimulation with IL-1β and EGF, and we demonstrate that this process is regulated by FZD8. In addition, we show that expression of IL1R1 and the EGFR is increased in fibroblasts of patients with GOLD stage IV COPD with CMH compared with patients without CMH. This shows that IL-1β and EGF lead to increased FZD8 expression in CMH in part via increased expression of their receptors, and in part based on FZD8 genotype.

For many WNT ligands, it is either not known or highly context dependent via which FZD receptor they exert their effect. FZD8 is a known receptor for WNT-5A[9] and WNT-5B[30] in airway smooth muscle cells and pulmonary fibroblasts. However, based on the crystal structure of the cysteine-rich domain of FZD8, it must be assumed that most if not all WNT ligands are potential agonists,[31] whereas R-spondins have also been described as FZD8 agonists.[32] Conversely, connective tissue growth factor[33] and insulin-like growth-factor-binding protein-4[34] may antagonise FZD8 by direct binding. WNT-5A and WNT-5B are highly expressed in fibroblasts,[8] and a role of FZD8 in their responses has been described.[9 30] Furthermore, the regulation by WNT-5A of CXCL8 release from human neutrophils has been described.[14] Therefore, WNT-5A and WNT-5B are potential ligands for FZD8 in chronic bronchitis, which is supported by our findings that WNT-5A and WNT-5B

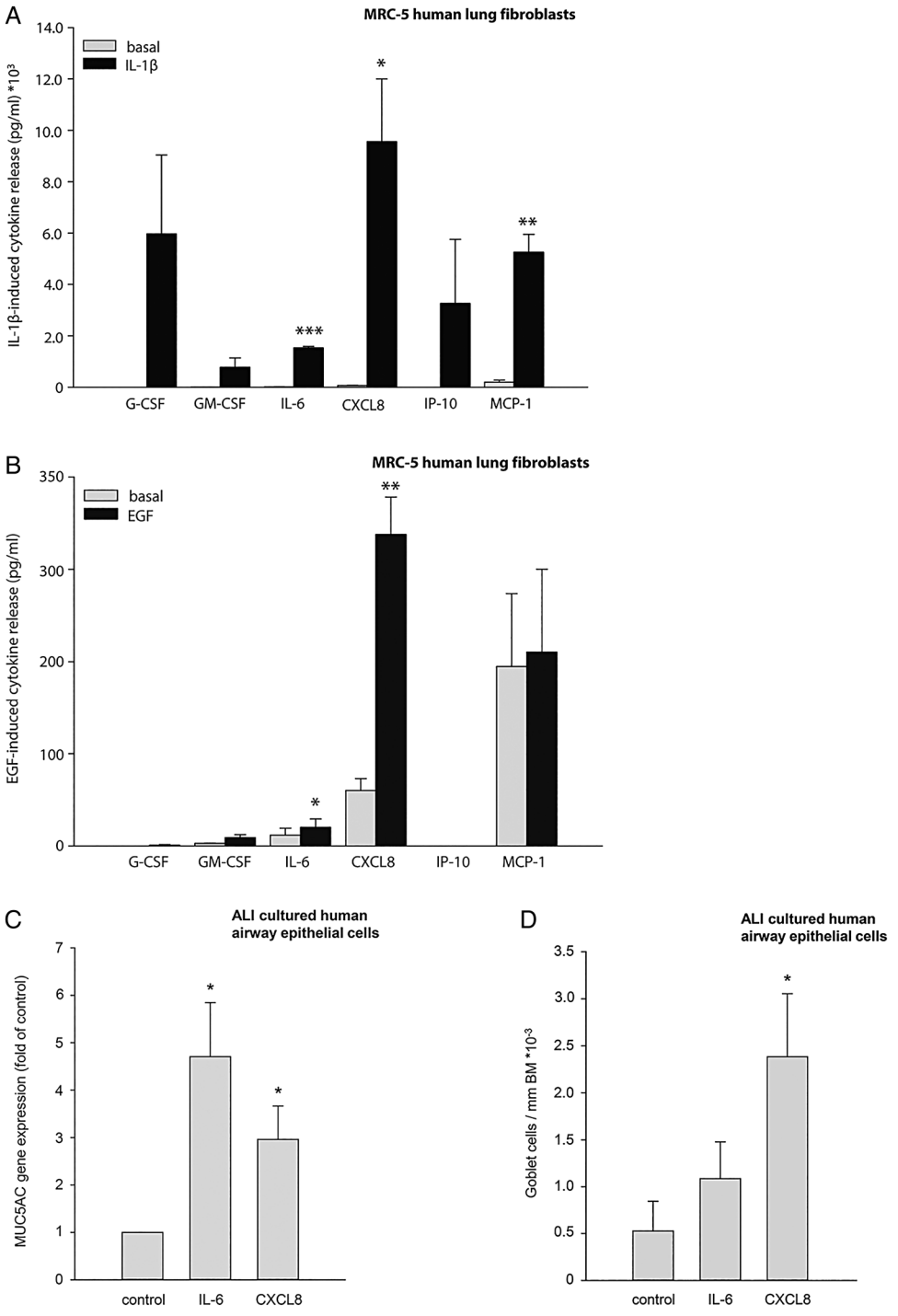


**Figure 5** Interleukin (IL)-6 and CXCL8 are released by primary MRC-5 human lung fibroblasts and induce MUC5AC gene expression in ALI-cultured primary human airway epithelial cells. Primary MRC-5 human lung fibroblasts were stimulated for 24 h with IL-1β (1 ng/mL) or epidermal growth factor (EGF) (10 ng/mL). A Milliplex was performed to screen for 26 cytokines to investigate which cytokines were produced. (A) IL-1β-induced cytokine secretion from primary MRC-5 human lung fibroblasts. (B) EGF-induced cytokine secretion from primary MRC-5 human lung fibroblasts. Data represent mean±SEM of five independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared with basal conditions (two-tailed Student's t test). Primary human airway epithelial cells were stimulated for 24 h with IL-6 (10 ng/mL) and CXCL8 (10 ng/mL) to study MUC5AC gene expression. (C) IL-6- and CXCL8-induced MUC5AC gene expression in differentiated primary human airway epithelial cells. Data represent mean ±SEM of five independent experiments. (D) IL-6- and CXCL8-induced number of goblet cells per mm basement membrane (BM) in paraffin-embedded sections of differentiated primary human airway epithelial cells. Data represent mean±SEM of four independent experiments. *p<0.05 compared with basal conditions (two-tailed Student's t test).

induce IL-6 and CXCL-8 release from human lung fibroblasts.[35] Clearly, a better appreciation of the ligands involved in FZD8 signalling, their cellular sources and their role in chronic bronchitis require further studies.

COPD is currently treated symptomatically and not phenotype specific. The awareness that patients with COPD need treatment according to their phenotype stimulated the investigation of new drug targets.[24] We found IL-6 and CXCL8 to be important in

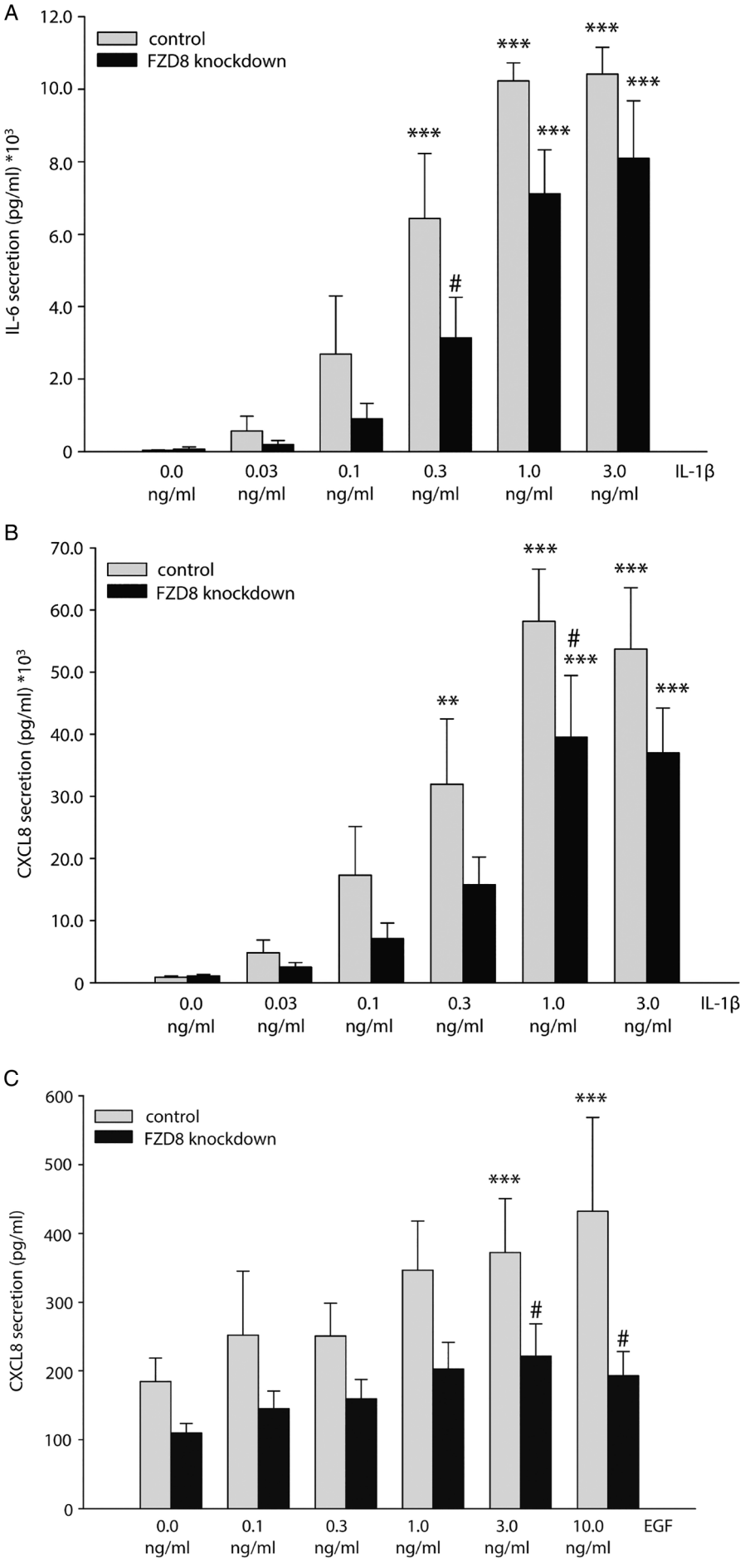


**Figure 6** FZD8 regulates interleukin (IL)-1β- and epidermal growth factor (EGF)-induced IL-6 and CXCL8 secretion. Primary MRC-5 human lung fibroblasts were transfected with specific FZD8 siRNA or non-targeting siRNA and stimulated for 24 h with increasing concentrations of IL-1β (0.03–3 ng/mL) and EGF (0.1–10 ng/mL). (A) IL-1β-induced IL-6 secretion by primary MRC-5 human lung fibroblasts; p=0.037 for the interaction of IL-1β stimulation and siRNA knock-down (two-way analysis of variance (ANOVA)). (B) IL-1β-induced CXCL8 secretion by primary MRC-5 human lung fibroblasts; p=0.008 for the interaction of IL-1β stimulation and siRNA knock-down (two-way ANOVA). (C) EGF-induced CXCL8 secretion by primary MRC-5 human lung fibroblasts; p<0.001 for the interaction of EGF stimulation and siRNA knock-down (two-way ANOVA). Data represent mean±SEM of five (CXCL8) or four (IL-6) independent experiments. **p<0.01, ***p<0.001 compared with control #p<0.05 compared with stimulated control siRNA (two-way ANOVA with Student–Newman–Keuls multiple comparisons test).

inducing mucus production. There are no biologicals used in COPD that target IL-6. Blocking CXCL8 by using a blocking antibody only or by blocking the CXCR2 receptor has been reported not to have a clinical impact.[36] Our data show that targeting FZD8 on fibroblasts could prevent both the secretion of IL-6 and CXCL-8, leading to a reduction in inflammatory processes and mucus secretion. Therefore, FZD8 may provide a rational drug target for chronic bronchitis and the development of FZD8 receptor antagonists may be warranted.

In conclusion, our results indicate that FZD8 plays an important pro-inflammatory role in chronic bronchitis. Interestingly, the fibroblast appears to play a major role herein. We provide genetic and functional data in vitro and in vivo supporting the role of FZD8 in pro-inflammatory cytokine production, inflammatory cell recruitment and mucus hypersecretion. These findings show a potential important role for WNT signalling via FZD8 in fibroblasts in chronic bronchitis. Therefore, targeting FZD8 is a strategy worth pursuing for the treatment of chronic bronchitis.

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**Contributors** Conception and design: AIRS, DSP, HM, IHH and RG. Laboratory work: AIRS (in vitro and in vivo studies); MHM (in vitro studies); C-AB and WT (primary fibroblast characterisation of patients with COPD). Data analysis and interpretation: AIRS, MHM, C-AB, DCN, DDS, WT, DSP, HM, IHH, RG, AED and HMB (genetic association study); MvdB and YB (eQTL analysis). Drafting of manuscript: AIRS, AED, MvdB, IHH and RG. Critically revising manuscript: MvdB, HMB, DCN, DDS, YB, C-AB, WT, DSP, HM, IHH and RG. All authors have read, reviewed and approved the final manuscript as submitted to take public responsibility for it.

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**Ethics approval** The study protocol followed national ethical and professional guidelines ('Code of conduct; Dutch federation of biomedical scientific societies'; http://www.federa.org) for all lung tissue and explant cell culture studies in Groningen. For the genetics study on CMH, approval by the local medical ethics committee and written informed consent from all patients were obtained (see online supplementary file).

**Provenance and peer review** Not commissioned; externally peer reviewed.

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# A pro-inflammatory role for the Frizzled-8 receptor in chronic bronchitis

## Supplementary File


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**Extended Methods section**

## Ethics statement

For the genetics study on chronic mucus hypersecretion (CMH) approval by the local medical ethics committee and written informed consent from all patients was obtained. At Laval, lung specimens were collected from patients undergoing lung cancer surgery and stored at the "Institut universitaire de cardiologie et de pneumologie de Québec" (IUCPQ) site of the Respiratory Health Network Tissue Bank of the "Fonds de recherche du Québec – Santé" (www.tissuebank.ca). Written informed consent was obtained from all subjects and the study was approved by the IUCPQ ethics committee. At Groningen, lung specimens were provided by the local tissue bank of the Department of Pathology and the study protocol was consistent with the Research Code of the University Medical Center Groningen and Dutch national ethical and professional guidelines ("Code of conduct; Dutch federation of biomedical scientific societies"; http://www.federa.org). At Vancouver, the lung specimens were provided by the James Hogg Research Center Biobank at St. Paul's Hospital and subjects provided written informed consent. The study was approved by the ethics committees at the UBC-Providence Health Care Research Institute Ethics Board.

## Animal studies

Heterozygous, inbred, specified-pathogen-free breeding colonies FZD8$^{+/-}$ mice (C57BL/6;129P2-*FZD8*$^{tm1Dgen}$/J), showing no obvious phenotype, were obtained from the Jackson Laboratory (USA). After breeding, homozygous FZD8$^{-/-}$ mice and wild-type$^{+/+}$ (WT) littermates were used for experiments. Animals were housed under a 12 hour light-dark cycle and received food and water *ad libitum*. Male FZD8$^{-/-}$ and WT mice were subjected for four

successive days to fresh air or cigarette smoke from Kentucky 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY, USA) by whole body exposure, as described previously.[1] In brief, cigarette smoke was directly circulated into a 6-liter Perspex box. After removing the filter, each cigarette was smoked in five minutes at a rate of 5L/hour in a ratio of 60L/hour air using a peristaltic pump (45 rpm, Watson Marlow 323 E/D, Rotterdam, the Netherlands). On the first day, mice were exposed to one cigarette in the morning and three cigarettes in the afternoon. On the second to fourth day, mice were exposed to five cigarettes in the morning and five cigarettes in the afternoon. Control animals were exposed at the same time intervals to fresh air. Eight mice were included in each group. Sixteen hours after the last cigarette smoke exposure, mice were euthanized by subcutaneous injection with a mixture of medetomidine (0.5 mg/kg Dormitor®, Orion Pharma, Mechelen, Belgium) and ketamine (40.0 mg/kg, Alfasan, Woerden, the Netherlands) followed by exsanguination. The lungs were lavaged five times with 1 mL PBS. The bronchial alveolar lavage fluid (BALF) fractions were pooled. From these, cytospins were prepared to determine total and inflammatory cell numbers. Cytospins were stained with May-Grünwald and Giemsa (Sigma, St. Louis, MO, USA). Differential cell count was performed by counting 400 cells in duplicate in a blinded manner. Preceding immunohistochemistry, the upper right lung lobe was taken up in formalin and paraffin-embedded. Preceding mRNA isolation, the post caval lobe was snap frozen, mechanically crushed under liquid nitrogen and taken up in lysis buffer.

**Fibroblast cell culture**

Human airway and parenchymal lung fibroblasts from ex-smoking GOLD stage IV COPD patients with and without CMH were isolated from transplanted lungs as has been described previously.[2] Presence of CMH was defined by patient records. Patient characteristics are shown in table 1. MRC-5 human lung fibroblasts[3] were obtained from Sigma (St. Louis,

MO, USA). Primary human airway and parenchymal lung fibroblasts and primary MRC-5 human lung fibroblasts were cultured in Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin and 1.5 μg/ml amphotericin B. Prior to the experiment, cells were grown to confluence in 6-well or 24-well cluster plates and placed in Ham's F12 medium with 0.5% (v/v) FBS for 24 hours. Subsequently, cells were stimulated with either 2 ng/ml recombinant human transforming growth factor (TGF)-$\beta_1$, 1 ng/ml recombinant human interleukin (IL)-1β, 10 ng/ml recombinant human tumor necrosis factor (TNF)-α, 10 ng/ml recombinant human epidermal growth factor (EGF) or 5% cigarette smoke extract (CSE) for several time points. To prepare CSE, two cigarettes were smoked sequentially using a peristaltic pump (323 E/D; Watson Marlow, Rotterdam, the Netherlands) at 45 rpm through 25 ml of Ham's F12 medium supplemented with 0.5% (v/v) FBS, referred to as 100% CSE. 100% CSE was diluted to a working concentration of 5% CSE. CSE was freshly prepared before each experiment.

**Air liquid interface (ALI) cell culture**

Human airway epithelial cells were isolated after incubating tracheal tissue from donor lungs for 2 hours at 37°C in Protease IX (Sigma, St. Louis, MO, USA) and plated on coated culture dishes. Coating consisted of 10 μg/ml bovine serum albumin, 10 μg/ml fibronectin (both from Sigma, St. Louis, MO, USA) and 30 μg/ml collagen (PureCol®, Advanced Biomatrix, San Diego, CA, USA) in phosphate buffered saline (PBS). Human airway epithelial cells were grown to approximately 70% confluence in keratinocyte serum-free medium (KSFM) supplemented with 25 μg/ml streptomycin, 25 U/ml penicillin, 1 μM isoproterenol, 0.2 ng/ml EGF, 25 μ/ml bovine pituitary extract. For the first week after isolation, KSFM medium was additionally supplemented with 1.5 μg/ml amphotericin B and 5 μg/ml ciproxin. Cells were plated on a 0.4 μm polyester membrane 12 mm inserts (Transwell® Permeable Supports,

Corning, NY, USA) coated well in 1:1 Bronchial Epithelial Cell Growth Medium (Lonza, Walkersville, MD, USA)/Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) (BEGM/DMEM) medium supplemented with 25 µg/ml streptomycin, 25 U/ml penicillin, 5.5 mg/ml sodium pyruvate (all from Gibco, Grand Island, NY, USA) 0.4% (w/v) bovine pituitary extract, 0.5 ng/ml EGF, 5 µg/ml insulin, 10 µg/ml transferrin, 1 µM hydrocortisone, 6.5 ng/ml T3, 0.5 µg/ml epinephrine (all from Lonza, Walkersville, MD, USA), 15 ng/ml retinoic acid and 1.5 µg/ml bovine serum albumin (Sigma, St. Louis, MO, USA). When confluence was reached, cells were air-exposed and allowed to differentiate into mucociliary epithelium for 14 days. Subsequently, cells were serum deprived for 16 hours in BEGM + ITS (5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium) and stimulated basolaterally for 24 hours with 10 ng/ml recombinant human IL-6 or 10 ng/ml recombinant human chemokine ligand (CXCL)8 to measure MUC5AC gene expression and to perform immunohistochemistry for goblet cells.

**Immunohistochemistry**

Inserts of ALI cell culture were taken up in formalin and paraffin-embedded according to the protocol 'Preparation of Costar® Transwell® Inserts for Histology'. Transverse cross-sections of 5 µm thick were used for morphometric analyses. Paraffin-embedded sections were stained for goblet cells with Periodic Schiff's (PAS, Sigma-Aldrich, Zwijndrecht, the Netherlands). PAS-positive cells were counted and expressed per mm basement membrane.

After sacrificing the mice, the upper right lung lobe was taken up in formalin and paraffin-embedded. Transverse cross-sections of 5 µm thick were used for morphometric analyses. Paraffin-embedded sections were stained for α-smooth muscle(sm)-actin using rabbit anti-α-sm-actin antibody (Abcam, Cambridge, UK) and visualized using a goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz

Biotechnology, CA, USA) and diaminobenzidine (Sigma-Aldrich, Zwijndrecht, the Netherlands). The presence of sm-α-actin around the airway was quantified using ImageJ.[4] The surface of positively stained tissue was expressed as $mm^2$ per $mm^2$ basement membrane. MUC5AC positive cells were stained in paraffin-embedded sections using a MUC5AC antibody staining (Neomarkers, Fremont, CA, USA).

**Small interfering (si)RNA transfection**

Human fibroblasts were grown to 90% confluence and transfected with specific siRNA against the FZD8 transcript to knockdown FZD8. Cells were transfected in serum-free Ham's F12 medium without supplements using 100 pmol FZD8-targeted siRNA or non-targeting control siRNA and Lipofectamine® 2000 Transfection Reagent. After 6 hours, the medium was changed to medium supplemented with 10% (v/v) FBS for 18 hours and subsequently to medium supplemented with 0.5% (v/v) FBS for 24 hours. Cells were stimulated with either recombinant human IL-1β or EGF in increasing concentrations (0.03-3 ng/ml IL-1β and 0.1-10 ng/ml EGF) for 24 hours. Knockdown was considered successful when FZD8 gene expression was reduced by 60%.

**Cytokine release from fibroblasts**

24 hours after stimulation, culture medium was collected for the determination of cytokines using enzyme-linked immunosorbent assay (ELISA) and Milliplex®. We used Milliplex® (MILLIPLEX MAP Human Cytokine/Chemokine - Premixed 26 Plex, Millipore, Billerica, MA, USA) to screen for primary MRC-5 human lung fibroblast cytokine release. We specifically measured concentrations of secreted IL-6 and CXCL8 in primary MRC-5 human

lung fibroblasts by ELISA according to the manufacturer's instructions (#M1916 (IL-6), #M1918 (CXCL8); Sanquin, Amsterdam, the Netherlands).

**mRNA isolation and real-time PCR analysis**

Total mRNA from primary MRC-5 human lung fibroblasts and from mice lung tissue was extracted using the NucleoSpin® RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Total mRNA from primary human airway and parenchymal lung fibroblasts was extracted using the miRNeasy Micro Kit (Qiagen, Venlo, the Netherlands). Total mRNA from differentiated primary human airway epithelial cells was extracted using Tri Reagent® Solution (Applied Biosystems, Life Technologies Europe BV, Bleiswijk, the Netherlands). The eluted mRNA was quantified using spectrophotometry (Nanodrop, Thermo Scientific™, Wilmington, DE, USA). Equal amounts of mRNA (1 µg) were then reverse transcribed using the Reverse Transcription System (Promega Benelux b.v., Leiden, the Netherlands) and the cDNA was stored at -20°C till further use. mRNA expression was determined using real-time PCR, which was performed with the Illumina Eco Personal QPCR System (Westburg, Leusden, the Netherlands). Primer sets are listed in table S1-2.

**Materials and reagents**

Recombinant human TGF-$\beta_1$, was obtained from R&D Systems (Minneapolis, MN, USA), recombinant human IL-1β, recombinant human TNF-α and recombinant human EGF were obtained from Sigma (St. Louis, MO, USA). Recombinant human IL-6 and recombinant human CXCL8 were obtained from ImmunoTools (Friesoythe, Germany). For CSE, Kentucky 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY, USA) were used. FZD8 targeted siRNA was obtained from Santa Cruz

Biotechnology Inc. (Heidelberg, Germany), non-targeting control siRNA from Qiagen (Venlo, the Netherlands). Lipofectamine® 2000 Transfection Reagent was obtained from Invitrogen (Paisley, UK). All other chemicals were of analytical grade.

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**Supplementary Figure legends**

**Figure S1: characterization of FZD8$^{-/-}$ mice.**

WT and FZD8$^{-/-}$ mice were exposed to PBS and paraffin-embedded sections were stained for α-sm-actin. (A) Quantification of the area positive for α-sm-actin around the airways in WT and FZD8$^{-/-}$ mice. (B) Quantification of the area positive for α-sm-actin around the vessels in WT and FZD8$^{-/-}$ mice. Data represent mean ± s.e.m. of 10 mice per group. (C) Representative staining for α-sm-actin in WT and FZD8$^{-/-}$ mice. WT and FZD8$^{-/-}$ mice were exposed to cigarette smoke for 4 days. mRNA expression was determined in whole lung homogenates. (D) FZD8 gene expression in whole lung homogenates of WT mice. (E) WNT-5A gene expression in whole lung homogenates of WT and FZD8$^{-/-}$ mice. (F) WNT-5B gene expression in whole lung homogenates of WT and FZD8$^{-/-}$ mice. (G) MUC5AC gene expression in whole lung homogenates of WT and FZD8$^{-/-}$ mice. Data represent mean ± s.e.m. of 8 mice per group. * $p < 0.05$ compared to air exposed WT mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons test).

**Figure S2: WNT/FZD gene expression in primary human parenchymal lung fibroblasts.**

Primary parenchymal lung fibroblasts of GOLD stage IV COPD patients with and without CMH were stimulated for 4 hours with 5% CSE, IL-1β (1 ng/ml), TNF-α (10 ng/ml), TGF-β (2 ng/ml) or EGF (10 ng/ml) to study WNT-5A, WNT-5B, WNT-16, FZD2, FZD6 and FZD8 gene expression. (A) IL-1β-induced gene expression. Basal delta Cq-values did not differ between groups: parenchymal fibroblasts GOLD stage IV COPD: 16.29±0.61, airway fibroblasts GOLD stage IV COPD: 16.28±0.28, parenchymal fibroblasts GOLD stage IV COPD with CMH: 15.96±0.38, airway fibroblasts GOLD stage IV COPD with CMH: 15.99±0.51. (B) EGF-induced gene expression. (C) CSE, IL-1β, TNF-α, TGF-β and EGF effects on FZD8 gene expression. Data represent mean ± s.e.m. of induction of FZD8 gene

expression of 8 patients per group. *** $p < 0.001$ compared to control (one-way ANOVA with Student-Newman-Keuls multiple comparisons test).

**Figure S3: IL-1β- and EGF-induced increase of FZD8 gene expression in parenchymal lung fibroblasts of GOLD stage IV COPD patients: correlation to SNP rs663700.** Correlation of the fold induction in FZD8 gene expression in parenchymal lung fibroblasts of GOLD stage IV COPD patients after stimulation with IL-1β or EGF to the genotype of the patients, concerning SNP rs663700. (A) Correlation between the fold induction in FZD8 gene expression after stimulation of parenchymal lung fibroblasts with IL-1β and patient genotype. (B) Correlation between the fold induction in FZD8 gene expression after stimulation of parenchymal lung fibroblasts with EGF and patient genotype. Data represent 5 patients with CC and 6 patients with CT; (one-tailed Mann-Whitney test).

**Figure S4: EGF effects on IL-6 secretion by MRC-5 human lung fibroblasts.** Primary MRC-5 human lung fibroblasts were transfected with specific FZD8 siRNA or non-targeting siRNA and stimulated for 24 hours with increasing concentrations of EGF (0.1-10 ng/ml). (A) EGF-induced IL-6 secretion by primary MRC-5 human lung fibroblasts. Data represent mean ± s.e.m. of 4 independent experiments. (B) FZD8 knockdown for the experiments shown in figures 6A-B. Data represent mean ± s.e.m. of 5 independent experiments. (C) FZD8 knockdown for the experiments shown in figure 6C and S4A. Data represent mean ± s.e.m. of 4 independent experiments. * $p < 0.05$ compared to control siRNA # $p < 0.05$ ## $p < 0.01$ compared to stimulated control siRNA (two-way ANOVA with Student-Newman-Keuls multiple comparisons test).

**Supplementary Tables**

**Table S1. Human primers used for the determination of specific genes of interest.**

| | | | Primer sequence | |
|---|---|---|---|---|
| FZD2 | Forward | 5' | CCC GACT TCAC GGT CTA CAT | 3' |
| | Reverse | 5' | CTG TTG GTG AGG CGA GTG TA | 3' |
| FZD6 | Forward | 5' | TTG TTG GCA TCT CTG CTG TC | 3' |
| | Reverse | 5' | CCA TGG ATT TGG AAA TGA CC | 3' |
| FZD8 | Forward | 5' | GAC ACT TGA TGG GCT GAG GT | 3' |
| | Reverse | 5' | CAA ATC TCG GGT TCT GGA AA | 3' |
| WNT-5A | Forward | 5' | GGG TGG GAA CCA AGA AAA AT | 3' |
| | Reverse | 5' | TGG AAC CTA CCC ATC CCA TA | 3' |
| WNT-5B | Forward | 5' | ACG CTG GAG ATC TCT GAG GA | 3' |
| | Reverse | 5' | CGA GGT TGA AGC TGA GTT CC | 3' |
| WNT-16 | Forward | 5' | GCT CCT GTG CTG TGA AAA CA | 3' |
| | Reverse | 5' | ACC CTC TGA TGT ACG GTT GC | 3' |
| MUC5AC | Forward | 5' | ATT TTT TCC CCA CTC CTG ATG | 3' |
| | Reverse | 5' | AAG ACA ACC CAC TCC CAA CC | 3' |
| 18S rRNA | Forward | 5' | CGC CGC TAG AGG TGA AAT TC | 3' |
| | Reverse | 5' | TTG GCA AAT GCT TTC GCT C | 3' |

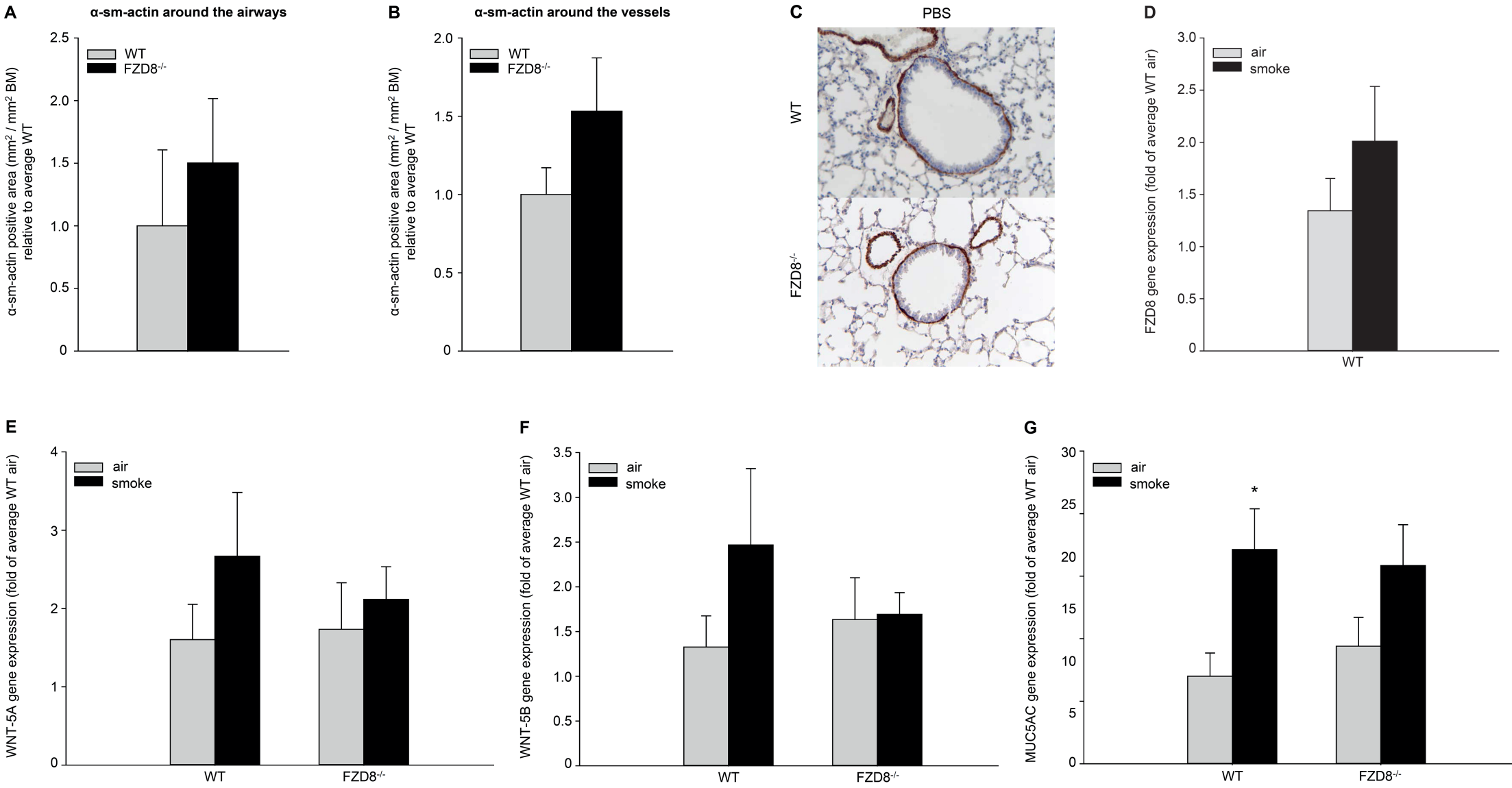
**Table S2. Mouse primers used for the determination of specific genes of interest.**

| | | | Primer sequence | |
|---|---|---|---|---|
| CXCL2 | Forward | 5' | AAG TTT GCC TTG ACC CTG AA | 3' |
| | Reverse | 5' | AGG CAC ATC AGG TAC GAT CC | 3' |
| CXCL5 | Forward | 5' | GAA AGC TAA GCG GAA TGC AC | 3' |
| | Reverse | 5' | GGG ACA ATG GTT TCC CTT TT | 3' |
| KC | Forward | 5' | GCT GGG ATT CAC CTC AAG AA | 3' |
| | Reverse | 5' | AGG TGC CAT CAG AGC AGT CT | 3' |
| FZD8 | Forward | 5' | TCC GTT CAG TCA TCA AGC AG | 3' |
| | Reverse | 5' | CGG TTG TGC TGC TCA TAG AA | 3' |
| WNT-5A | Forward | 5' | CAA ATA GGC AGC CGA GAG AC | 3' |
| | Reverse | 5' | CTC TAG CGT CCA CGA ACT CC | 3' |
| WNT-5B | Forward | 5' | GGT TCC ACT GGT GTT GCT TT | 3' |
| | Reverse | 5' | AGA CTT TTG TGA GGC GGA GA | 3' |
| MUC5AC | Forward | 5' | GAG ATG GAG GAT CTG GGT CA | 3' |
| | Reverse | 5' | GCA GAA GCA GGG AGT GGT AG | 3' |
| 18S rRNA | Forward | 5' | AAA CGG CTA CCA CAT CCA AG | 3' |
| | Reverse | 5' | CCT CCA ATG GAT CCT CGT TA | 3' |

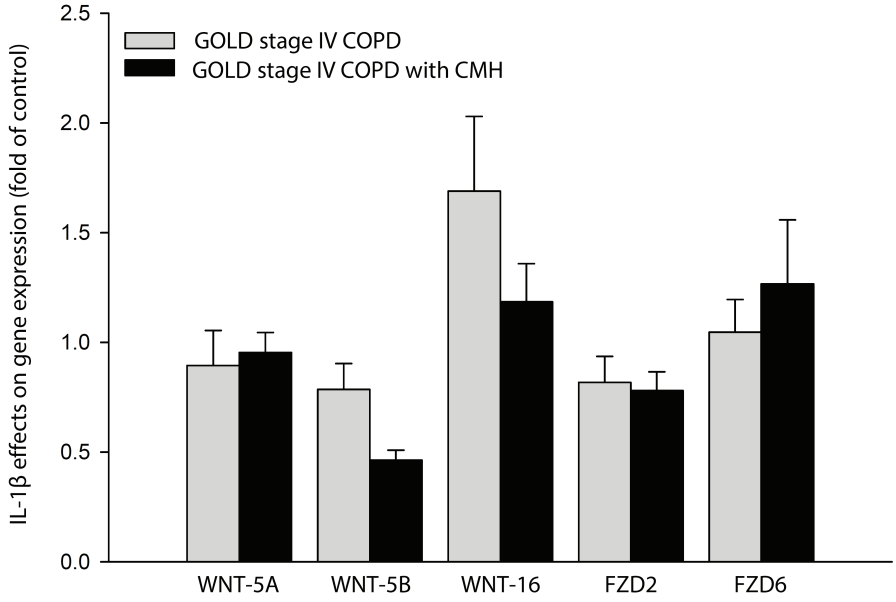
**Table S3. OR for CMH for the 6 tested SNPs in the *FZD8* region.**

| SNP (CHR* 10) | base pair position | OR | SE | L95† | U95‡ | p-value | left gene | right gene |
|---|---|---|---|---|---|---|---|---|
| rs640827 | 35941721 | 0.8499 | 0.07413 | 0.735 | 0.9828 | $2.82 * 10^{-02}$ | GJD4 | FZD8 |
| rs10827519 | 35952397 | 0.7996 | 0.08744 | 0.6737 | 0.9491 | $1.05 * 10^{-02}$ | GJD4 | FZD8 |
| rs663700 | 35956378 | 0.8095 | 0.08367 | 0.684 | 0.9495 | $9.89 * 10^{-03}$ | GJD4 | FZD8 |
| rs596642 | 35976759 | 0.8652 | 0.07958 | 0.7403 | 1.011 | $6.89 * 10^{-02}$ | FZD8 | LOC439954 |
| rs11010252 | 35987557 | 1.143 | 0.1164 | 0.9102 | 1.437 | $2.49 * 10^{-01}$ | FZD8 | LOC439954 |
| rs618443 | 35988716 | 1.128 | 0.1019 | 0.9238 | 1.377 | $2.37 * 10^{-01}$ | FZD8 | LOC43995 |

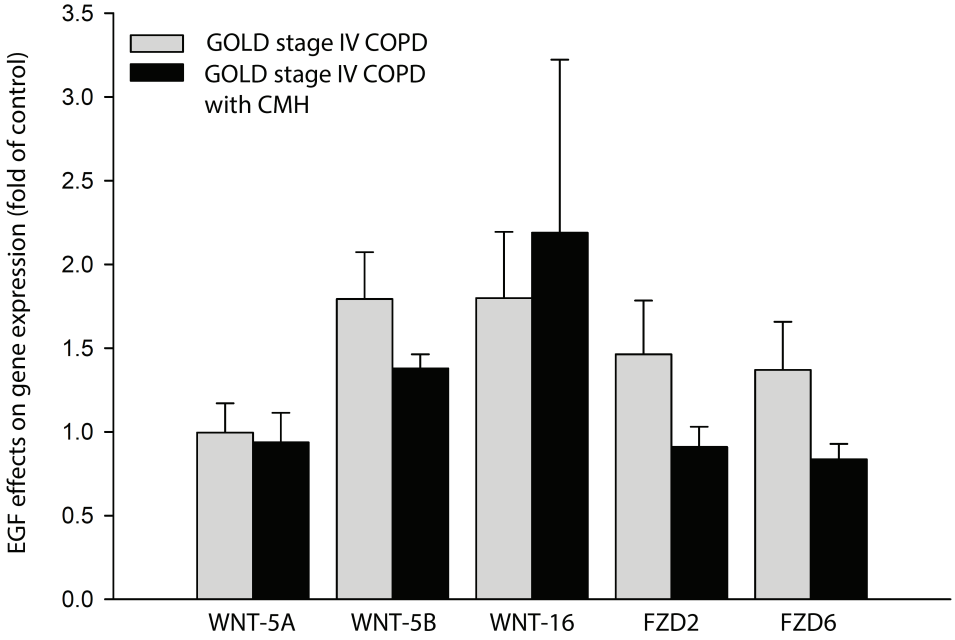
*CHR = chromosome
† L95 = lower limit
‡ U95 = upper limit


A
α-sm-actin around the airways
α-sm-actin positive area (mm² / mm² BM) relative to average WT
WT
FZD8-/-
B
α-sm-actin around the vessels
α-sm-actin positive area (mm² / mm² BM) relative to average WT
WT
FZD8-/-
C
PBS
WT
FZD8-/-
D
FZD8 gene expression (fold of average WT air)
air
smoke
WT
E
WNT-5A gene expression (fold of average WT air)
air
smoke
WT
FZD8-/-
F
WNT-5B gene expression (fold of average WT air)
air
smoke
WT
FZD8-/-
G
MUC5AC gene expression (fold of average WT air)
air
smoke
*
WT
FZD8-/-

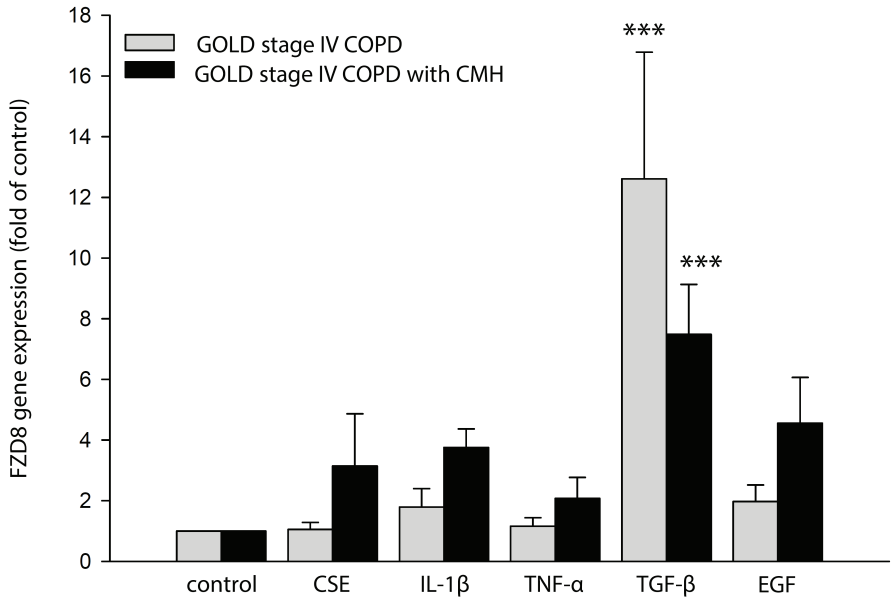
**A**

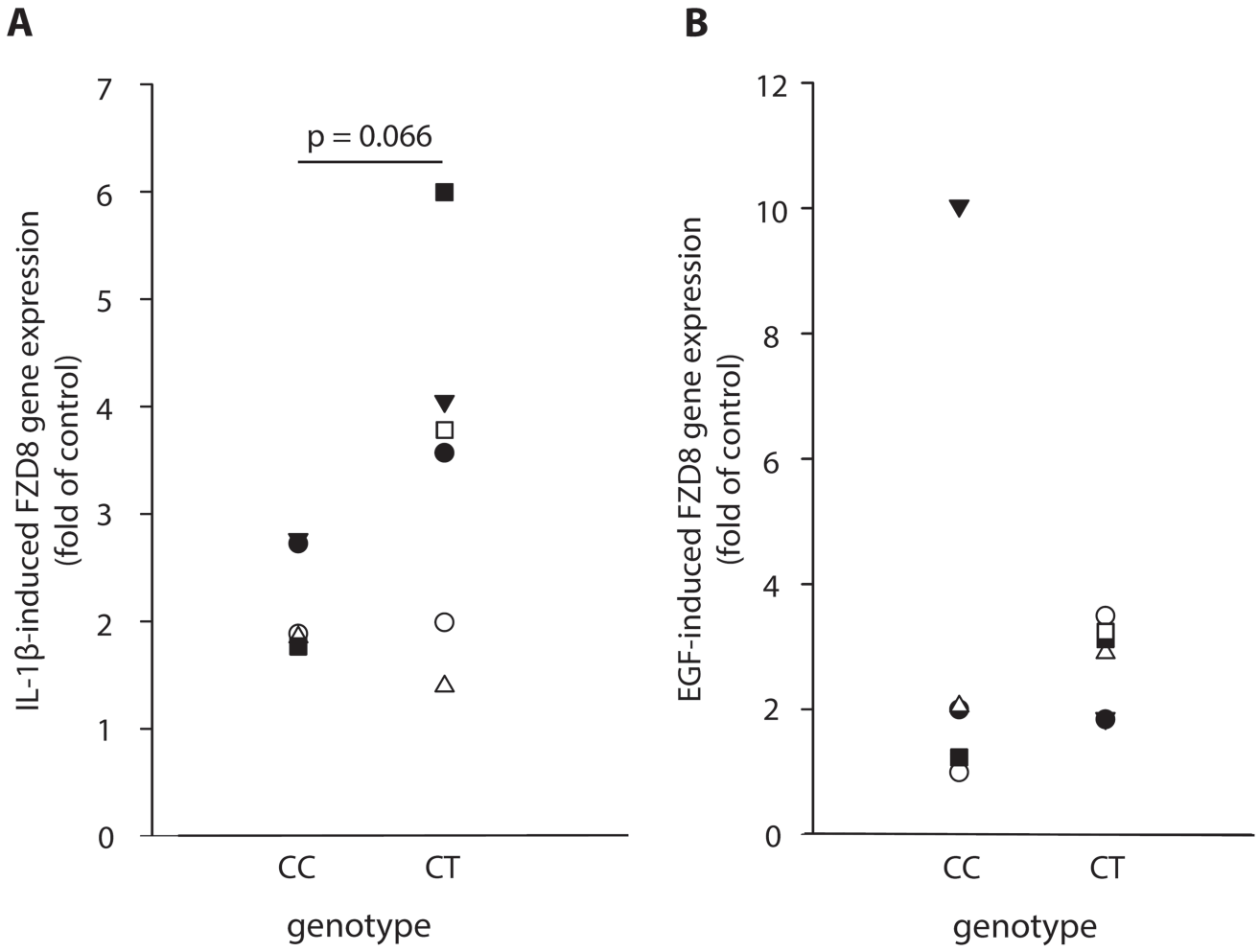

GOLD stage IV COPD
GOLD stage IV COPD with CMH
IL-1β effects on gene expression (fold of control)
2.5
2.0
1.5
1.0
0.5
0.0
WNT-5A
WNT-5B
WNT-16
FZD2
FZD6

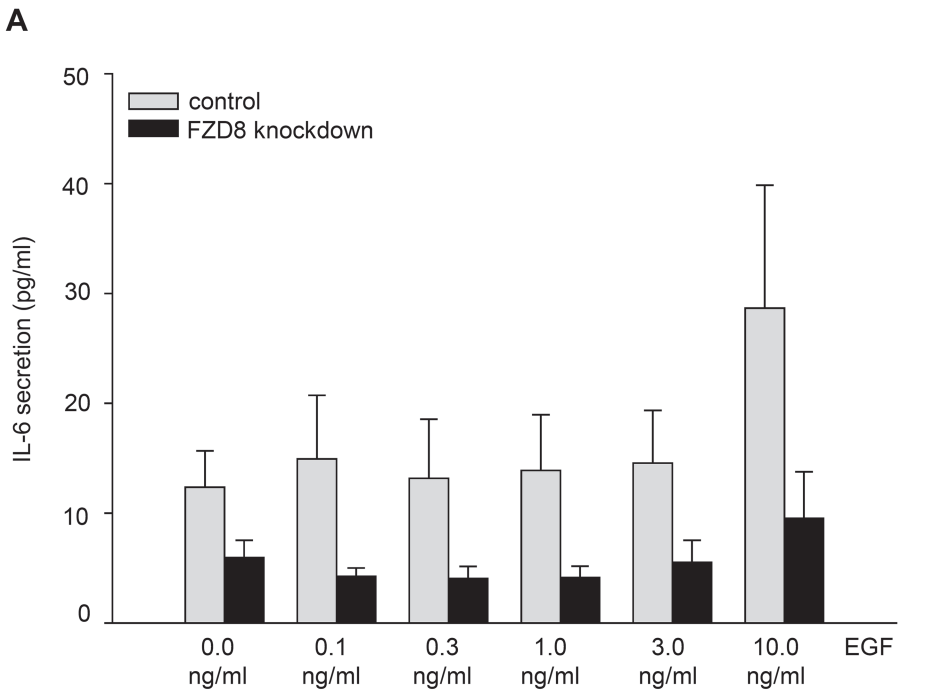
**B**

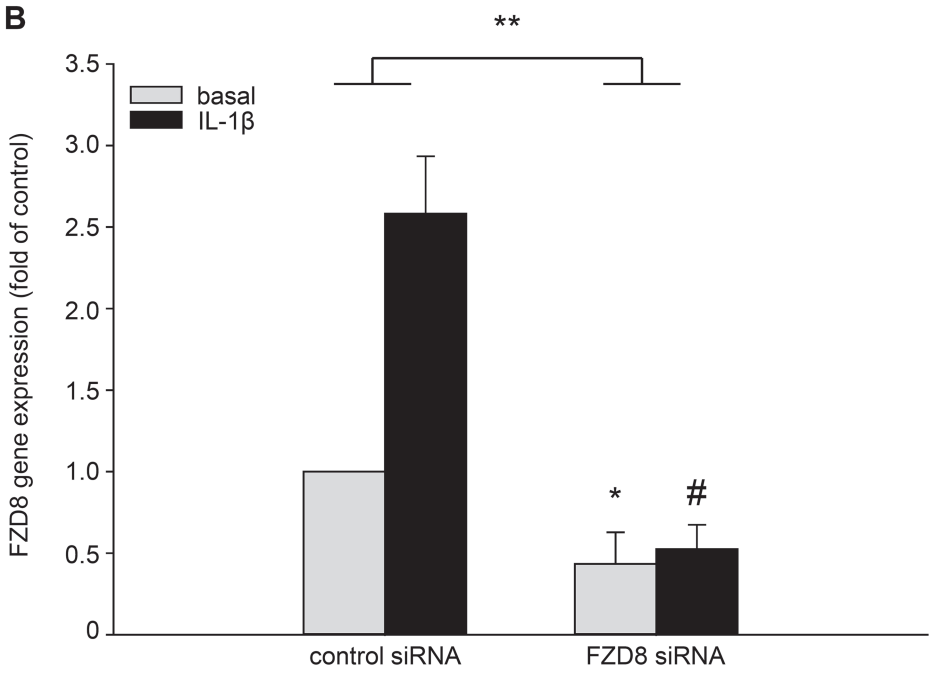

GOLD stage IV COPD
GOLD stage IV COPD with CMH
EGF effects on gene expression (fold of control)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WNT-5A
WNT-5B
WNT-16
FZD2
FZD6

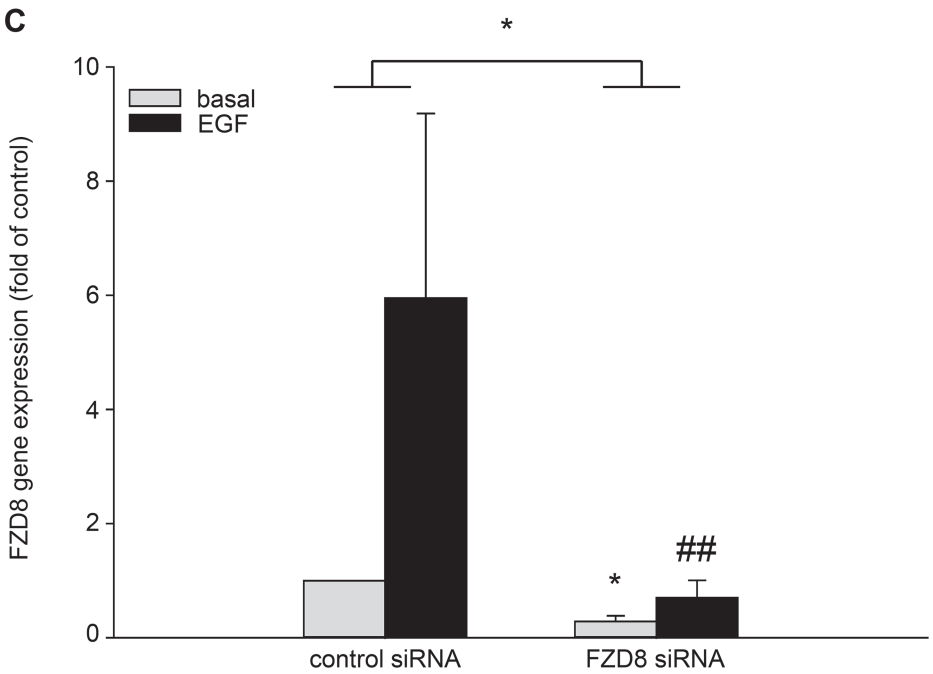
**C**


GOLD stage IV COPD
GOLD stage IV COPD with CMH
FZD8 gene expression (fold of control)
18
16
14
12
10
8
6
4
2
0
***
***
control
CSE
IL-1β
TNF-α
TGF-β
EGF


A
B
p = 0.066
IL-1β-induced FZD8 gene expression
(fold of control)
EGF-induced FZD8 gene expression
(fold of control)
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
CC
CT
genotype


A
control
FZD8 knockdown
IL-6 secretion (pg/ml)
50
40
30
20
10
0
0.0 ng/ml
0.1 ng/ml
0.3 ng/ml
1.0 ng/ml
3.0 ng/ml
10.0 ng/ml
EGF


B
**
basal
IL-1β
FZD8 gene expression (fold of control)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
#
control siRNA
FZD8 siRNA


C
*
basal
EGF
FZD8 gene expression (fold of control)
10
8
6
4
2
0
*
##
control siRNA
FZD8 siRNA