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Reduced Frizzled receptor 4 expression prevents WNT/β-catenin-driven alveolar lung repair in COPD

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Abstract

Rationale: Chronic obstructive pulmonary disease (COPD), in particular emphysema, is characterized by loss of parenchymal alveolar tissue and impaired tissue repair. WNT/β-catenin signaling is reduced in COPD, however, the mechanisms thereof, specifically the role of WNT receptors Frizzled (FZD), remain unexplored. **Objective**: To identify and functionally characterize specific FZD receptors that control downstream WNT signaling in impaired lung repair in COPD. **Methods**: FZD expression was analyzed in lung homogenates and alveolar epithelial type II (ATII) cells of never-smokers, smokers, and COPD patients, and two experimental COPD models by qRT-PCR, immunoblotting, and immunofluorescence. The functional effects of cigarette smoke on FZD4, WNT/β-catenin signaling, and elastogenic components were investigated in primary ATII cells *in vitro* and 3D lung tissue cultures (3D-LTCs) *ex vivo*. Gain- and loss-of-function approaches were applied to determine the effects of FZD4 signaling on alveolar epithelial cell wound healing and repair, and on expression of elastogenic components. **Measurements and Main Results**: FZD4 expression was reduced in human and experimental COPD lung tissues as well as primary human ATII cells from COPD patients. Cigarette smoke exposure downregulated FZD4 expression *in vitro* and *in vivo*, along with reduced WNT/β-catenin activity. Inhibition of FZD4 decreased WNT/β-catenin-driven epithelial cell proliferation, wound closure and interfered with ATII-to-ATI cell trans-differentiation and organoid formation, which was augmented by FZD4 overexpression. Moreover, FZD4 restoration by overexpression or pharmacological induction led to induction of WNT/β-catenin signaling and expression of elastogenic components in 3D-LTCs *ex vivo*. **Conclusions**: Reduced FZD4 expression in COPD contributes to impaired alveolar repair capacity.

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of death worldwide and is associated with a poor outcome and high socioeconomic burden (1). COPD is characterized by chronic airway inflammation and remodeling as well as loss of parenchymal alveolar tissue, called emphysema (2, 3). COPD initiation and development has been linked to a variety of genetic and environmental insults with cigarette smoking being one of the major risk factors (4). The mechanisms driving disease pathogenesis include oxidative stress, a protease/antiprotease imbalance and altered innate immune response leading to pulmonary inflammation, cell death and alveolar destruction (5-7). More recent data suggest that impaired endogenous lung tissue maintenance and, in particular, damaged alveolar epithelial cell repair processes further contribute to emphysema development and progression, however, the molecular mechanisms are not fully understood (6-9). The identification of novel therapeutic targets that are suitable to induce lung repair is of major interest, as to date no causal therapy is available for this devastating disease. Signal pathways that govern normal lung development represent potential targets. WNT/β-catenin signaling is known to be critical for lung morphogenesis (10, 11). The WNT pathway consists of at least 19 different WNT ligands, which are secreted glycoproteins that can bind to 10 distinct Frizzled (FZD) receptors and low density lipoprotein-related protein (LRP) 5/6 co-receptors (10, 12). WNT signaling can be divided into canonical (i.e. β-catenin dependent) and non-canonical signaling. In the case of canonical WNT/β-catenin signaling, ligand-receptor binding prevents β-catenin phosphorylation, and thus degradation. β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it binds to T-cell specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF). This in turn leads to the expression of various target genes involved, among others, in cell proliferation (12).

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We and others have demonstrated that canonical WNT/β-catenin signaling is reduced in the lung epithelium of emphysematous lung tissue from COPD patients (13-16). Activation of WNT/β-catenin by a GSK-3β inhibitor (Lithium Chloride) led to reversal of experimental emphysema *in vivo* (13), and importantly, to alveolar epithelial cell activation in COPD patient-derived 3D lung tissue cultures (3D-LTCs) (14). The mechanism underlying WNT/β-catenin signal reduction in COPD, however, has not yet been explored. Here, we investigated the role of FZD receptors that control downstream WNT signaling. We demonstrate that FZD4 is downregulated by cigarette smoke (CS) in experimental and human COPD, preventing WNT/β-catenin signal activity and thus alveolar tissue repair in COPD. These data suggest that the induction of canonical WNT signaling by targeting the WNT receptor FZD4 in the lung might lead to improved alveolar epithelial cell function and thus represents a potential beneficial approach for tissue repair in COPD patients.

Material and Methods

Human lung tissue study population

Lung resection specimens were obtained from 92 subjects, of which 78 were from surgery for solitary pulmonary tumors (Ghent University Hospital, Ghent, Belgium) and 14 were from explant lungs of end-stage COPD patients undergoing lung transplantation (University Hospital Gasthuisberg, Leuven, Belgium). Lung tissue from the resection specimen was harvested by a pathologist at maximum distance from the tumor. The cohort of 92 patients was divided into 4 subgroups: 18 never smokers, 26 smokers without airflow limitation, 34 patients with COPD GOLD stage II and 14 patients with COPD GOLD stage III-IV (Table 1). Patients were considered ex-smokers when they had ceased smoking for more than 1 year. COPD diagnosis and severity was defined using pre-operative spirometry according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification. All patients with COPD had stable disease as patients with exacerbations within 2 months before the study were excluded. Other exclusion criteria were chemotherapy or radiotherapy in the last 6 months, diagnosis of mesothelioma or asthma and infection of the upper or lower respiratory tract in the preceding 4 weeks. Our study was approved by the medical ethics committee of the Ghent University Hospital (2011/114) and the University Hospital Gasthuisberg (S51577). All subjects provided written informed consent. Primary human alveolar epithelial type II (phATII) cells were isolated from non-COPD (n=7) or COPD (n=3) lung tissue biopsies from the Comprehensive Pneumology Center (CPC) cohort at the University Hospital Grosshadern of the Ludwig Maximilian University (LMU). Participants provided written informed consent to participate in this study, in accordance with approval by the local ethics committee of the LMU, Germany (Project 333-10, 455-12).

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Animals

Six- to eight-week-old pathogen-free female C57BL/6N mice were obtained from Charles River and housed in rooms with constant temperature and humidity with 12h light cycles. Mice had free access to water and rodent chow. For the mouse model of elastase-induced emphysema, pancreatic porcine elastase was dissolved in sterile phosphate-buffered saline and applied orotracheally (80 U/kg body weight) in a total volume of 80 µl. Control mice received 80 µl of sterile phosphate-buffered saline (PBS). For the mouse model of CS-induced emphysema, animals were exposed to filtered air (FA) or mainstream CS, generated from 3R4F Research Cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY, USA), twice per day for 50 minutes, for the indicated time, as described previously (17). Mice were anesthetized and sacrificed at indicated times post-elastase instillation or CS exposure. Lung tissue was flushed, excised, snap-frozen in liquid nitrogen and stored at -80°C until further analyses. All animal studies were conducted under strict governmental and international guidelines and approved by the local government for the administrative region of Upper Bavaria (Project 55.2-1-54-2532-129-14).

Generation of human and mouse three-dimentional (3D) *ex vivo* lung tissue cultures (LTCs)

Generation of the 3D-LTCs was performed as previously described (14). In brief, for the patient-derived 3D-LTCs, lung segments were cannulated through the bronchus and filled with warm agarose (3 %, A9414; Sigma). Segments were cut with a vibratome (Hyrax V55; Zeiss, Jena, Germany) to 500 µm slices (speed 6-10 µm/s, frequency of 100 Hz, amplitude 1.2 mm). Slices were treated with FzM1 (2.5 µM) or respective DMSO control for 24h or 72h in sterile medium (DMEM/Ham's F12; Gibco, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B; Sigma). For murine 3D-LTCs, healthy six-

to eight-week-old pathogen-free female C57BL/6N mice were used. Lungs were flushed via the heart with sterile sodium chloride solution and filled with warm, low gelling temperature agarose (2 %) in sterile medium (DMEM/Ham's F12). Further, lobes were cut with a vibratome to a thickness of 300 µm slices (speed 10–12 µm/s, frequency 80 Hz, amplitude of 1 mm). 3D-LTCs were treated with FzM1 (2.5 µM), valproic acid (VA 0.5 mM; Sigma Aldrich P4543), combination of FzM1 and VA or respective DMSO control for 24h or 72h in sterile cultivation medium containing 0.1% FCS. RNA was isolated and gene expression was analyzed by qPCR. Supernatants were collected and stored at -80°C before analysis.

Detailed description of material and methods, including cigarette smoke extract preparation, cell culture and functional readouts can be found in the Supplemental Methods.

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Results

FZD4 is expressed by the alveolar epithelium and decreased in COPD

We first analyzed the expression of all FZD receptors in the mouse model of elastase-induced emphysema, which has been previously shown to exhibit reduced WNT/β-catenin signaling (13) (Fig. 1A). We found that in particular *Fzd4* and *Fzd7* expression was significantly decreased. *Fzd4* and *Fzd7* transcripts were also highly expressed in primary mouse alveolar epithelial type II (pmATII) cells and we detected FZD4 protein in cultured pmATII cells (Supplementary Fig. E1A and E1B). In order to elucidate the potential clinical relevance for altered FZD expression, we further screened for FZD expression in available microarray datasets from whole lung homogenates (GSE47460) as well as primary human alveolar epithelial type II (phATII) cell isolates from COPD and non-COPD patients (18). In both data sets, *FZD4*, but not *FZD7*, were significantly reduced (Supplementary Fig. E1C and E1D, respectively). We detected FZD4 protein expression on healthy phATII cells (Fig. 1B) and confirmed *FZD4* reduction in phATII cells obtained from lung tissue explants from COPD patients compared to donors (Fig. 1C, relative *FZD4* mRNA expression (mean ± s.d.): non-COPD -1.2±0.38, COPD -2.36±0.35, P=0.106). Notably, we confirmed *FZD4* reduction in a large cohort of patients with COPD (n=48) compared to never-smokers (n=18) as well as for severe COPD (n=14) compared to smokers without COPD (n=26) (Fig. 1D). *FZD4* mRNA expression further positively correlated with lung function (% FEV1, post-bronchodilator) (Fig. 1E and Supplementary Fig. E2E) and negatively correlated with smoking pack years (Fig. 1F) and aging (Supplementary Fig. E2) in this cohort (even when excluding samples from non-cancerous GOLD IV patients, as presented in Supplementary Fig. E2). In line with this, we observed that oxidative stress-induced senescence reduced *Fzd4* in epithelial cells *in vitro* (Supplementary Fig. E1E).

We thus focused our further studies on FZD4 as a potential receptor contributing to impaired tissue repair observed in COPD pathogenesis.

FZD4 expression is downregulated by cigarette smoke (CS) *in vivo* and *in vitro*.

Given that cigarette smoking is an important risk factor for COPD, and our data suggesting a link between CS and FZD4 expression (Fig. 1D and F), we next addressed if FZD4 is regulated by CS *in vivo* and *in vitro*. We found reduced *Fzd4* receptor mRNA expression in lung homogenates from mice exposed to CS compared to filtered air (FA) at early (3 days) as well as late (4 months) time points (Fig. 2A, relative *Fzd4* mRNA expression (mean ± s.d.): FA 3d 2.13±0.1, CS 3d 1.35±0.19, FA 4m 1.49±0.09, CS 4m 0.5±0.14). At baseline, *Fzd4* mRNA expression was also significantly reduced over time (Fig. 2A), and in old *versus* young mice (Supplementary Fig. E3A), suggesting a potential aging-associated reduction. In agreement with the mRNA expression, we found reduced FZD4 protein as early as 3 days upon CS exposure as analyzed by immunoblotting, suggesting an immediate effect of CS on FZD4 expression, which was accompanied by reduced active β-catenin (ABC) protein expression (Fig. 2B). Similarly, we found reduced FZD4 expression in the alveolar epithelium in tissue specimens of smokers compared to non smokers (Supplementary Fig. E3B). In order to investigate the effect of CS in more detail, we exposed primary mouse alveolar epithelial type II (pmATII) cells directly to CS extract (CSE) (Fig. 2D, E). CSE led to the induction of the well-known marker *Cyp1a1* as well as reduction of *Sparc* expression (19) in pmATII cells (Fig. 2D). Moreover, WNT/β-catenin target gene *Axin2* and *Fzd4* mRNA expression were decreased upon stimulation with CSE in pmATII cells as well as in MLE12 cells (Fig. 2D and Supplementary Fig. E3C, respectively) (Fig. 2D, fold change (relative mRNA expression (mean + s.d.): Axin2 -1.44+0.25, Fzd4 -1.66+0.71, $P<0.01$). FZD4 was also reduced on protein

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level in pmATII cells upon CSE exposure, as assessed by immunofluorescence staining (Fig. 2E, FZD4 expression relative to DAPI, % of 0% CSE (100%) (mean + s.d.): 25% CSE 76+10%, P<0.05) and immunoblotting (Supplementary Fig. E3D).

FZD4 is a positive regulator of canonical WNT signaling

Given that CS exposure reduced FZD4 expression along with decreased WNT/β-catenin signal activity in alveolar epithelial cells, we next aimed to elucidate if FZD4 expression directly influences WNT/β-catenin signal activity. To this end, we used the recently described inhibitor FzM1, which is an allosteric ligand of FZD4, described to hamper FZD4-Dishevelled complex that is required for β-catenin nuclear translocation (20). Treatment of pmATII cells with FzM1 led to decreased WNT/β-catenin signaling, as analyzed by target gene expression (*Axin2, Lgr5*) (Fig. 3A, log fold change (mean + s.d.): *Axin2* -1.76+0.59, *Lgr5* -2.12+0.82, P<0.05). In line with these findings, FzM1-mediated inhibition of FZD4 led to the attenuation of WNT3A-induced TCF/LEF-dependent gene transcription in MLE12 cells (Fig. 3B, TOP/FOP activation, fold of control (mean + s.d.): FzM1 1.46+0.8, WNT3A 8.55+2.3, FzM1+WNT3A 2+1.2, P<0.01). Furthermore, FzM1 decreased ABC level and reduced LRP6 phosphorylation (pLRP6) upon WNT3A treatment in MLE12 cells, as assessed by immunoblotting (Fig. 3C). Moreover, we also found decreased *AXIN2* expression by FzM1 in 3D human lung tissue cultures (3D-LTCs) *ex vivo* (Supplementary Fig. E4A). We further corroborated our findings using a gain-of-function approach (Fig. 3D and Supplementary Fig. E5). Overexpression of FZD4 led to enhanced WNT/β-catenin signal activity upon WNT3A stimulation in MLE12 cells (Fig. 3D, TOP/FOP activation FZD4 overexpression (OE) compared to empty vector (EV) (mean + s.d.): OE 1.3+0.11, EV+WNT3A

3.5+1.6, OE+WNT3A 12.1+2.5, P<0.001). Altogether, these data suggest that FZD4 is a direct regulator of canonical WNT signaling in the mouse and human alveolar epithelium.

FZD4 expression regulates alveolar epithelial wound healing and repair

Canonical WNT signaling has been implicated in lung repair, therefore we next investigated the role of FZD4 on alveolar epithelial cell function. We assessed cellular proliferation and wound healing, crucial processes involved in tissue repair upon gain- and loss-of-function of FZD4. Inhibition of FZD4 signaling by FzM1 led to decreased cell viability (Fig. 4A, compared to 100% control (mean + s.d.): FzM1 73+7%, P<0.001), decreased total cell counts (Fig. 4B, compared to 100% control (mean + s.d.): FzM1 62+17.6%, P<0.05), reduction of Ki67 in MLE12 cells (Fig. 4D, % of FZD4 positive cells (mean + s.d.): control 78.8+0.01%, FzM1 67.6+3%, P<0.05). In addition, FzM1 significantly delayed wound closure in a scratch assay (Fig. 4F, closed scratch in % (mean + s.d.): Control 73.1%+2.9%, FzM1 44.3+6.5%, P<0.05). These data were further confirmed by FZD4 overexpression which led to increased cell viability (Fig. 4C, FZD4 OE compared to EV (mean + s.d.): 123.6+11.6%, P<0.01), percentage of Ki67 positive cells (Fig. 4E, % of FZD4 positive cells (mean + s.d.): EV 45.5+0.9%, OE 56.7+6.4%, P<0.05) as well as accelerated wound closure (Fig. 4G, closed scratch in % (mean + s.d.): EV 74.9+10.3%, OE 80.7+10.4%, P<0.01).

ATII cells are capable of self-renewal and exert progenitor function for ATI cells upon alveolar epithelial injury. We next elucidated whether FZD4 expression impacts ATII cell marker expression, trans-differentiation and organoid formation. FZD4 inhibition by FzM1 led to a reduction of alveolar epithelial cell marker transcripts, such as *E-cadherin* and the ATI cell marker *T1α* (Supplementary Fig. E6A). Moreover, in the well-characterized *in vitro* model, in which ATII cells spontaneously activate WNT/β-catenin signaling and trans-

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differentiate into ATI-like cells during primary culture *in vitro* (21, 22), we observed that blocking of FZD4 by FzM1 led to a significant decrease of the ATI cell marker T1α on protein level. This was accompanied by decreased ABC and pLRP6 (both indicators of WNT/β-catenin activity) (Fig. 5A), indicating that loss of FZD4 activity results in a defect of ATII-to-ATI cell trans-differentiation, which is presumably mediated by reduced canonical WNT signaling. In addition, we observed a significantly lower number of organoids formed by ATII cells upon FzM1 treatment (Supplementary Fig. E6B). Altogether, these results indicate that FZD4 acts as a positive regulator of lung epithelial wound healing, (trans)differentiation, and organoid formation, and thus might be a potential regulator of tissue repair in the lung.

FZD4 regulates WNT/β-catenin-driven repair and elastogenic components

WNT/β-catenin signaling has been recently linked to extracellular and elastin remodeling (14, 23). Loss of elastic fibers from small airways and alveolar walls represents a main feature of COPD and elastin, which constitutes the main component of elastic fibers (24), has been shown to be regulated by cigarette smoke (25). Notably, we detected decreased elastin (*Eln*) gene expression induced by CS both, in whole lung homogenate *in vivo* and in epithelial cells *in vitro* (Fig. 6A and 6B, C respectively). While lung epithelial cells most likely do not present the main source of elastin, protein was also detected in cultured ATII cells (Supplementary Fig. E7A), suggesting that they might contribute to the elastic matrix upon injury. We further analyzed expression of elastogenic components and found that both CSE and FZD4 inhibition by FzM1 reduced gene expression of elastin fiber genes, such as *Eln*, Insulin-like growth factor 1 (*Igf1*), Lysyl Oxidase (*Lox*), Lysyl Oxidase Like 1 (*Loxl*), Fibulin1 (*Fbln1*) and Fibulin 5 (*Fbln5*) (26) (Fig. 6C and 6D, respectively) in pmATII cells. Remarkably, IGF1, which has been implicated in elastogenesis processes and shown to be

secreted by ATII cells in previous studies (27, 28) was also significantly reduced on protein level upon CSE as well as FZD4 inhibition in pmATII cells (Fig. 6E and 6F). Furthermore, we confirmed decreased expression of both *Eln* and *Igf1* in *ex vivo* 3D-LTCs upon CSE and FzM1 treatment (Fig. 6G and H, and Supplementary Fig. E8A).

Finally, we aimed to restore FZD4 function and found that FZD4 overexpression in MLE12 cells induced *Eln* transcript and was able to rescue the CSE-induced elastin reduction (Fig. 6I), however, *Igf1* expression was below detection limit in this cell line. As such, we used pmATII cells and utilized valproic acid (VA), which has been reported to induce FZD4 expression (29). Treatment of pmATII cells with VA led to a significant increase in *Fzd4* (but not *Fzd5* or *8)* transcript (Supplementary Fig. E9A), along with *Axin2* induction (Supplementary Fig. E9A). As expected, induction of Wnt/β-catenin signaling by VA was diminished by co-treatment with the FZD4 inhibitor as measured with TOP/FOP flash activity (Supplementary Fig. E9B). Accordingly, we found an increase in *Fzd4* and *Axin2* by VA treatment in *ex vivo* mouse 3D-LTCs (Fig. 6J) and while *Igf1* transcript was not significantly induced at this time point, we detected a significant increase in IGF1 protein upon VA treatment *ex vivo* (Fig. 6K). Taken together, these data support the notion that FZD4 signaling is involved in elastic matrix composition.

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Discussion

Here, we report reduced expression of the FZD4 receptor in COPD patients as well as two independent animal models of COPD. We demonstrate for the first time to our knowledge that downregulation of FZD4 within the alveolar epithelium is mediated by cigarette smoke *in vitro* and *in vivo*, leading to loss of WNT/β-catenin signal activity and subsequently reduced lung tissue repair. We show that FZD4 reduction diminishes alveolar epithelial cell wound repair capacity and contributes to reduced elastogenic component expression, features of COPD pathogenesis, and thus might represent a potential therapeutic target (Fig. 7).

Loss of functional parenchymal lung tissue is a main feature of COPD, however, the underlying mechanisms are still poorly understood. Altered WNT signal activity has been linked to COPD (13-16, 30, 31). It has been shown that WNT/β-catenin signaling is reduced in the lung epithelium in COPD and that this contributes to diminished tissue repair (13, 14) as well as airway inflammation (32). Notably, we have recently demonstrated that activation of WNT/β-catenin is feasible in patient-derived human lung tissue cultures (3D-LTCs) and induces alveolar epithelial cell activation (14). The cause for WNT/β-catenin signal reduction in emphysema and a potential involvement of specific WNT/β-catenin receptors, however, remains elusive.

WNT signaling is regulated by a large number of different, but structurally similar, WNT ligands and FZD receptors. At least 19 WNT ligands and 10 membrane-bound FZD receptors are described to transduce intracellular WNT signaling via a number of co-receptors (12). WNT/FZD interactions are proposed to be highly context dependent, however, our knowledge of specific WNT/FZD pairs remains limited (33). To date, there are

no studies investigating the functional influence of FZD4 on WNT signaling neither in the lung, nor in general. FZD4 has been reported to be involved in angiogenesis in the eye with FZD4 mutation in humans being responsible for incomplete retinal vascularization in familial exudative vitreoretinopathy (34, 35). Here, we initially found FZD4 expression to be enriched in the alveolar epithelium and show that FZD4 expression is altered by CS *in vitro* and *in vivo*. Similarly, Guo *et al*. have recently reported reduced *FZD4* mRNA in a CS-exposed bronchial epithelial cell line *in vitro* (30). Notably, expression analysis in our cohort of never-smokers, smokers and COPD patients, revealed that FZD4 expression is even further decreased in late stage COPD patients compared to smokers, suggesting additional COPD-related mechanisms driving FZD4 reduction.

COPD has been proposed to be a disease of accelerated aging (36). Interestingly, *FZD4* expression correlated with age in the human and murine lung, further indicating that WNT signaling also impacts aging processes and might be involved in cellular senescence (36, 37). We have recently reported a canonical to non-canonical WNT signal switch in COPD (38), which might be driven by aging (39). We provide several lines of evidence that FZD4 is mainly mediating canonical WNT/β-catenin signaling, which is in line with previous reports (33, 40), and thus suggesting that FZD4 reduction might contribute to a WNT signal shift by inhibiting WNT/β-catenin signaling. Nevertheless, we cannot rule out that FZD4 reduction also impacts non-canonical WNT signaling and further studies are needed to shed light onto WNT receptor mediating non-canonical WNT signaling in alveolar epithelial cell repair.

ATII cells play a major role in lung homeostasis and can furthermore serve as a progenitor pool for ATII and ATI cells after alveolar injury (41, 42). Gain-and loss of function studies of FZD4 not only modulated WNT/β-catenin signaling in alveolar epithelial cells, but further led to several functional effects, such as wound healing, differentiation of the

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alveolar epithelium as well as organoid formation. These data are in line with previous findings describing an imbalance of alveolar epithelial cell apoptosis/proliferation in end-stage emphysema (43) and strongly indicate that FZD4 signaling is relevant for alveolar epithelial cell repair.

Breakdown of elastic fibers in the lung is one of the main features of COPD and overall alveolar fiber content has been shown to positively correlate with lung function parameters (44). Elastic fibers are insoluble extracellular matrix (ECM) assemblies that consist of an elastin core and a mantle of fibrillin-rich microfibrils (45). Cross-linking of tropoelastin monomers, a critical step to generate an insoluble elastin polymer, is initiated by the lysyl oxidase family of enzymes (Lox and Loxl 1-4). Inflammatory mediators such as macrophage elastase (MMP-12) can modulate elastin gene expression and subsequently decrease accumulation of elastic fibers in tissues (6, 7). CS is known to increase the elastolytic activity of macrophages (25, 46), impair the production of lysyl oxidase and alter elastin re-synthesis in elastase-induced emphysema (47) and has been shown to decrease protein elastin content *in vivo (48)* Similarly, we observed that CS decreased elastin expression in a COPD model *in vivo* and downregulated expression of elastogenic components, such as Loxl, *in vitro.* Notably, blocking of FZD4 mimicked this alteration in alveolar epithelial cells *in vitro*. Moreover, IGF1, which is an epithelial cell-derived proelastogenic cytokine reduced in COPD (49-51) , was also reduced upon both CSE and FZD4 blockade. To evaluate the potential role for FZD4 signaling to enhance lung repair processes, we applied two different approaches, FZD overexpression *in vitro* and induction of FZD4 by valproic acid (VA) *ex vivo*. FZD4 overexpression was able to rescue the CSE-induced decrease in elastin expression, however we could not detect all elastogenic components in the used cell line. VA is an FDA approved drug for seizures, and manic or

mixed episodes associated with bipolar disorder. It has been linked to increases in FZD4 expression (29) and could be used for treatment of primary cells and tissue. VA treatment led to FZD4-dependent induction of WNT signal activity and increased expression of IGF1, decreased in COPD patients (49-51). However, we cannot exclude that VA additionally acts on other signaling pathways contributing to the observed effects.

FZD4 is the first WNT receptor that we identified to be involved in alveolar epithelial cell repair. Recently, it has been reported that FZD8 is expressed by lung fibroblast and exhibits a pro-inflammatory role in chronic bronchitis (31). Moreover, we found that FZD8 on fibroblasts also mediates pro-fibrotic TGF-β signaling in lung fibrosis (52). Our data suggest that other FZD receptor are also differentially regulated in COPD and thus might very well be involved in mechanisms of lung injury and repair. One can hypothesize that these FZDs might be enriched and/or regulated on other cell types, such as mesenchymal or inflammatory cells, that are further needed for proper lung repair. Distinct WNT/FZD signals seem to be cell-specific, thus highlighting the potential of these membrane-bound receptors as therapeutic targets.

The study presented here identifies reduced FZD4 signaling as an important contributor to impaired lung repair in COPD using several different experimental setups *in vivo*, *ex vivo* and *in vitro*. Certain limitations of the study, however, require further investigations. COPD is a very complex disease with several factors contributing to the pathogenesis. In this study, we mainly focus on the impact of the main risk factor cigarette smoke on FZD4 expression and subsequent alveolar epithelial cell repair, however, the role of FZD4 needs to be explored further in the context of other hallmarks of COPD, such as inflammation and tissue remodeling. We confirmed that FZD4 impacts WNT/β-catenin signaling and elastogenic mediators in 3D *ex vivo* lung tissue cultures (3D-LTCs) from

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precision lung cut slices (PCLS), which enables the investigation within the intact 3D lung architecture and presence of several structural cell types (14). Moreover, this novel method represents an important alternative to *in vivo* animal models. The application of 3D-LTCs holds promise to reduce overall animal experiments, thus fulfilling 3Rs (Replacement, Reduction and Refinement) recommendations from the European Parliament (53). Nevertheless, studies genetically modifying FZD4 signaling in mice *in vivo* will be of high interest to further elucidate feature of lung tissue repair including changes in elastic fiber composition and corroborate the FZD4 as a potential therapeutic target for COPD.

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Table 1

Characteristics of study population (RT-PCR study) (n = 92)

	never smokers	smokers without COPD	COPD GOLD II	COPD GOLD III-IV
Number	18	26	34	14
Gender ratio (male/female)	6/12 #	19/7 #	31/3 #	8/6 #
Age (years)	65 (56-70)	63 (55-70)	66 (58-69)§	56 (54-60)*§†
Current- / ex-smoker	-	16/10	22/12	0/14
Smoking history (PY)	0 (0-0)	28 (15-45)*	45 (40-60)*§	30 (25-30)*†
FEV1 post (L)	2,7 (2,3-3,2)	2,7 (2,3-3,3)	2,0 (1,8-2,4)*§	0,7 (0,7-0,9)*§†
FEV1 post (% predicted)	102 (92-116)	95 (93-112)	68 (61-75)*§	26 (20-32)*§†
FEV1 / FVC post (%)	78 (75-83)	75 (71-79)*	56 (53-60)*§	32 (27-35)*§†
D_{LCO} (% predicted)	90 (80-105)	80 (61-102)	67 (51-87)*	35 (33-41)*§†
KCO (% predicted)	103 (88-123)	91 (68-107)*	87 (62-108)*	59 (50-65)*§†
ICS (yes/no)	0/18 #	1/25 #	15/19 #	13/1 #

Footnote

PY (pack years); FEV1 (forced expiratory volume in 1 second); FVC (forced vital capacity); DLCO (diffusing capacity of the lung for carbon monoxide); ICS (inhaled corticosteroids)

Data are presented as median (IQR)

Mann-Whitney U test: * $P < 0.05$ versus never smokers; § $P < 0.05$ versus smokers without COPD; † $P < 0.05$ versus COPD GOLD I-II

Fisher's exact test: # $P < 0.001$

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Figure legends

Figure 1: FZD4 is expressed in the alveolar epithelium and decreased in COPD

(A) *Fzd* expression in whole lung homogenate obtained at day 2, 5 or 7 post PBS or Elastase instillation in C57BL/6 mice; relative gene expression ΔCt (ΔCt = Ct Hprt - Ct gene of interest) is presented as mean ± s.d. (n=3-6). Means were compared using one-way Anova followed by Newman-Keuls's multiple comparison test. (B) Immunofluorescence staining for FZD4 protein expression in phATII cells. The scale bar represents 20 µm. (C) *FZD4* gene expression in phATII cells isolated from non-COPD and COPD patients; relative gene expression ΔCt is presented as mean ± s.d. (non COPD n=7; COPD n=3). Means were compared using the Student's t-test. (D) *FZD4* expression in lung resection specimens from 92 subjects: 18 never smokers, 26 smokers without airflow limitation, 34 patients with COPD GOLD stage II and 14 patients with COPD GOLD stage III-IV; relative gene expression ΔCt (corrected by a normalization factor that was calculated based on the expression of the three reference genes) is presented as mean ± s.e.m. Means were compared using Mann-Whitney U test. (E) Correlation between *FZD4* expression in the lung and the lung function parameter $\%FEV_1$. (F) Correlation between *FZD4* expression in the lung and pack-years (PY). Significance: *p<0.05, **p<0.01, ***p<0.001.

Figure 2: FZD4 expression is downregulated by cigarette smoke *in vivo* and in *vitro*

(A) *Fzd4* gene expression in whole lung homogenate obtained from C57BL/6 mice exposed to filtered air (FA) or CS for 3 days or 4 months; relative gene expression ΔCt is presented as mean ± s.d. (n=4-6). Means were compared using one-way Anova followed by Newman-Keuls's multiple comparison test. (B) Representative immunoblot showing FZD4 and active β-catenin (ABC) protein expression in whole lung homogenate obtained from C57BL/6 mice

exposed to filtered air (FA) or cigarette smoke (CS) for 3 days, quantified relatively to β-actin expression; data are presented as mean ± s.d. (n=4). Means were compared using the Student's t-test. (C) Representative image of FZD4 immunofluorescence stainings on pmATII cells at day 2 of culture. The scale bar represents 20 µm. (D) Gene expression in pmATII cells exposed to 0% or 25% cigarette smoke extract (CSE) for 24h. Gene expression ΔΔCt is presented as mean + s.d. (n=4). Means were compared using the paired Student's t-test. (E) Representative images of immunofluorescence staining for FZD4 and CDH1 (E-CAD) in pmATII cells exposed to 0% or 25% CSE for 24h; overall expression of the FZD4 protein was quantified in comparison to the number of DAPI nuclear staining, presented as mean + s.d. (n=4), 0% CSE serves as 100%. Means were compared using the paired Student's t-test. Significance: *p<0.05, **p<0.01, ***p<0.001.

Figure 3: FZD4 is a positive regulator of canonical WNT signaling

(A) mRNA expression of canonical WNT target genes *Axin2* and *Lgr5* in pmATII cells treated with FzM1 (2.5 µM) for 24h. Gene expression ΔΔCt is presented as mean + s.d. (n=3). Means were compared using paired Student's t-test. (B) Determination of β-catenin-dependent gene transcription (TOP/FOP flash assay) in MLE12 cells pre-treated with FzM1 (10 µM) and stimulated with WNT3A (100 ng/ml); data are presented as mean + s.d. (n=3). Means were compared using one-way Anova followed by Newman-Keuls's multiple comparison test. (C) Representative immunoblot showing active β-catenin (ABC) protein level and LRP6 phosphorylation (pLRP6) in MLE12 cells pre-treated with FzM1 for 20 minutes and stimulated with WNT3A for 3h; quantified relatively to β-actin expression; data presented as mean + s.d. (n=5). Means were compared using one-way Anova followed by Newman-Keuls's multiple comparison test. (D) Determination of β-catenin-dependent gene

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transcription in MLE12 cells transfected with empty vector (EV) or FZD4 overexpressing plasmid (OE) and treated with WNT3A (100 ng/ml); data are presented as mean + s.d. (n=3). Means were compared using one-way Anova followed by Newman-Keuls's multiple comparison test. Significance: *p<0.05, **p<0.01, ***p<0.001.

Figure 4: FZD4 expression regulates alveolar epithelial cell proliferation and wound closure

(A) Influence of FZD4 expression on the viability of lung epithelial cells determined by WST-1 assay in MLE12 cells treated with FzM1 (5 µM) for 24h, data are presented as mean + s.d. (n=4). (B) MLE12 cell number after 24h treatment without or with FzM1 (5 µM); data are presented in comparison to control (100%) as mean + s.d. (n=3). Means were compared using the Student's t-test. (C) Influence of FZD4 expression on the viability of lung epithelial cells determined by WST-1 assay in MLE12 cells transfected with EV or OE; data are presented as mean + s.d. (n=4). (D) Representative images and quantification of Ki67 immunofluorescence staining on MLE12 cells treated with DMSO (Ctrl) or FzM1; data are presented as mean + s.d. (n=3). The scale bar represents 50 µm. (E) Representative images and quantification of Ki67 immunofluorescence staining on MLE12 cells transfected with EV or OE; data are presented as mean + s.d. (n=3). Please note the reduced baseline proliferation upon transfection in comparison to DMSO control in (D). The scale bar represents 50 µm. (F) Representative images and quantification of wound size in MLE12 cells treated with DMSO or FzM1; data are presented as mean + s.d. (n=3). (G) Representative images and quantification of wound size in MLE12 cells transfected with EV or OE; data are presented as mean + s.d. (n=4). Significance: *p<0.05, **p<0.01, ***p<0.001.

Figure 5: FZD4 alters ATII-to-ATI cell trans-differentiation processes

(A) Representative immunoblot showing protein levels of active β-catenin (ABC), pLRP6, T1α (ATI epithelial marker) in pmATII cells treated with DMSO or FzM1 (2.5 µM) for 24h or 72h; quantified relatively to β-actin expression; data are presented as mean + s.d. relatively to respective control (n=3-4). Means were compared using Student's t-test. Significance: *p<0.05, **p<0.01, ***p<0.001.

Figure 6: FZD4 expression influences elastogenic components

(A) *Eln* gene expression in whole lung homogenate of cigarette smoke exposed mice. Relative gene expression ΔCt is presented as mean ± s.d. (n=3-5). Means were compared using one-way Anova followed by Newman-Keuls's multiple comparison test. (B) *Eln* gene expression in MLE12 cells exposed to CSE or treated with FzM1. Gene expression ΔΔCt is presented as mean + s.d. (n=3-4). Means were compared using the paired Student's t-test. (C) *Eln, Igf1, Lox, Loxl, Fbln1, Fbln5* gene expression in pmATII cells treated with FzM1. Gene expression ΔΔCt is presented as mean + s.d. (n=3). Means were compared using the paired Student's t-test. (D) *Eln, Igf1, Lox, Loxl, Fbln1, Fbln5* gene expression in pmATII cells exposed to CSE. Gene expression ΔΔCt is presented as mean + s.d. (n=3). Means were compared using the paired Student's t-test. (E) Relative IGF1 protein secretion in pmATII cells exposed to CSE. Data presented as mean + s.d. (n=4). Means were compared using the paired Student's t-test. (F) Relative IGF1 protein secretion in pmATII cells treated with FzM1. Data presented as mean + s.d. (n=5). Means were compared using the paired Student's t-test. (G) *Eln* and *Igf1* gene expression in mouse PCLS exposed to CSE for 24h or 72h. Gene expression ΔΔCt is presented as mean + s.d. (n=3). Means were compared using the paired Student's t-test. (H) *Eln* and *Igf1* gene expression in mouse PCLS treated with FzM1 for 24h or 72h. Gene expression ΔΔCt is presented as mean + s.d. (n=3). Means were compared using the paired

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Student's t-test. (I) *Eln* gene expression in MLE12 cells transfected with OE FZD4 and exposed to CSE; gene expression ΔΔCt is presented as mean + s.d. (n=3-5). Means were compared using one-way Anova followed by Newman-Keuls's multiple comparison test. (J) Gene expression upon VA treatment in mouse 3D-LTCs. ΔΔCt is presented as mean + s.d. (n=3). (K) Relative IGF1 protein secretion in mouse 3D-LTCs. Data presented as mean ± s.d. (n=3). Means were compared using the paired Student's t-test. Significance: *p<0.05, **p<0.01, ***p<0.001.

Figure 7: Schematic representation of the proposed contribution of FZD4 signaling to impaired tissue repair in COPD.

Heathy lung: in the presence of FZD4 receptor in the alveolar lung epithelium WNT/β-catenin signaling maintains cell proliferation and tissue repair. COPD: decreased expression of FZD4 receptor induced by cigarette smoke and additional environmental/genetic factors, leads to reduced WNT/β-catenin signaling and inhibition of epithelial cell function and components of the elastic matrix, thus contributing to impaired tissue repair and emphysema development.

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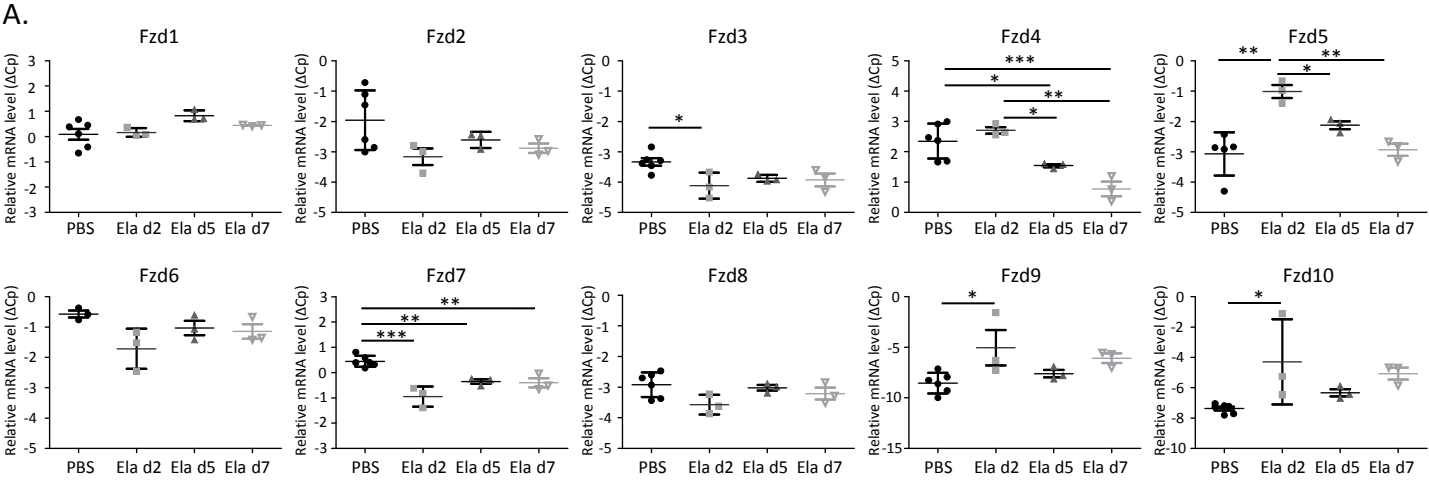
A.



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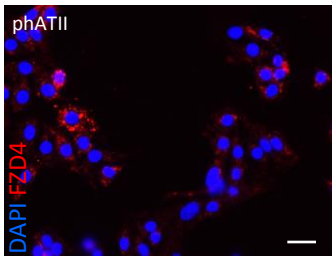

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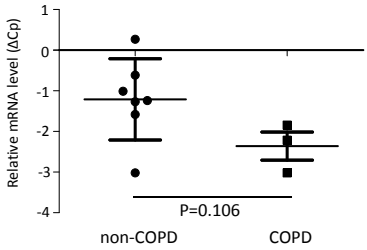

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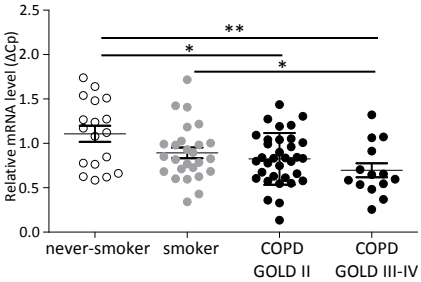

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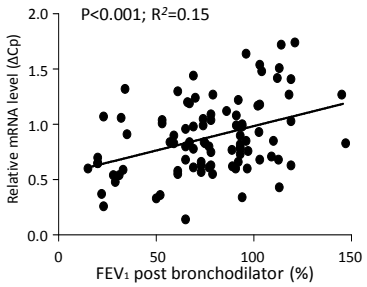

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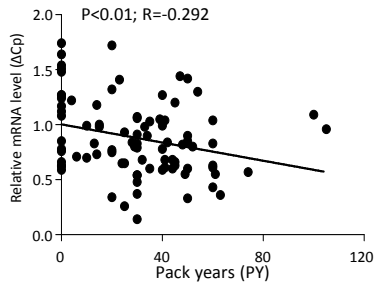

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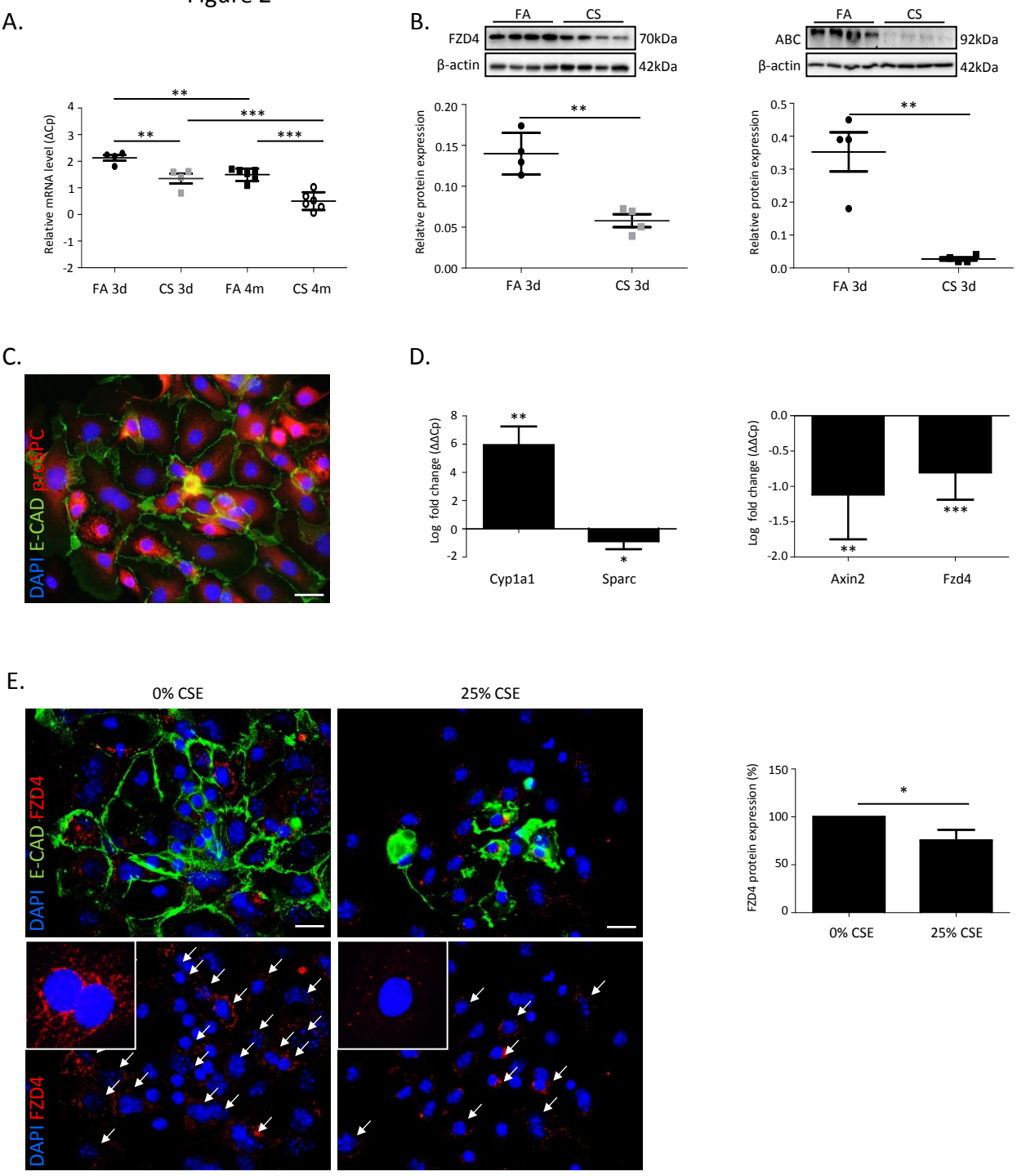
Figure 2



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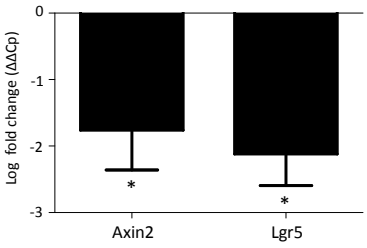

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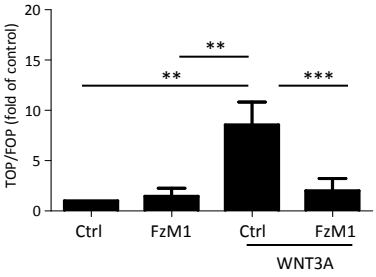

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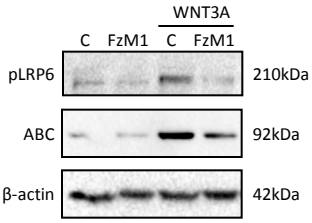

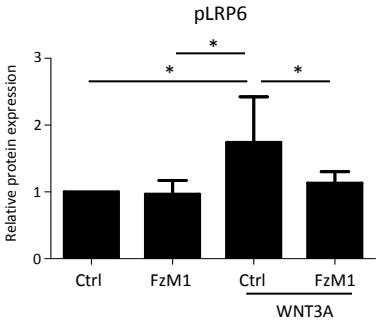

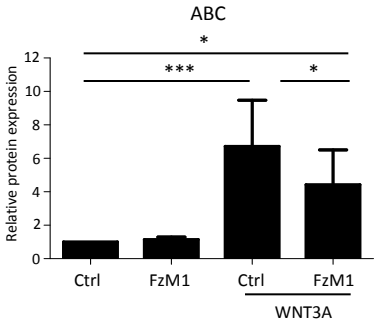

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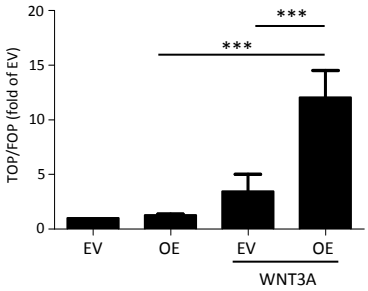

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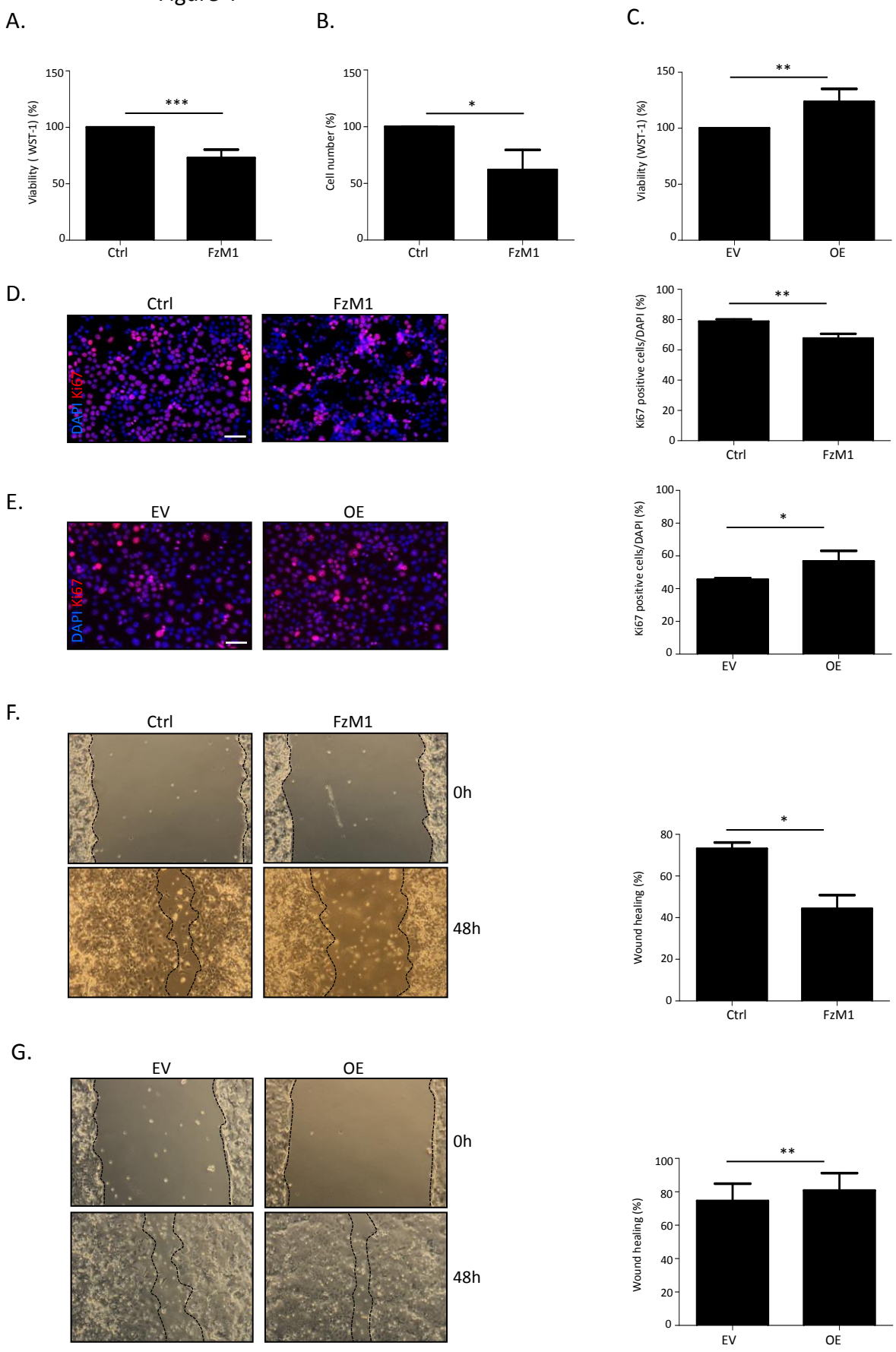
Figure 4



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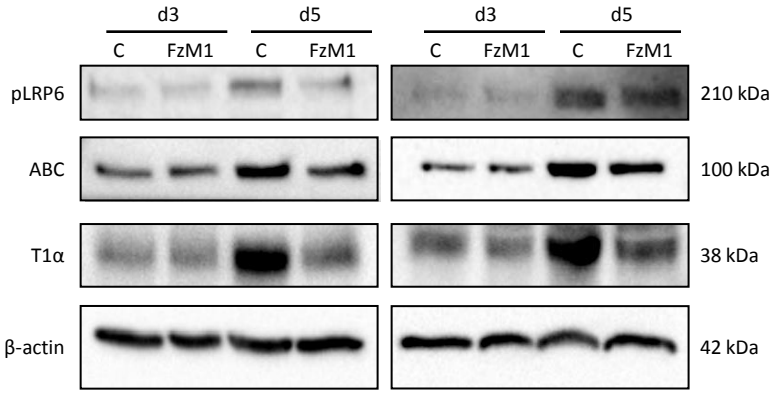

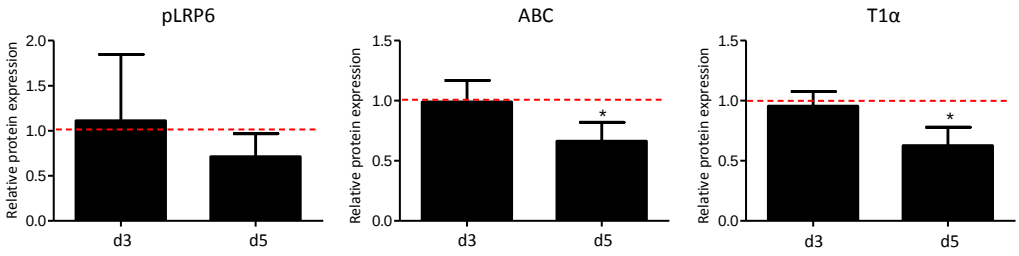

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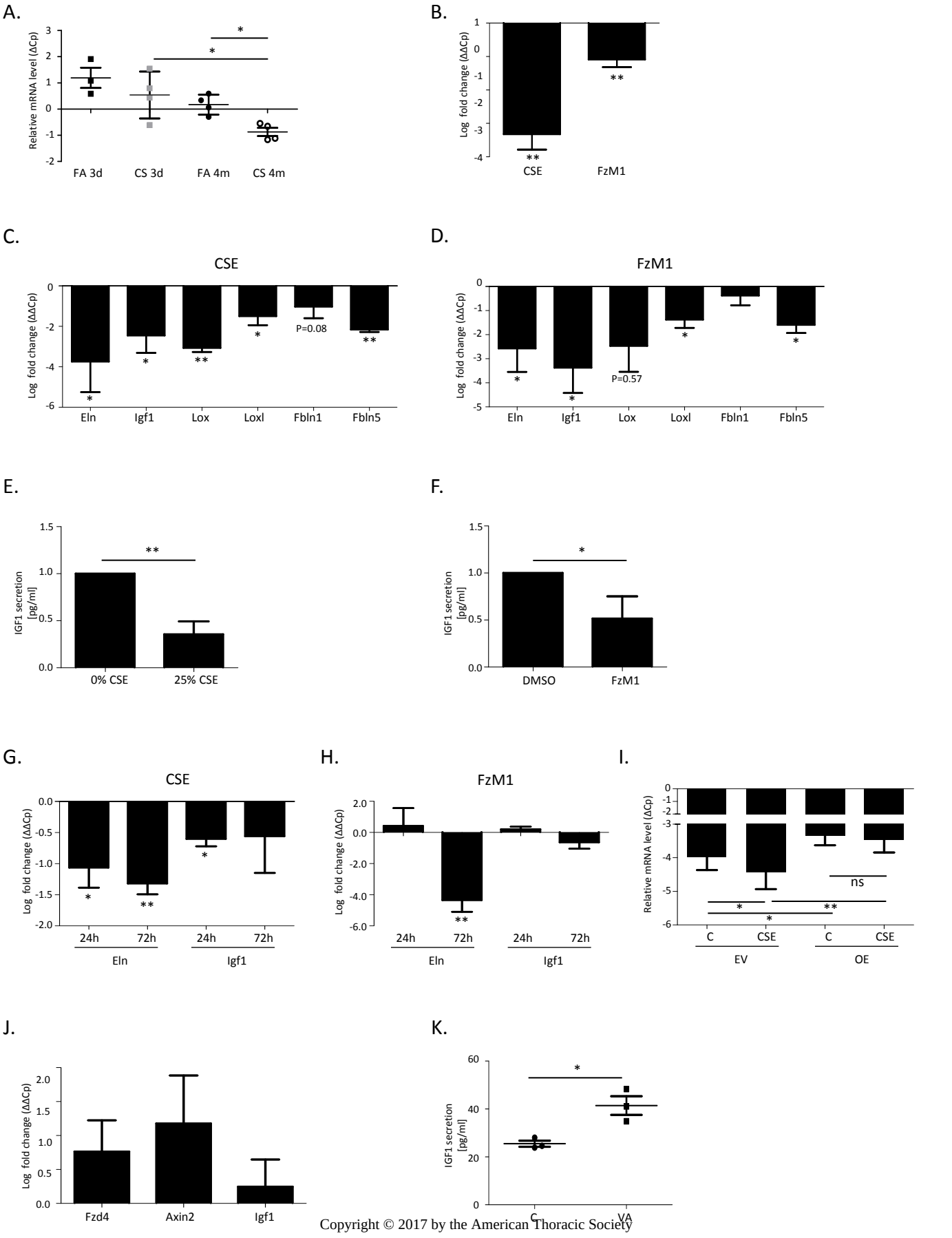
Figure 6



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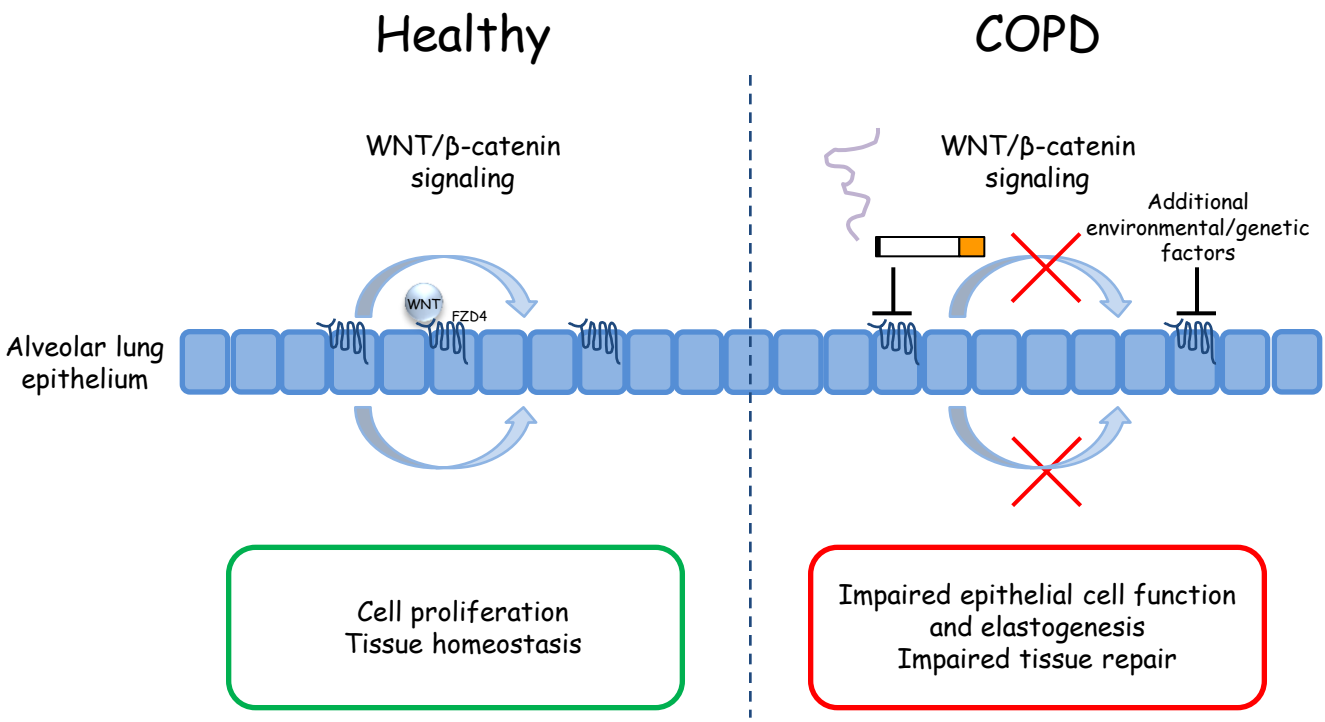

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Online Data Supplement

Reduced Frizzled receptor 4 expression prevents WNT/β-catenin-driven alveolar lung repair in COPD

Wioletta Skronska-Wasek, Kathrin Mutze, Hoeke A. Baarsma, Ken R. Bracke, Hani N. Alsafadi, Mareike Lehmann, Rita Costa, Mariano Stornaiuolo, Ettore Novellino, Guy G. Brusselle, Darcy E. Wagner, Ali Ö. Yildirim, Melanie Königshoff

Table E1

Sequences of primers

Gene name	Gene sequence
mAxin2	5‘-AGCAGAGGGACAGGAACCA-3‘ 5‘-CACTTGCCAGTTTCTTTGGCT-3‘
mCdh1	5‘-CCATCCTCGGAATCCTTGG-3‘ 5‘-TTTGACCACCGTTCTCCTCC-3‘
mCyp1a1	5‘-TCCTGAAGAGTGCTCTGGGT-3‘ 5‘-TAAACCATTTGGGAAGGCTG-3‘
mElastin	5‘-GGCGTCTTGCTGATCCTCT-3‘ 5‘-ATAATAGACTCCACCGGGAACT-3‘
mFzd1	5‘-AAACAGCACAGGTTCTGCAAAA-3‘ 5‘-TGGGCCCTCTCGTTCCTT-3‘
mFzd2	5‘-TCCATCTGGTGGGTGATTCTG -3‘ 5‘-CTCGTGGCCCCACTTCATT -3‘

mFzd3	5‘-GCCTATAGCGAGTGTTCAAAACTCA -3‘ 5‘-TGGAAACCTACTGCACTCCATATCT -3‘
mFzd4	5‘-TCCAGCCAGCTGCAGTTCTTCC -3‘ 5‘-CTGAAAGGCACATGCCACCGC -3‘
mFzd5	5‘-CCCACCGCACGTTTTCC -3‘ 5‘-GCTTTTCATTTCGCTTCTTGTTATC -3‘
mFzd6	5‘-GTTCTACCCTGTCGGAAATTGTG -3‘ 5‘-GTGGATGAGAAGTTACAGGAACAGTGT -3‘
mFzd7	5‘-GCCAGGTGGATGGTGACCTA -3‘ 5‘-CCGCAATGCATCCACACTAG -3‘
mFzd8	5‘-GCAAGGAGGCCCAACTAAGAC -3‘ 5‘-GAGGCCCAAGCGGATCA -3‘
mFzd9	5‘-CCCATCATGGAGCAATTCAATT -3‘ 5‘-GGGAGCCGGGCACAGT -3‘
mFzd10	5‘-ACGAGATGCTGGGACTGACC-3‘ 5‘-AACCGCATTTGGCGTTACAT-3‘
mHprt	5‘-CCTAAGATGAGCGCAAGTTGAA-3‘ 5‘-CCACAGGACTAGAACACCTGCTAA-3‘
mIgf1	5‘-ACACCTCTTCTACCTGGCGCTC-3‘ 5‘-ATAGCCTGTGGGCTTGTTGAAGT-3‘
mLgr5	5‘-GCGTTCACGGGCCTTCACAG-3‘

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	5‘-GGCATCTAGGCGCAGGGATTGA-3‘
mLox	5‘-GTCACCAACATTACCACAGCATGG-3‘ 5‘-GCCTTCAGCCACTCTCCTCTGT-3‘
mLoxl	5‘-ACTTTCTCCCCAACCGGCCA-3‘ 5‘-CCTTGTGTCCCTCGGCTACCTT-3‘
mSparc	5‘-AAACATGGCAAGGTGTGTGA-3‘ 5‘-AAGTGGCAGGAAGAGTCGAA-3‘
mT1α	5‘-ACAGGTGCTACTGGAGGGCTT-3‘ 5‘-TCCTCTAAGGGAGGCTTCGTC-3‘
huAXIN2	5‘-AGAAATGCATCGCAGTGTGAAG-3‘ 5‘-GGTGGGTTCTCGGGAAATG-3‘
huELN	5‘-GGCCATTCCTGGTGGAGTTCC-3‘ 5‘-AACTGGCTTAAGAGGTTTGCCTCC-3‘
huFZD4	5‘-GACAACTTTCACACCGCTCATC-3‘ 5‘-CCTTCAGGACGGGTTCACA-3‘
huHPRT	5‘-AAGGACCCCACGAAGTGTTG-3‘ 5‘-GGCTTTGTATTTTGCTTTTCCA-3‘

Table E2

Gene Expression Assays for the housekeeping genes Applied Biosystems

Gene	ID
huGAPDH	Hs99999905_m1
huHPRT1	Hs99999909_m1
huSDHA	Hs00417200_m1

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Table E3

Antibodies

Antibody	Company and number
Active β-catenin (ABC)	Millipore 05-665
β-actin	Sigma A3854
CDH1 (E-CAD)	BD 610181
Tropoelastin	Kindly provided by Dr. Robert Mecham, Washington University
FZD4	Millipore 07-2166
Ki67	Dako M7249
pLRP6	Cell signalling 2568S
T1α	R&D AF3244

Online Data Supplement

Supplementary Figure E1: FZD4 is highly expressed in the lung epithelium and decreased in whole homogenate and phATII cells from COPD patients

(A) *Fzd* expression in pmATII cells; relative gene expression ΔCt presented as mean + s.d. (n=3). (B) Immunofluorescence staining for FZD4 protein expression in pmATII cells. The scale bar represents 20 µm. (C) *FZD* expression in whole lung homogenate of COPD patients (published microarray GSE47460); data are presented as mean ± s.e.m. (non-COPD n=91; COPD n=144) of normalized array values. Means were compared using the Student's t-test. (D) *FZD* expression in phATII cells from non-COPD and COPD patients (published microarray GSE29133); expression of genes in COPD patients is presented relatively to non-COPD patients as mean + s.e.m. (non-COPD n=3; COPD n=3). (E) pmATII cells treated with H_2O_2, relative gene expression ΔCt presented as mean + s.d. (n=5). Significance: *p<0.05, **p<0.01, ***p<0.001

Supplementary Figure E2: Correlation between FZD4 expression and different parameters in human lung tissue (excluding COPD GOLD IV patients)

Correlations were performed in a cohort of 92 subjects: 18 never smokers, 26 smokers without airflow limitation, 34 patients with COPD GOLD stage II and 14 patients with COPD GOLD stage III-IV; Correlation between *FZD4* mRNA expression and (A) % FEV_1, (B) FEV_1/FVC, (C) DLCO, (D) pack-years (PY), and (E) age, are depicted.

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Supplementary Figure E3: Cigarette smoke decreases canonical WNT signaling and FZD4 expression *in vivo* and *in vitro*

(A) *Fzd4* expression in the whole lung homogenates obtained from young (≤3 months, n=8) or old (≥12 months, n=10) mice. Relative gene expression ΔCt is presented as mean ± s.e.m. Means were compared using the Student's t-test. (B) Immunofluorescence staining for FZD4 protein expression in human lung tissue from non smokers and smokers. The scale bar represents 20 µm. (C) Gene expression in MLE12 cells exposed to 0% or 25% CSE for 24h. Gene expression ΔΔCt is presented as mean + s.d. (n=4). Means were compared using the paired Student's t-test. Significance: *p<0.05, **p<0.01, ***p<0.001. (D) Immunoblot showing FZD4 expression in pmATII cells exposed to CSE for 24h and its quantification (n=2).

Supplementary Figure E4: Blockade of FZD4 decreases Axin2 in human 3D-LTCs

(A) Axin2 gene expression in human 3D-LTCs upon FZD4 blockade with FzM1, presented as ΔCt (n=3) and the effect assessed with Student's t-test. Significance: *p<0.05, **p<0.01, ***p<0.001.

Supplementary Figure E5: FZD4 overexpression efficiency

FZD4 overexpression efficiency was evaluated by (A) qPCR, gene expression is presented as ΔCt and (B) immunofluorescence staining on MLE12 cells. Means were compared using one-way Anova followed by Newman-Keuls's multiple comparison test. Significance: *p<0.05, **p<0.01, ***p<0.001.

Supplementary Figure E6: FZD4 inhibition by FzM1 negatively regulates the expression of epithelial markers in pmATII cells and inhibits organoid formation

(A) *E-cadherin (Cdh1)* and *T1α* epithelial marker expression was measured in pmATII cells stimulated with FzM1 (n=3) by qPCR. Gene expression is presented as mean + s.d. Means were compared using the paired Student's t-test. (B) Representative pictures of organoids formed by pmATII cells at day 7 of culture and quantification of organoid numbers. Data presented as mean + s.d. (n=3). Means were compared using the paired Student's t-test. Significance: *p<0.05, **p<0.01, ***p<0.001.

Supplementary Figure E7: Elastin expression in the lung

(A) Immunoblot showing Tropoelastin protein expression in cultured pmATII cells at day 4 and in mouse lung homogenate.

Supplementary Figure E8: Cigarette smoke effect on 3D-LTCs

(A) *Cyp1α1* and *Sparc* gene expression in mouse 3D-LTCs generated from precision cut lung slices exposed to CSE for 24h or 72h. Gene expression ΔΔCt presented as mean + s.d. (n=3). Means were compared using the paired Student's t-test. Significance: *p<0.05, **p<0.01, ***p<0.001.

Supplementary Figure E9: VA induces FZD4 expression and WNT signaling in lung epithelial cells

(A) *Fzd 4, 5, 8* and *Axin2* gene expression in pmATII cells treated with VA for 24h. Relative gene expression ΔCt presented as mean + s.d. (n=3). (B) Determination of β-catenin-dependent gene transcription (TOP/FOP flash assay) in MLE12 cells

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stimulated with FzM1**,** VA or combination thereof for 24h. Data presented as mean + s.d. (n=3). Means were compared using one-way Anova followed by Newman-Keuls's multiple comparison test. Significance: *p<0.05, **p<0.01, ***p<0.001.

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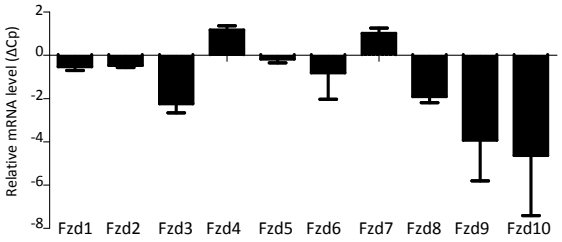

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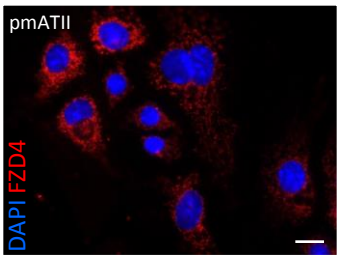

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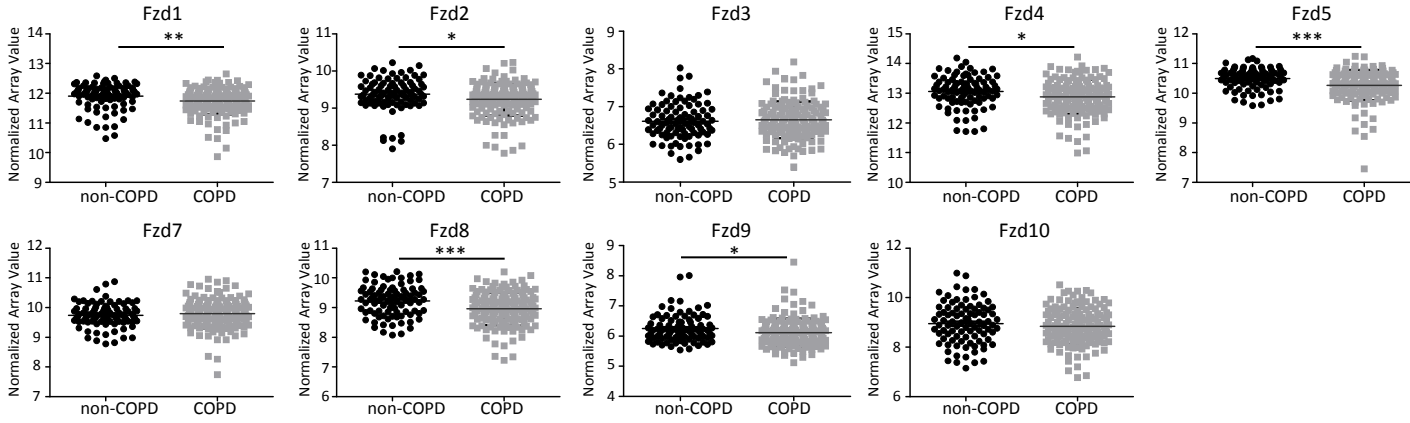

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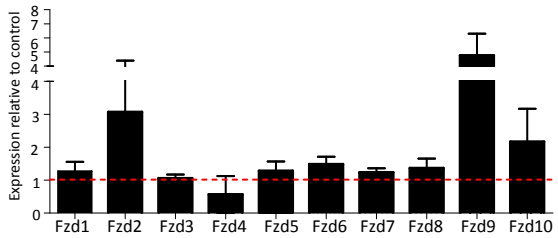

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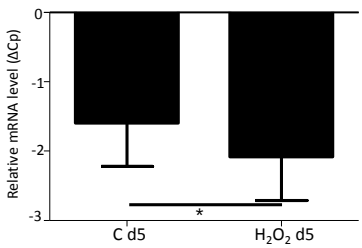

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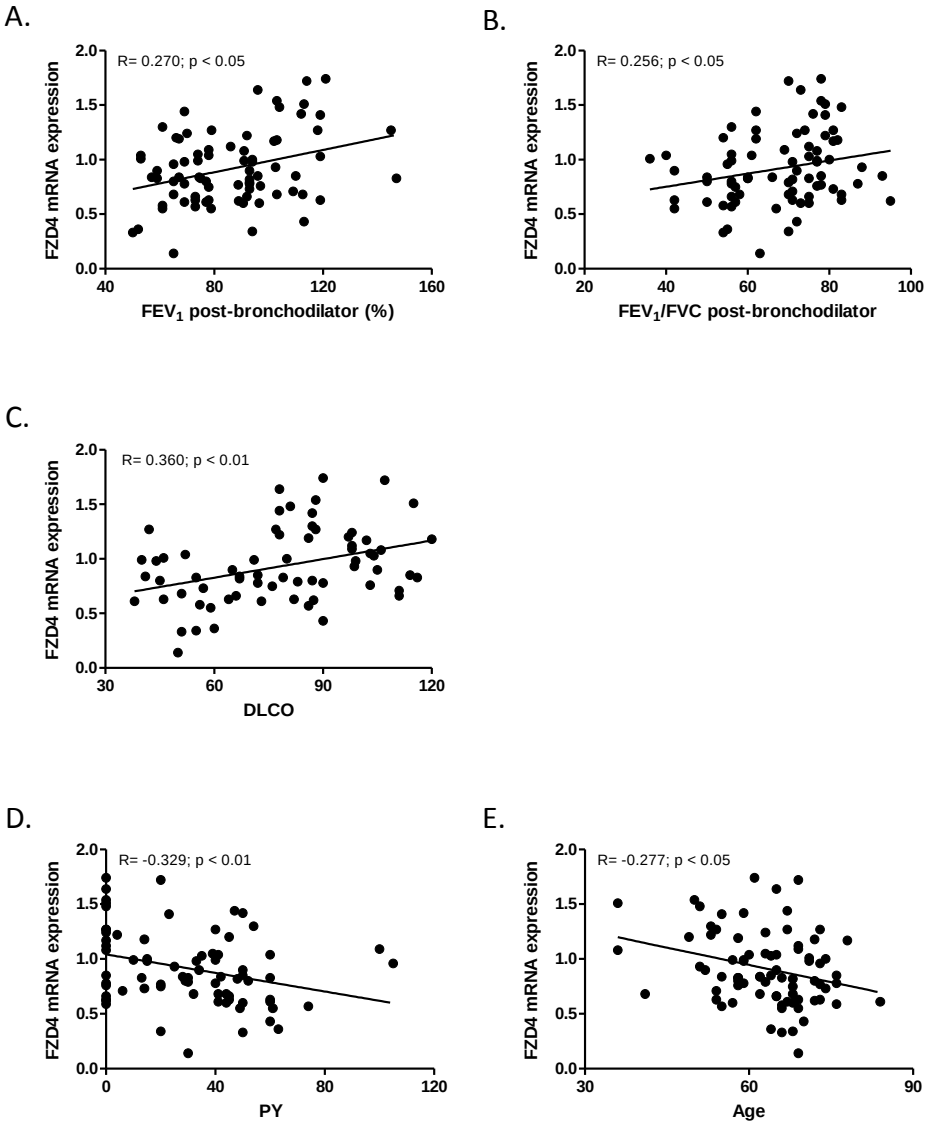
Supplementary Figure E2



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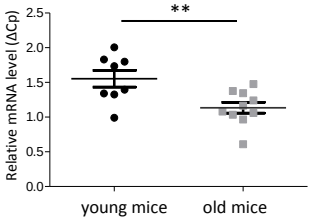

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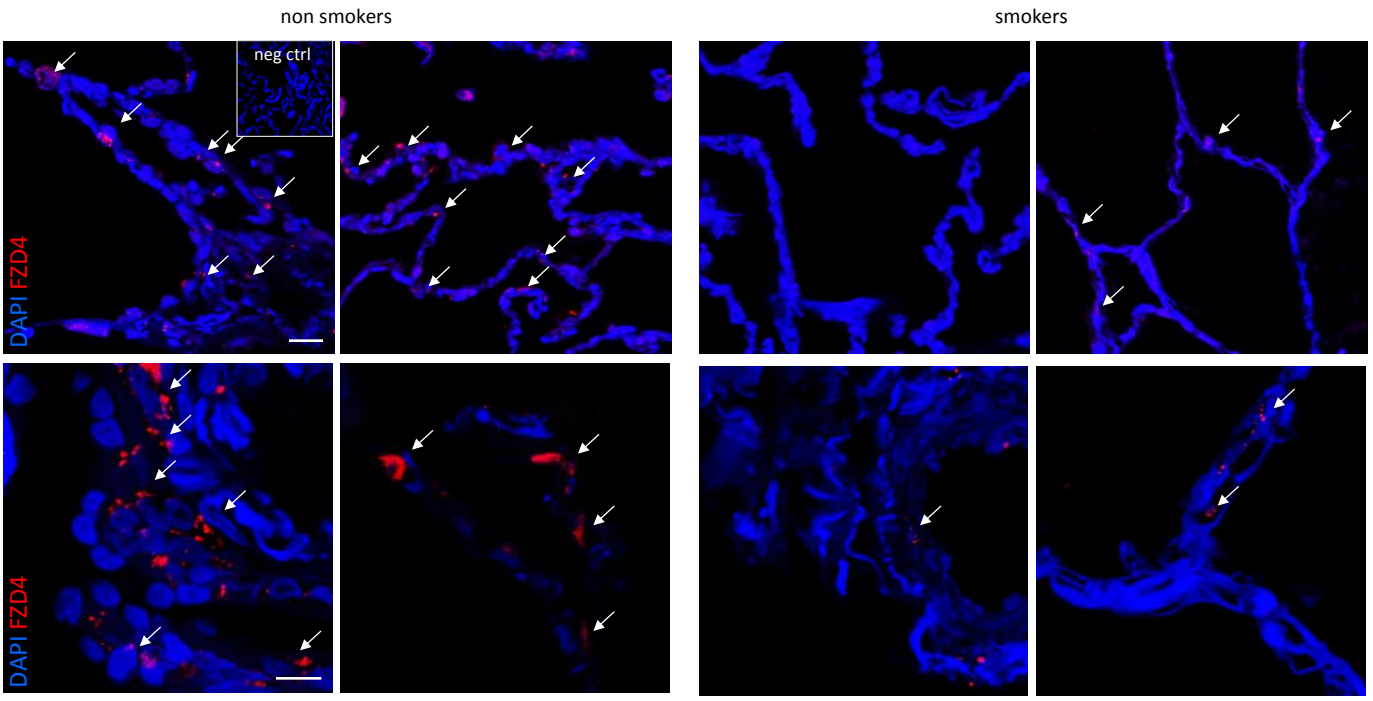

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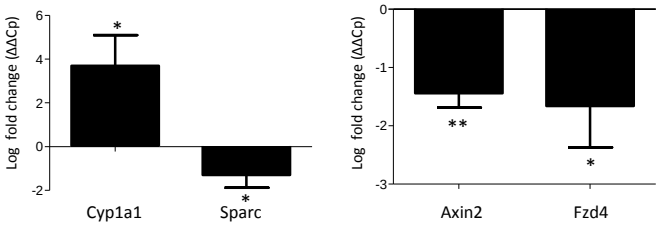

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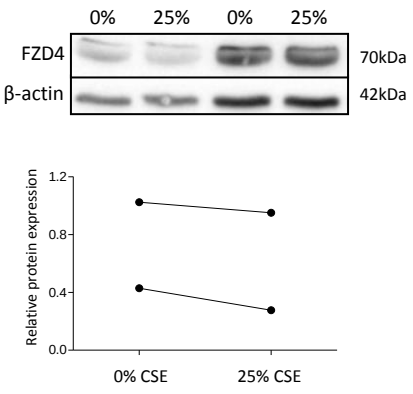

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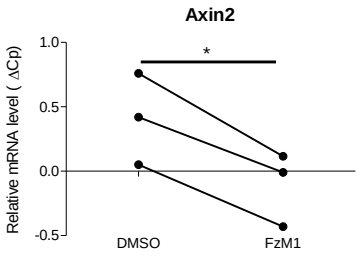

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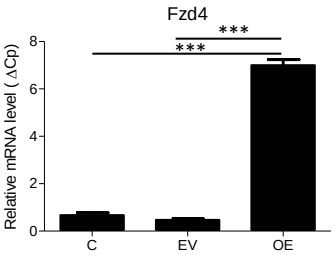

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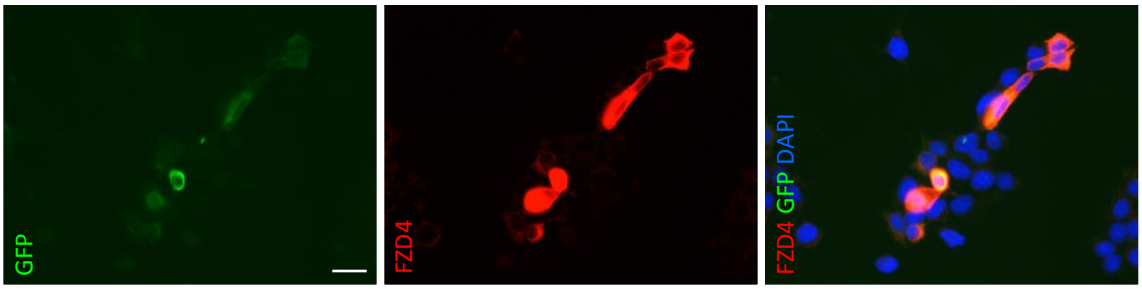

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Supplementary Figure E6

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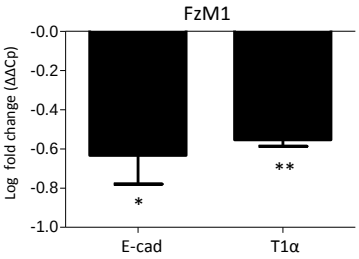

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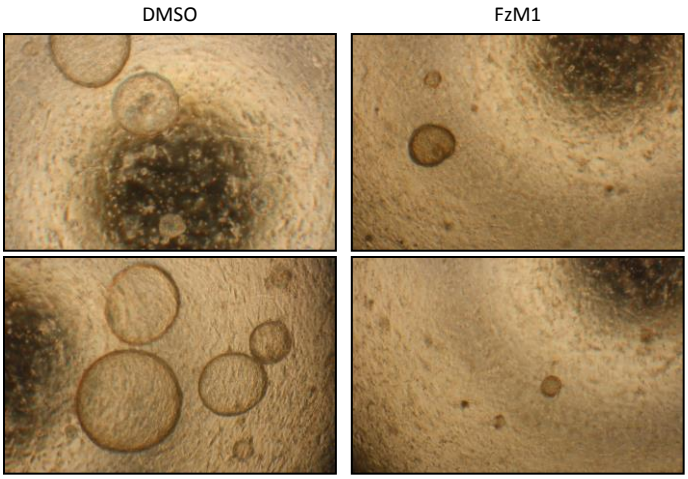

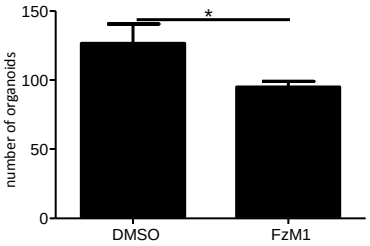

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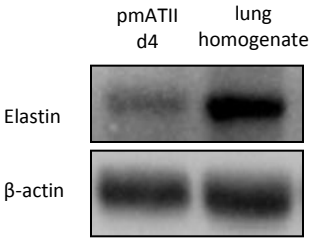

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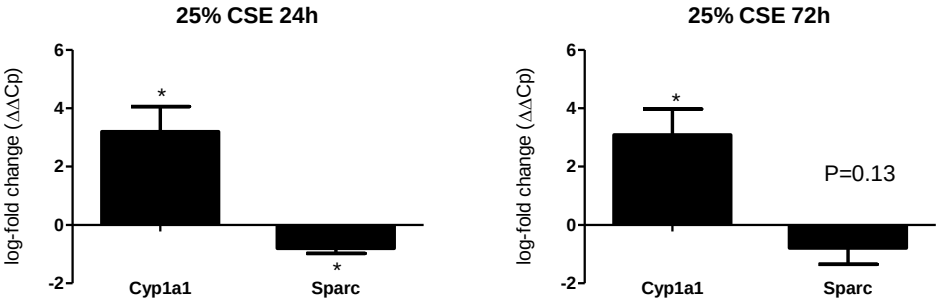
A. Supplementary Figure E8



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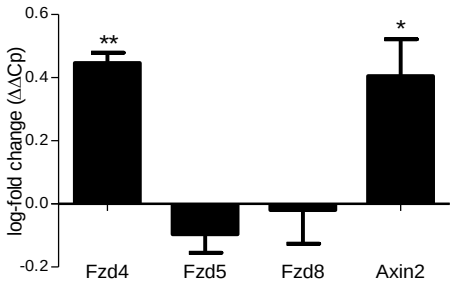

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