Online Data Supplement

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

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Supplemental Material and Methods

Isolation of primary alveolar epithelial type II (pATII) cells

Isolation of primary human ATII (phATII) cells was performed as previously described (1), with some modifications. In brief, lung tissue was minced and digested with dispase/collagenase (Roche) at 37°C for 2h. Next, samples were filtered through nylon meshes and the suspension was centrifuged at 400 g, 4°C for 10 minutes. Subsequently, the cell pellet was re-suspended in DMEM/F12 medium and layered onto a discontinuous Percoll density gradient (1.04 – 1.09 g/ml) and centrifuged at 300 g for 20 minutes. Macrophages and alveolar epithelial cells in the interphase were recovered and depleted for macrophages with CD45 specific magnetic beads (Miltenyi Biotec) according to the manufacturer's recommendations. The primary mouse (pmATII) cells were isolated from pathogen-free C57BL/6 mice as previously described (1, 2). Lungs were lavaged with PBS and filled with dispase (BD Bioscience, San Jose, CA, US) for digestion. Tissue was minced and the cell suspension was filtered through 100-, 20-, and 10 µm nylon meshes (Sefar, Heiden, Switzerland). The single cell suspension was then centrifuged for 10 minutes at 200 g and the pellet was re-suspended in DMEM cell culture medium (Sigma Aldrich). To further purify the ATII cell population, cells were incubated on CD16/CD32 and CD45 (BD Bioscience)-coated Petri dishes for negative selection of lymphocytes and macrophages. Negative selection of fibroblasts was performed by adherence on non-coated plates. Cell purity was assessed routinely by analysis of epithelial (EpCAM, panCK and pro-SPC), mesenchymal (αSMA, CD90), endothelial (CD31) and hematopoietic cell (CD45) markers by immunofluorescence and flow cytometry.

Cigarette smoke extract (CSE) preparation

CSE was prepared as published previously (3). In short, smoke from six cigarettes (Research-grade cigarettes 3R4F; Kentucky Tobacco Research and Development Center at the University of Kentucky) was bubbled through 100 ml of fetal bovine serum (FBS)-free cell culture medium at a constant airflow. This medium was then sterile filtered through a 0.2 µm filter (Minisart; Satorius Stedim Biotech, Göttingen, Germany), aliquoted and stored at −20°C. Medium served as the 100% CSE stock solution.

Cell culture

MLE12 murine lung epithelial cells (ATCC CRL-2110) were cultured in RPMI medium supplemented with 10% (v/v) FBS, 100 µg/ml streptomycin and 100 U/ml penicillin. Unless described otherwise, cells were grown, washed with PBS and starved for synchronization in respective medium supplemented with 0.1% (v/v) FBS, 100 µg/l streptomycin and 100 U/ml penicillin. Cells were treated with vehicle (PBS) as a control, recombinant protein WNT3A (R&D Systems) for indicated time periods (2, 6 or 24h). For the experiments using a small molecule inhibitor of FZD4 - FzM1 (4), cells were pre-treated for 30 minutes with FzM1 and then stimulated with CSE or WNT3A for 24h together with FzM1. For the experiments with CSE exposure, cells were grown until 70% confluency and stimulated for 24h with 25% CSE in 0% FBS medium without a prior starvation period. In experiments using primary epithelial cells, isolated pmATII cells were seeded, cultured until the following day, then starved with medium containing 0.1% FBS for 12h for synchronization. For CS experiments, pmATII cells were cultured until day 3 and then stimulated with phenol red-free DMEM 25% CSE

media, supplemented with 1% Glutamax, without FBS for 24h. For experiments with the FzM1 inhibitor, cells were cultured until day 2 and treated without a starvation period with DMSO or FzM1 (5 μM) in medium supplemented with 10% FBS for 24 or 72h (until day 3 or day 5). Cells cultured until day 5 received fresh treatment at day 3.

Transfection of plasmids

MLE12 cells were transfected with empty vector (EV) (Amsbio, pCMV6KN) or *Fzd4* overexpressing plasmids (OE) (5) (Addgene plasmid #42197) using Lipofectamine LTX and Plus Reagent (Thermo Fisher Scientific), according to the manufacturer's directions. For the β-catenin-driven gene transcription luciferase reporter assay (TOP/FOPflash assay), MLE12 cells were transfected with luciferase reporter plasmids (Super 8x TOPFlash with the wild-type TCF binding sites; Super 8X FOPflash with the mutant-type TCF binding sites, Addgene clone M50, M51) with lipofectamine 2000 (Thermo Fisher Scientific) in starvation media (0.1% FBS) without antibiotics. 6h after TOP/FOP transfection, cells were treated with WNT3A recombinant protein for 24h in the absence or presence of FzM1. For experiments with both the luciferase reporter and *Fzd4* OE plasmids, MLE12 cells were transfected reversely together with both plasmids with the usage of Lipofectamine LTX and Plus Reagent. To detect the β-catenin-driven luciferase activity we used the Glo lysis buffer and Bright-Glo™ reporter assay system (Promega, Madison, Wisconsin, USA). Measurements for each individual experiment were performed at least in duplicates with the use of the TriStar LB 941 plate reader (Berthold Technologies).

RNA isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR, qPCR)

Total RNA was extracted using the Peqlab Total RNA extraction Kit (Peqlab, Erlangen, Germany), for human tissue with the miRNeasy Mini kit (Qiagen) according to the manufacturer's protocol and cDNAs were generated by reverse transcription using SuperScriptTM II (Invitrogen), for human tissue with iScript Advanced kit (BioRad). Quantitative (q)RT-PCR was performed using fluorogenic SYBR Green and the Light Cycler 480 detection system (Roche) and for human tissue with the LightCycler96 detection system (Roche). Hypoxanthine phosphoribosyltransferase (*HPRT)* was used as a reference gene. PCR was performed using the primers listed in Table E1. For human tissue *HPRT1,* Glyceraldehyde-3-phosphate dehydrogenase *(GADPH)* and Succinate Dehydrogenase Complex, Subunit A (SDHA) Gene Expression Assays (Table E2) were used for the housekeeping genes (Applied Biosystems).

Relative gene expression is presented as ΔCt value (ΔCt = Ct Hprt - Ct gene of interest). Relative change in transcript level upon treatment is expressed as ΔΔCt value (ΔΔCt = ΔCt of treated sample - ΔCt of control). For human tissue, expression of genes was corrected by a normalization factor that was calculated based on the expression of the three reference genes and presented as relative gene expression.

Immunofluorescence staining

For immunofluorescence staining experiments, MLE12 or ATII cells were seeded on poly-l-lysine coated coverslips. After treatments, cells were stopped at the indicated time points and fixed with ice-cold acetone-methanol (1:1) for 10 minutes and washed 3 times with 0.1% BSA in PBS. Next, cells were permeabilized with 0.1% Triton X-100 solution in PBS, blocked with 5% BSA in PBS for 30 minutes and incubated with primary antibodies, followed by appropriate secondary antibodies (Table E3), 1h each, and

DAPI (Roche, Basel, Switzerland) for 10 minutes to visualize cell nuclei. Coverslips were fixed with 4% PFA for 10 minutes, mounted with fluorescent mounting medium (Dako), visualized with an Axio Imager microscope (Zeiss) and analyzed using the Axiovision software.

Immunoblotting

Cells or pulverized lung tissue were lysed with radioimmunoprecipitation assay buffer (RIPA) or Tissue Protein Extraction Reagent (T-Per, Thermo Fisher) supplemented with phosphatase and protease inhibitors (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using the BCA assay (Pierce, Thermo Fisher Scientific). Each sample was mixed with 4x Laemmli loading buffer (150 mM Tris HCl, 275 mM SDS, 400 nM dithiothreitol, 3.5% (w/v) glycerol, 0.02% bromophenol blue) and equal amounts of protein were subjected to electrophoresis in polyacrylamide gels, and transferred to nitrocellulose or PVDF membranes. Next, membranes were blocked with 5% non-fat dried milk solution in TRIS-buffered saline containing 0.01% (v/v) Tween (TBS-T) (Applichem) for 1h and incubated with primary antibodies at 4°C overnight and for 1h at RT with secondary, HRP-conjugated, antibodies (Table E3). Bands were visualized using chemiluminescence reagents (Pierce ECL, Thermo Scientific, Ulm, Germany), recorded with ChemiDocTMXRS+ system and analyzed with Image Lab 5.0 software (Biorad, Munich, Germany).

Enzyme-linked immunosorbent assay (Elisa)

Supernatants were collected from cigarette smoke extract- and FzM1-treated ATII cells. Assay was performed according to the manufacturer's instructions (IGF1 DuoSet ELISA DY791; R&D, Minneapolis, Minnesota, USA).

WST-1 assay

MLE12 cells were seeded into 96-well plates. For experiments with FzM1, cells were synchronized with 0.1% FBS media for 24h and then treated with FzM1 (5µM) in 0.1% FBS media for 24h. For the experiments with Fzd4 OE plasmid, cells were transfected reversely and allowed to adhere for 24h. Next, cells were starved for 6h with 0.1% FBS media and then media was exchanged to 1% FBS media and cells were cultured for an additional 24h. Proliferation was monitored using the WST-1 assay kit according to the manufacturer's protocol. In short, media was aspirated and 100 µl of 10% WST-1 solution was added to each well and cells were incubated for 2h. Absorbance (wavelength 450nm) was measured using microplate reader (Tecan Sunrise, Switzerland).

Cell counting

To investigate the influence of the small molecule inhibitor FzM1 on cell proliferation, MLE12 cells were seeded in triplicates in 12 well plates in RPMI media supplemented with 10% FBS, starved in 0.1% FBS media for 24h and treated with DMSO or FzM1 in 0.1% media for 24h. Next, cells were detached with trypsin, neutralized with 10% FBS media and counted using the CASY Cell Counter & Analyzer.

Scratch assay

MLE12 cells were grown in 24-well plates with DMEM/F12 cultivation medium supplemented with 10% (v/v) FBS and antibiotics. Next, cells were synchronized in

0.1% FCS medium for 24 h. Confluent monolayers of cells were wounded by scraping with a pipette tip. Cells were washed twice with PBS and stimulated in quadruplicates with FzM1 (5 µM) or DMSO control. For experiments with Fzd4 overexpressing plasmid, cells were transfected reversely while seeding with Fzd4 OE or empty vector and left for 24h. Confluent monolayers of cells were wounded by scraping with a pipette tip. Images of wounds were captured at the initial time of wounding and 48h post scraping. The size of each wound was measured in triplicates per image and analyzed using ImageJ software.

Organoid formation assay

Freshly isolated ATII cells in cultivation media were mixed 1:1 with CCL206 cells (ATCC, Mlg 2908) in matrigel (BD, 354230). CCL206 cells were pretreated with mitomycin C (Sigma, M4287) to inhibit their further proliferation. Inserts (Falcon, FALC353095) were palced into 24-well plates and mixture with the cells was added on top of each insert. Organoid media consisting of DMEM/F-12 media (Sigma, 31330-095) containing FBS, antibiotics, glutamine, insulin-transferrin-selenium (Gibco, 51300-044), cholera toxin (0.1 µg/ml, Sigma, C8052), EGF (0.025 µg/ml, Sigma, SRP3196-500UG), bovine pituitary extract (30 µg/ml, Sigma, P1476) and retinoic acid (0.01 µM, Sigma, R2625-50MG) was added to the well, outside of insert. For first 2 days media was supplemented with Rock inhibitor (10 µM, Sigma, Y0503-1MG). Media was exchanged every second day and at day 7 of culture number of organoids was quantified.

GEO analyses

For FZD receptor expression analysis in clinical cohorts, normalized data were extracted from published microarray datasets: whole lung homogenate from COPD and non-COPD patients of the Lung Genome Research Consortium (GSE47460), phATII cells from COPD and non-COPD patients (GSE29133) (6).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data are expressed as mean ± s.d. (n<5) or mean ± s.e.m. Means between two groups were compared using the Student's t-tests. Comparisons between more than two groups were performed by one-way Anova followed by Newman-Keuls's multiple comparison test or Mann-Whitney U test. P values were considered to indicate a statistically significant difference when <0.05.

References for Online Methods

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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‘

	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

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

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.

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 *T1a* 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) *Cyp1a1* 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

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.

Supplementary Figure E1

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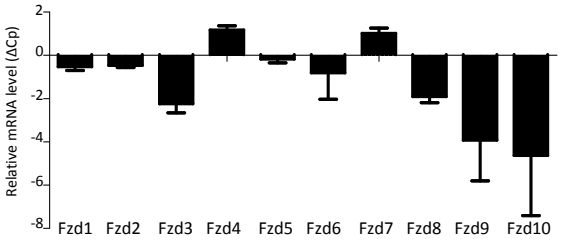

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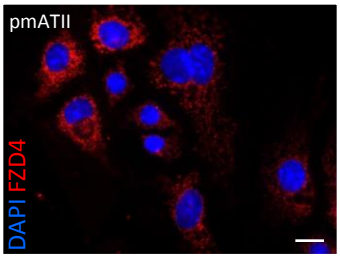

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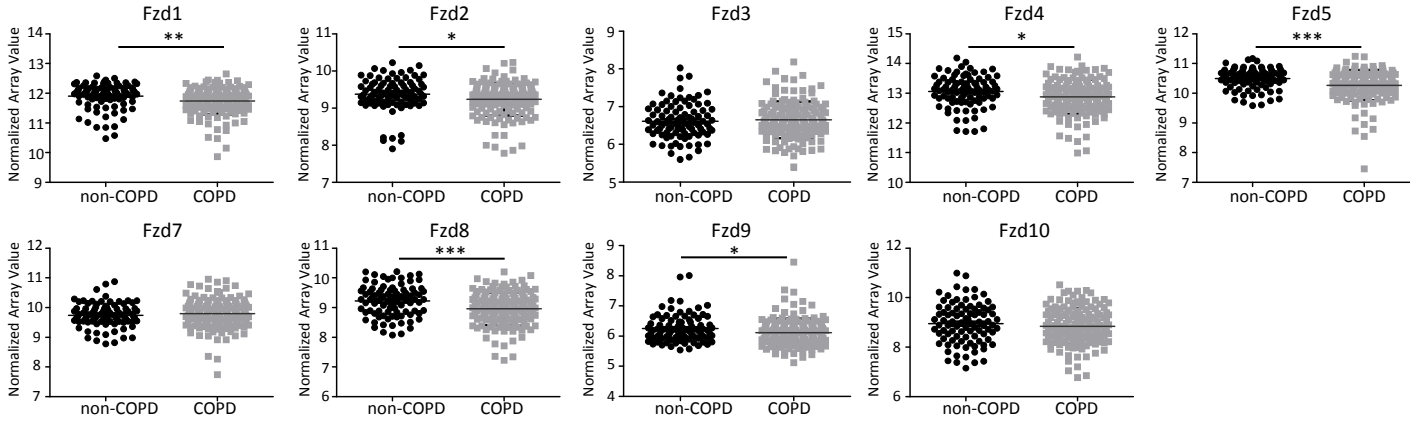

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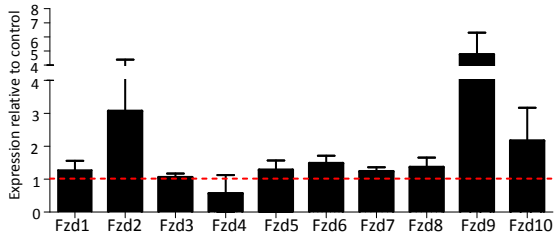

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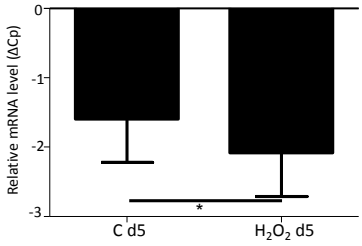

Supplementary Figure E2

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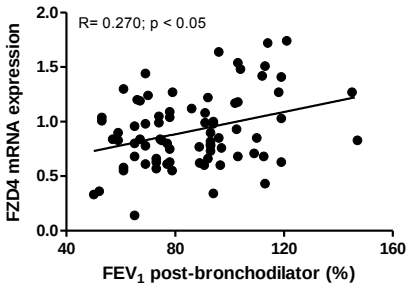

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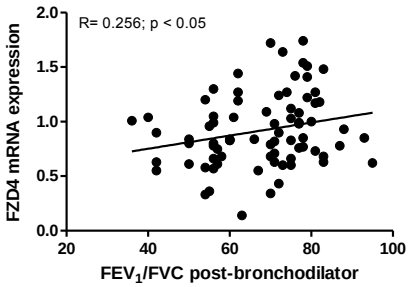

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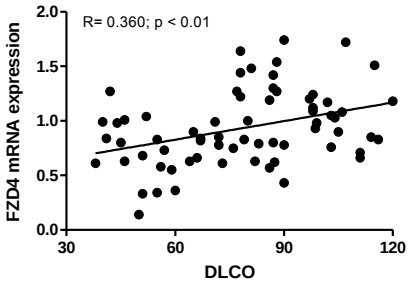

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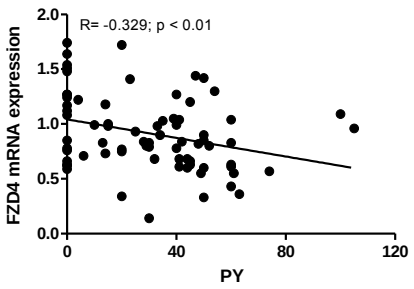

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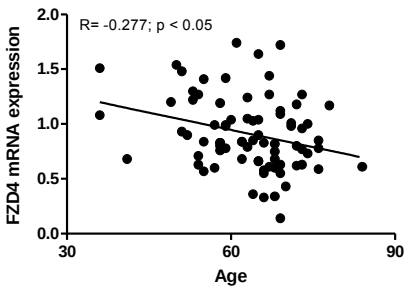

Supplementary Figure E3

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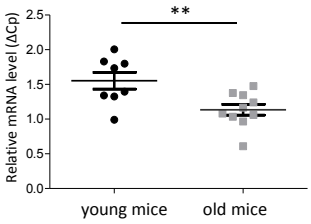

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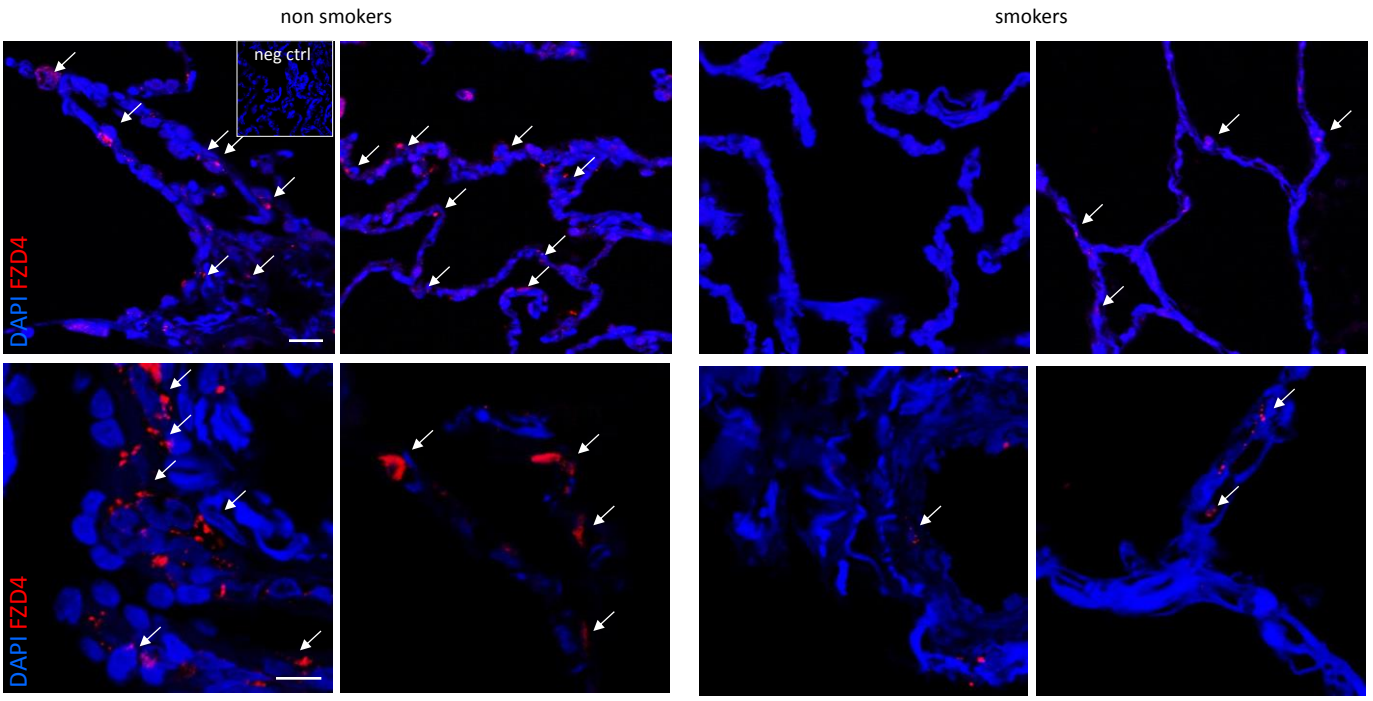

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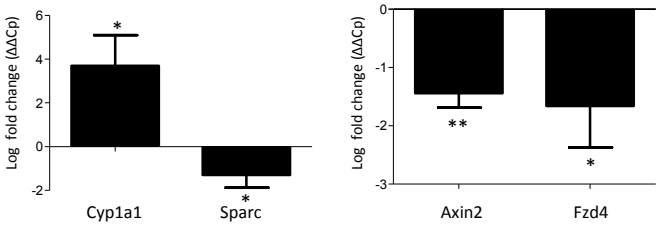

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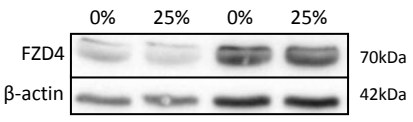
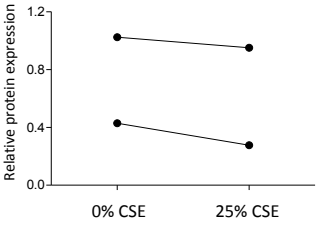
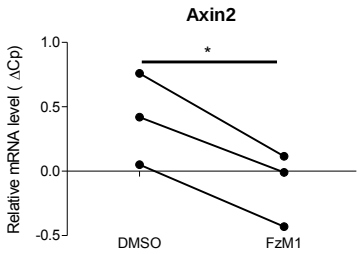




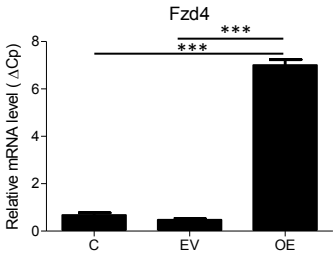
Supplementary Figure E4

A.

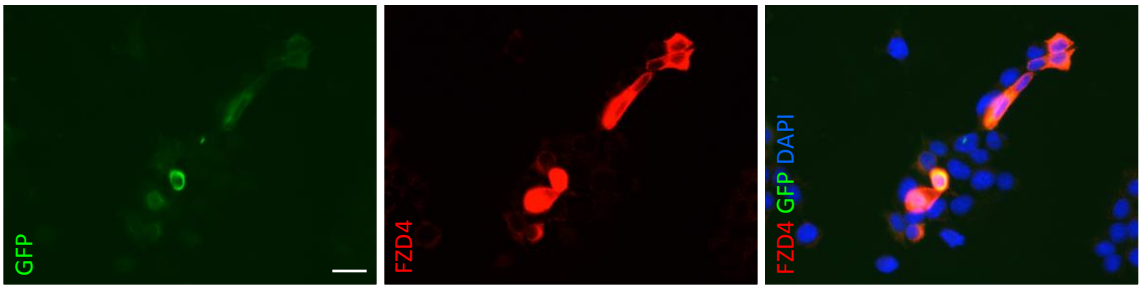


Supplementary Figure E5

A.

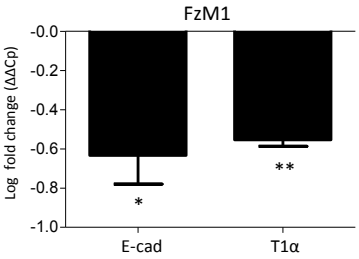


B.



Supplementary Figure E6

A.



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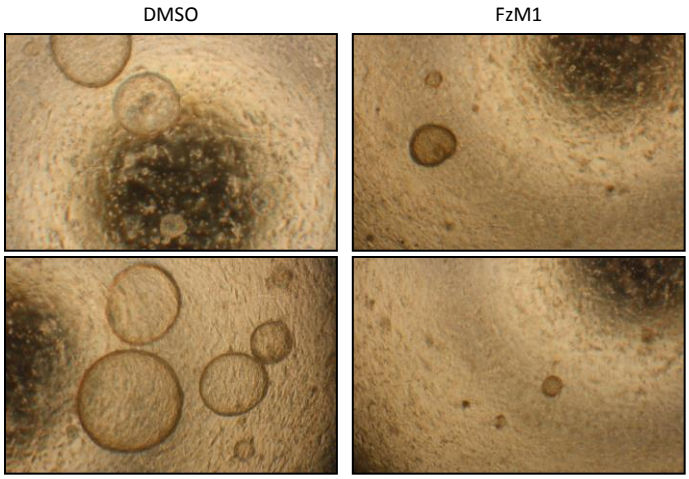
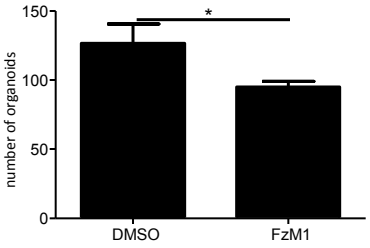
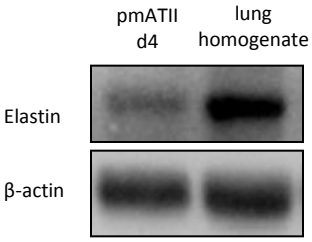




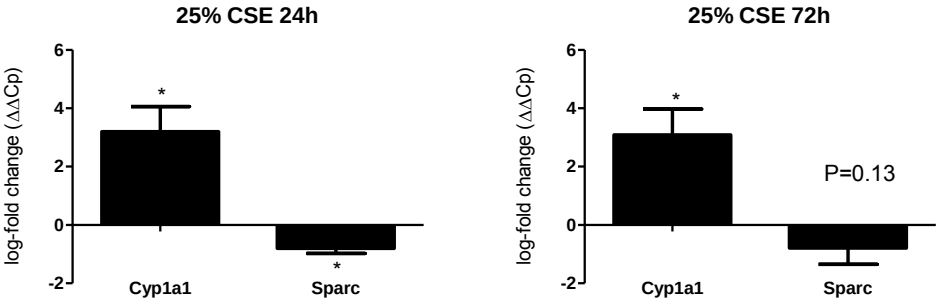
Supplementary Figure E7

A.



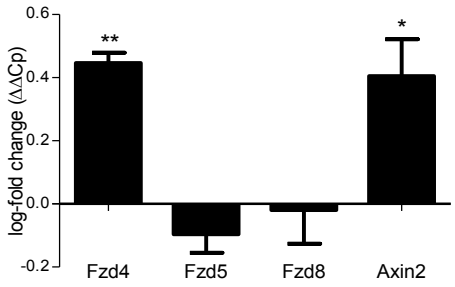
A.

Supplementary Figure E8



Supplementary Figure E9

A.



B.