

Supplemental Information

Macropinocytosis inhibition attenuates pro-fibrotic responses in lung fibroblasts and pulmonary fibrosis models

Ivan O. Rosas^{1,†}, Aaron K. McDowell-Sanchez¹, Santiago Sanchez¹, Juan D. Cala-Garcia¹, Alan R. Waich Cohen¹, Elisa Ruiz-Echartea², Scott A. Ochsner², Daniel Kraushaar³, Lindsay J. Celada¹, Dandan Sun^{4, 5}, Francesca Polverino¹, Cristian Coarfa², Neil J. McKenna², and Konstantin Tsoyi^{1,6†}.

¹Section of Pulmonary, Critical Care, and Sleep Medicine, Department of Medicine, Baylor College of Medicine, Houston, TX.

²Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX.

³Genomic and RNA Profiling Core, Baylor College of Medicine, Houston, TX.

⁴Department of Neurology, the Pittsburgh Institute for Neurodegenerative Diseases, University of Pittsburgh, Pittsburgh, PA 15260

⁵Veteran's Affairs Research Center, Pittsburgh, PA 15240, USA

⁶Division of Pulmonary and Critical Care, Department of Medicine, University of Maryland School of Medicine, Baltimore, MD.

[†]Corresponding Authors:

Ivan O. Rosas, M.D., Section of Pulmonary, Critical Care, and Sleep Medicine, Department of Medicine, Baylor College of Medicine, 7200 Cambridge Street, Houston, TX 77030.

Email: Ivan.Rosas@bcm.edu.

Konstantin Tsoyi, Ph.D., Division of Pulmonary and Critical Care, Department of Medicine, University of Maryland School of Medicine, 20 Penn Street, HSRF II, Baltimore, MD, 21201.

Email: KTsoyi@som.umaryland.edu.

Supplemental Methods:

Reagents

Antibodies against α -SMA (Cat #: ab5694), collagen 1 (Cat #: ab316222), and fibronectin (Cat #: ab2413) were from Abcam. Anti-GAPDH, and anti-MEOX1 antibodies were obtained from Santa Cruz Biotechnology (Cat#: sc-365062, and sc-398845 respectively). Antibodies against phospho-S6 ribosomal protein (Ser235/236, Cat #: 2211), S6 ribosomal protein (Cat #: 2217), phospho-p70 S6 kinase (Thr389, Cat #: 9206), p70 S6 kinase (Cat #: 2708) were from Cell Signaling Technologies. Recombinant TGF- β 1 was obtained from Sino Biological (Cat. # 10804-HNAC). Rapalink-1, collagenase type 1 and type 2 were obtained from Cell Signaling Technology (Cat#: 88626, 62648, 77336 respectively). Fluorescein isothiocyanate (FITC)-labeled dextran (70 kDa, Cat #: 46945) was obtained Millipore Sigma. Alexa Fluor-488 (AF488)-labeled transferrin from human Serum was from ThermoFisher Scientific. 5-(N-ethyl-N-isopropyl)-Amiloride (EIPA) (Cat #: 14406), and Imipramine (Cat #: 15890) were obtained from Cayman Chemical. All other reagents, including 4-hydroxy-tamoxifen (4-OHT, Cat #: H6278), were from Millipore Sigma.

Primary Lung Fibroblasts

Deidentified human lung fibroblasts (HLFs) were derived from patients who were subjected to lung transplantation with IPF. Control (Con) HLFs were derived from nonfibrotic lung samples lacking any evidence of disease which were unsuitable for transplantation. Mouse lung fibroblasts from *Slc9a1^{fl/fl}* Col1a2-Cre-ER(T)^{+/0}, *Slc9a1^{fl/fl}* mice were obtained as previously described (1). Fibroblasts were cultured in complete media (DMEM; Corning) containing 10% FBS (Corning), 100 IU of penicillin and 100 μ g/ml streptomycin (Corning), 292 μ g/ml L-glutamine (Corning), and 100 μ g/ml Primocin (InVivoGen) in humidified incubators at 37°C and 10% CO₂. To induce Cre

recombinase expression in *Slc9a1^{fl/fl}* / Col1a2-Cre-ER(T)^{+/0} or *Slc9a1^{fl/fl}* lung fibroblasts, cells were treated with 4-hydroxytamoxifen (4-OHT, 1 μ M) for 24 hours.

Macropinocytosis assay

Macropinocytosis levels were measured by the uptake of high molecular weight (70 kDa) FITC-labeled dextran by fibroblasts as described with modifications (2). Briefly, cells were cultured on a 6-well plate (flow cytometry) or on the slide chamber in complete media. When cells reached 70-80% confluence, they were conditioned in low serum conditions (1% of FBS) for 12-16 hours. Then, cells were treated with 70 kDa FITC-labeled dextran (0.5 mg/ml) for 1 hour. After incubation, cells were washed three times with PBS and either fixed with 4% paraformaldehyde, additionally stained with DAPI for fluorescent microscopy imaging or harvested, stained with DAPI and subjected to flow cytometry. Images were analyzed by ImageJ (Image Processing and Analysis in Java Edition: 1.29 URL: <http://rsb.info.nih.gov/ij/> NIH, Maryland) and flow cytometry data by FlowJo.

Flow cytometry

Cells were harvested and incubated in Fc receptor blocking solution (Biolegend, Cat. #: 422302) for 5 minutes, spun down and resuspended in cell stain buffer (Biolegend, Cat. #: 420201). Then, samples were stained with anti-CD45-FITC (Biolegend, Cat. #: 368507), anti-EpCAM-PE (Biolegend, Cat. #: 324206), anti-CD31-FITC (Biolegend, Cat. #: 303104), anti-CD90-PE (Biolegend, Cat. #: 328110), anti-PDGFR α -PE (Biolegend, Cat. #: 323506), anti-CD44-APC-Cy7 (Biolegend, Cat. #: 103028) or relevant isotype control Ab for 30 min. After incubation, cells were washed in cell stain buffer and passed through the cell strainer (40 μ m). Cells were subjected to flow cytometry for at least 10,000 events and analyzed using FlowJo software.

Mice

WT C57BL/6 mice were obtained from JAX laboratory and used at 10-12 weeks of age. Conditional *Slc9a1*-knockout mice of both sexes were bred as follows on a C57BL/6 background. Transgenic *Col1a2*^{Cre-ER(T)+/0} mice were obtained from JAX Laboratory (Strain #: 029567) and *Slc9a1*^{fl/fl} mice were donated by Dr. Dandan Sun (University of Pittsburgh) (3). To generate fibroblast-specific *Slc9a1*-deficient mice, *Slc9a1*^{fl/fl} mice were crossbred with *Col1a2*^{Cre-ER(T)+/0} (heterozygous allele) transgenic mice to generate mice heterozygous for both alleles. Progeny from the second cross between *Slc9a1*^{fl/fl} mice and heterozygous *Slc9a1*^{fl/WT} *Col1a2*^{Cre-ER(T)+/0} mice (from the first cross) were used for further experiments. All mice were genotyped by PCR techniques as described previously (3). For treatment of mice, a stock solution of tamoxifen was diluted in corn oil to 20 mg/ml. To selectively delete *Slc9a1* in activated fibroblasts, adult *Slc9a1*^{fl/fl} *Col1a2*^{Cre-ER(T)+/0} mice (10-12 weeks old) and control *Slc9a1*^{fl/fl} mice were administered a tamoxifen suspension (0.1 ml of diluted stock) *via* intraperitoneal (*i.p.*) injection (75 mg/kg), for 5 days before administration of bleomycin sulfate (Bleo) (0.5 mg/kg) and every 5 days thereafter until sacrifice.

Bleomycin (Bleo) model of pulmonary fibrosis

Lung fibrosis was elicited in mice by intratracheal (*i.t.*) injection of a single dose of 0.5 mg/kg body weight of Bleo as described previously (4); control mice received a volume of sterile saline equal to the volume of Bleo. Mice were sacrificed 21 days after saline or Bleo instillation. Bronchoalveolar lavage fluid (BALF) was collected by lavaging lungs with 1 ml of PBS. BALF was used for protein concentration using bicinchoninic acid (BCA) protein assay, and monocyte chemoattractant protein-1 (MCP1) by ELISA. The right middle lobe was subjected to hydroxyproline assay, the left lung was fixed and subjected to immunohistochemistry, the rest of the right lung was assessed for expression of fibrotic marker mRNAs (*Acta2*, *Colla1*, and *Meox1*) and histology.

***In vivo* macropinocytosis assay**

To measure macropinocytosis *in vivo*, the collected lungs were infused with 500 µl of 0.5 mg/ml of (70 kDa) FITC-labeled dextran solution and kept in 1% FBS DMEM for 1 hour. Then, single cell suspension were obtained by enzymatic digestion (0.1% collagenase I and 0.25% collagenase II solution). Cell suspension samples were stained with CD90.2-APC (Biolegend, Cat. #: 140311), CD45-PE (Biolegend, Cat. #: 103105), EpCAM-PE (Biolegend, Cat. #: 118205), CD31-PE (Biolegend, Cat. #: 102407) and DAPI (Biolegend, Cat. #: 422801) and subjected to flow cytometry to measure macropinocytosis (**Supplemental Figure 6A**). Data were analyzed using FlowJo.

Hydroxyproline assay

To quantify collagen deposition, the right lung (middle lobe) from each mouse was hydrolyzed in 6N HCl for 24 h at 110°C, and hydroxyproline levels were quantified as previously described (5). Each sample was tested in triplicate. Data are expressed as micrograms of hydroxyproline per lung.

Histology

Lung sections were fixed by inflation with buffered 10% formalin solution and embedded in paraffin. Thin (4 µm) sections were deparaffinized and rehydrated. Tissue slides were then stained by hematoxylin-eosin (H&E) and Masson's Trichrome staining to evaluate histopathologic changes and collagen deposition, respectively (5).

Precision Cut Lung Slices (PCLS)

PCLS from IPF lungs were prepared as previously described. Briefly, using a syringe pump, lungs were infiltrated with warm, 2% (37°C) low-melting agarose–HBSS solution (MilliporeSigma, no. A9414; kept at 37°C). After complete solidification of agarose in the inflated lobes on ice, tissue blocks of approximately 10 mm in diameter were prepared. Lung slices (300 µm thick) were cut

perpendicularly to the visible airway with a vibratome (Precisionary Instruments, no. VF-300, Greenville, NC) at room temperature in HBSS. Then, slices were cultured in 24-well plates supplemented with DMEM/F12 media containing 1% FBS and antibiotics. Lung slices were incubated with or without EIPA (12.5 μ M) or Imipramine (Imi, 10 μ M) for another 72 h. Culture media was changed at 24 and 48 hours and treated with drugs or DMSO with total of 2 treatments. After treatment, slices were subjected to total RNA isolation to measure profibrotic gene expression, *e.g.*, *ACTA2*, *COL1A1*, or immunofluorescent staining.

Single Cell RNA sequencing

PCLS from IPF lungs were treated Single cell lung data from was mapped using 10x Genomics Cell Ranger v7.0.1 onto the human genome reference provided by 10x Genomics. Doublet detection was performed using scrubblet (6). Data was processed using the Python Scanpy library (7) we performed guided cell annotation using CellTypist (8) and references provided by the Human Lung Cell Atlas (HLCA) (9) augmented with an aberrant basaloid reference (10). UMAP plots were generated using Scanpy. Objects were converted to the Seurat format using the zellkonverter package. Cell type marker plots were generated using Seurat (11). Differential gene expression was generated using MAST (12) with significance achieved at FDR-adjusted p-value<0.1 and fold change exceeding 1.25x. Relative differences in abundance of cell types within specific compartments were derived using ChiSquare as implemented in the R statistical system.

Bulk RNA sequencing

MRC-5 cells were treated with EIPA (12.5 μ M) or Vehicle (DMSO) with or without TGF- β 1 (10 ng/ml) for 24 hours. Then total RNA isolation and elimination of DNA was done using RNeasy kit (QIAGEN). A cDNA library was prepared with 250ng of RNA using the Illumina TruSeq Stranded mRNA kit according to the manufacturer's protocol. Libraries were quantified and

pooled equimolarly. Sequencing was performed on an Illumina NextSeq 500 targeting around 40 million read pairs per sample. Sequencing was run on NextSeq 500 Sequencing System. FastQ file generation was executed using Illumina's cloud-based informatics platform, BaseSpace Sequencing Hub at BCM Genomic and RNA Profiling Core.

Western Blot

Polyacrylamide gel electrophoresis and immunoblotting were performed according to standard methods as previously described (13). The electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membranes by semidry electrophoretic transfer at 15 V for 60–75 min. The membranes were blocked overnight at 4°C in 5% bovine serum albumin (BSA). The cells were incubated with primary antibodies diluted 1:500 in Tris-buffered saline/Tween 20 (TBS-T) containing 5% BSA for 2 h and then incubated with the secondary antibody at room temperature for 1 h. Suitable horseradish peroxidase (HRP)-conjugated secondary antibodies were used (1:5000 dilution in TBST containing 1% BSA). The signals were detected by ECL (Thermo Fisher Scientific). Quantification of protein bands was performed with the computer software ImageJ and was expressed as a ratio of band intensity with respect to the loading control.

Quantitative Real-Time PCR (qPCR)

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized from RNA (1 µg) using a SuperScript First-strand synthesis system for reverse transcription (RT) PCR (Invitrogen, Carlsbad, CA). Primer sequences are shown in Table S1. RT-PCR, with SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA, USA), was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). The relative quantity of target mRNA was calculated by use of the CT method, or $2^{-\Delta\Delta CT}$, as described (14), and

normalized by use of GAPDH as an endogenous control (Sequence Detection System software, version 1.7; Applied Biosystems).

Amino acid (AA)-free and -supplemented conditions and amino acid assay

Cells were plated on 24-well plates at a density of 1×10^4 per well. Then, cells were incubated in AA-free and AA-supplemented DMEM media. AA-free media was prepared from commercially available AA-free DMEM powder (MyBiosource, Cat #: MBS6120661) and supplemented with antibiotics or supplemented with MEM amino acid solution (ThermoFisher Scientific, Cat #: 11130051) and glutamine (referred as AA-supplemented media) in the presence or absence of EIPA ($12.5 \mu\text{M}$) $\pm$ TGF- β 1 (10 ng/ml) for 4 hours. Then, cells were washed with PBS twice, lysed and subjected to L-amino acid assay using a commercially available kit (Abcam, Cat #: ab65347).

Gel contraction assay

The assay was performed as previously described (5). Gel sizes were measured by the ruler at 0 and 24 h.

Mitochondrial stress assay

HLFs were treated with EIPA ($12.5 \mu\text{M}$) $\pm$ TGF- β 1 (10 ng/ml) for 24 hours. Then, cells were replated onto Seahorse XFe24 plates at 3×10^4 per well, cultured in Seahorse XF DMEM medium, pH 7.4 (Agilent, Cat. # 103575-100). Media was supplemented with 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate. Injections of oligomycin A ($1 \mu\text{M}$), carbonyl cyanide-4-trifluoromethoxyphenylhydrazone (FCCP, $2 \mu\text{M}$), and antimycin A and rotenone ($1 \mu\text{M}$) were supplied by Agilent (Seahorse XFp Cell Mito Stress Test Kit, Cat #: 103010-100). Oxygen consumption rate (OCR) was detected using Seahorse XFe24 Analyzer.

Lentiviral Transfection

For TSC1, RAPTOR, MEOX1 silencing experiments, the pLKO.1 plasmid, carrying the human shRNA against target genes (consortium numbers TRCN0000039736 for shTSC1(a), TRCN0000010453 for shTSC1(b), TRCN0000039734 for shTSC1(c), TRCN0000332886 for shRAPTOR(a), TRCN0000010415 for shRAPTOR(b), TRCN0000039770 for shRAPTOR(c), TRCN0000016109 for shMEOX1(a), TRCN0000016110 for shMEOX1(b), TRCN0000016111 for shMEOX1(c) respectively) and pLKO.1, carrying a Scr sequence, were purchased from MilliporeSigma. Lentiviral particles were generated by use of a commercially available packaging mix, provided by Millipore Sigma (Cat. #: SHP001) in human embryonic kidney 293 T cells, according to the manufacturer's instructions. To overexpress MEOX1, the open reading frame (ORF) of MEOX1 was cloned into pLenti-C-DDK Lentiviral Expression Vector (Origene Inc., Cat #: PS100092). Lenti-C-MEOX1-DDK and Lenti-C-DDK (empty vector, EV) lentiviral particles were generated using Lentiviral Packaging Kit (Origene Inc., Cat. #: TR30037). Lung fibroblasts were infected with the lentiviral particles, and stably infected cells were selected by use of puromycin (10 µg/ml).

Cell viability

Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (15).

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed by use of an enzymatic Chromatin IP kit from Cell Signaling Technology (Cat#: 9002S), according to the manufacturer's instructions. The samples were incubated with an antibody to MEOX1 following immunoprecipitation and elution of DNA. 10 µl of DNA input was obtained before incubation with MEOX1 ab. Binding of MEOX1 to DNA was assessed by qRT-PCR, by use of the CT method ($2^{-\Delta\Delta CT}$), normalized with the $2^{-\Delta\Delta CT}$ from the

input from the same samples, and graphed as a fold change of MEOX1 ab incubated/input. DNA-protein cross-links were reversed, and 10 µl of each sample was used as a template for qRT-PCR. COL1A1 oligonucleotide sequences for PCR primers were forward 5'-ATG GTC GGG ATA ATT GAT GAA-3' and reverse 5'-GGA AAA AAA AGG AAC AGA AGG-3' which encompasses the COL1A1 promoter segment from nucleotide –1854 to –1658, or 5'-CTT TAC TAA TAC TGC TGT CCA-3' and reverse 5'-TAC ACC AAG AGG CTG AGA TGT-3' which encompasses the ACTA2 promoter segment from nucleotide –1084 to –834.

References:

1. Tsoyi K, Hall SR, Dalli J, Colas RA, Ghanta S, Ith B, et al. Carbon Monoxide Improves Efficacy of Mesenchymal Stromal Cells During Sepsis by Production of Specialized Proresolving Lipid Mediators. *Crit Care Med.* 2016;44(12):e1236-e45.
2. Comisso C, Flinn RJ, and Bar-Sagi D. Determining the macropinocytic index of cells through a quantitative image-based assay. *Nat Protoc.* 2014;9(1):182-92.
3. Begum G, Song S, Wang S, Zhao H, Bhuiyan MIH, Li E, et al. Selective knockout of astrocytic Na(+)/H(+) exchanger isoform 1 reduces astrogliosis, BBB damage, infarction, and improves neurological function after ischemic stroke. *Glia.* 2018;66(1):126-44.
4. Tsoyi K, Liang X, De Rossi G, Ryter SW, Xiong K, Chu SG, et al. CD148 Deficiency in Fibroblasts Promotes the Development of Pulmonary Fibrosis. *Am J Respir Crit Care Med.* 2021.
5. Tsoyi K, Chu SG, Patino-Jaramillo NG, Wilder J, Villalba J, Doyle-Eisele M, et al. Syndecan-2 Attenuates Radiation-induced Pulmonary Fibrosis and Inhibits Fibroblast Activation by Regulating PI3K/Akt/ROCK Pathway via CD148. *Am J Respir Cell Mol Biol.* 2018;58(2):208-15.
6. Wolock SL, Lopez R, and Klein AM. Scrublet: Computational Identification of Cell Doublets in Single-Cell Transcriptomic Data. *Cell Syst.* 2019;8(4):281-91 e9.
7. Wolf FA, Angerer P, and Theis FJ. SCANPY: large-scale single-cell gene expression data analysis. *Genome Biol.* 2018;19(1):15.
8. Xu C, Prete M, Webb S, Jardine L, Stewart BJ, Hoo R, et al. Automatic cell-type harmonization and integration across Human Cell Atlas datasets. *Cell.* 2023;186(26):5876-91 e20.
9. Sikkema L, Ramirez-Suastegui C, Strobl DC, Gillett TE, Zappia L, Madissoon E, et al. An integrated cell atlas of the lung in health and disease. *Nat Med.* 2023;29(6):1563-77.

10. Adams TS, Schupp JC, Poli S, Ayaub EA, Neumark N, Ahangari F, et al. Single-cell RNA-seq reveals ectopic and aberrant lung-resident cell populations in idiopathic pulmonary fibrosis. *Sci Adv.* 2020;6(28):eaba1983.
11. Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM, 3rd, et al. Comprehensive Integration of Single-Cell Data. *Cell.* 2019;177(7):1888-902 e21.
12. Finak G, McDavid A, Yajima M, Deng J, Gersuk V, Shalek AK, et al. MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data. *Genome Biol.* 2015;16:278.
13. Tsoyi K, Lee TY, Lee YS, Kim HJ, Seo HG, Lee JH, et al. Heme-oxygenase-1 induction and carbon monoxide-releasing molecule inhibit lipopolysaccharide (LPS)-induced high-mobility group box 1 release in vitro and improve survival of mice in LPS- and cecal ligation and puncture-induced sepsis model in vivo. *Mol Pharmacol.* 2009;76(1):173-82.
14. Schmittgen TD, and Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008;3(6):1101-8.
15. Tsoyi K, Kim HJ, Shin JS, Kim DH, Cho HJ, Lee SS, et al. HO-1 and JAK-2/STAT-1 signals are involved in preferential inhibition of iNOS over COX-2 gene expression by newly synthesized tetrahydroisoquinoline alkaloid, CKD712, in cells activated with lipopolysacchride. *Cell Signal.* 2008;20(10):1839-47.

Figure legends:

Supplemental Figure 1. EIPA and Imipramine inhibit macropinocytosis but not other types of endocytosis.

(A) Human control (Con) and IPF-derived lung fibroblasts were treated with EIPA (12.5 μ M) and imipramine (Imi, 10 μ M) for 24 hours. After incubation culture media was changed and cells were incubated with FITC-Dextran (70 kDa, 0.5 mg/ml) for 1 hour. Then, cells were washed three times with PBS and subjected to flow cytometry to measure fluorescence intensity of FITC in cells (n=4 each condition). (B) Cells were treated with EIPA and Imi as described in (A). After incubation, cells were exposed to AF488-labeled transferrin (1 mg/ml) for 1 hour. Then cells were washed, harvested and subjected to flow cytometry (n=4 each condition). (C) Cells were treated with EIPA and Imi as described in (A). After treatment cells were lysed and subjected to

western blot to determine caveolin-1 (CAV1) (n=4 each condition). (D) Con HLFs were stimulated with TGF- β 1 (10 ng/ml) in the presence or absence of EIPA (12.5 μ M) and imipramine (Imi, 10 μ M) for 24 hours. Then RNA was isolated and FN1 expression was measured by qRT-PCR as described in Supplemental Methods. (E) Control HLFs were stimulated with TGF- β 1 (10 ng/ml) for different time point. After stimulation, cells were additionally incubated with FITC-Dextran (70 kDa, 0.5 mg/ml) for 1 hour. Then, cells were subjected to flow cytometry to measure macropinocytosis as described in Methods (n=4 each condition). $P < 0.05$; significant comparisons by one-way ANOVA: *vs. Control HLF or unstimulated, † vs. IPF HLF.

Supplemental Figure 2: Effect of EIPA on metabolic reprogramming and cell viability.

(A) Control HLFs were treated with EIPA (12.5, and 25 μ M) or imipramine (Imi, 10 and 20 μ M) for 24 hours. Then, cell viability was measured using MTT assay as described in Methods (n=4 each condition). (B and C) Control HLFs were treated with EIPA (12.5 μ M) with or without TGF- β 1 (10 ng/ml) for 24 hours. Then cells were replated to Seahorse plates and subjected to the mito-stress assay as described in Methods (n=5 each condition). (D and E) HLFs were lentivirally-transfected with scramble (Scr) or shTSC1 as described in Methods. Then, total RNA was isolated and mRNA and protein level of *MEOX1* were determined by RT-qPCR and western blot respectively (n=3 each condition). (F) Cells were lentivirally-transfected with scramble (Scr), shTSC1 as described in Methods. Then cells were stimulated with TGF- β 1 (10 ng/ml) for 24 hours, harvested and subjected to western blot as described in Methods (n=3 each condition). $P < 0.05$; significant comparisons by one-way ANOVA (A-C and F) or Student t-test (D and E): *vs. unstimulated or Scr, † vs. TGF- β 1 alone.

Supplemental Figure 3: Effect of EIPA on collagen1a1 expression in AA and albumin-supplemented HLFs, effect of RAPTOR and MEOX1 silencing on MEOX1, collagen1 and α SMA expression.

(A) Control HLFs were conditioned in AA-free (AA-), AA-supplemented (AA+) or in 5% of bovine serum albumin (BSA+) in the presence or absence of EIPA (12.5 μ M) for 4 hours. Then, cells were harvested and subjected to qRT-PCR as described in Methods (n=4 each condition).

(B) Control HLFs were lentivirally transfected with Scr or shRAPTOR as described in Methods. Then, cells were treated with TGF- β 1 (10 ng/ml) for 24 hours or left untreated. Protein levels of MEOX1 were measured by western blot as described in Methods (n=4 each condition). (C)

Control HLFs were lentivirally transfected with Scr or shMEOX1 as described in Methods.

Then, cells were treated with TGF- β 1 (10 ng/ml) for 24 hours or left untreated. Protein levels of COL1 and α SMA were measured by western blot as described in Methods (n=4 each condition).

(D) Control HLFs were stimulated with TGF- β 1 (10 ng/ml) for 24 hours in the presence or absence of EIPA (12.5 μ M) or imipramine (10 μ M). Then, cells were fixed with 10% formalin and subjected to ChIP assay as described in Methods to measure the binding of MEOX1 to ACTA2 and COL1A1 promoters (n=4 each condition). Data are mean $\pm$ SEM. $P < 0.05$;

significant comparisons by one-way ANOVA: * vs. unstimulated or Scr alone, † vs. TGF- β 1 alone.

Supplemental Figure 4: Effect of Slc9a1 deficiency on macropinocytosis in mouse lung fibroblasts.

Mouse lung fibroblasts (MLFs) were isolated from *Slc9a1*^{f/f} and *Slc9a1*^{f/f} *Col1a2*^{Cre-ER(T)/0} mice as described in Methods. (A) *Slc9a1* mRNA expression was determined with qRT-PCR (n=3 each condition). (B, C and D) Cells were subjected to low AA conditions for 24 hours. Then,

cells were incubated with 70kDa FITC-Dextran (0.5 mg/ml) (B), AF488-Transferrin (1 mg/ml) (C), or lysed for western blot to measure CAV1 (n=3 each condition). $P < 0.05$; significant comparisons by Student t-test: * vs. *Slc9a1^{fl/fl}* (WT).

Supplemental Figure 5: H&E staining of the Bleo injured lungs.

(A, B and C) Mouse lungs were harvested and stained with H&E on the same area shown for Trichrome staining in Fig. 4 A, F, and 6 D respectively as described in Methods.

Supplemental Figure 6: Macropinocytosis inhibitors inhibit macropinocytosis in mesenchymal cells and do not affect lung inflammation in Bleo-injured mice.

(A) Mouse lungs were subjected to in vivo macropinocytosis assay as described in Methods. Live mesenchymal cells were considered as DAPI negative, CD90.2 positive and CD45/EpCAM/CD31 negative. (B) Mice were subjected to Bleo and treated with EIPA or imipramine (Imi) as described in Figure 4 F, and 6 D respectively. At day 21, lungs were harvested and subjected to in vivo macropinocytosis assay as described in Methods (n=5 each condition). (C and D) BALF fluid samples were collected as described in Methods and total protein levels and Mcp1 were measured by BCA assay or ELISA respectively (n=5 each condition). $P < 0.05$; significant comparisons by one-way ANOVA (B-D): *vs. No Bleo (Sham), †vs. Bleo alone.

Supplemental Figure 7: Effect of macropinocytosis inhibition on profibrotic gene expression in Bleo-injured mice and effect of imipramine on cell contractility.

(A) The lungs from WT and *Slc9a1* fCKO mice were subjected to RNA isolation as described in Methods. The mRNA levels of *Colla1*, *Acta2* and *Meox1* were measured with qRT-PCR as described in Methods (n=5 each condition). (B) The lungs from WT mice exposed to EIPA treatment with or without Bleo injury were subjected to RNA isolation as described in Methods.

The mRNA levels of *Colla1*, *Acta2* and *Meox1* were measured with qRT-PCR as described in Methods (n=5 each condition). (C) Control HLFs were mixed with collagen 1 solution as described in Methods and treated with Imi (10 μ M) with or without TGF- β 1 (10 ng/ml). Gel size was measured at 0 and 24 h after collagen gelation (n=4 for each condition). (D) IPF PCLS were treated with EIPA (12.5 μ M) or Imi (10 μ M) as described in Methods. Then, PCLS were lysed and subjected to qRT-PCR to assess *MEOX1* expression (n= 5 for each condition). Data are mean $\pm$ SEM. $P < 0.05$; significant comparisons by one-way ANOVA: *vs. No Bleo (Sham) or unstimulated, † vs. Bleo alone or TGF- β 1 alone.

Supplemental Figure 8: The effect of EIPA on different mesenchymal cell populations in IPF PCLS.

(A-F) IPF-derived PCLS were treated with EIPA (12.5 μ M) as described in Supplemental Methods. (G) CTHRC1 differential gene expression was assessed in different fibroblast subpopulations in Control and IPF PCLS as described in Supplemental Methods. Data analyzed by the hypergeometric test.

Supplemental Figure 9: The effect of EIPA on CTHRC1^{hi} cells with in different fibroblast subpopulations in IPF PCLS.

(A-D) IPF-derived PCLS were treated with EIPA (12.5 μ M) as described in Supplemental Methods. Data analyzed by the hypergeometric test.

Supplemental Figure 10: The knockdown efficiency of MEOX1, RAPTOR and TSC1 shRNAs.

(A, B and C) Control HLFs were lentivirally transfected with Scr or target shRNAs as described in Methods. Then, cells were harvested and lysed for western blot to determine the knockdown efficiency of each shRNA against its protein target by western blot (n of 3 each condition). (D, E

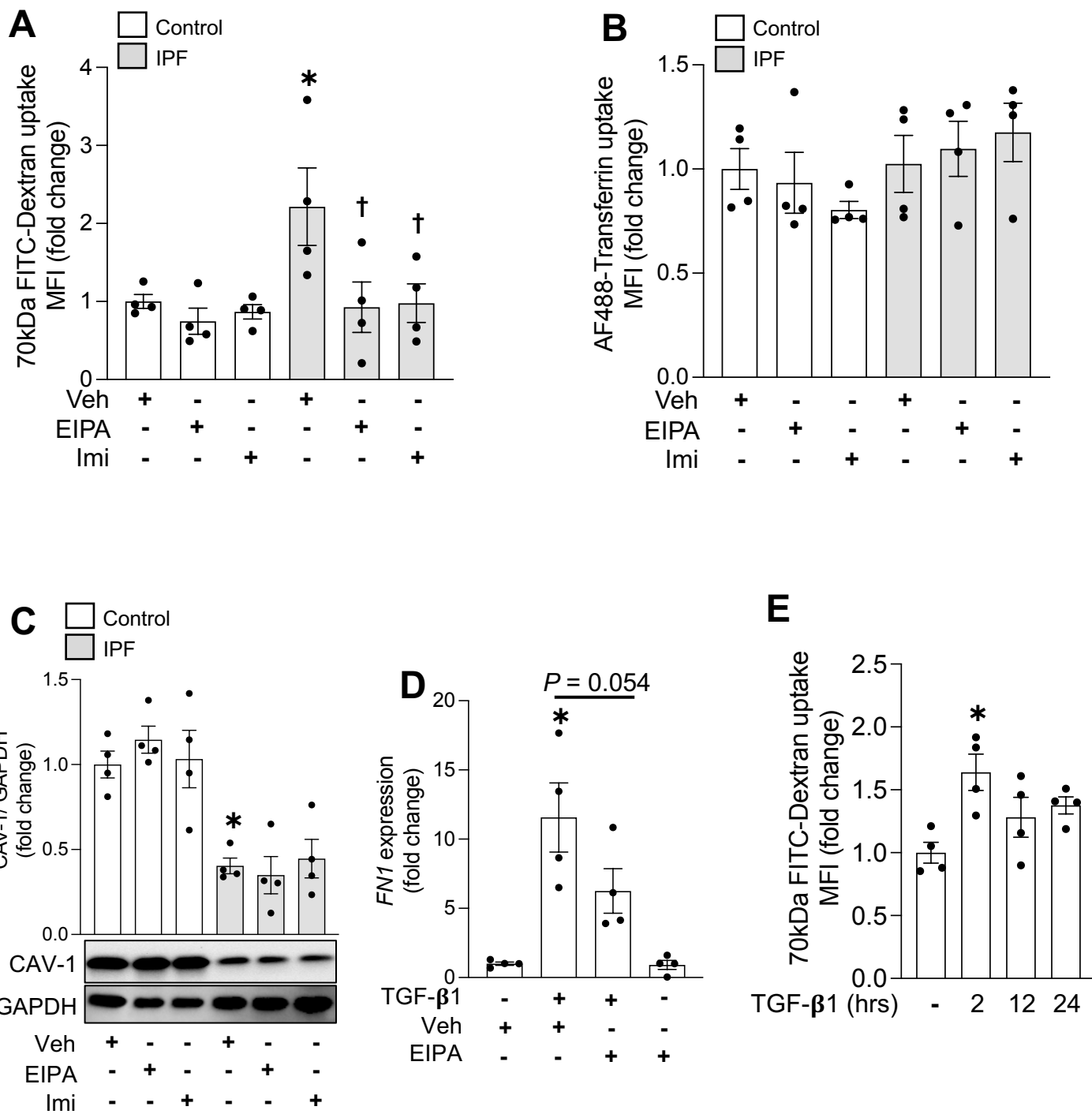
and F) Cells were lentivirally transfected with Scr or shRNA against its targets. Then, cells were with TGF- β 1 (10 ng/ml) for 24 hours. After stimulation, cells were harvested and subjected to qRT-PCR to measure COL1A1, ACTA2 or MEOX1 mRNA levels (n of 3 each condition). Data are mean $\pm$ SEM. $P < 0.05$; significant comparisons by one-way ANOVA: *vs. Scr alone, [†]vs. Scr + TGF- β 1 alone.

Supplemental Figure 11: The expression of mesenchymal markers in primary HLFs.

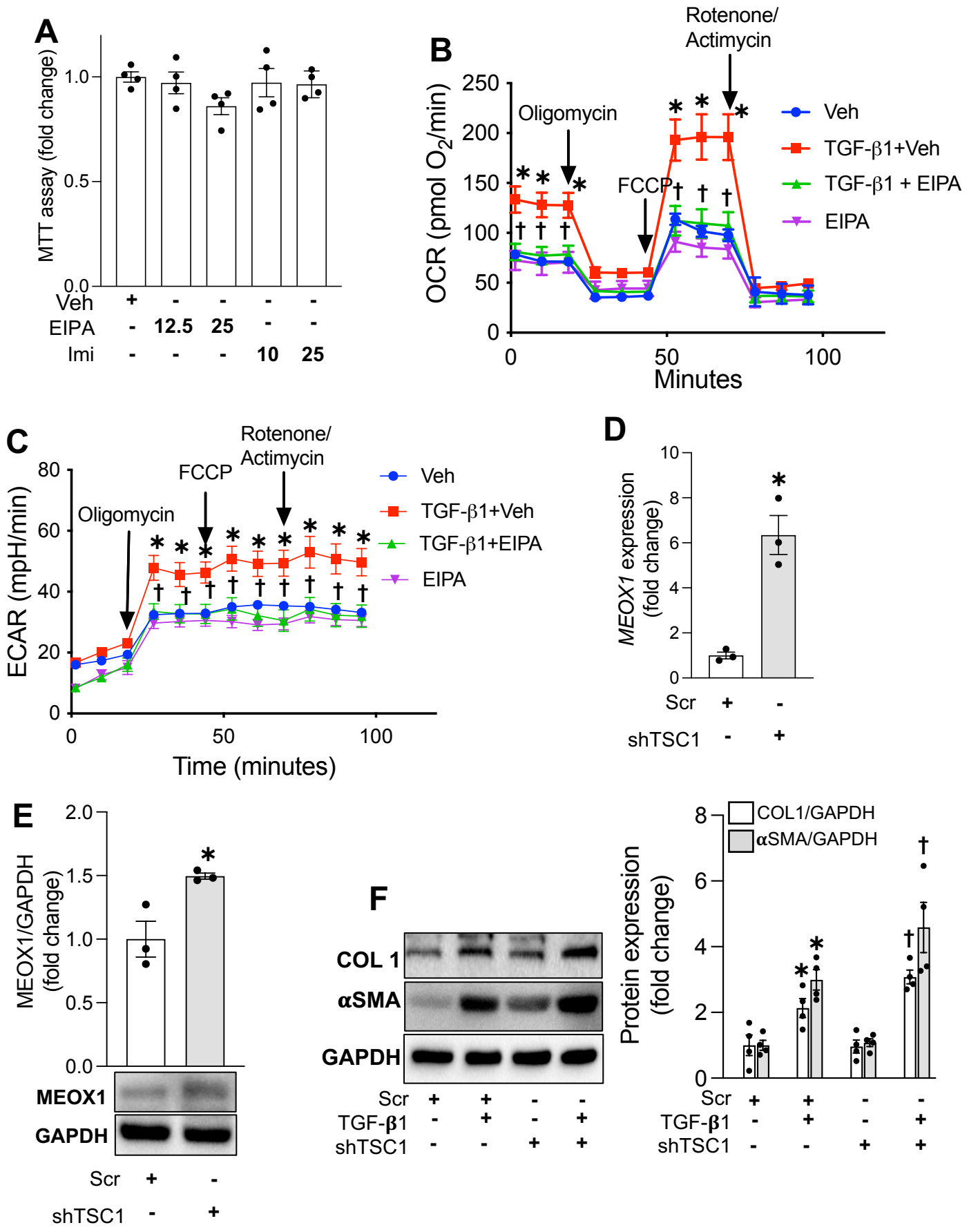
Control HLFs were harvested and stained with isotype Ab or target Ab as described in Methods and subjected to flow cytometry. The depicted histograms are the representative of 3 independent experiments.

Supplemental Table 1: List of Primers used for qRT-PCR.

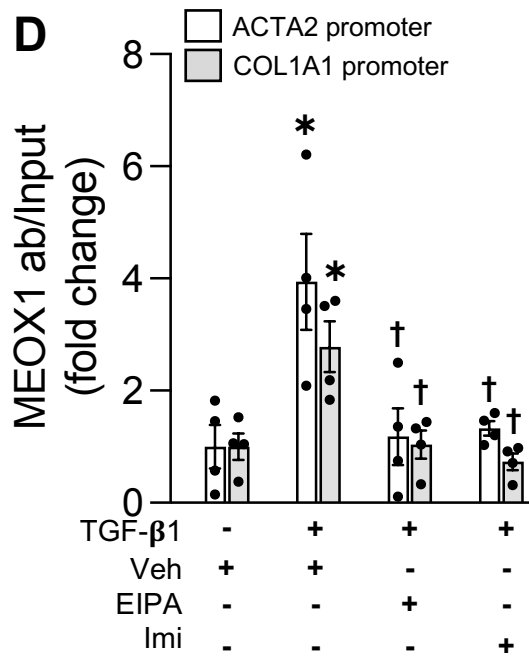
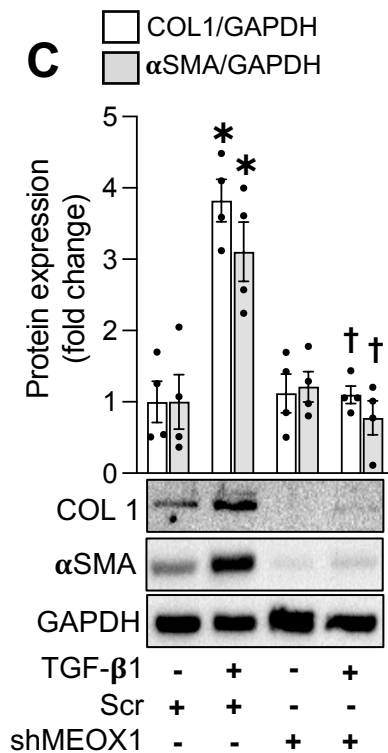
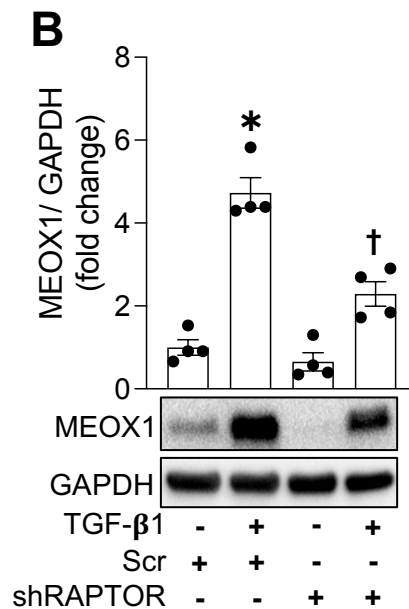
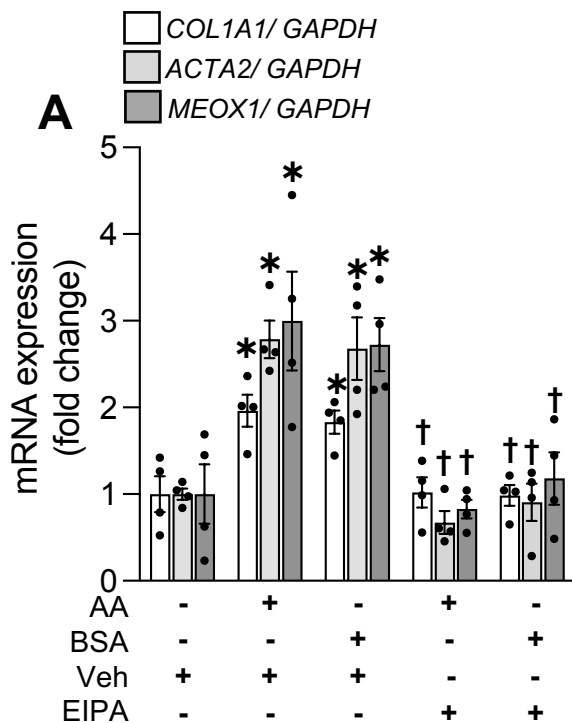
Supplemental Figure 1



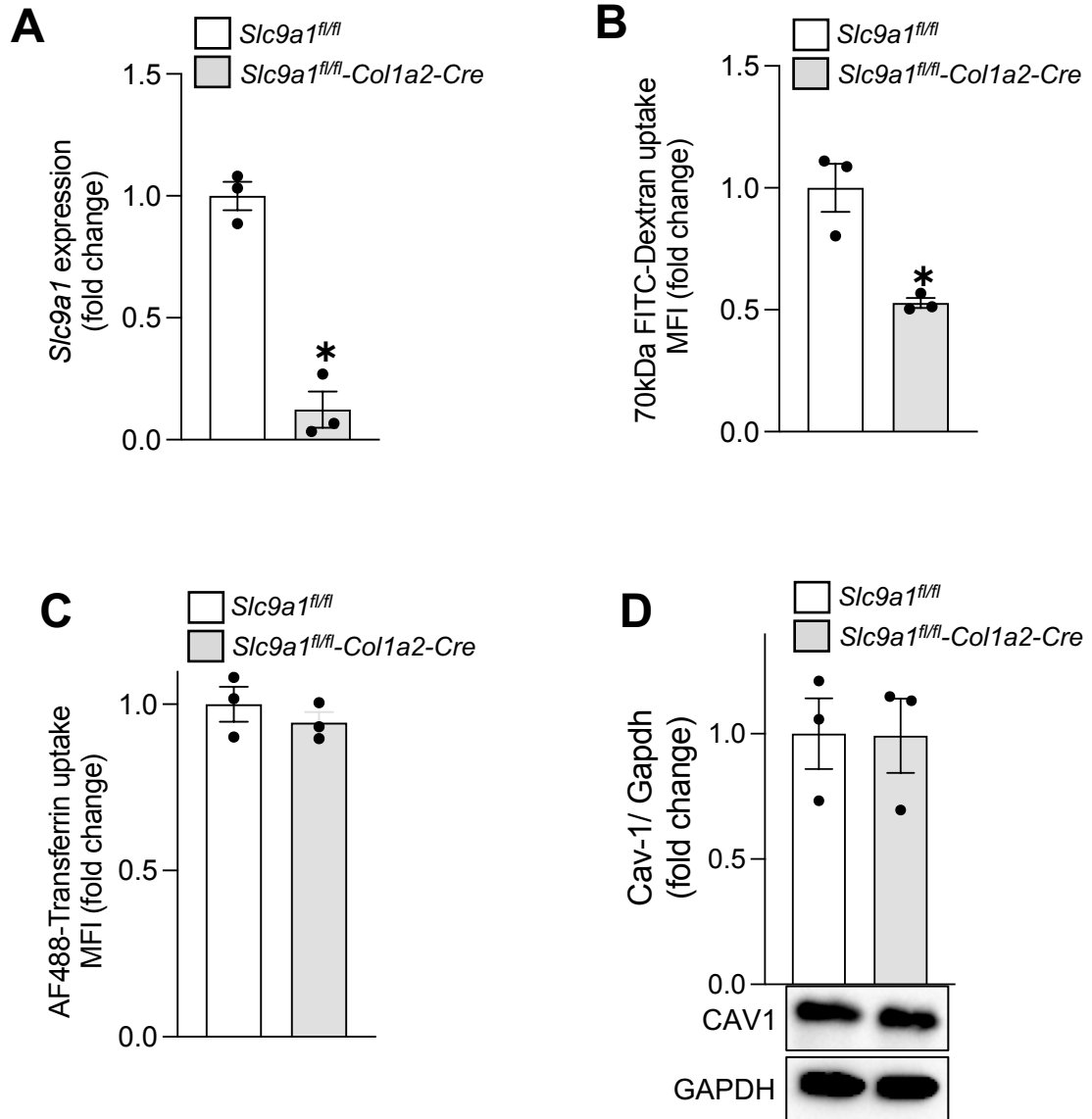
Supplemental Figure 2



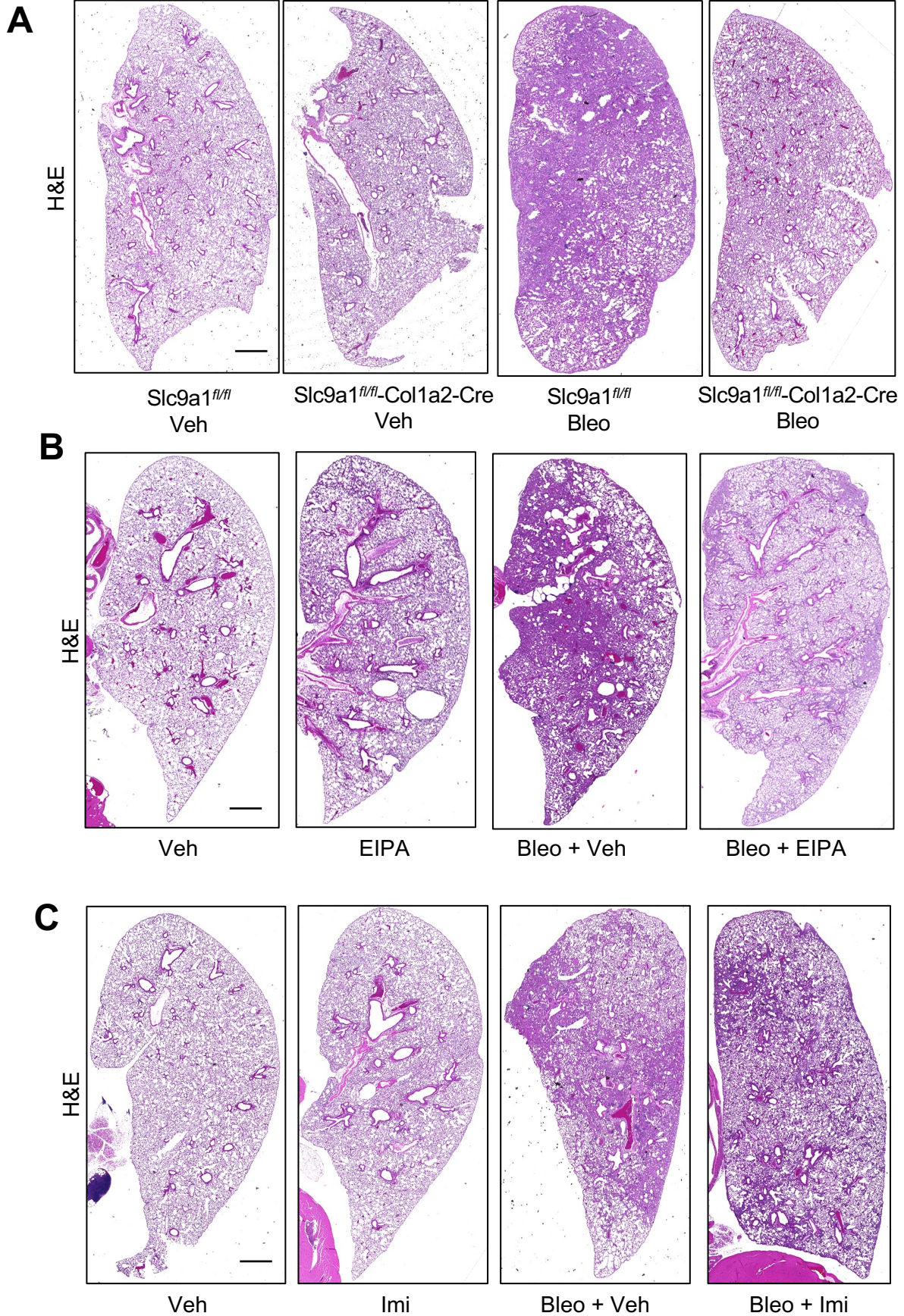
Supplemental Figure 3



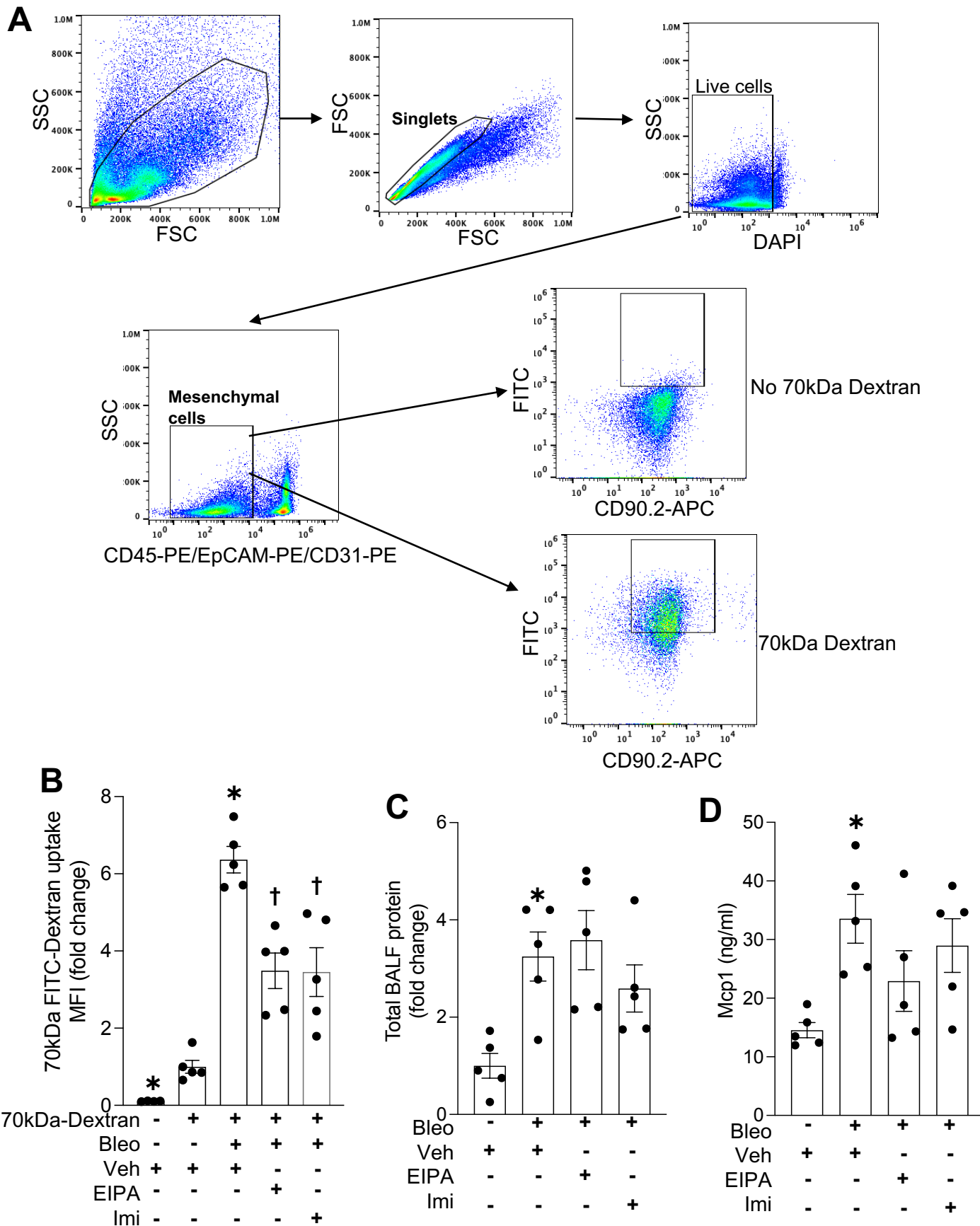
Supplemental Figure 4



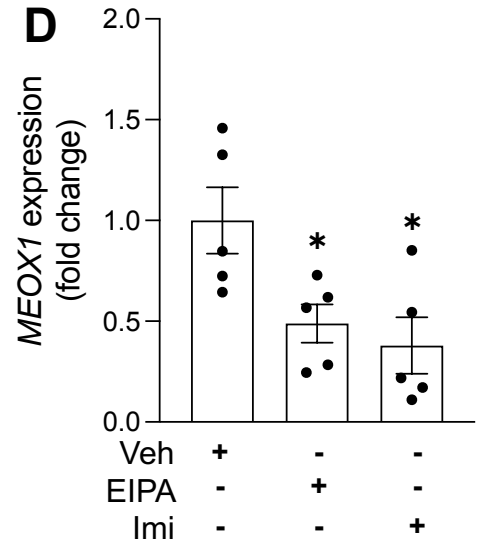
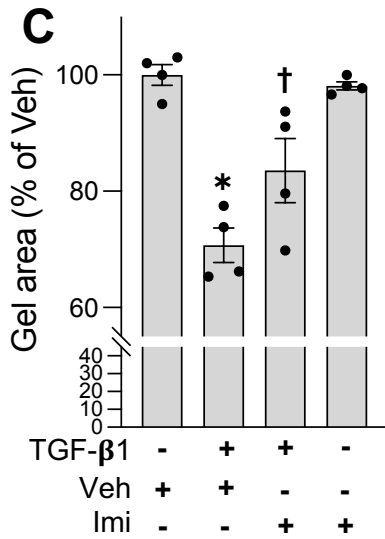
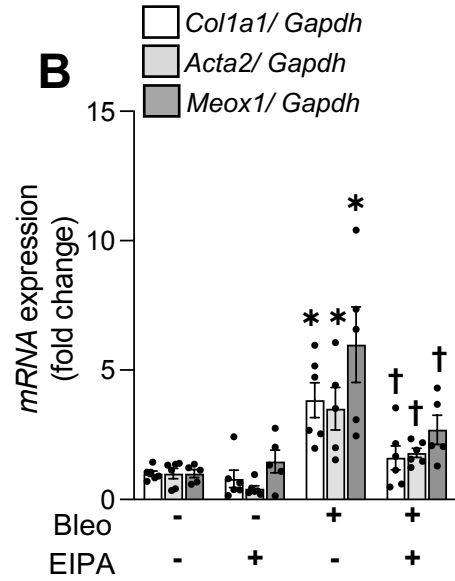
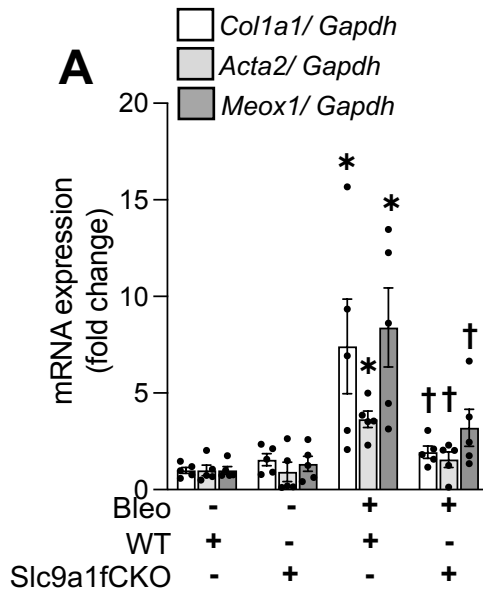
Supplemental Figure 5



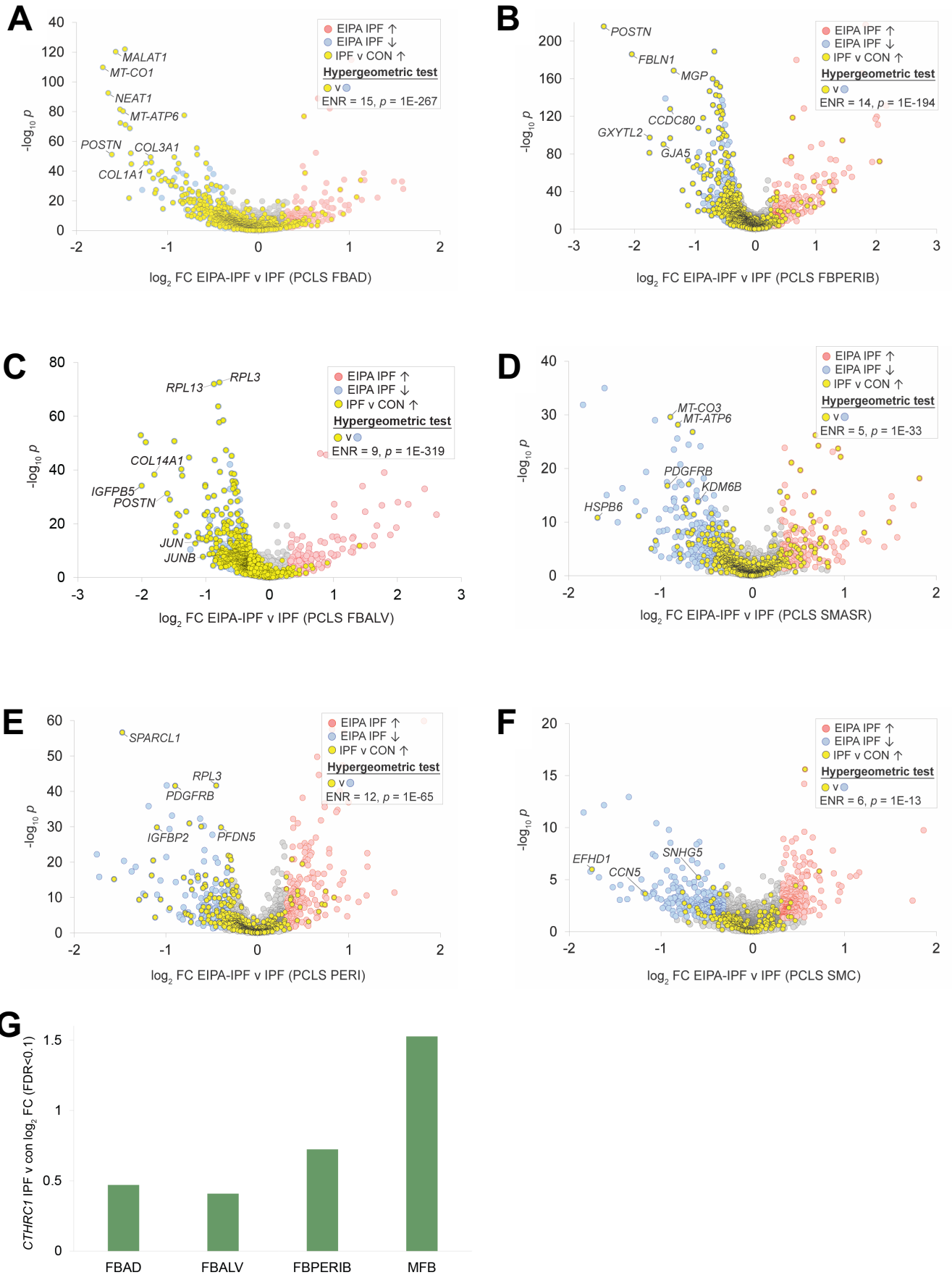
Supplemental Figure 6



Supplemental Figure 7

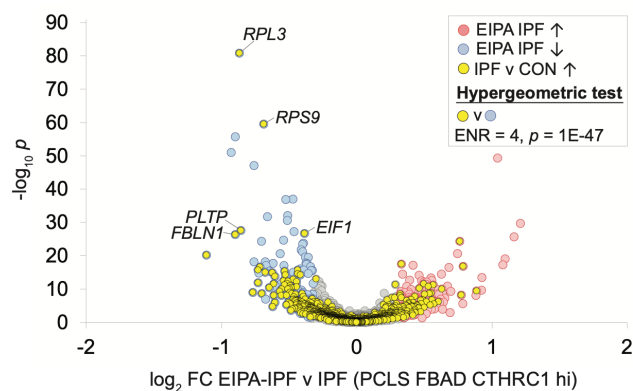


Supplemental Figure 8

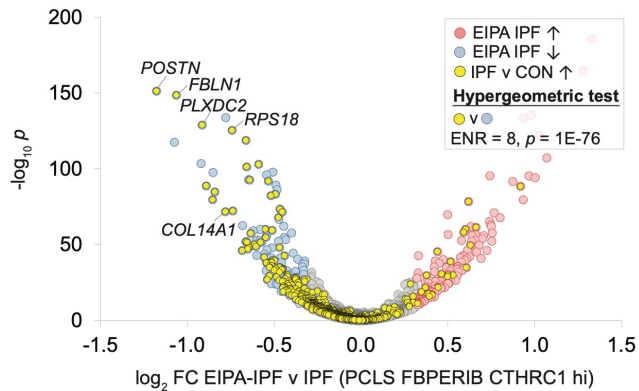


Supplemental Figure 9

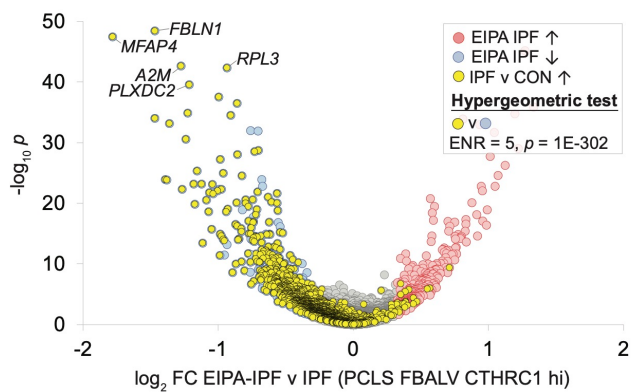
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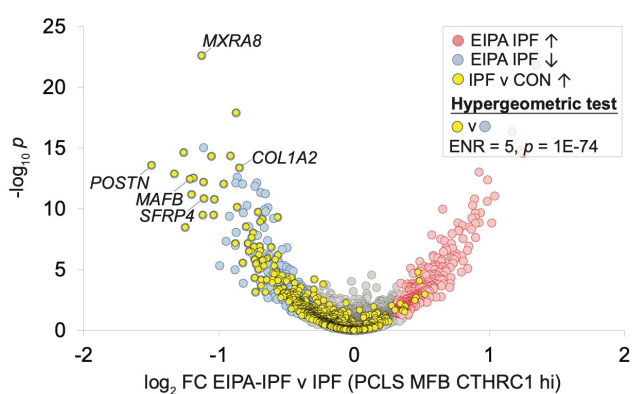
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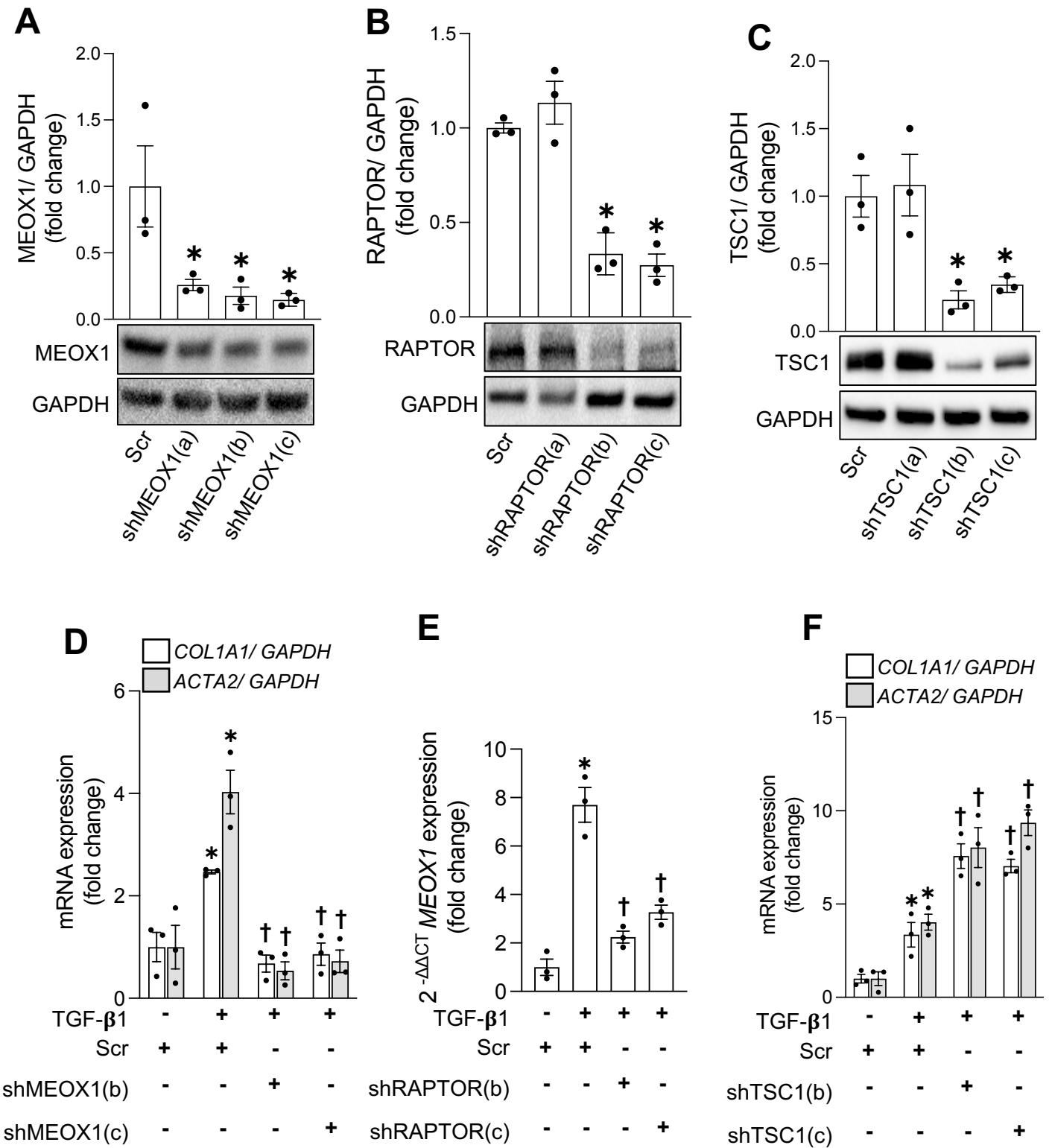
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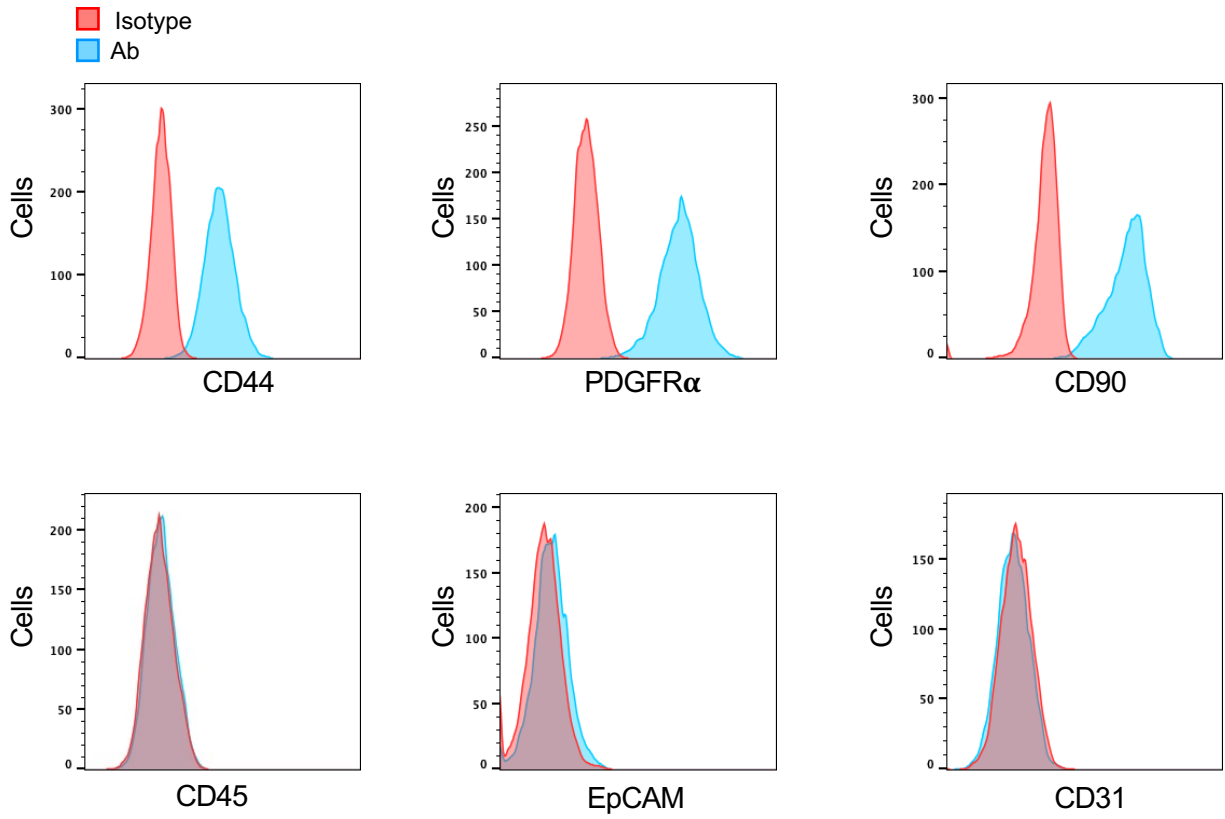
D



Supplemental Figure 10



Supplemental Figure 11



Supplemental Table 1

Primers:		
Gene	Forward (5'-3')	Reverse (5'-3')
Human GAPDH	GGATTTGGTCGTATTGGG	GGAAGATGGTGATGGGATT
Human COL1A1	AAGGGACACAGAGGTTTCAGTGG	CAGCACCAGTAGCACCATCATTTTC
Human FN1	GCCAGATG TGAGCTGCAC	GAGCAAATGGCACCGAGATA
Human ACTA2	GTGAAGAAGAGGACAGCACTG	CCCATTCCCACCATCACC
Human MEOX1	GAAACCCCCACTCGGAAGG	GGGTGCTGCTCAGTGAAGAT
Human JUN	CCTTGAAAGCTCAGAACTCGGAG	TGCTGCGTTAGCATGAGTTGGC
Human POSTN	CAGCAAACCACCTTCACGGATC	TTAAGGAGGCGCTGAACCATGC
Human IGFBP5	CGTGCTGTGTACCTGCCCAATT	ACTTGTCCACGCACCAGCAGAT
Mouse Gapdh	GTTGTCTCCTGCGACTTCA	GGTGGTCCAGGGTTTCTTA
Mouse Col1a1	ACCTCAGGGTATTGCTGGAC	CACCACTTGATCCAGAAGGA
Mouse Slc9a1	CCTGACCTGGTTCATCAACA	TCATGCCCTGCACAAAGACG
Mouse Acta2	ATCACCAACTGGGACGACAT	CATACATGGCTGGGACATTG
Mouse Meox1	GGAGGATTGCATGGTACTTGGG	CTTTGCTGCTGCCTTCTGGCTT