Supplemental material and methods

Recombinant adenovirus

The construction of adenoviral vectors has previously been described (1, 2). Briefly, Cre-recombinase cDNA and TGF-β1 cDNA were cloned into a shuttle vector downstream a human CMV promoter and co-transfected with a shuttle plasmid in 293 cells. The control vector (AdDL) with no insert in the E1 region was produced in the same way (1). The TGF-β1 cDNA has cysteine to serine mutations at position 223 and 225, rendering the expressed TGF-β1 biologically active.

Immunochemistry

Paraffin embedded sections of human lung tissue were pretreated in citrate buffer pH6 for 40 minutes for antigen retrieval. Section were then incubated with TRIM33 (sc-101179, Santa Cruz), α-SMA (A2547, Sigma Aldrich) and CD68 (HPA048382, Sigma Aldrich) monoclonal antibody respectively (. Positive cells were revealed using the Chromogen DAB (K3468, Dako) for TRIM33 and Green Chromogen (BRR807A, Biocare Medical Vina Green) or Red Chromogen for CD68, α-SMA, CD326 (WR806, Biocare Medical Warp Red). Rabbit and Mouse Ig were used to test the specificity of immunostaining.

Immunofluorescence

The lungs from mice were insufflated with a mix of 1:1 Water/OCT and quickly freeze at -80°C. Slide from those lungs were fixed in methanol for 15 min before being saturated in 5% FBS.

Primary antibodies were incubated over-night at 4°C for TRIM33 (301-059A Biothyl laboratory, Texas, USA), Vimentin (ab8978, Abcam), CD68 (NPB2-33337, Novus Bio), GFP (#2956), CD326 (ab221552, abcam), ER-TR7 (NB100-64932, Novus Bio). Goat anti-rabbit and goat anti-mouse conjugated with Alexa Fluor 594 or Alexa Fluor 488 (Molecular Probe, InVitrogen, Cergy Pontoise, France) were used as secondary antibodies at a dilution of 1 : 2000.

TGF- β1, TNFα, IL-6 quantification (ELISA)

Total mouse TGF-β1 from BALFs was determined using ELISA (R&D Systems, Lille, France), according to the recommendations of the manufacturer.

Cell culture

A549 lung epithelial cells (ATCC) were grown as monolayers in 5% CO2 at 37°C in DMEM High glucose medium (Lonza, Paris, France), supplemented with 10% of Fetal Bovine Serum (FBS, Lonza). Cells were seeded at 80% confluence one day prior starting the treatment and then stimulated with recombinant human TGF-β1 (rTGF-β1, R&D Systems, Minneapolis, MN) in complete medium at 10 ng/ml.

Primary cell culture

Mouse fibroblasts were isolated as previously described (3, 4) and cultured in DMEM + 10% serum, 1% L-glutamine and 1% penicillin/streptomycin. BMDM were isolated as previously described (5) and cultured in RPMI + 10% serum, 1% L-glutamine and 1% penicillin/streptomycin.

Bone Marrow Isolation and Bone marrow-derived Macrophages

Wild type mice or Floxed *Trim33* mice femurs and tibiae were carefully cleaned of flesh. The tip of each bone was cut off. Then the marrow was collected by inserting a syringe into the medullary cavity at one end of the bone and flushing with Dulbecco's Modified Eagle's Medium (DMEM). The cells were filtered before being centrifuged at 10 000 rpm at 4°C for 10 min. Next the supernatant was replaced by a culture medium DMEM containing macrophage colony-stimulating factor (M-CSF). Cultures were kept at 37°C with 5% CO2 for seven days. The medium was changed every two days.

RNA interference, transfection and infection

Plasmids used were : pcDNA3.1 B myc empty vector, pcDNA3.1 HSPB5, pcDNA3.1 6 his-SUMO-1, pcDNA3.1 TRIM33. TRIM33 was subcloned from pSG5-Flag-TRIM33, a gift from R.Losson (Strasbourg, France). Plasmids constructs were transiently transfected using TransIT-X2® transfection reagent (Mirus bio, Merck KGaA, Darmstadt, Germany) following the manufacturer's recommendations. HSPB5 siRNA and TRIM33 siRNA were purchased from Applied Biosystems (Courtaboeuf, France). SiRNAs (50 nM) were transfected using INTERFERin (Polyplus, Illkirch, France) following the manufacturer's recommendations. Six hours after

transfection, the medium was removed. Adenoviral vectors were used to infect BMDM, primary fibroblasts and 3D-lung tissue slices for 24h with a Multiplicity of infection of 5, 4 and 8 respectively.

q-PCR analysis

Total mRNA from total lungs, mouse fibroblasts and 3D-lung tissue slices was extracted using TRIzol (Invitrogen, Carlsbad, USA). Reverse transcription was performed on total mRNA using the M-MLV kit (Promega, Charbonnieres, France). Quantitative RT-PCR (ViiA 7 Real-Time PCR System, ThermoFischer Scientific, Waktham, USA) was performed on the cDNA using SYBR green master mix (ThermoFisher Scientific) using the following primers for mouse :

Serpine1	Forward	5'-GGCCGTGGAACAAGAATGAGAT-3'
	Reverse	5'-GCTTGAAGAAGTGGGGCATGAAG-3'
Tgfb1	Forward	5'-CGTGGCTTCTAGTGCTGACGC-3'
	Reverse	5'-CCATGTCGATGGTCTTGCAGGT-3'
Hspb5	Forward	5'-GCCTCTTCGACCAGTTCTTCG-3'
	Reverse	5'-AGGGAAGTGGCTGTTGAGAAG-3'
Acta2	Forward	5'-GCAAGAGAGGGATCCTGACG-3'
	Reverse	5'-TCGTCCCAGTTGGTGATGATG-3'
Rpl32 (60S ribosomal protein L32) House keeping gene	Forward	5'-GAAACTGGCGGAAACCCA-3'
	Reverse	5'-GGATCTGGCCCTTGAACCTT-3'
Col1a1	Forward	5'-GCTCCTCTTAGGGGCCACT-3'
	Reverse	5'-CCACGTCTCACCATTGGGG-3'
Twist1	Forward	5'-GGACAAGCTGAGCAAGATTCA-3'
	Reverse	5'-CGGAGAAGGCGTAGCTGAG-3'
Lef1	Forward	5'-TGTTTATCCCATCACGGGTGG-3'
	Reverse	5'-CATGGAAGTGTCGCCTGACAG-3'
Trim33	Forward	5'-CTTCTGCCTGCGCTGTCT-3'
	Reverse	5'-TGCACTTGCATTATCTTCACAA-3'

Or human :

TRIM33	Forward	5’-CTGTTTTCTGCCCTGTACACA -3’
	Reverse	5’-CGCCAGTAGATTCTCAATTGCA-3’
UBC House keeping gene	Forward	5’-GTGGTGCGTCCAGAGAGAC-3’
	Reverse	5’-GGCCTTCGCCATATCCTTTTC-3’

Western blotting

30 µg of proteins were loaded on 12% polyacrylamide gels. Membranes were incubated overnight at 4°C with specific antibodies at a dilution of 1:1000 for the detection of HSPB5 (mouse clone 1B6.1-3G4, Enzo life science, New York, USA), Smad4 (mouse clone B-8, Santa Cruz biotechnologiy, Dallas, USA), TRIM33 (rabbit NBP1-83747, Novus Biologicals, Centennial, USA), PAI-1 (mouse clone 3A120, Thermo Fisher Scientific, Waltham, USA), Col1a1 (HPA008405 Sigma-Aldrich, St. Louis, USA). HSC70 (mouse clone B-6, Santa Cruz Biotechnology) or β-Actin (mouse, a1978, Sigma-Aldrich) antibody was used as a loading control. HRP-conjugated goat anti-rabbit or goat anti-mouse antibodies (Jackson Immunoresearch Laboratories, Suffolk, UK) were used as secondary antibodies.

SUMOylation assay

A549 cells were transfected with 5 µg of each plasmid encoding SUMO-1-his, TRIM33 and HSPB5. Cells were treated overnight with the non-selective DUB inhibitors PR616 (Sigma-Aldrich) before cell lysis. Sumoylated proteins (SUMO-1-His) were pulled-down following the manufacturer's instructions, using Protino ® Ni-NTA agarose (Macherey-Nagel, Bethlehem, USA), an affinity chromatography matrix designed to purifying proteins carrying a His-tag and revealed using TRIM33 antibodies.

FLIM-FRET

Cells were fixed with paraformaldehyde (4% PFA for 10 min) and permeated with a PBS-Triton (0.1%, 5 min) solution. After saturation of nonspecific sites with BSA (5%, 20 min), cells were incubated with primary antibodies overnight in a humidified chamber at 4°C. Cells were stained for SMAD4 with a mouse antibody (clone B-8, Santa Cruz biotechnology), and for TRIM33 with a rabbit antibody (NBP1-83747). Goat anti-rabbit and goat anti-mouse conjugated with Alexa Fluor 594 and Alexa Fluor 488 respectively (Molecular Probe, InVitrogen, Cergy Pontoise, France) were used as secondary antibodies at a dilution of 1: 2000. The interaction between Smad4 and TRIM33 was studied using FLIM-FRET measurements. The fluorescence lifetime is defined as the average time a molecule remains in an excited state prior to return to the ground state. Fluorescence lifetime imaging microscopy (FLIM) is a well-established technique to study molecular interactions and visualize FRET. The presence of acceptor molecules in the close environment of the donor allows FRET to occur, resulting in a decrease of donor fluorescence lifetime.

We have measured the fluorescence lifetime of Smad4-Alexa 488 (donor) in the presence and the absence of TRIM33-Alexa 494 (acceptor) to determine the occurrence of FRET. Imaging was carried out with a ×100 PlanApo objective (NA: 1.4, oil, Nikon, Japan) using a time-correlated single-photon counting (TCSPC) module (PicoQuant) on a Nikon Eclipse TE-200 confocal scanning microscope from DImaCell imaging facility (Dijon, France). Excitation at 485 nm was provided by a pulsed diode laser (40 MHz). Fluorescence emission of Alexa 488 was collected using a Single-Photon Avalanche Diode detector, using a FF01-520/35 band-pass emission filter (Semrock). We performed a global lifetime analysis on regions of interest of the FLIM images using the SymPhoTime software (PicoQuant). Fluorescence lifetimes were calculated by fitting the tail of Alexa 488 fluorescence decay with a bi-exponential model, with two lifetime constants. For this analysis, we used the mean value of these two constants as the Alexa 488 lifetime.

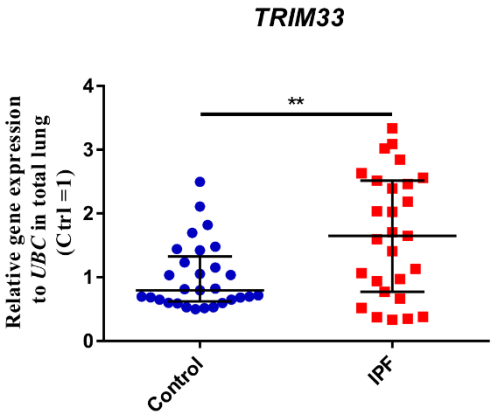
Bio Layer Interferometry

For in vitro interaction assays, we used Biolayer interferometry technology (Octet Red96, Forté-Bio). This is an optical analytical technique that analyzes the interference pattern of white light reflected from biosensor tip surface. The change in thickness of the biological layer resulting from analyte binding on his immobilized ligand was measured by a wavelength shift.

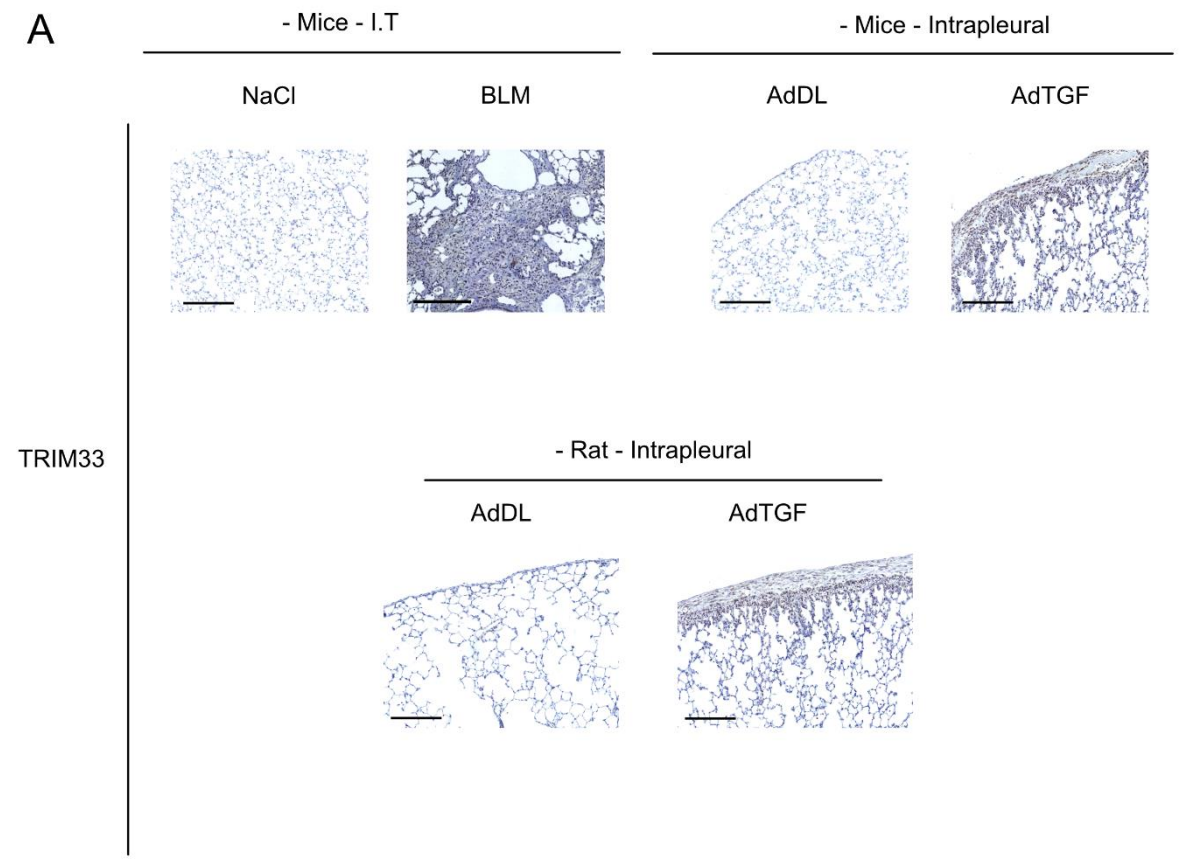
BLI was performed using an OctetRED96 instrument from PALL/ForteBio (San Jose, USA). Recombinant SMAD4 and HSPB5 were biotinylated with NHS-PEG4-biotin reagent (Thermo Fisher Scientific, 21330) in a ratio of 1:3. The excess of reagent was removed using Zeba Spin Desalting Columns (Thermo Fisher Scientific). Proteins were loaded on streptavidin biosensors

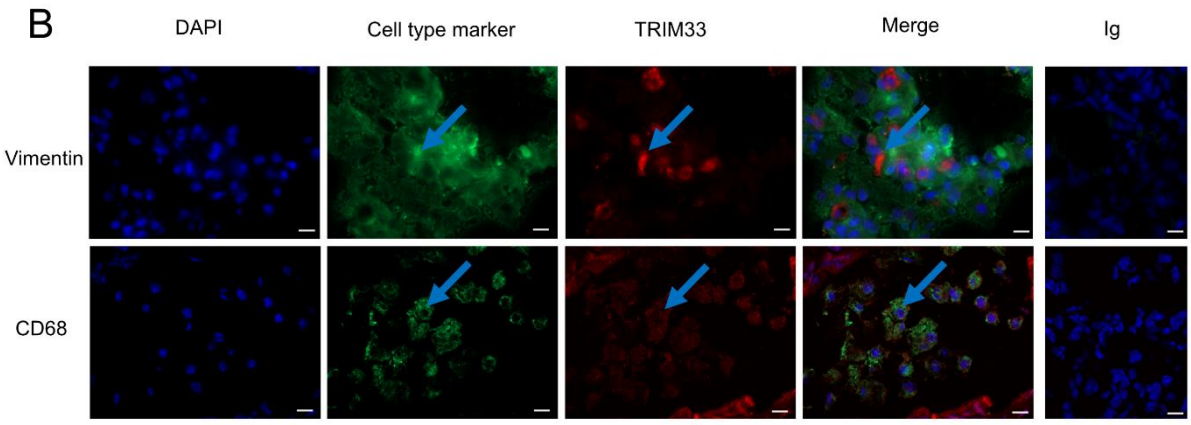
(ForteBio) at 5µg/ml. Association step was performed for 600 sec in wells containing the indicated concentration of rhSMAD4 or rhTRIM33 diluted in PBS. The experiment was performed in black 96-well plates with 200 µL volume/well, under constant shaking (400 g) at 25°C. The Kd values were calculated by the software provided by the manufacturer (version 7.1.0).

Supplementary Figure S1. *TRIM33* is up-regulated in lung of IPF patients. mRNA levels of *TRIM33* were analyzed by quantitative PCR of samples from 27 IPF patients undergoing lung transplantation and 29 normal lung tissues flanking lung cancer resections (Control). Results are expressed as median with interquartile range; **P = 0.0028, unpaired t test with Welch's correction.

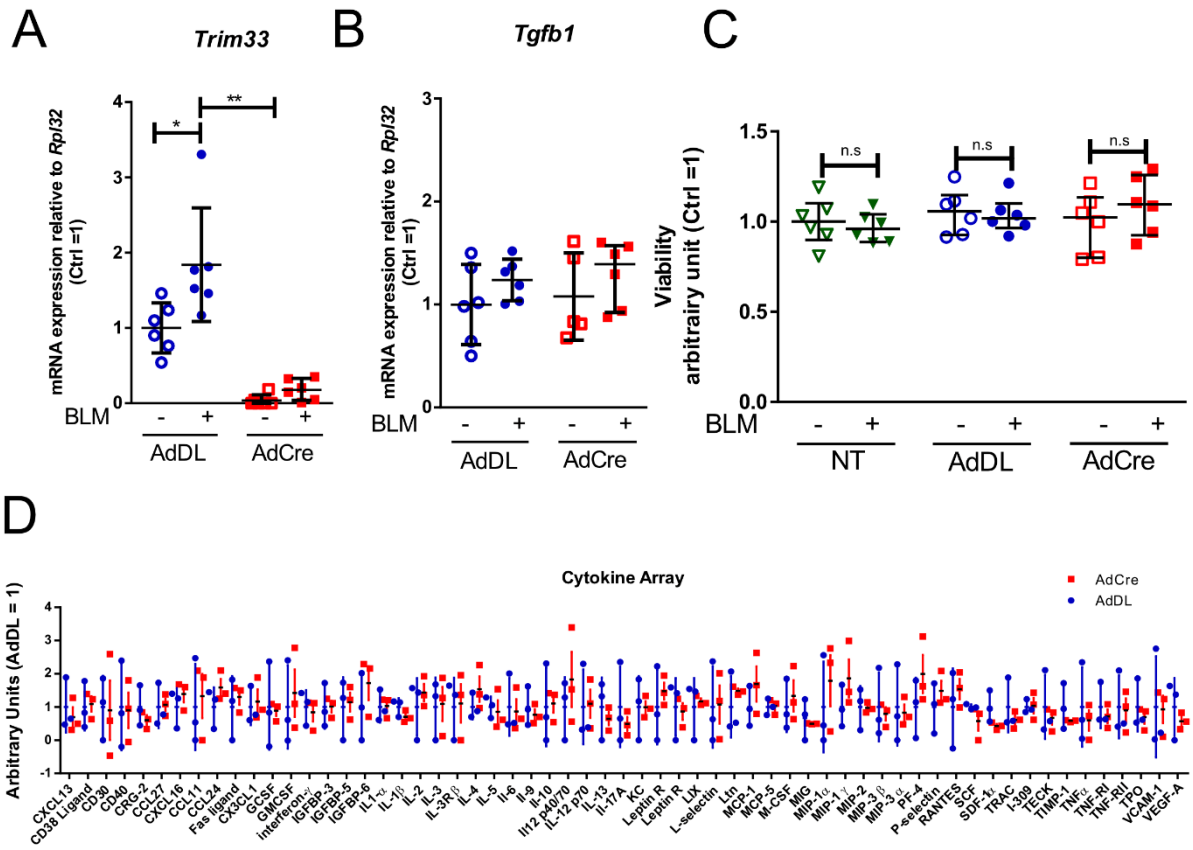


Supplementary Figure S2: TRIM33 is up-regulated in rodent models of experimental lung fibrosis. (A) Representative images of TRIM33 immunostaining on lung sections from mice challenged with either intratracheal instillation of bleomycin (BLM) (upper left), or intrapleural administration of AdTGF-β1 (upper right) or from rats exposed to AdTGF-β1 by intrapleural injection (lower part), scale bar 250 μm. (B) Representative images (n=5) of TRIM33 immunofluorescence (red) with a co-staining of either Vimentin (Green) for fibroblasts (arrow head) or CD68 (Green) for macrophages (arrow head) on lung sections from mice 21 days after instillation of bleomycin, scale bar: 10 μm.

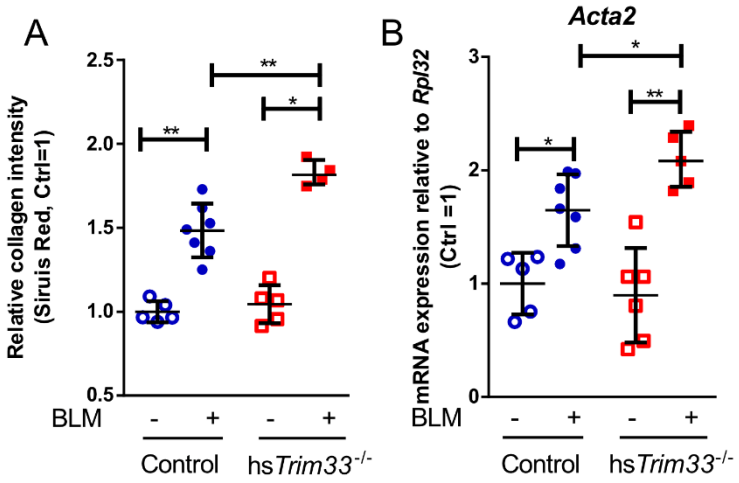

A
- Mice - I.T
- Mice - Intrapleural
NaCl
BLM
AdDL
AdTGF
TRIM33
- Rat - Intrapleural
AdDL
AdTGF


B
DAPI
Cell type marker
TRIM33
Merge
Ig
Vimentin
CD68

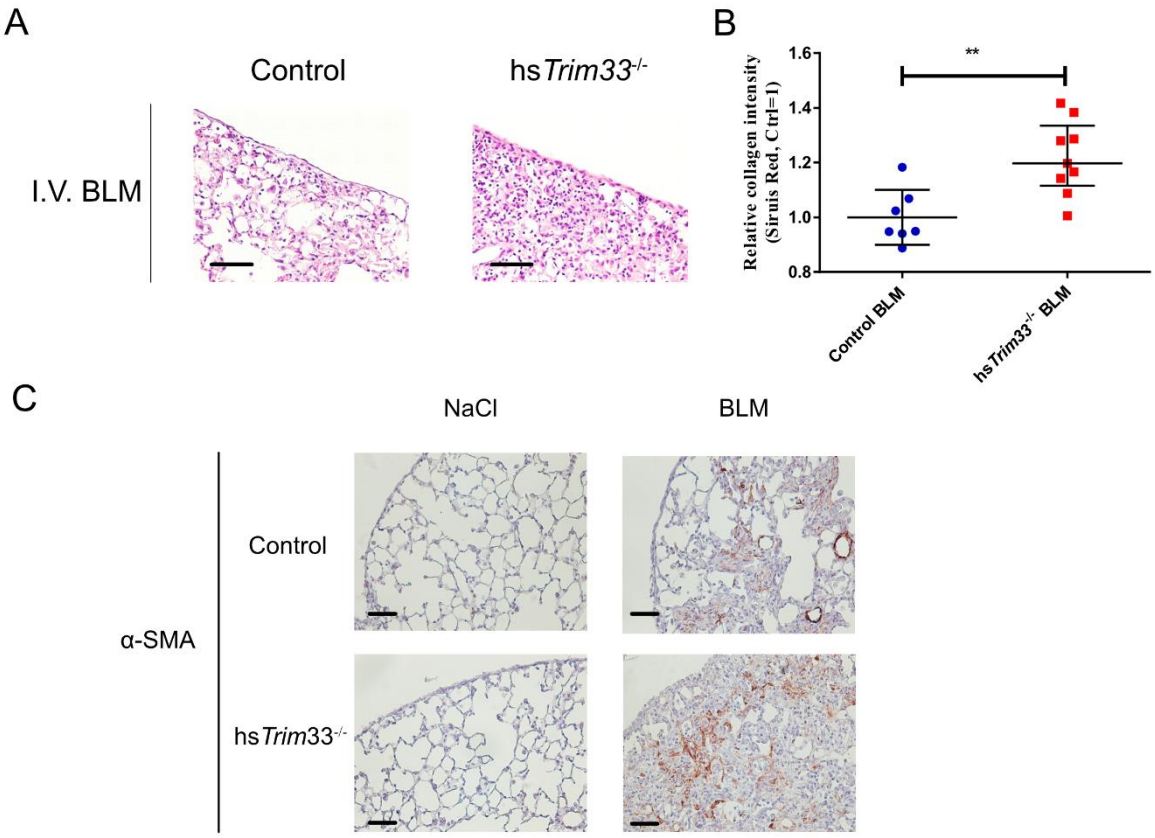
Supplementary Figure S3: *Trim33* deletion does not affect cytokine expression in BMDM. (A-B) mRNA levels of *Trim33, Tgfb1* were analyzed by quantitative PCR on BMDM infected with AdDL or AdCre and exposed to bleomycin (5mg /ml, 48h) (n=3). (C) Viability of BMDM assessed using methylene blue after bleomycin treatment (5mg/ml, 48h). Results are expressed as median with interquartile range (n=3 with 2 biological duplicates). (D) Cytokine array was performed on the supernatant from BMDM infected with AdDL or AdCre and exposed to bleomycin (5mg /ml, 48h) (n=3).


A
Trim33
mRNA expression relative to Rpl32 (Ctrl =1)
*
**
BLM
AdDL
AdCre
B
Tgfb1
mRNA expression relative to Rpl32 (Ctrl =1)
C
Viability arbitrairy unit (Ctrl =1)
n.s
NT
AdDL
AdCre
D
Cytokine Array
AdCre
AdDL
Arbitrary Units (AdDL = 1)
CXCL13
CD30 Ligand
CD30
CD40
CRG-2
CCL27
CXCL16
CCL11
CCL24
Fas ligand
CX3CL1
GCSF
GMCSF
IGFBP-3
IGFBP-5
IGFBP-6
IL-2
IL-3
IL-4
IL-5
Il-6
Il-9
Il-10
Il12 p40/70
IL-12 p70
IL-13
Il-17A
KC
Leptin R
L-selectin
LIX
Ltn
MCP-1
MCP-5
M-CSF
MIG
MIP-2
PF-4
P-selectin
RANTES
SCF
TRAC
I-309
TECK
TIMP-1
TNF-RI
TNF-RII
TPO
VCAM-1
VEGF-A

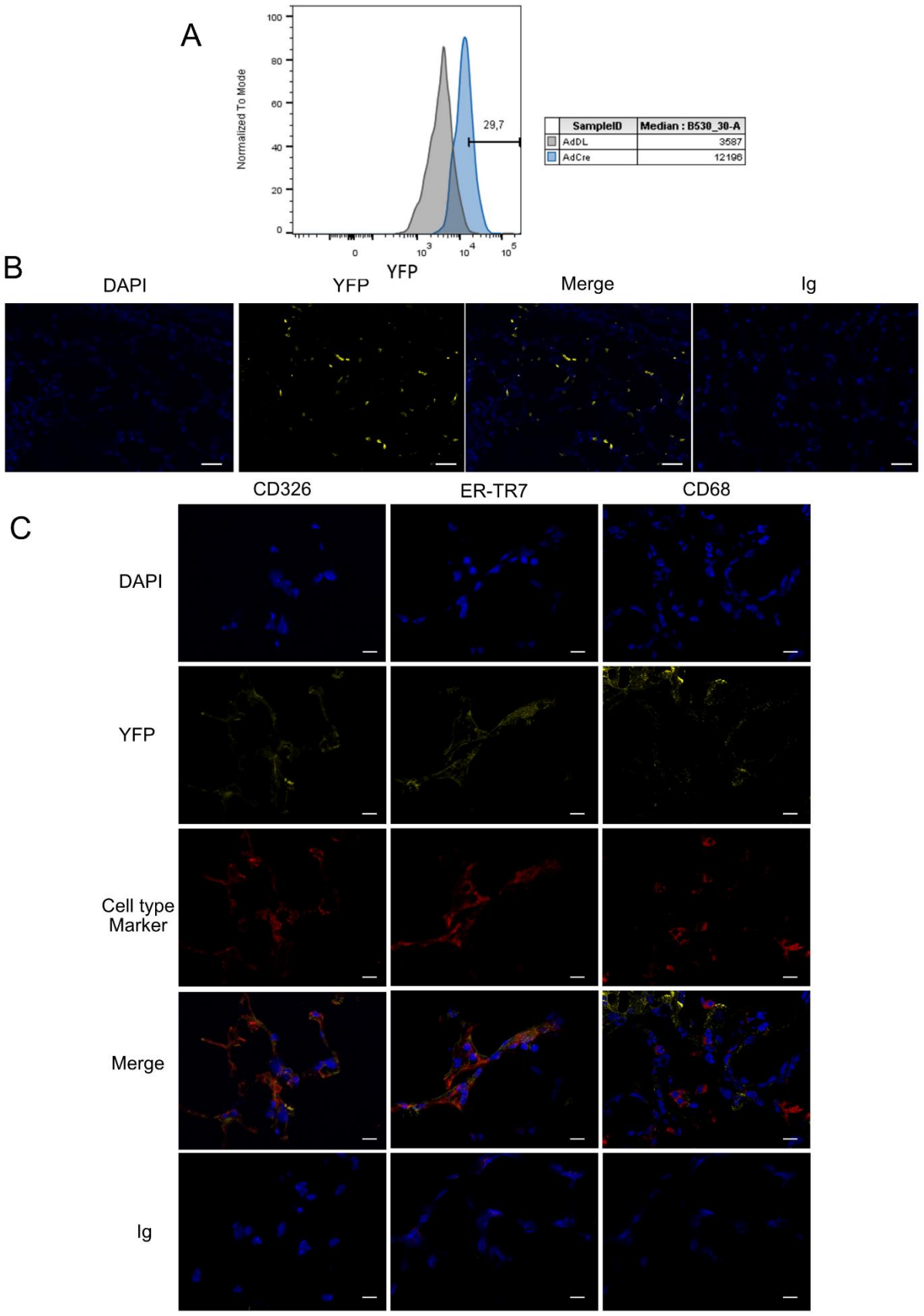
Supplementary Figure S4: hs*Trim33*-/- mice are more sensitive to bleomycin-induced fibrosis. (A) Collagen quantification of lung sections stained with Picrosirius red from control and hs*TRIM33*-/- mice at day 21 after BLM-treatment. Results are expressed as median with interquartile range; *P < 0.05, **P<0.01, non-parametric Mann-Whitney test, n= 4-8 per group. Measurements were made on the parenchyma areas. (B) *Acta2* mRNA levels analyzed by quantitative PCR in lungs from control and hs*Trim33*-/- mice at day 21 after BLM-treatment. Results are expressed as median with interquartile range. *P < 0.05, **P < 0.01, non-parametric Mann-Whitney test, n= 4-8 per group.


A
Relative collagen intensity
(Siruis Red, Ctrl=1)
2.5
2.0
1.5
1.0
0.5
**
**
*
BLM
-
+
-
+
Control
hsTrim33-/-
B
Acta2
mRNA expression relative to Rpl32
(Ctrl =1)
3
2
1
0
*
*
**
BLM
-
+
-
+
Control
hsTrim33-/-

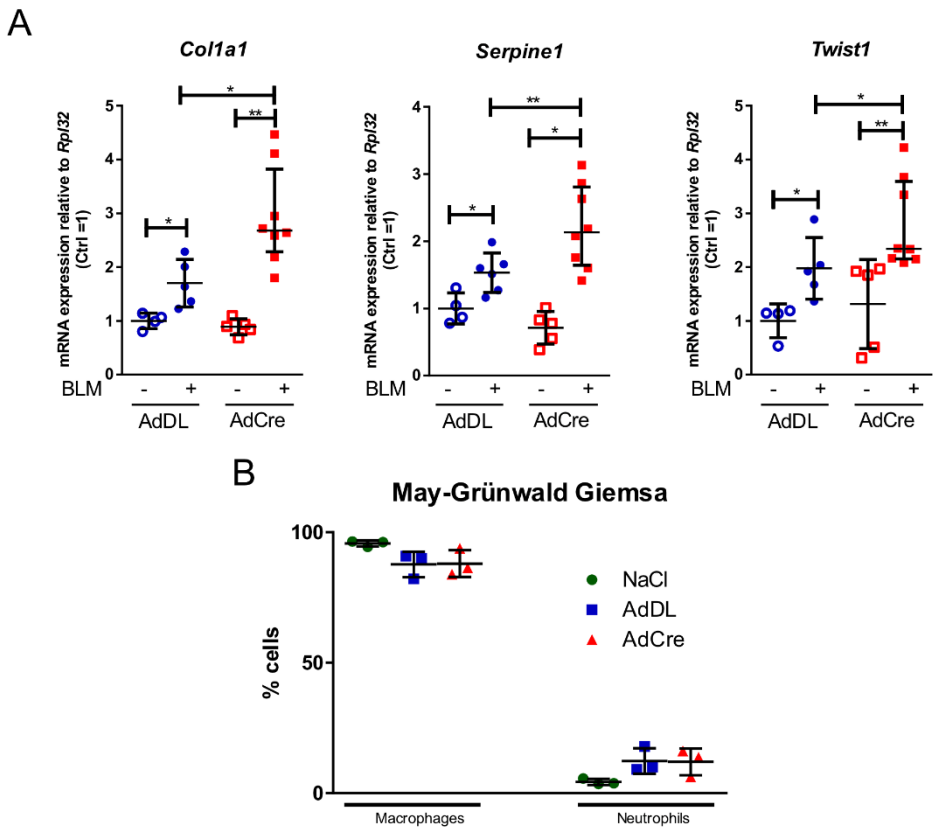
Supplementary figure S5: hs*Trim33*-/- mice are more sensitive to intravenous bleomycin-induced fibrosis. (A) Histological analysis of control (hs*Trim33*-/-) and hs*Trim33*-/- mouse lungs 14 days after intravenous injection of bleomycin (BLM). Hematoxylin and eosin staining (n= 6), scale bar 250 µm. (B) Collagen quantification using lung sections stained with Picrosirius red from control and hs*Trim33*-/- mice at day 14 after BLM exposure. Measurements were made on the parenchyma areas. Results are expressed as median with interquartile range. **P< 0.01, non-parametric Mann-Whitney test; n= 7-9 per group. (C) Representative images (×500) of α-SMA immunostaining on lung sections from control and hs*Trim33*-/- mice at day 21 after BLM-treatment, scale bar: 250 µm.


A
Control
hsTrim33-/-
I.V. BLM
B
Relative collagen intensity (Sirius Red, Ctrl=1)
1.6
1.4
1.2
1.0
0.8
**
Control BLM
hsTrim33-/- BLM
C
NaCl
BLM
Control
α-SMA
hsTrim33-/-

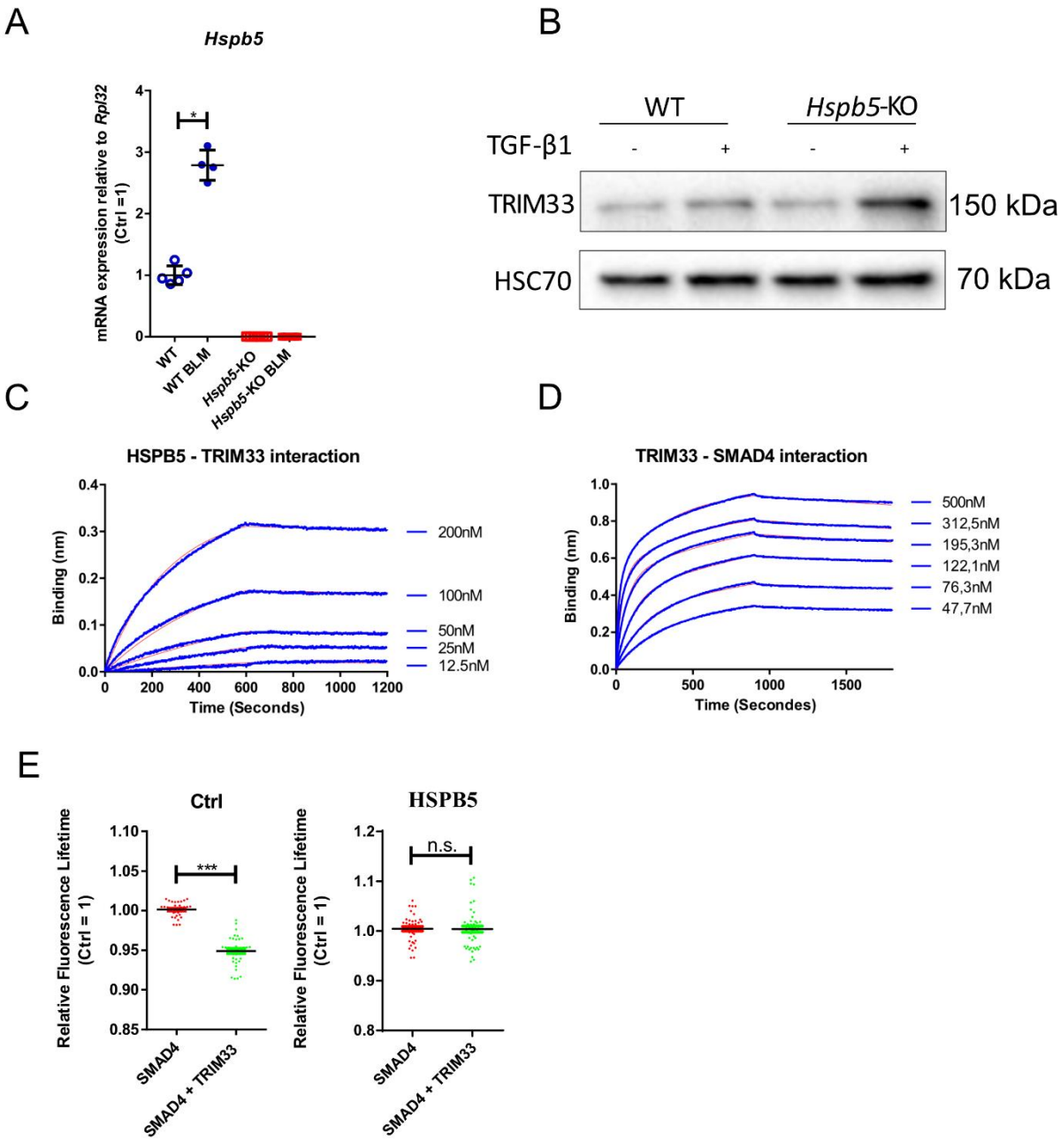
Supplementary Figure S6: Intra-tracheal injection of AdCre allows creation of a partial *Trim33* lung knock-out. (A) Cytometry analysis on the YPF signal on cells from whole lung of R26-stop-EYFP mutant mice lung, 3 days after AdDL or AdCre intra-tracheal injection (n=3). (B) Representative images (×300) of YFP immunofluorescence (Yellow) on lung sections from mice 3 days after instillation of AdCre (n=3), scale bar: 20 µm. (B) Representative images (×500) of YFP immunofluorescence (Yellow) with a co-staining of either CD326 (Red) for epithelial cells or ER-TR7 (Red) for fibroblast or CD68 (Red) for macrophages on lung sections from mice 3 days after instillation of AdCre (n=3), scale bar: 10 µm.


A
Normalized To Mode
100
80
60
40
20
0
29,7
0
10^3
10^4
10^5
YFP
SampleID | Median : B530_30-A
AdDL | 3587
AdCre | 12196
B
DAPI
YFP
Merge
Ig
C
CD326
ER-TR7
CD68
DAPI
YFP
Cell type Marker
Merge
Ig

Supplementary Figure S7: Pulmonary inhibition of *Trim33* worsens bleomycin-induced fibrosis in mice. (A) mRNA levels of C*ol1a1, Serpine1* and *Twist1* were analyzed by quantitative PCR in the lungs from control and Trim33f/l mice exposed to AdDL or AdCre and day 21 after bleomycin (BLM) or NaCl instillation. Results are expressed as median with interquartile range. *P < 0.05, **P < 0.01, non-parametric Mann-Whitney test, n= 4-8 per group. (B) May-Grünwald Giemsa quantification of the BALF cells isolated from mice, 3 days after injection with either NaCl, AdDL or AdCre (n=3 per group).


A
Col1a1
Serpine1
Twist1
mRNA expression relative to Rpl32 (Ctrl =1)
BLM
AdDL
AdCre
B
May-Grünwald Giemsa
% cells
NaCl
AdDL
AdCre
Macrophages
Neutrophils

Supplementary Figure S8: TRIM33 is able to interact directly with SMAD4. (A) *Hspb5* mRNA levels analyzed by quantitative PCR in lungs from control and *Hspb5*-KO mice at day 21 after BLM-treatment. Results are expressed as median with interquartile range. *P < 0.05, non-parametric Mann-Whitney test, n= 4-8 per group. (B) Protein levels of TRIM33 analyzed by Western Blot in primary pulmonary fibroblasts from SV129 control mice of SV129 *Hspb5* deficient mice (*Hspb5*-KO) cultured with rTGF-β1 (48h, 10ng/ml) (n=3). (C) The binding of recombinant TRIM33 to recombinant HSPB5 immobilized on a SA biosensor was determined by biolayer interferometry (graph representative of 3 independent experiments). (D) The binding of recombinant SMAD4 to a recombinant TRIM33 immobilized on a SA biosensor was determined by biolayer interferometry (graph representative of 3 independent experiments). (E) The proximity between TRIM33 and SMAD4 was asset by FLIM-FRET in A549 cells transfected with HSPB5 or an empty vector as control (Ctrl). The fluorescence lifetime of SMAD4 (Alexa 488, donor) in presence or the absence of TRIM33 (Alexa 494, acceptor) was determined to assess FRET (n=3). Results are shown as median with interquartile range; ***P < 0.001, non-parametric Mann-Whitney test. N.S; nonsignificant.


A
Hspb5
mRNA expression relative to Rpl32 (Ctrl =1)
WT
WT BLM
Hspb5-KO
Hspb5-KO BLM
B
WT
Hspb5-KO
TGF-β1
TRIM33
150 kDa
HSC70
70 kDa
C
HSPB5 - TRIM33 interaction
Binding (nm)
Time (Seconds)
200nM
100nM
50nM
25nM
12.5nM
D
TRIM33 - SMAD4 interaction
Binding (nm)
Time (Secondes)
500nM
312,5nM
195,3nM
122,1nM
76,3nM
47,7nM
E
Ctrl
HSPB5
Relative Fluorescence Lifetime (Ctrl = 1)
***
n.s.
SMAD4
SMAD4 + TRIM33

Supplementary Figure S9: uncropped Western Blots.

Fig 2 B - TRIM33

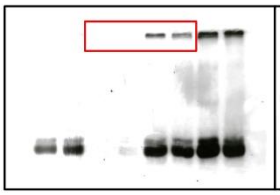


Fig 2 B - HSC70

Fig 5 B TRIM33

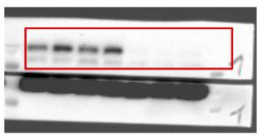


Fig 5 B HSC70

Fig 6 D TRIM33

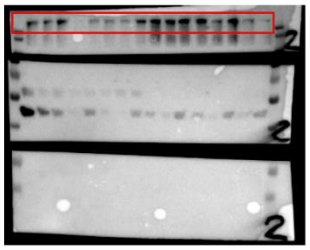


Fig 6 D HSC70

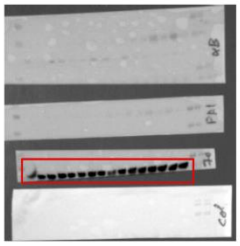


Fig 6 D HSPB5, PAI-1, Type1 Collagen

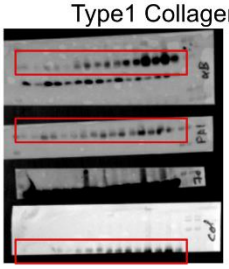


Fig 7 A TRIM33

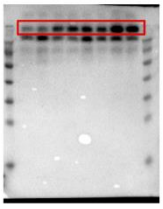


Fig 7 A HSC70

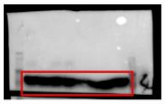


Fig 7 C TRIM33, ACTIN HSPB5

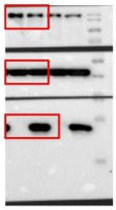


Fig7 E TRIM33

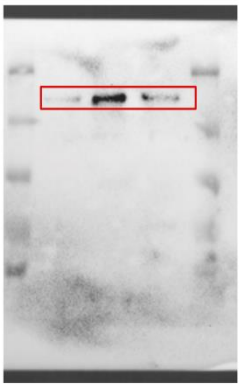


Fig7 E HSC70, HSPB5

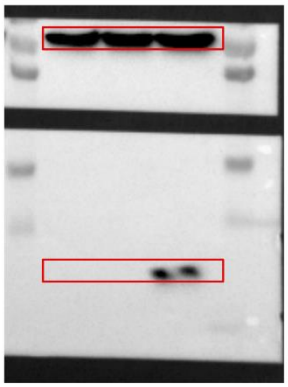
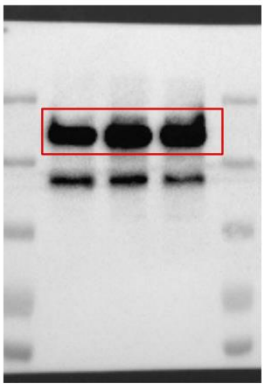
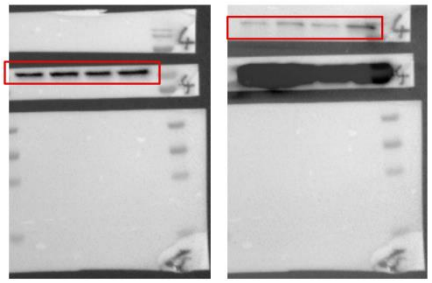


Fig7 E TRIM33



S8 - TRIM33 - HSC70



Supplemental Reference

1. Bett AJ, Haddara W, Prevec L, Graham FL. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proceedings of the National Academy of Sciences of the United States of America* 1994;91:8802-8806.
2. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *The Journal of clinical investigation* 1997;100:768-776.
3. Bonniaud P, Martin G, Margetts PJ, Ask K, Robertson J, Gauldie J, Kolb M. Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" balb/c mouse lungs. *American journal of respiratory cell and molecular biology* 2004;31:510-516.
4. Janssen LJ, Farkas L, Rahman T, Kolb MR. Atp stimulates ca(2+)-waves and gene expression in cultured human pulmonary fibroblasts. *The international journal of biochemistry & cell biology* 2009;41:2477-2484.
5. Lv J, Xiong Y, Li W, Yang W, Zhao L, He R. Blt1 mediates bleomycin-induced lung fibrosis independently of neutrophils and cd4+ t cells. *The Journal of Immunology* 2017:1600465.