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Supplementary Materials for

LOXL4, but not LOXL2, is the critical determinant of pathological collagen cross-linking and fibrosis in the lung

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This PDF file includes:

Figure S1

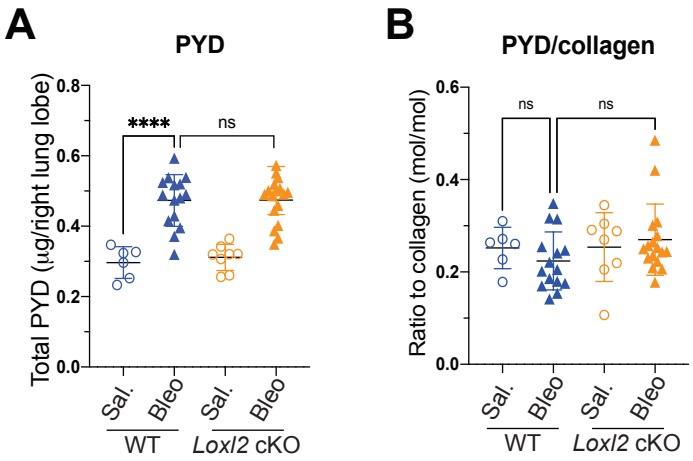
A
PYD
Total PYD (μg/right lung lobe)
0.8
0.6
0.4
0.2
0.0
****
ns
Sal.
Bleo
Sal.
Bleo
WT
Loxl2 cKO
B
PYD/collagen
Ratio to collagen (mol/mol)
0.6
0.4
0.2
0.0
ns
ns
Sal.
Bleo
Sal.
Bleo
WT
Loxl2 cKO

Figure S2

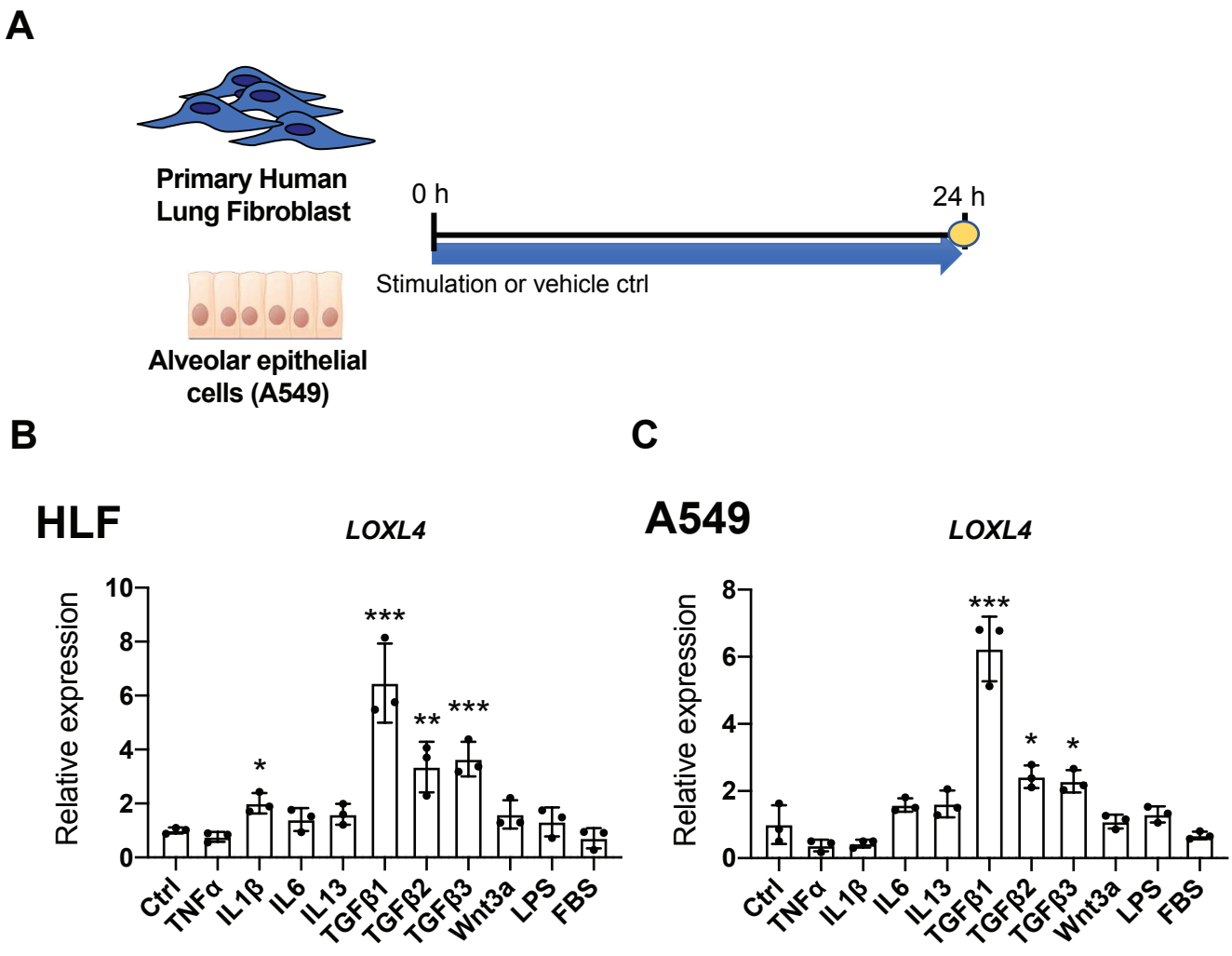
A
Primary Human Lung Fibroblast
Alveolar epithelial cells (A549)
0 h
24 h
Stimulation or vehicle ctrl
B
HLF
LOXL4
Relative expression
10
8
6
4
2
0
*
***
**
***
Ctrl
TNFα
IL1β
IL6
IL13
TGFβ1
TGFβ2
TGFβ3
Wnt3a
LPS
FBS
C
A549
LOXL4
Relative expression
8
6
4
2
0
***
*
*
Ctrl
TNFα
IL1β
IL6
IL13
TGFβ1
TGFβ2
TGFβ3
Wnt3a
LPS
FBS

Figure S3

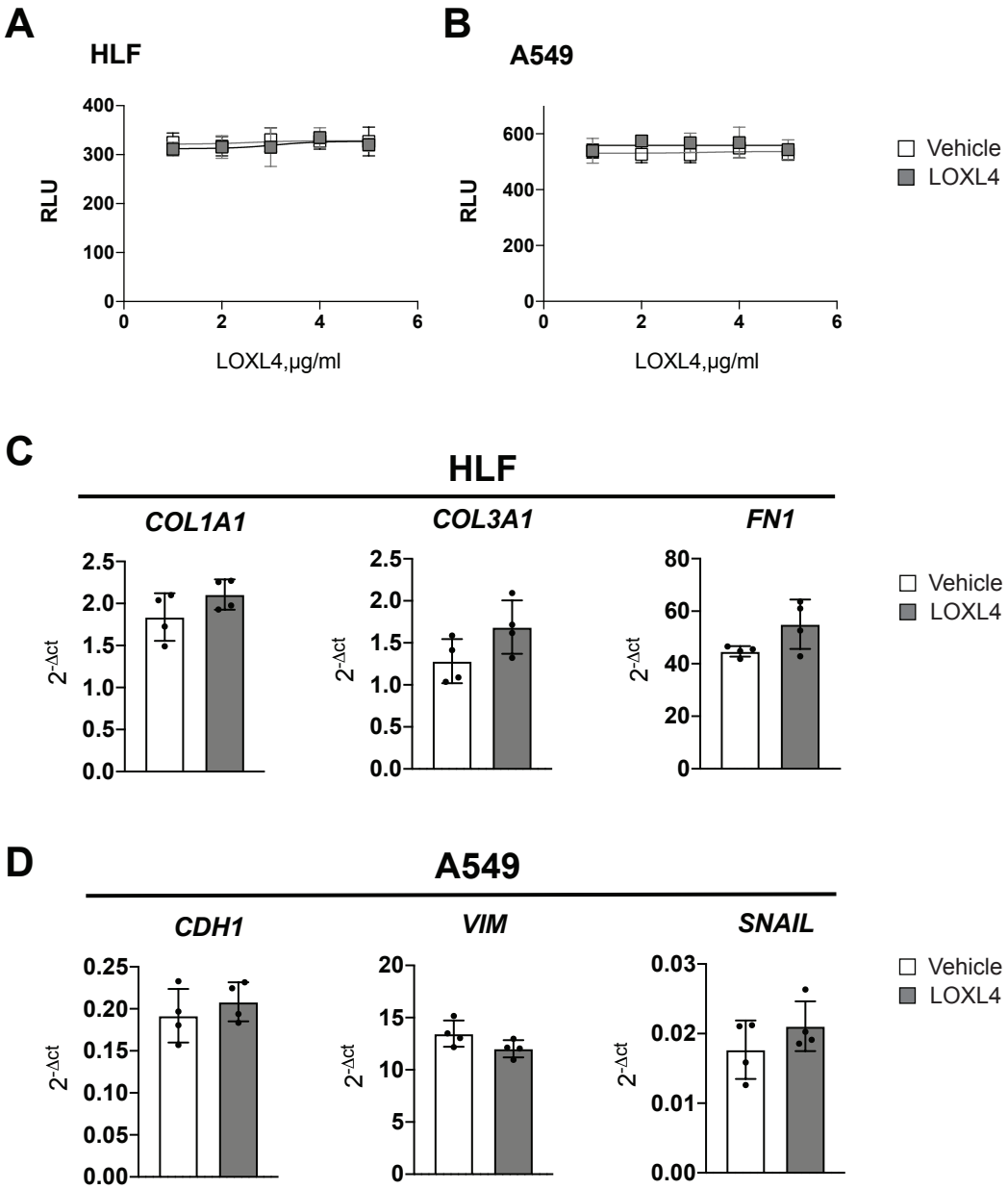
A
HLF
RLU
LOXL4,μg/ml
B
A549
RLU
LOXL4,μg/ml
Vehicle
LOXL4
C
HLF
COL1A1
COL3A1
FN1
2-Δct
D
A549
CDH1
VIM
SNAIL
2-Δct

Figure S4

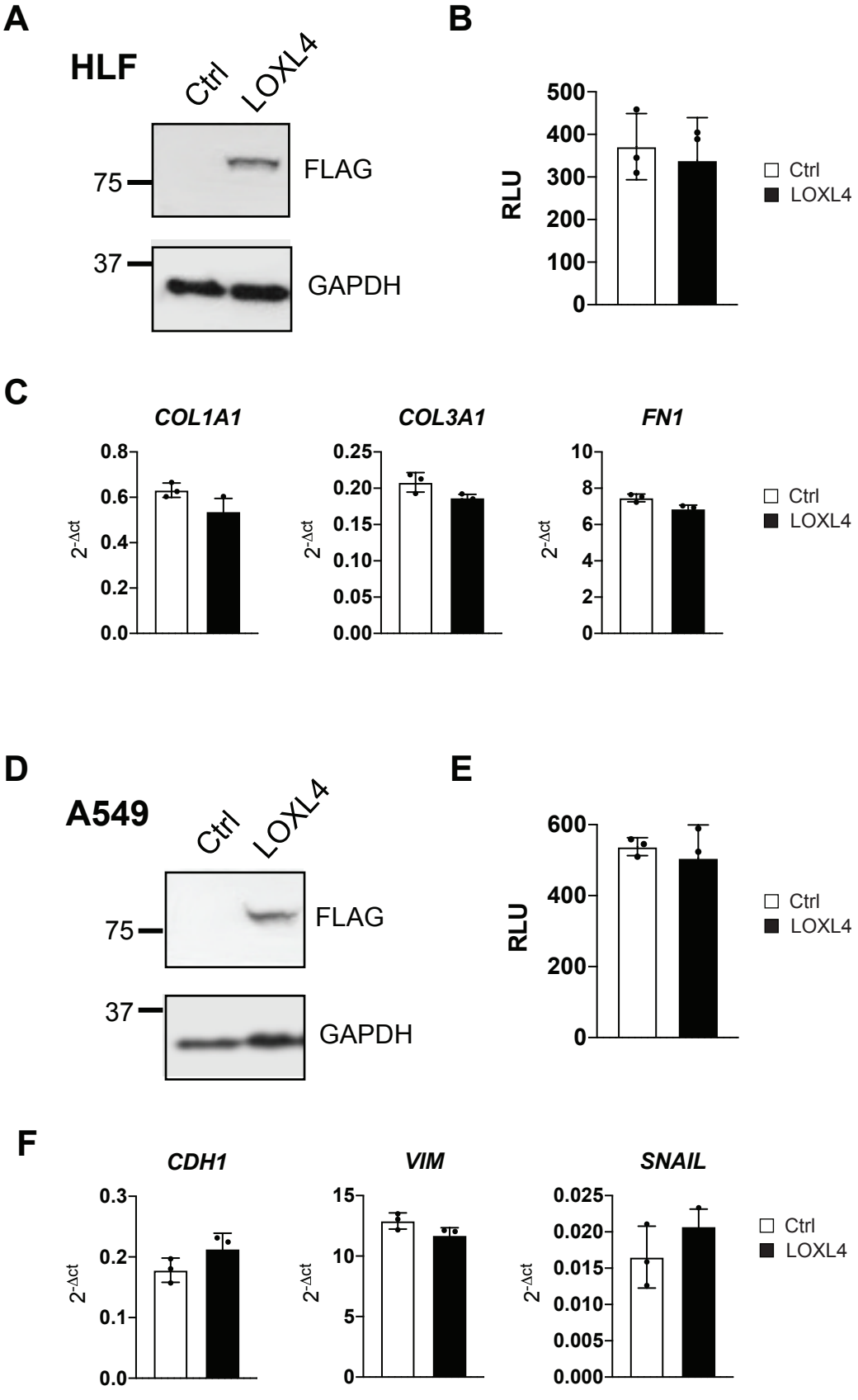
A
HLF
Ctrl
LOXL4
75
FLAG
37
GAPDH
B
RLU
500
400
300
200
100
0
Ctrl
LOXL4
C
COL1A1
COL3A1
FN1
2-Δct
0.8
0.6
0.4
0.2
0.0
0.25
0.20
0.15
0.10
0.05
0.00
10
8
6
4
2
0
Ctrl
LOXL4
D
A549
Ctrl
LOXL4
75
FLAG
37
GAPDH
E
RLU
600
400
200
0
Ctrl
LOXL4
F
CDH1
VIM
SNAIL
2-Δct
0.3
0.2
0.1
0.0
15
10
5
0
0.025
0.020
0.015
0.010
0.005
0.000
Ctrl
LOXL4

Figure S5

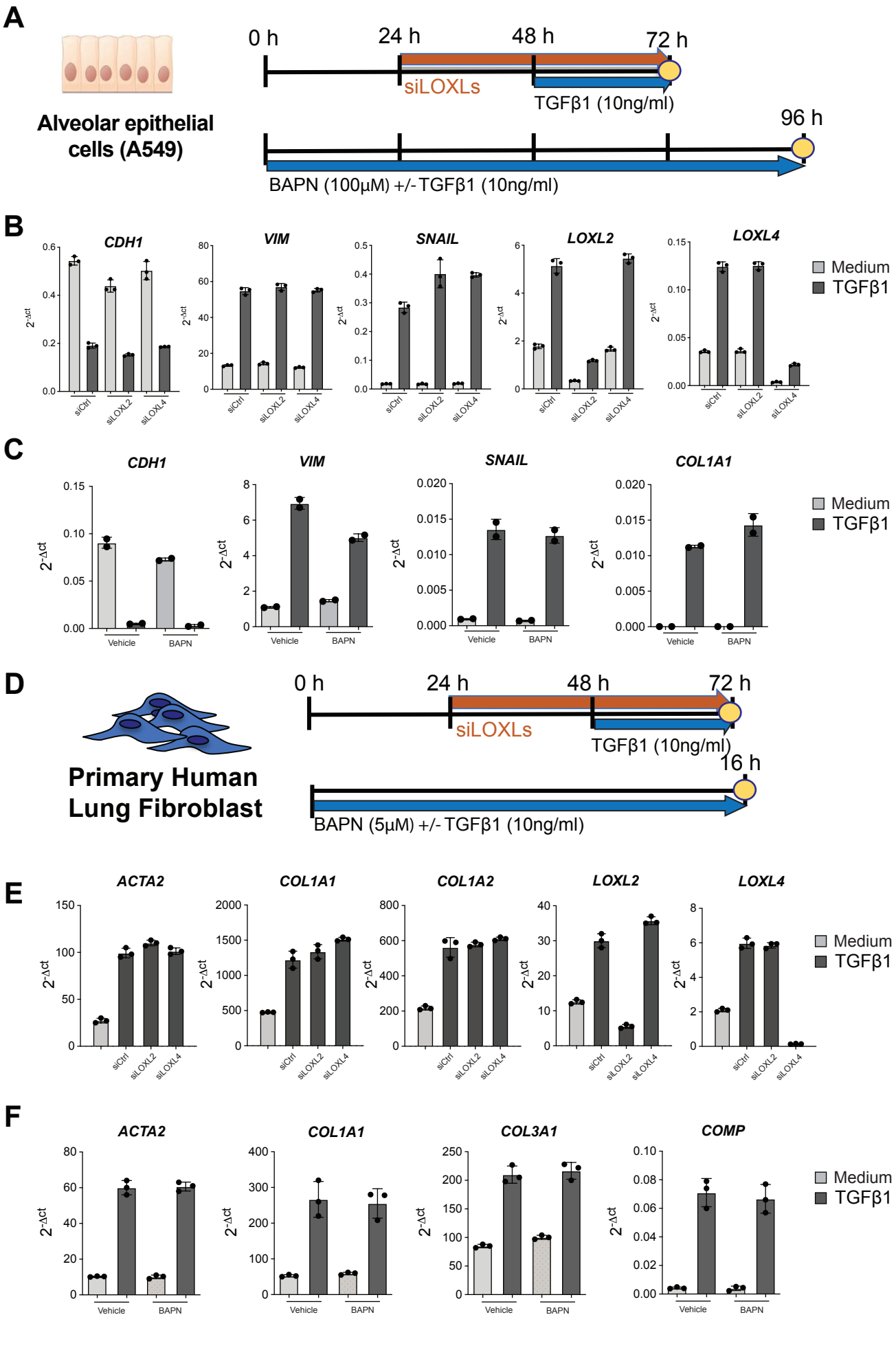
A
Alveolar epithelial cells (A549)
0 h
24 h
48 h
72 h
siLOXLs
TGFβ1 (10ng/ml)
96 h
BAPN (100μM) +/- TGFβ1 (10ng/ml)
B
CDH1
VIM
SNAIL
LOXL2
LOXL4
2-Δct
siCtrl
siLOXL2
siLOXL4
Medium
TGFβ1
C
CDH1
VIM
SNAIL
COL1A1
2-Δct
Vehicle
BAPN
Medium
TGFβ1
D
Primary Human Lung Fibroblast
0 h
24 h
48 h
72 h
siLOXLs
TGFβ1 (10ng/ml)
16 h
BAPN (5μM) +/- TGFβ1 (10ng/ml)
E
ACTA2
COL1A1
COL1A2
LOXL2
LOXL4
2-Δct
siCtrl
siLOXL2
siLOXL4
Medium
TGFβ1
F
ACTA2
COL1A1
COL3A1
COMP
2-Δct
Vehicle
BAPN
Medium
TGFβ1

Figure S6

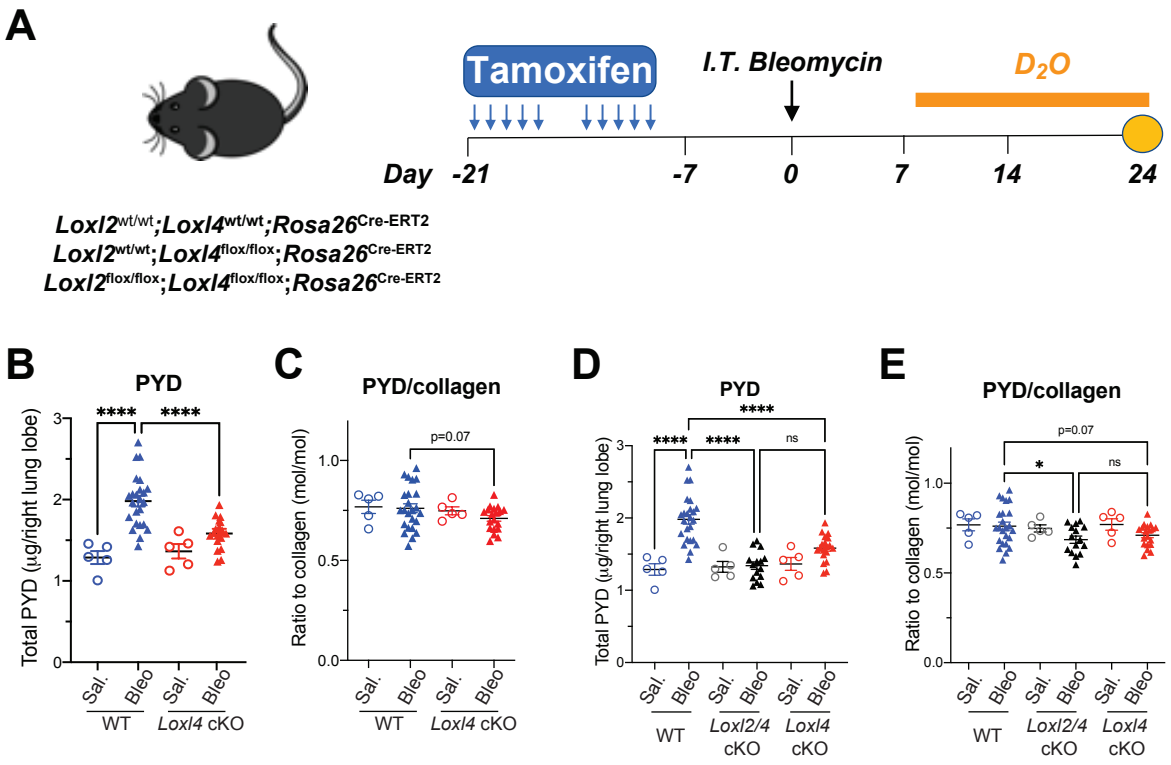
A
Tamoxifen
I.T. Bleomycin
D2O
Day -21 -7 0 7 14 24
Loxl2wt/wt;Loxl4wt/wt;Rosa26Cre-ERT2
Loxl2wt/wt;Loxl4flox/flox;Rosa26Cre-ERT2
Loxl2flox/flox;Loxl4flox/flox;Rosa26Cre-ERT2
B
PYD
Total PYD (µg/right lung lobe)
****
****
Sal.
Bleo
Sal.
Bleo
WT
Loxl4 cKO
C
PYD/collagen
Ratio to collagen (mol/mol)
p=0.07
Sal.
Bleo
Sal.
Bleo
WT
Loxl4 cKO
D
PYD
Total PYD (µg/right lung lobe)
****
****
****
ns
Sal.
Bleo
Sal.
Bleo
Sal.
Bleo
WT
Loxl2/4 cKO
Loxl4 cKO
E
PYD/collagen
Ratio to collagen (mol/mol)
p=0.07
*
ns
Sal.
Bleo
Sal.
Bleo
Sal.
Bleo
WT
Loxl2/4 cKO
Loxl4 cKO

Figure S7

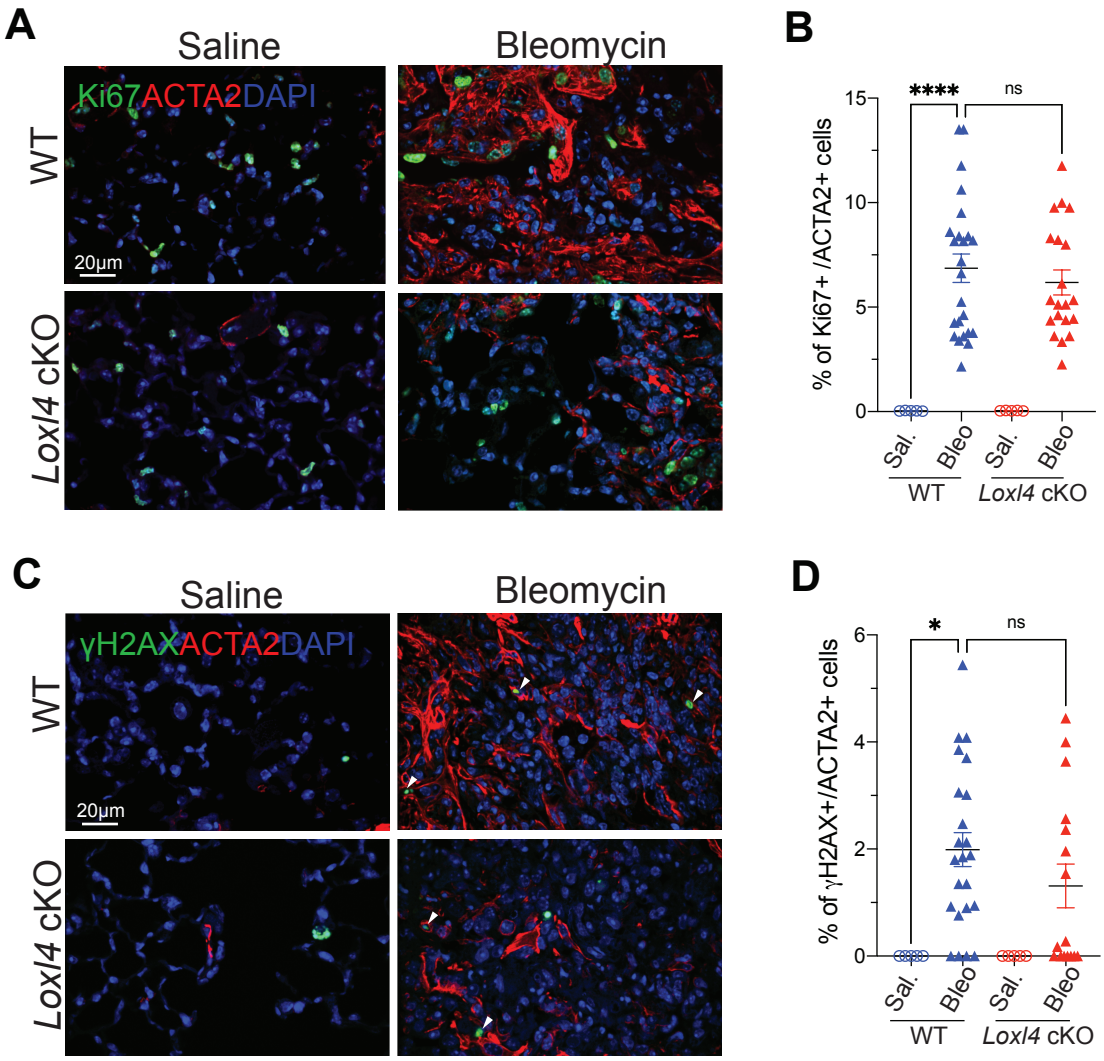
A
Saline
Bleomycin
Ki67ACTA2DAPI
WT
Loxl4 cKO
20μm
B
****
ns
% of Ki67+ /ACTA2+ cells
15
10
5
0
Sal.
Bleo
Sal.
Bleo
WT
Loxl4 cKO
C
Saline
Bleomycin
γH2AXACTA2DAPI
WT
Loxl4 cKO
20μm
D
*
ns
% of γH2AX+/ACTA2+ cells
6
4
2
0
Sal.
Bleo
Sal.
Bleo
WT
Loxl4 cKO

Figure S8

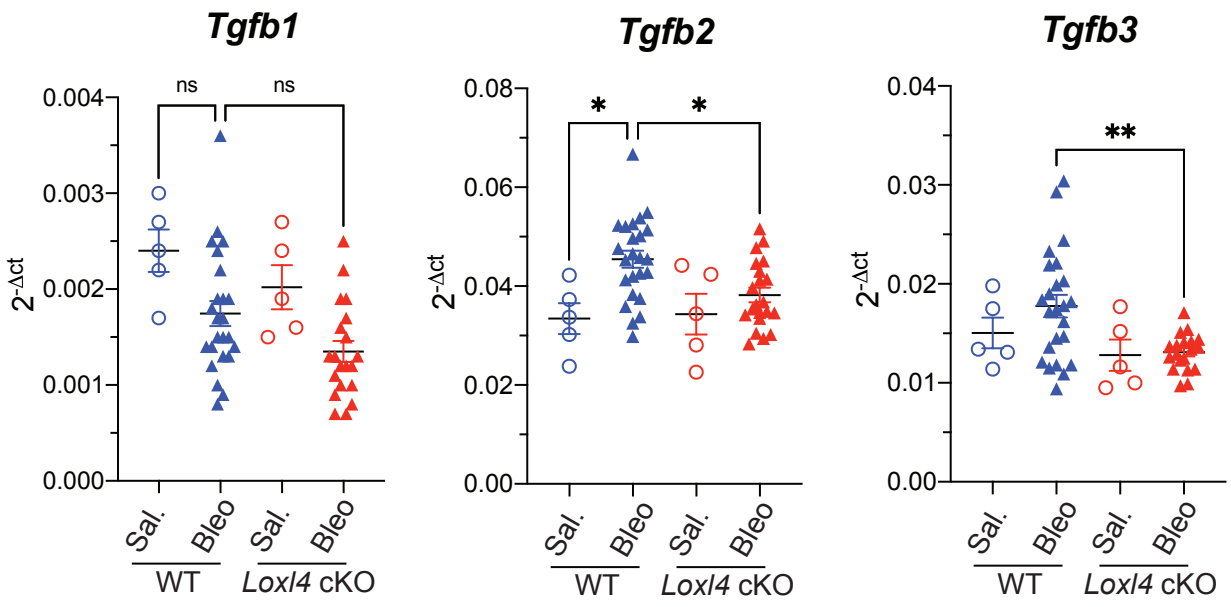
Tgfb1
Tgfb2
Tgfb3
2-Δct
ns
ns
*
*
**
0.004
0.003
0.002
0.001
0.000
0.08
0.06
0.04
0.02
0.00
0.04
0.03
0.02
0.01
0.00
Sal.
Bleo
Sal.
Bleo
WT
Loxl4 cKO

Table S1. List of primers used for qPCR

Mouse Primer	Sequence
mAnkrd1-F	GCTGGTAACAGGCAAAAAGAAC
mAnkrd1-R	CCTCTCGCAGTTTCTCGCT
mCol1a1-F	GCTCCTCTTAGGGGCCACT
mCol1a1-R	CCACGTCTCACCATTGGGG
mCol1a2-F	GTAACTTCGTGCCTAGCAACA
mCol1a2-R	CCTTTGTCAGAATACTGAGCAGC
mComp-F	ACTGCCTGCGTTCTAGTGC
mComp-R	CGCCGCATTAGTCTCCTGAA
mCTGF-F	GGGCCTCTTCTGCGATTTC
mCTGF-R	ATCCAGGCAAGTGCATTGGTA
mFn1-F	ATGTGGACCCCTCCTGATAGT
mFn1-R	GCCCAGTGATTTCAGCAAAGG
mLox-F	TCTTCTGCTGCGTGACAACC
mLox-R	GAGAAACCAGCTTGGAACCAG
mLoxl1-F	GAGTGCTATTGCGCTTCCC
mLoxl1-R	GGTTGCCGAAGTCACAGGT
mLoxl2-F	ATTAACCCCAACTATGAAGTGCC
mLoxl2-R	CTGTCTCCTCACTGAAGGCTC
mLoxl3-F	CTACTGCTGCTACACTGTCTGT
mLoxl3-R	GACCTTCATAGGGCTTTCTAGGA
mLoxl4-F	AGCGGACAGACCAGAGGAG
mLoxl4-R	CCTTGACCATACTTGGCACTG
mTGFb1-F	CTCCCGTGGCTTCTAGTGC
mTGFb1-R	GCCTTAGTTTGGACAGGATCTG
mTGFb2-F	CTTCGACGTGACAGACGCT
mTGFb2-R	GCAGGGGCAGTGTAAACTTATT
mTGFb3-F	CCTGGCCCTGCTGAACTTG
mTGFb3-R	TTGATGTGGCCGAAGTCCAAC
mTnc-F	ACGGCTACCACAGAAGCTG
mTnc-R	ATGGCTGTTGTTGCTATGGCA
mSerpine1-F	TTCAGCCCTTGCTTGCCTC
mSerpine1-R	ACACTTTTACTCCGAAGTCGGT
Human Primer	**Taqman Probe ID**
hACTA2	Hs00426835_g1
hCDH	Hs01023894_m1
hCOL1A1	Ha00164004_m1
hCOL1A2	Hs01028956_m1
hCOL3A1	Hs00943809_m1
hCOMP	Hs00164359_m1
hFN1	Hs00365052_m1
hLOXL2	Hs00158757_m1
hLOXL4	Hs00260059_m1
hSNAIL	Hs00195591_m1
hVIM	Hs00958111_m1

Supplementary figure legends

Figure S1. LOXL2 deficiency does not affect PYD levels in the bleomycin model

(A-B) Quantification of lung collagen crosslinking by (A) total PYD and (B) PYD normalized by collagen. WT: Saline n=6, bleomycin n=15; *Loxl2* cKO: saline n=8, bleomycin n=17. Data represents mean ± S.D. **** $p < 0.0001$. ns, not significant. p-value is calculated using one-way ANOVA.

Figure S2. TGFβ is the main upstream regulator of LOXL4 expression in human lung epithelial cells and lung fibroblasts *in vitro*.

(A) Experiment scheme of screening a variety of pro-fibrotic and pro-inflammatory cytokines and stimuli in human lung fibroblasts (HLFs) and human lung epithelial cells (A549) *in vitro*. (B-C) mRNA expression of *LOXL4* in response to various stimulations in (B) HLFs and (C) A549 cells. mRNA levels are expressed relative to mean mRNA levels in cells with control treatment, which was arbitrarily assigned a value of 1. Data represents mean ± S.D. Similar results were seen in three independent experiments. * $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$, p-value is calculated using unpaired t-test.

Figure S3. Recombinant LOXL4 protein does not impact EMT or lung fibroblast activation *in vitro*.

(A-B) (A) HLFs and (B) A549 cells were treated with recombinant LOXL4 protein at the indicated concentrations for 24 hours followed by CellTiter-Glo assay. (C) mRNA expression of *COL1A1*, *COL3A1* and *FN1* in response to recombinant LOXL4 (5μg/ml) treatment on HLFs. (D) mRNA expression of *CDH1*, *VIM* and *SNAIL* in response to recombinant LOXL4 (5μg/ml) treatment on

A549 cells. Data represents mean ± S.D. Similar results were seen in three independent experiments.

Figure S4. Overexpression of LOXL4 does not impact EMT or lung fibroblast activation *in vitro*.

(A) Western blotting confirmed overexpression of LOXL4 in HLFs. (B) Viability of HLFs transfected with LOXL4 overexpression construct after 48 hours examined by CellTiter-Glo assay. (C) mRNA expression of *COL1A1*, *COL3A1* and *FN1* in response to overexpression of LOXL4 on HLFs. (D) Western blotting confirmed overexpression of LOXL4 in human lung epithelial cells (A549). (E) Viability of A549 cells transfected with LOXL4 overexpression construct after 48 hours examined by CellTiter-Glo assay. (F) mRNA expression of *CDH1*, *VIM* and *SNAIL* in response to overexpression of LOXL4 on A549 cells. Data represents mean ± S.D. Similar results were seen in three independent experiments.

Figure S5. Blocking LOXL2/4 activity does not affect EMT or lung fibroblast activation *in vitro*.

(A) Experiment scheme of examining the impact of blocking LOXL2/4 activity on EMT *in vitro*. (B) mRNA expression of *CDH1*, *VIM, SNAIL, LOXL2* and *LOXL4* in response to TGFβ1 stimulation in A549 cells transfected with control, *LOXL2* or *LOXL4* siRNA. (C) mRNA expression of *CDH1*, *VIM*, *SNAIL* and *COL1A1* in response to TGFβ1 stimulation in A549 cells treated with or without BAPN (pan-LOX inhibitor). (D) Experiment scheme of examining the impact of blocking LOXL2/4 activity on lung fibroblast activation *in vitro*. (E) mRNA expression of *ACTA2*, *COL1A1*, *COL1A2*, *LOXL2* and *LOXL4* in response to TGFβ1 stimulation in HLFs transfected with control, *LOXL2* or *LOXL4* siRNA. (F) mRNA expression of *ACTA2*, *COL1A1*,

COL3A1 and *COMP* in response to TGFβ1 stimulation in HLFs treated with or without BAPN. Data represents mean ± S.D. Similar results were seen in three independent experiments.

Figure S6. PYD levels in *Loxl4* cKO and *Loxl2/4* cKO mice in the bleomycin model.

(A) Schematic regime of tamoxifen-induced *Loxl4 and Loxl2/4* deletion followed by IT bleomycin challenge. Mice were treated with deuterated water two weeks before the end of the study. Lung tissues were harvested for terminal analyses on day 24 after bleomycin challenge. (B-C) Quantification of lung collagen crosslinking by (B) total PYD and (C) PYD normalized by collagen from *Loxl4* cKO mice. (D-E) Quantification of lung collagen crosslinking by (D) total PYD and (E) PYD normalized by collagen from *Loxl2/4* cKO mice. Data represents mean ± S.D. **** $p < 0.0001$. ns, not significant. p-value is calculated using one-way ANOVA.

Figure S7. LOXL4 deficiency does not affect myofibroblasts proliferation or apoptosis in the bleomycin model.

(A-B) (A) Representative immunofluorescent images of Ki67 and ACTA2 in lung tissue with quantification of (B) percentage of Ki67+/ACTA2+ cells per field. (C-D) (C) Representative immunofluorescent images of γH2AX and ACTA2 in lung tissue with quantification of (B) percentage of γH2AX+/ACTA2+ cells per field. Arrow heads present (C) γH2AX+ nuclei/ACTA2+ cells. Data in (B) and (D) represent mean value of quantification from five random fields/image. * $p < 0.05$, **** $p < 0.0001$. ns, not significant. p-value is calculated using one-way ANOVA.

Figure S8. TGFβ ligand expression in the bleomycin model.

mRNA expression of *Tgfb1*, *Tgfb2* and *Tgfb3* in lungs from WT or *Loxl4* cKO mice challenged by saline or bleomycin. Data represents mean ± S.D. * $p < 0.05$, ** $p < 0.01$. ns, not significant. p-value is calculated using one-way ANOVA.

Table S1. List of primers used for qPCR.