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Supplemental Information for

MEKK3-TGFβ crosstalk regulates inward arterial remodeling

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This PDF file incudes:

Supplemental Figure Legends

Figure S1. Loss of MEKK3 in ECs induces cardiac hypertrophy. (**A**) Q-PCR analysis of *Mekk3* expression in isolated lung ECs, aortic smooth muscle cells and cardiomyocytes from Ctrl and *Mekk3*^{iECKO} mice, n=3 per group. Data represent mean ± SD. ****$P < 0.0001$, ns: not significant, calculated by unpaired *t*-test. (**B**) Representative higher magnification H&E images of RV and LV for Ctrl and *Mekk3*^{iECKO} mice at 4 weeks after tamoxifen injection. Scale bar: 500μm. (**C**) Representative right ventricle systolic pressure (RVSP) tracing for Ctrl and *Mekk3*^{iECKO} mice at 4 weeks after tamoxifen injection. (**D**) Representative left ventricle systolic pressure (LVSP) tracing for Ctrl and *Mekk3*^{iECKO} mice at 8 weeks after tamoxifen injection. (**E**) Representative systolic blood pressure (SBP) and diastolic blood pressure (DBP) tracing for Ctrl and *Mekk3*^{iECKO} mice at 8 weeks after tamoxifen injection.

Figure S2. Heart rate and ECG for Ctrl and *Mekk3*^{iECKO} mice. (**A**) Heart rate (BPM) for Ctrl and *Mekk3*^{iECKO} mice at 2, 4 and 8 weeks after tamoxifen injection. n=5 mice per time point and per group. Data represent mean ± SEM. ns: not significant, calculated by two-way ANOVA with Tukey's multiple comparison tests. (**B**) Representative electrocardiogram (ECG) tracing for Ctrl and *Mekk3*^{iECKO} mice at 4 and 8 weeks after tamoxifen injection.

Figure S3. *Mekk2* knockout mice don't develop hypertension. (**A**) Western blot analysis of MEKK2 expression in Ctrl and *Mekk2*$^{-/-}$ lungs. (**B**) RVSP of Ctrl and *Mekk2*$^{-/-}$ mice at 6 months old. (**C**) LVSP of Ctrl and *Mekk2*$^{-/-}$ mice at 6 months old. (**B-C**) n=4 male mice for each group. Data represent mean ± SEM. ns: not significant, calculated by unpaired *t*-test.

Figure S4. MEKK3 deletion in ECs induces TGFβ signaling. (**A**) Representative TGFβR2 staining of Ctrl and *Mekk3*^{iECKO} entire lungs at 4 weeks after tamoxifen

injection. Scale bar: 1mm. (**B-C**) Representative immunostaining and quantification of SM22α (**B**), and Collagen I (**C**) in lung sections from Ctrl and *Mekk3*^{iECKO} mice at 4 weeks after tamoxifen injection. Scale bar: 25μm. (**D**) Representative Smad2/3 immunostaining and nuclear translocation quantification in lung sections from Ctrl and *Mekk3*^{iECKO} mice at 4 weeks after tamoxifen injection. Scale bar: 25μm. n=4 mice per group. Data represent mean ± SEM. **P* < 0.05, calculated by Mann-Whitney U-test.

Figure S5. Knockdown of MEKK3 in HPAECs induces EndMT. (**A**) Q-PCR analysis of EndMT markers expression in human pulmonary artery endothelial cells (HPAECs) treated with Ctrl or MEKK3 siRNA. n=3 independent experiments. Data represent mean ± SD. ***P* < 0.01, ****P* < 0.001, calculated by unpaired *t*-test. (**B**) Western blot analysis of EndMT markers expression in HPAECs treated with Ctrl or MEKK3 siRNA. (**C**) Immunostaining of LIN28 in Ctrl and *Mekk3*^{iECKO} mice lungs. Scale bar: 50μm. Arrowheads point to endothelial cells expressing LIN28.

Figure S6. Loss of MEKK3 in ECs impairs FGF2-ERK1/2-Let7 signaling pathway. (**A-B**) ERK1/2 activation upon (**A**) FGF2 (100ng/ml) and (**B**) VEGF165 (50ng/ml) treatment in HUVECs treated with Ctrl or MEKK3 siRNA. (**C-D**) Smad1/5/9 activation upon (**C**) BMP9 (10ng/ml) and (**D**) BMP6 (50ng/ml) treatment in HUVECs treated with Ctrl or MEKK3 siRNA. (**E**) Q-PCR analysis of FGFR1 (n=6) expression in HUVECs treated with Ctrl or MEKK3 siRNA. Data represent mean ± SEM. (**F**) Western blot analysis of FGFR1 expression in HUVECs treated with Ctrl or MEKK3 siRNA. ns: not significant, calculated by unpaired *t*-test.

Figure S7. Additional images showing EndMT. (**A-C**) Representative GFP and SMA staining in lung (**A**), kidney (**B**), and liver (**C**) from mTmG Ctrl and mTmG *Mekk3*^{iECKO} mice at 4 weeks after tamoxifen injection. Scale bar: 50μm. Arrowheads point to endothelial cells expressing SMA.

Figure S8. F4/80 staining in atherosclerotic plaque.

(**A**) Representative F4/80 staining in brachiocephalic artery lesion from *Apoe*$^{-/-}$ mice and *Apoe*$^{-/-}$ *Mekk3*^{iECKO} mice. Scale bar: 100μm.

Figure S9. Suppression of TGFβR signaling rescues MEKK3-knockout-induced EndMT. (**A**) Q-PCR analysis of MEKK3, SM22α, fibronectin (FN) and N-Cadherin expression in HUVECs treated with Ctrl or MEKK3 siRNA in addition to TGFβR inhibitor. (**B**) Western Blot and (**C**) Q-PCR analysis of EndMT markers expression in HUVECs treated with Ctrl or MEKK3 siRNA in addition to TGFβR1/R2 siRNA. n=3 independent experiments. Data represent mean ± SD. ***P* < 0.01, ****P* < 0.001, ns: not significant, calculated by one-way ANOVA with Tukey's multiple comparison tests.

Figure S10. Negative control for immunohistochemistry staining with non-immune species-matched isotype IgG. Lung sections were blocked and incubated with indicated primary antibodies and its non-immune species-matched IgG at 4°C overnight. Sections then were incubated with secondary antibodies at room temperature for 2h, finally mounted with DAPI. (**A**) Representative immunostaining of mouse isotype IgG, fibronectin and TGFβ with CD31 and DAPI. (**B**) Representative immunostaining of rabbit isotype IgG, p-Smad2 Ser465/467 and p-Smad3 Ser423/425 with CD31 and DAPI. Scale bar: 25μm.

Figure S1

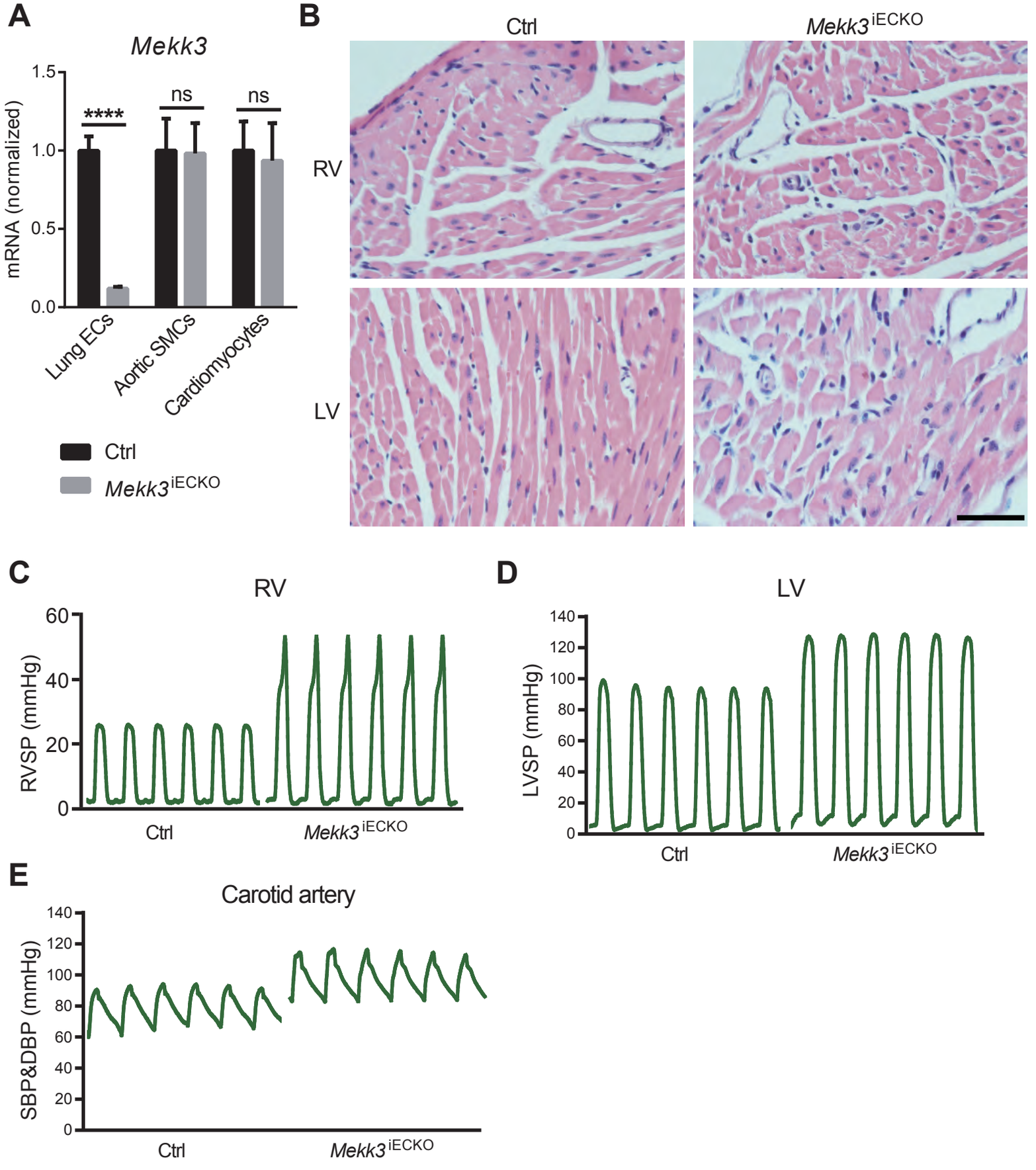

A
Mekk3
mRNA (normalized)
1.5
1.0
0.5
0.0
****
ns
ns
Lung ECs
Aortic SMCs
Cardiomyocytes
Ctrl
Mekk3iECKO
B
Ctrl
Mekk3iECKO
RV
LV
C
RV
RVSP (mmHg)
60
40
20
0
Ctrl
Mekk3iECKO
D
LV
LVSP (mmHg)
140
120
100
80
60
40
20
0
Ctrl
Mekk3iECKO
E
Carotid artery
SBP&DBP (mmHg)
140
120
100
80
60
40
20
0
Ctrl
Mekk3iECKO

Figure S2

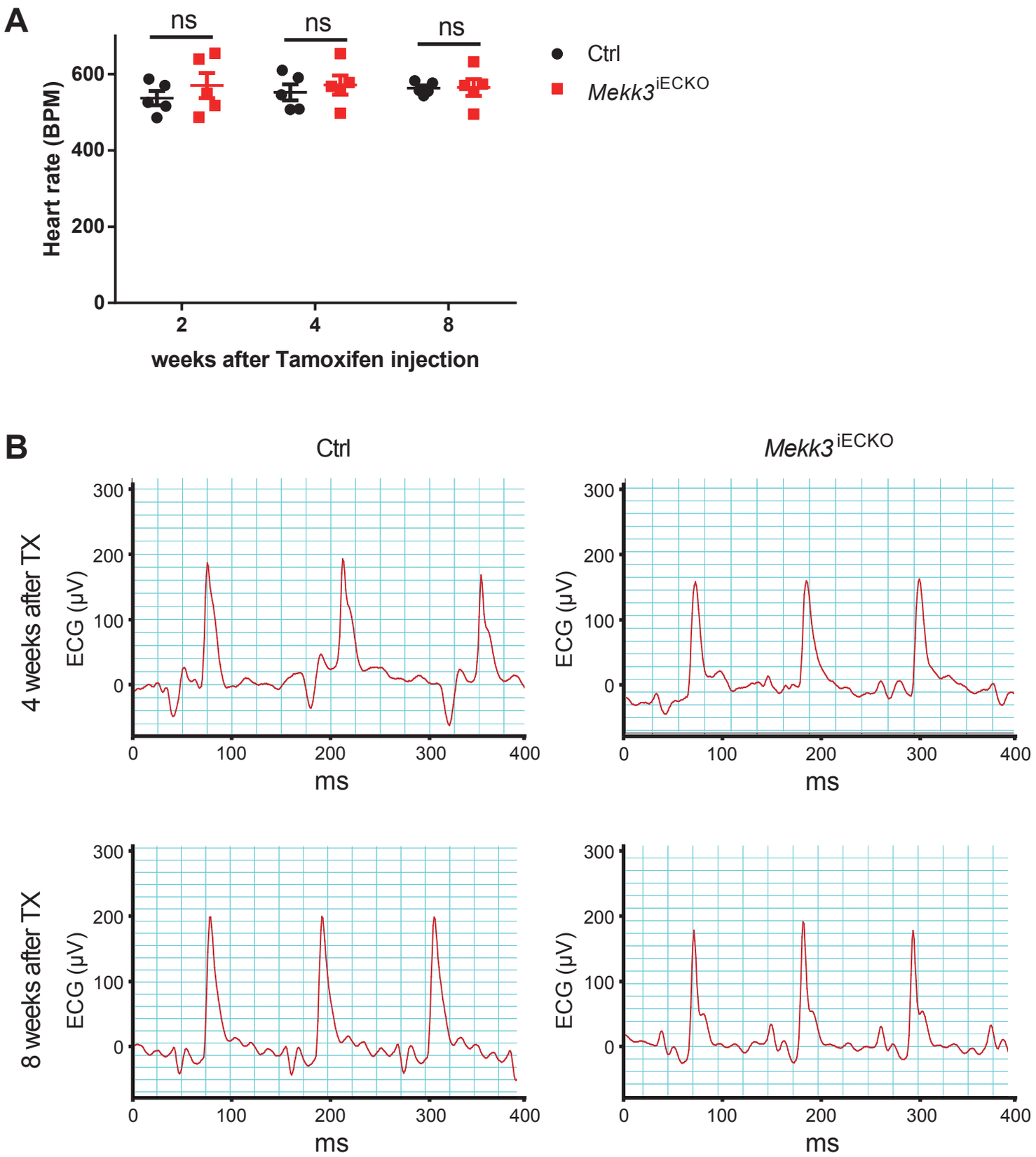

A
ns
ns
ns
Heart rate (BPM)
600
400
200
0
2
4
8
weeks after Tamoxifen injection
Ctrl
Mekk3iECKO
B
Ctrl
Mekk3iECKO
4 weeks after TX
8 weeks after TX
ECG (μV)
300
200
100
0
0
100
200
300
400
ms

Figure S3

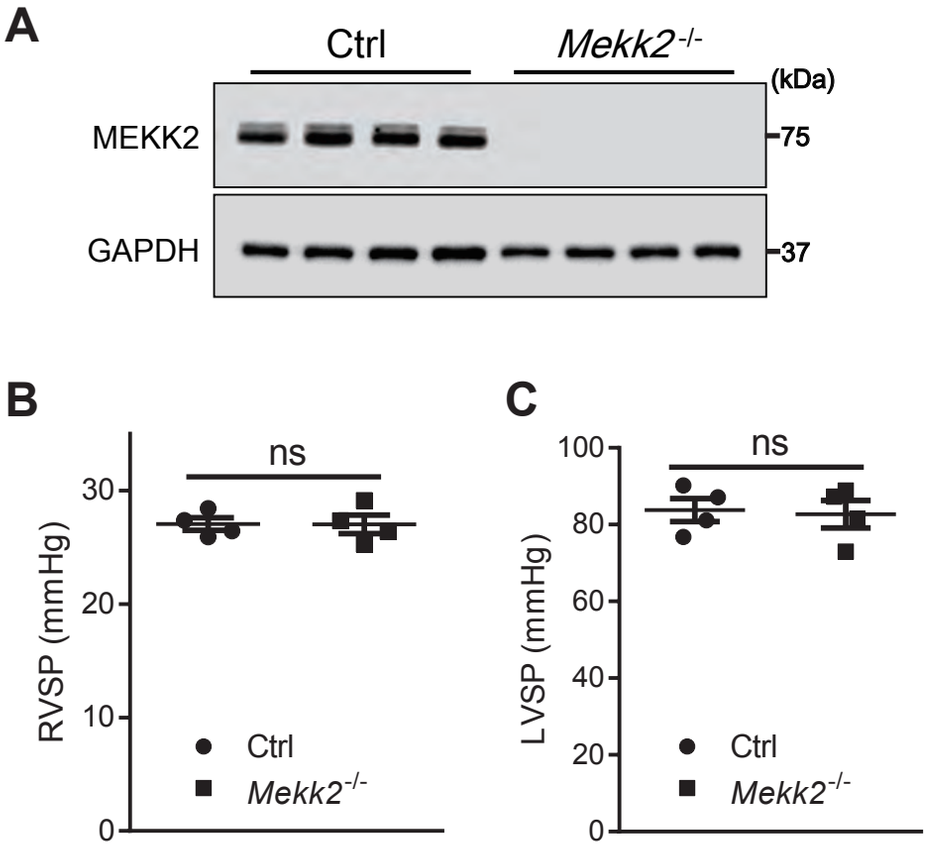

A
Ctrl
Mekk2-/-
(kDa)
MEKK2
75
GAPDH
37
B
ns
RVSP (mmHg)
30
20
10
0
Ctrl
Mekk2-/-
C
ns
LVSP (mmHg)
100
80
60
40
20
0
Ctrl
Mekk2-/-

Figure S4

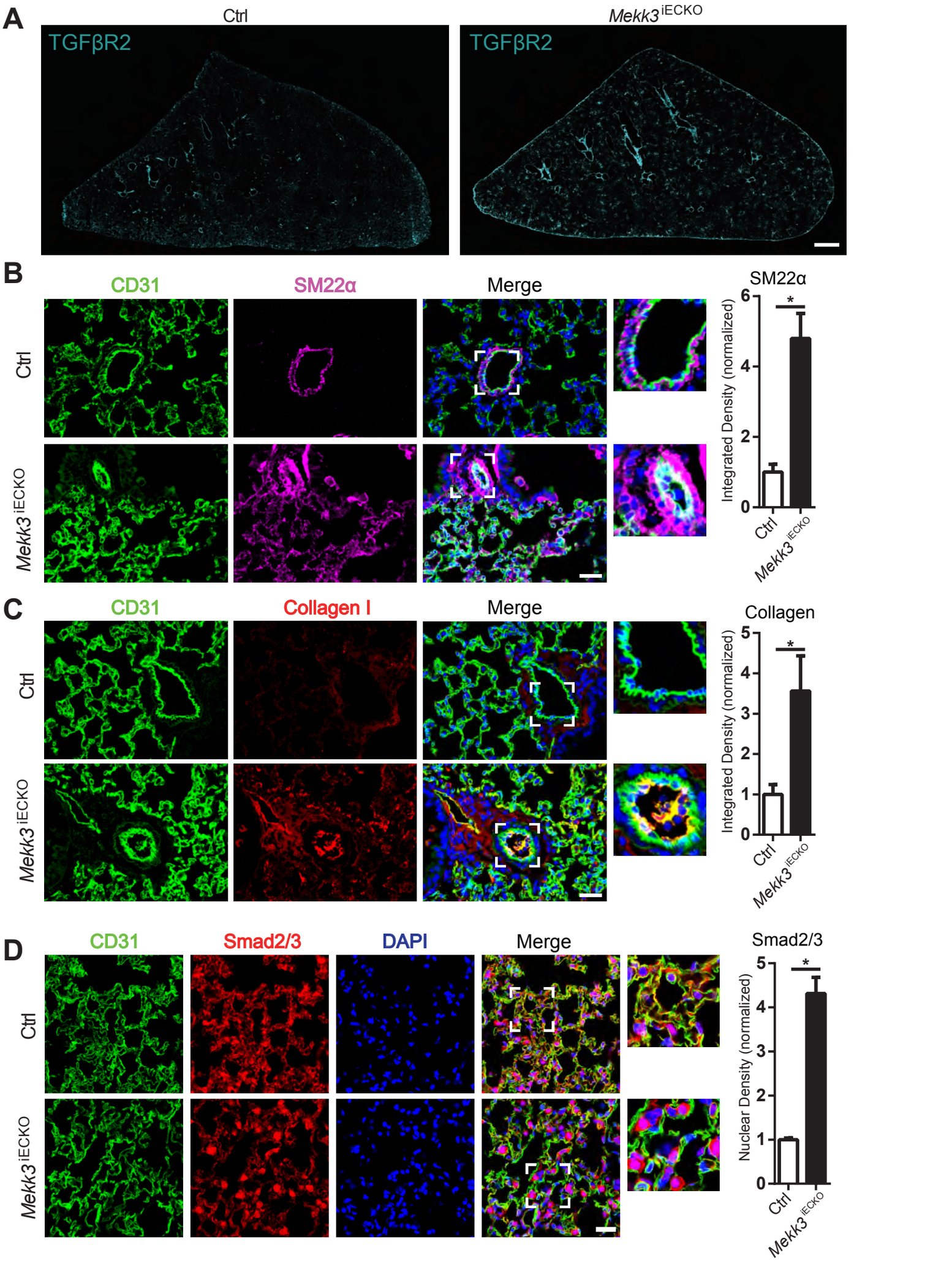

A
Ctrl
Mekk3 iECKO
TGFβR2
TGFβR2
B
CD31
SM22α
Merge
Ctrl
Mekk3 iECKO
SM22α
Integrated Density (normalized)
6
4
2
0
*
Ctrl
Mekk3 iECKO
C
CD31
Collagen I
Merge
Ctrl
Mekk3 iECKO
Collagen
Integrated Density (normalized)
5
4
3
2
1
0
*
Ctrl
Mekk3 iECKO
D
CD31
Smad2/3
DAPI
Merge
Ctrl
Mekk3 iECKO
Smad2/3
Nuclear Density (normalized)
5
4
3
2
1
0
*
Ctrl
Mekk3 iECKO

Figure S5

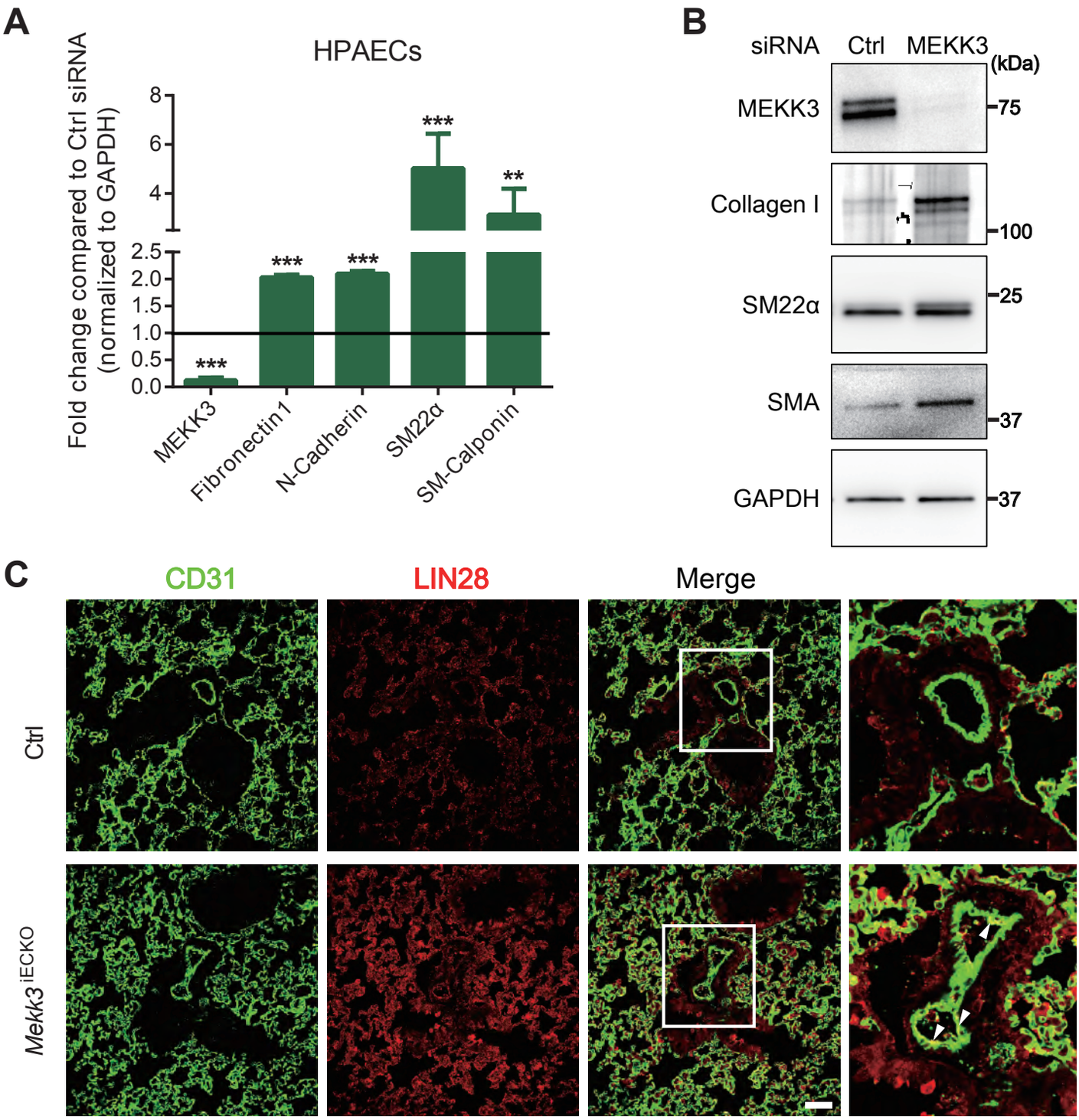

A
HPAECs
Fold change compared to Ctrl siRNA (normalized to GAPDH)
MEKK3
Fibronectin1
N-Cadherin
SM22α
SM-Calponin
B
siRNA
Ctrl
MEKK3
(kDa)
MEKK3
Collagen I
SM22α
SMA
GAPDH
75
100
25
37
37
C
CD31
LIN28
Merge
Ctrl
Mekk3 iECKO

Figure S6

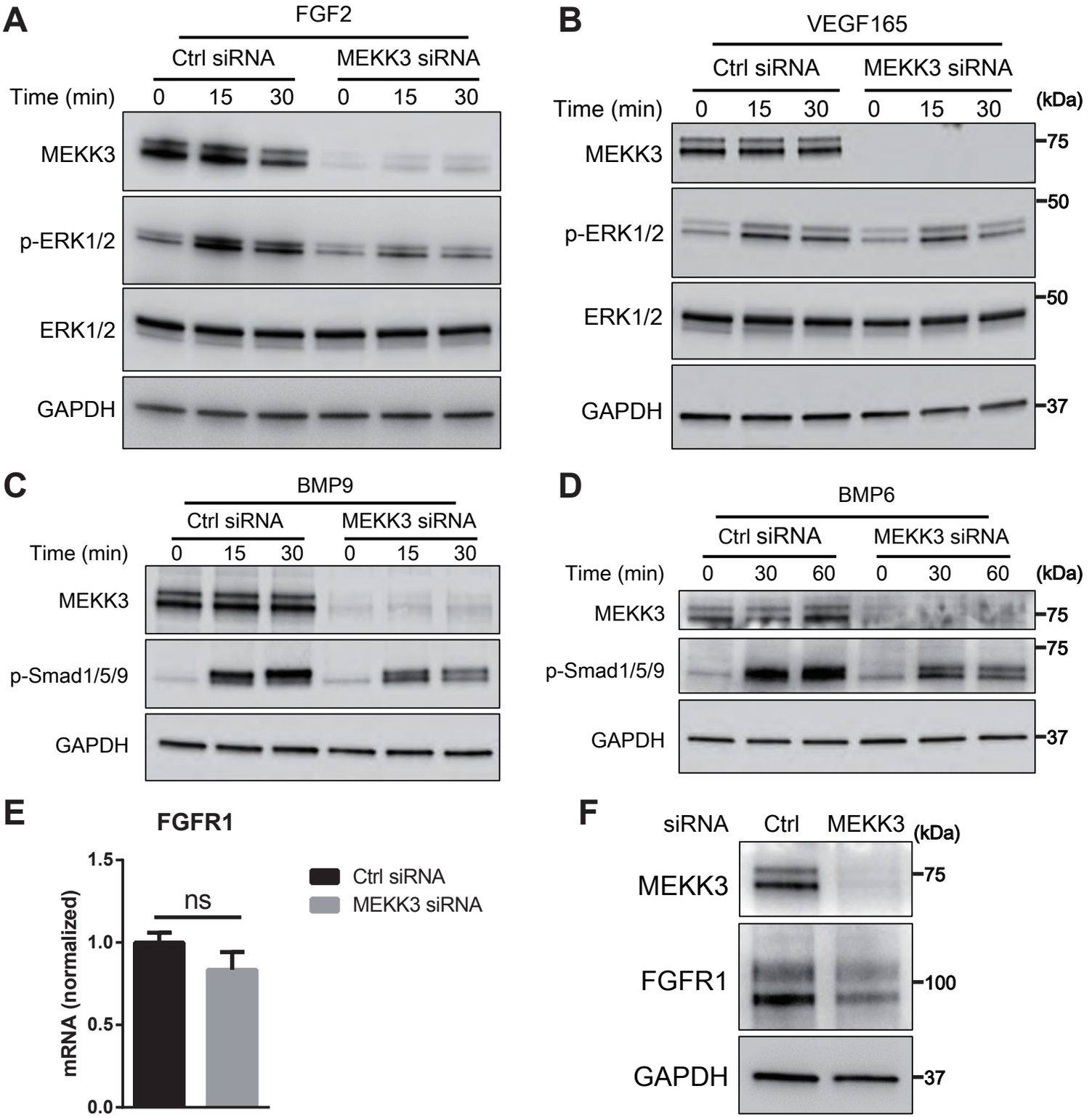

A
FGF2
Ctrl siRNA
MEKK3 siRNA
Time (min)
0 15 30 0 15 30
MEKK3
p-ERK1/2
ERK1/2
GAPDH
B
VEGF165
Ctrl siRNA
MEKK3 siRNA
Time (min)
0 15 30 0 15 30
(kDa)
MEKK3
p-ERK1/2
ERK1/2
GAPDH
75
50
50
37
C
BMP9
Ctrl siRNA
MEKK3 siRNA
Time (min)
0 15 30 0 15 30
MEKK3
p-Smad1/5/9
GAPDH
D
BMP6
Ctrl siRNA
MEKK3 siRNA
Time (min)
0 30 60 0 30 60
(kDa)
MEKK3
p-Smad1/5/9
GAPDH
75
75
37
E
FGFR1
mRNA (normalized)
1.5
1.0
0.5
0.0
ns
Ctrl siRNA
MEKK3 siRNA
F
siRNA
Ctrl
MEKK3
(kDa)
MEKK3
FGFR1
GAPDH
75
100
37

Figure S7

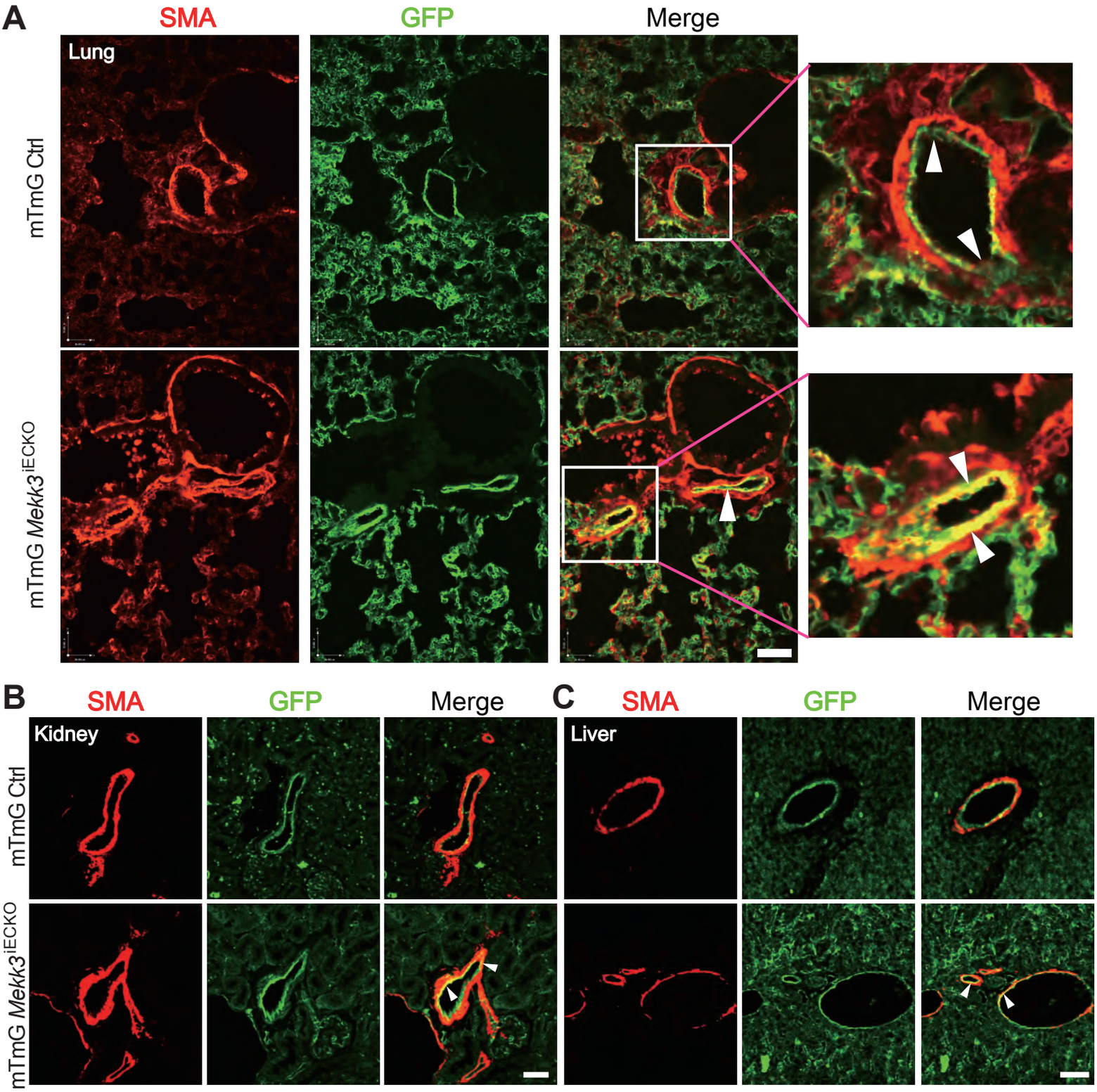

A
SMA
GFP
Merge
Lung
mTmG Ctrl
mTmG Mekk3iECKO
B
SMA
GFP
Merge
Kidney
mTmG Ctrl
mTmG Mekk3iECKO
C
SMA
GFP
Merge
Liver

Figure S8

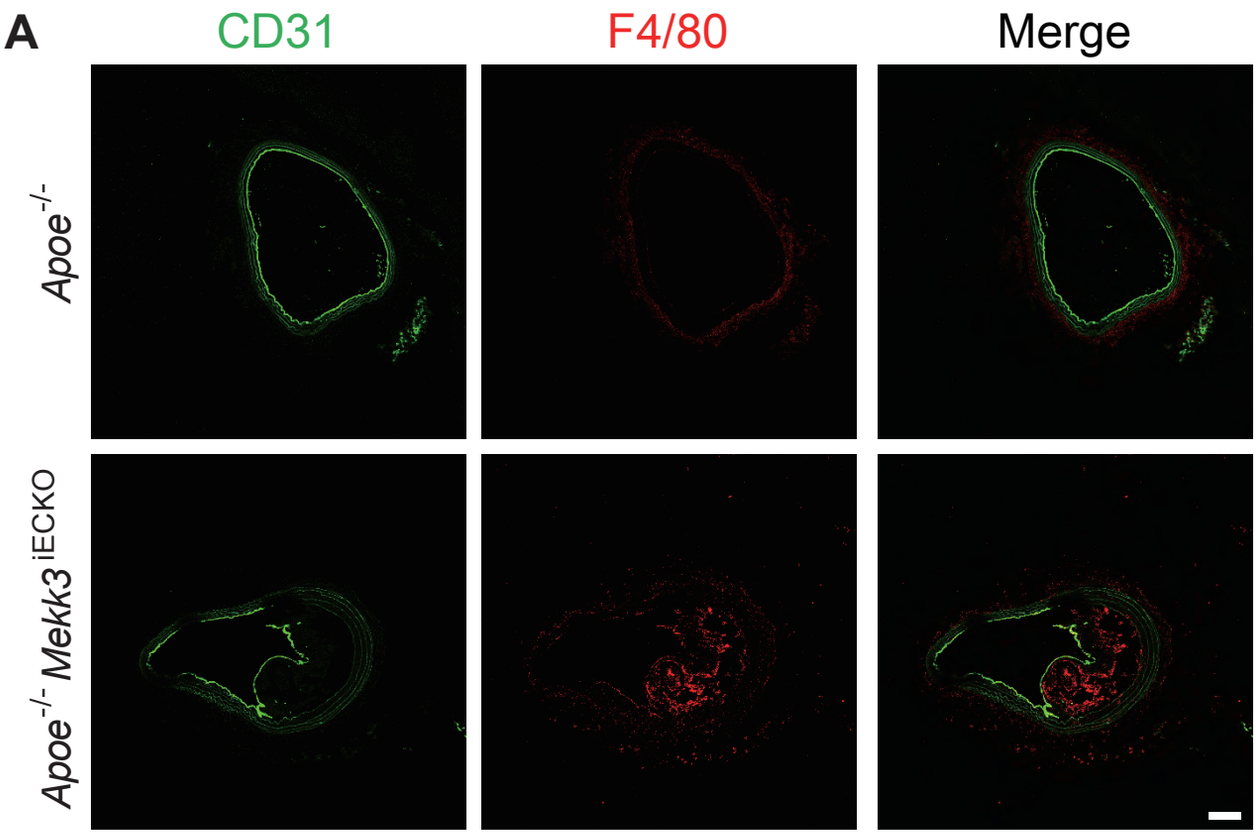
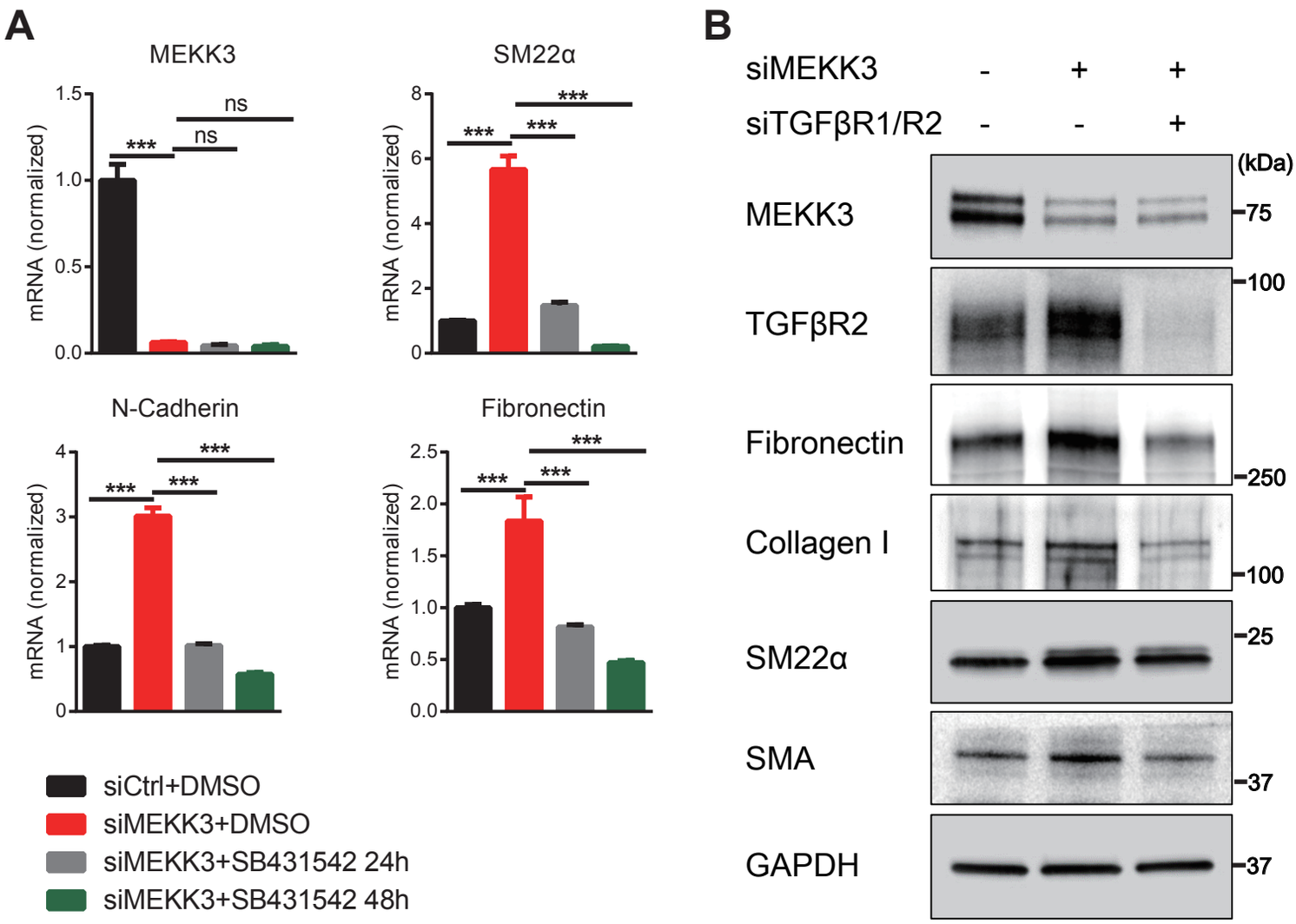


Figure S9


A
MEKK3
SM22α
N-Cadherin
Fibronectin
mRNA (normalized)
ns
***
siCtrl+DMSO
siMEKK3+DMSO
siMEKK3+SB431542 24h
siMEKK3+SB431542 48h
B
siMEKK3 - + +
siTGFβR1/R2 - - +
(kDa)
MEKK3
TGFβR2
Fibronectin
Collagen I
SM22α
SMA
GAPDH
75
100
250
100
25
37
37

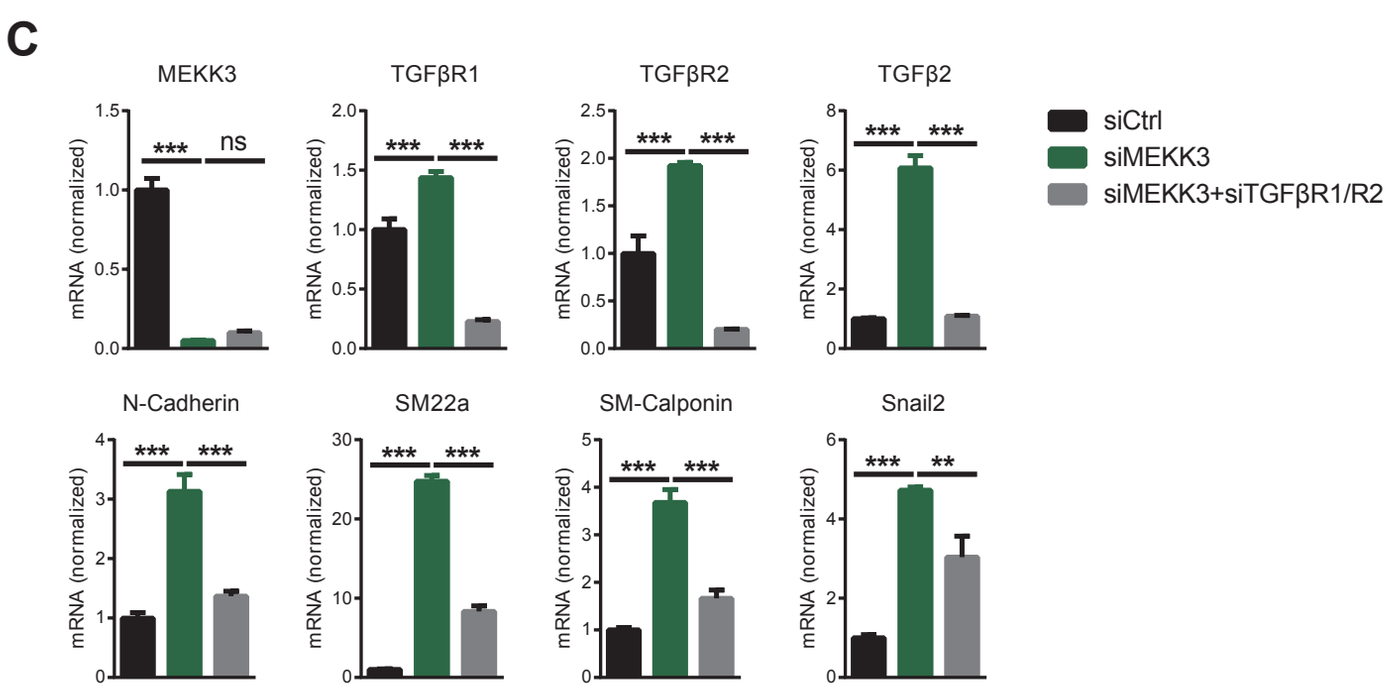
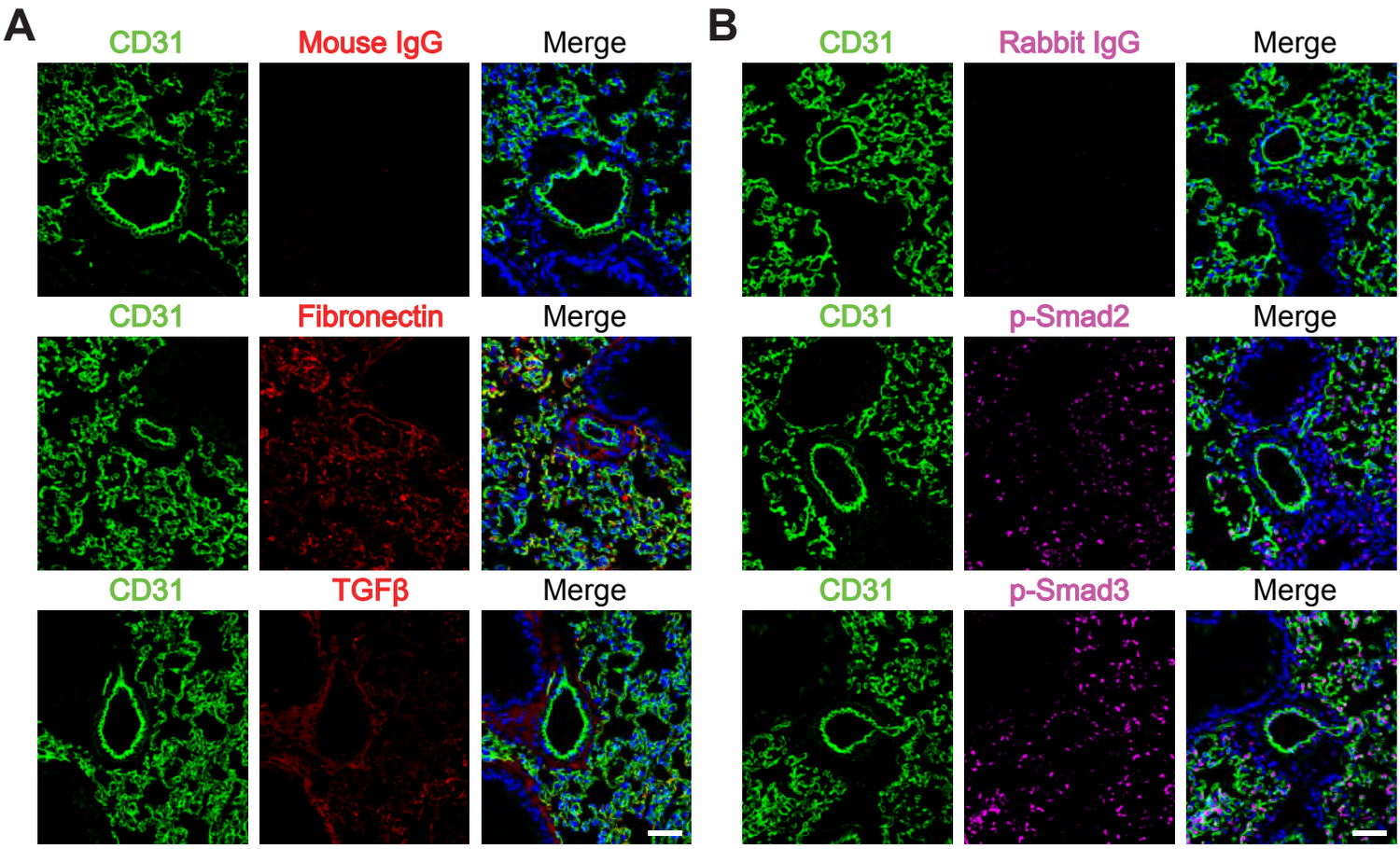

C
MEKK3
TGFβR1
TGFβR2
TGFβ2
N-Cadherin
SM22a
SM-Calponin
Snail2
mRNA (normalized)
siCtrl
siMEKK3
siMEKK3+siTGFβR1/R2

Figure S10


A
CD31
Mouse IgG
Merge
CD31
Fibronectin
Merge
CD31
TGFβ
Merge
B
CD31
Rabbit IgG
Merge
CD31
p-Smad2
Merge
CD31
p-Smad3
Merge

Supplemental Table 1. List of qPCR primers used.

Genes	Sequences (5' to 3')
hMEKK3-F	CAGACAGGAATACTCAGATCGGG
hMEKK3-R	TCTTCTGCCATCACTGTAGTCC
hGAPDH-F	GGAGCGAGATCCCTCCAAAAT
hGAPDH-R	GGCTGTTGTCATACTTCTCATGG
hFN1-F	GAGAATAAGCTGTACCATCGCAA
hFN1-R	CGACCACATAGGAAGTCCCAG
hCdh2-F	AGCCAACCTTAACTGAGGAGT
hCdh2-R	GGCAAGTTGATTGGAGGGATG
hNOS3-F	TGATGGCGAAGCGAGTGAAG
hNOS3-R	ACTCATCCATACACAGGACCC
hSnail2-F	TGTGACAAGGAATATGTGAGCC
hSnail2-R	TGAGCCCTCAGATTTGACCTG
hTGFβ1-F	GTACCTGAACCCGTGTTGCT
hTGFβ1-R	GTATCGCCAGGAATTGTTGC
hTGFβ2-F	ATGCGGCCTATTGCTTTAGA
hTGFβ2-R	GTTGGCATTGTACCCTTTGG
hTGFβ3-F	GCCTCAGTCTTTGGGATCTG
hTGFβ3-R	GTGTGAGCTGGGAAGAGAGG
hTGFβR1-F	CAGCTCTGGTTGGTGTCAGA
hTGFβR1-R	ATGTGAAGATGGGCAAGACC
hTGFβR2-F	TGAGTTCAACCTGGGAAACC
hTGFβR2-R	GGTTGATGTTGTTGGCACAC
hSM α-actin-F	CAAAGCCGGCCTTACAGAG
hSM α-actin-R	AGCCCAGCCAAGCACTG
hSM22α-F	GATTTTGGACTGCACTTCGC
hSM22α-R	GTCCGAACCCAGACACAAGT
hSM-calponin-F	CTGGCTGCAGCTTATTGATG
hSM-calponin-R	CTGAGAGAGTGGATCGAGGG
hLIN28a-F	AGCGCAGATCAAAAGGAGACA
hLIN28a-R	CCTCTCGAAAGTAGGTTGGCT
hLIN28b-F	CATCTCCATGATAAACCGAGAGG
hLIN28b-R	GTTACCCGTATTGACTCAAGGC
mGapdh-F	AGGTCGGTGTGAACGGATTTG
mGapdh-R	TGTAGACCATGTAGTTGAGGTCA
mMekk3-F	GCCAATATCCTCCGAGACTCAGCTGGGAAT
mMekk3-R	CTTGAGAGCTCAGTACACTAGCTG
mNos3-F	TCAGCCATCACAGTGTTCCC
mNos3-R	ATAGCCCGCATAGCGTATCAG
mFn1-F	ATGTGGACCCCTCCTGATAGT

mFn1-R	GCCCAGTGATTTCAGCAAAGG
mSnail2-F	TGGTCAAGAAACATTTCAACGCC
mSnail2-R	GGTGAGGATCTCTGGTTTTGGTA
mCdh2-F	AGCGCAGTCTTACCGAAGG
mCdh2-R	TCGCTGCTTTCATACTGAACTTT
mCol3a1-F	ACGTAGATGAATTGGGATGCAG
mCol3a1-R	GGGTTGGGGCAGTCTAGTG
mSm22a-F	CAACAAGGGTCCATCCTACGG
mSm22a-R	ATCTGGGCGGCCTACATCA
mTgfb2-F	TCGACATGGATCAGTTTATGCG
mTgfb2-R	CCCTGGTACTGTTGTAGATGGA
mTgfbr1-F	CAGCTCCTCATCGTGTTGGTG
mTgfbr1-R	GCACATACAAATGGCCTGTCTC
mTgfbr2-F	CCGCTGCATATCGTCCTGTG
mTgfbr2-R	AGTGGATGGATGGTCCTATTACA