Supplementary materials

Simvastatin attenuates abdominal aortic aneurysm formation favoured by lack of Nrf2 transcriptional activity

Aleksandra Kopacz[1], Ewa Werner[1,2], Anna Grochot-Przęczek[1], Damian Klóska[1], Karolina Hajduk[1], Christoph Neumayer[3], Alicja Józkowicz[1], Aleksandra Piechota-Polanczyk[1*]

[1]Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland.

[2]Department of Animal Reproduction and Anatomy, Faculty of Animal Science, University of Agriculture, Krakow, Poland

[3]Department of Surgery, Division of Vascular Surgery, Medical University of Vienna, Austria.

***Corresponding author:**

Aleksandra Piechota-Polanczyk, PhD, Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland, e-mail: aleksandra.piechota-polanczyk@uj.edu.pl, phone: +48 12 664-64-12, Fax: +48 12 664-69-02

Supplementary Figure Legends:

Fig. S1

Confirmation of the model. Mice of both genotypes were divided into the following groups: 1) sham (saline, n=8); 2) angiotensin II (AngII group; n=10 or 14); 3) simvastatin + saline (Sim group; n=12); 4) simvastatin + angiotensin II (Sim+AngII group; n=13). Simvastatin was administered daily for 7 consecutive days before osmotic pump placement and during Ang II infusion for another 14 or 28 days. **(A-B)** Assessment of NFE2L2 mRNA level in the abdominal aorta**.** (**A**) Relative expression of *NFE2L2* verified using primers recognizing the fragment, encoding the DNA binding domain (exon 5). *eEF2* was used as a reference gene. 3-way ANOVA with Tukey's post hoc test. **(B**) Relative expression of *NFE2L2* verified using primers recognizing the fragment, encoding the N terminal fragments of Nrf2, present in tKO mice (exon 3). *eEF2* was used as a reference gene. 3-way ANOVA with Tukey's post hoc test.; (**C-D**) Verification of statin administration through assessment of HMG-CoA reductase mRNA level. (**C**) Relative expression of *HMGCR* in the abdominal aorta. *eEF2* was used as a reference gene. 3-way ANOVA with Tukey's post hoc test. **(D**) Relative expression of *NFE2L2* verified using primers recognizing the fragment, encoding the N terminal fragments of Nrf2). Relative expression of *HMGCR* in the abdominal aorta. *eEF2* was used as a reference gene. 3-way ANOVA with Tukey's post hoc test. $^{**}p<0.01$; $^{***}p<0.001$ vs saline; $^{\#}p<0.05$; $^{\#\#}p<0.01$ vs AngII, $^{@@@}$ $p<0.001$ vs WT.

Fig. S2

The scheme of simvastatin and angiotensin II dosage to WT and tKO mice together with time points of *in vivo* measurements of blood pressure (BP) and USG.

Fig. S3

The schematic analysis of aortic area and diameter from the B-mode (A) **and M-mode** (B) for representative mice at day 0 and day 28 after osmotic pump placement. MA- the mesenteric artery; LA- the left renal artery; RA- the right renal artery.

Fig. S4

Morphological and histological changes in the aorta of WT and tKO mice from control groups. (**A**) Representative image of aortas isolated from WT and tKO mice treated with saline and simvastatin+saline (Sim). Scale bar = 4 mm (**B**) Time-dependent changes in the aortic inner diameter [mm] of control groups measured with USG. 3-way ANOVA with Tukey's post hoc test, $^{**}p<0.01$ vs saline.

Fig. S5

The inflammatory response is attenuated at day 28. Mice of both genotypes were divided into the following groups: 1) sham (saline group, (n=4); 2) angiotensin II (AngII group; n=5); 3) simvastatin + saline (Sim group; n=6); 4) simvastatin + angiotensin II (Sim+AngII group; n=7). Simvastatin was administered daily for 7 consecutive days before osmotic pump placement and during Ang II infusion for another 28 days. (**B**) Relative expression of *VCAM1*, *SELE* in the aortic wall. *eEF2* was used as a reference gene. 3-way ANOVA with Tukey's post hoc test. $^{*}p<0.05$ vs saline.

Fig. S6

Supplementary information for the assessment of metalloproteinase activity. (**A**) Relative expression of *MMP9* within the abdominal aortic wall. *eEF2* was used as a reference gene. 3-way ANOVA with Tukey's post hoc test. Rectangle – mice, which developed the aneurysm. (**B**) Negative controls for *in situ* zymography (Fig. 6A) performed on abdominal aorta in mice of both genotypes. Gelatinases activity was muted with 1 h incubation of aortic specimens with 1,10 phenanthroline (Phe, 10 μM). Green-gelatinases activity, Blue-nuclei. L – lumen. Scale bar 0.1 mm.

Fig. S7

Negative controls for AT1R (A) and P-ERK (B) immunofluorescent stainings (Fig. 7A-B). L – lumen. Scale bar 50 μm.

Supplementary Table 1

Table. 1 The sequence of primers used in the study.

Gene	Primer sequence
Mouse *Eef2*	Forward: 5'- GACATCACCAAGGGTGTGCA -3' Reverse: 5'- TCAGCACACTGGCATAGACC -3'
Mouse *NFE2L2 (exon 3)*	Forward: CAGAGACATTCCCATTTGTAG Reverse: ATTCGGGAATGGAAAATAGC
Mouse *NFE2L2 (exon 5)*	Forward: TCACACGAGATGAGCTTAGGGCAA Reverse: TACAGTTCTGGGCGGCGACTTTAT
Mouse *HMGCR*	Forward: GATAGCTGATCCTTCTCCTC Reverse: ATGCTGATCATCTTGGAGAG
Mouse *Vcam1*	Forward: CCGGCATATACGAGTGTGAA Reverse: GATGCGCAGTAGAGTGCAAG
Mouse *Sele*	Forward: ATGCCTCGCGCTTTCTCTC Reverse: GTAGTCCCGCTGACAGTATGC
Mouse *IL1β*	Forward: 5'- CTGGTGTGTGACGTTCCCATTA-3' Reverse: 5'- CCGACAGCACGAGGCTTT -3'
Mouse *IL4*	Forward: 5'- CTGGTGTGTGACGTTCCCATTA -3' Reverse: 5'- CCGACAGCACGAGGCTTT -3'
Mouse *IL6*	Forward: 5'-AAAGAGTTGTGCAATGCAATGGCAATTCT-3' Reverse: 5'- AAGTGCATCATCGTTGTTCATACA -3'
Mouse *Hmox1*	Forward: 5'- TTCTTCACCTTCCCCAACATTG -3' Reverse: 5'- CAGCTCCTGCAACTCCTCAAA -3'
Mouse *NQO1*	Forward: 5'-GCTCGTAGCAGGATTTTGCCT -3' Reverse: 5'-CAGGATGCCACTTCTGAATCG -3'
Mouse *Col1α1*	Forward: 5'- ACTACCGGGCCGATGATGCTAACG -3' Reverse: 5'- CGATCCAGTACTCTCCGCTCTTCC -3'
Mouse *Col1α2*	Forward: 5'- GCCACCATTGATAGTCTCTCC -3' Reverse: 5'- CACCCCAGCGAAGAACTCATA -3'
Mouse *Col3α1*	Forward: 5'- ATCTATGAATGGTGGTTTTCAGTT -3' Reverse: 5'- TTTTGCAGTGGTATGTAATGTTCT -3'
Mouse *MMP2*	Forward: 5'- ACAGGACATTGTCTTTGATG -3'

	Reverse: 5’- TACACAGCGTCAATCTTTTC – 3’
Mouse *MMP3*	Forward: 5’- CTAAAAGCATTCACACCCTG -3’ Reverse: 5’- TTTCTTCTCATCAAACCTCC -3’
Mouse *TGFβ1*	Forward: 5’-TGATAAAGTGGAGTGAAGAGAG -3’ Reverse: 5’-GGCTTTTTGTAGTTTCCTAGAG -3’

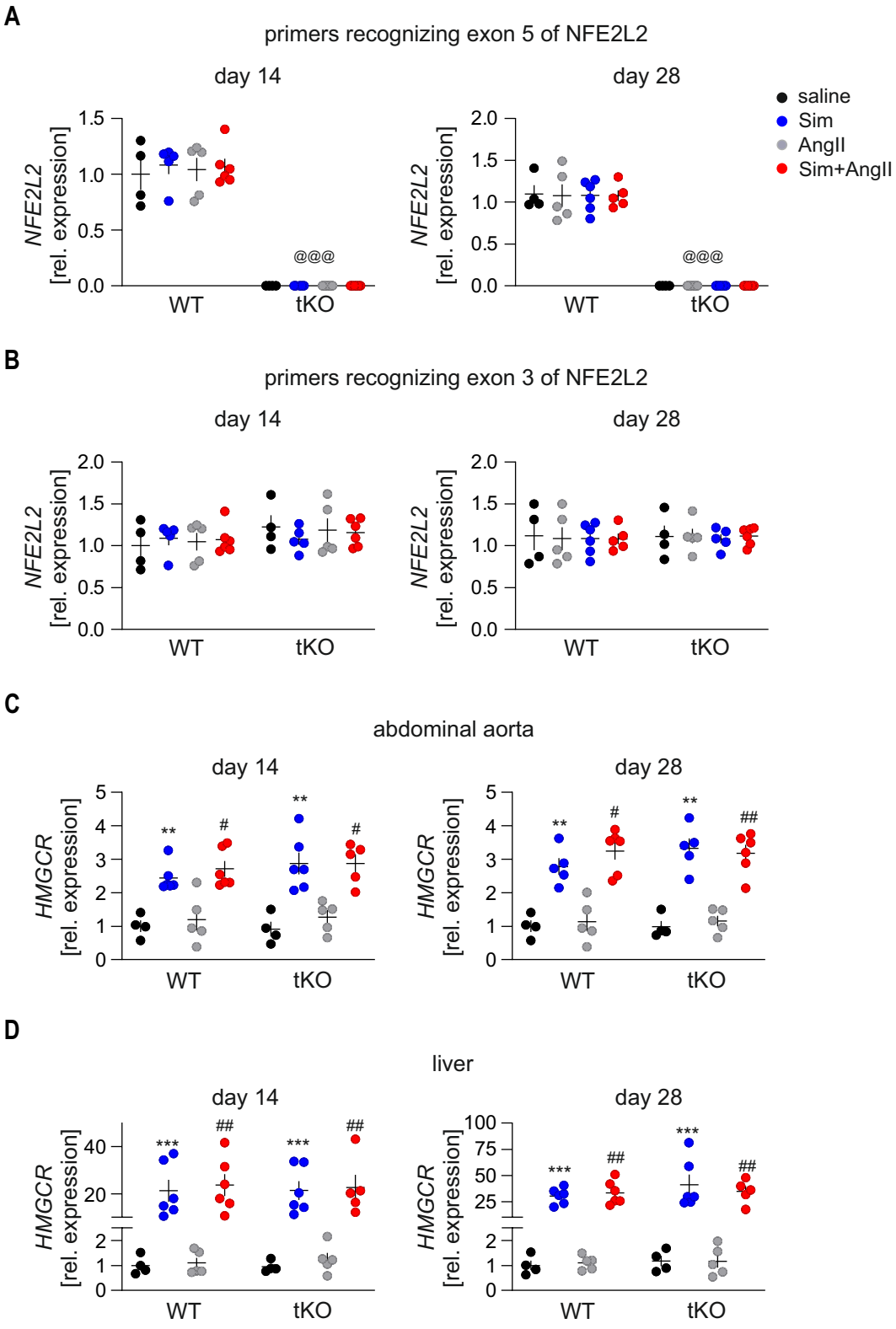
A
primers recognizing exon 5 of NFE2L2
day 14
day 28
saline
Sim
AngII
Sim+AngII
NFE2L2 [rel. expression]
@@@
WT
tKO
B
primers recognizing exon 3 of NFE2L2
C
abdominal aorta
HMGCR [rel. expression]
D
liver

Fig. S1

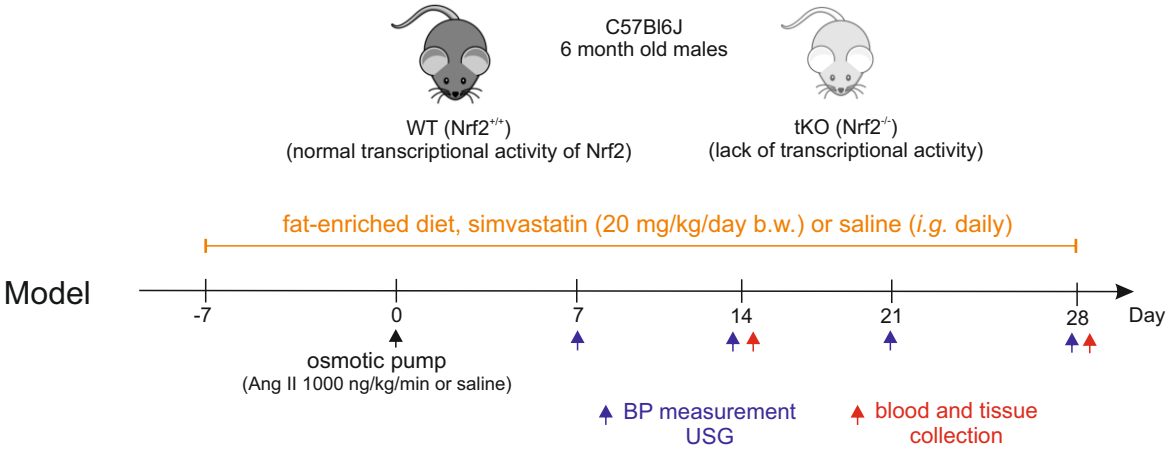
C57Bl6J
6 month old males
WT (Nrf2+/+)
(normal transcriptional activity of Nrf2)
tKO (Nrf2-/-)
(lack of transcriptional activity)
fat-enriched diet, simvastatin (20 mg/kg/day b.w.) or saline (i.g. daily)
Model
-7
0
7
14
21
28
Day
osmotic pump
(Ang II 1000 ng/kg/min or saline)
BP measurement
USG
blood and tissue
collection

Fig. S2

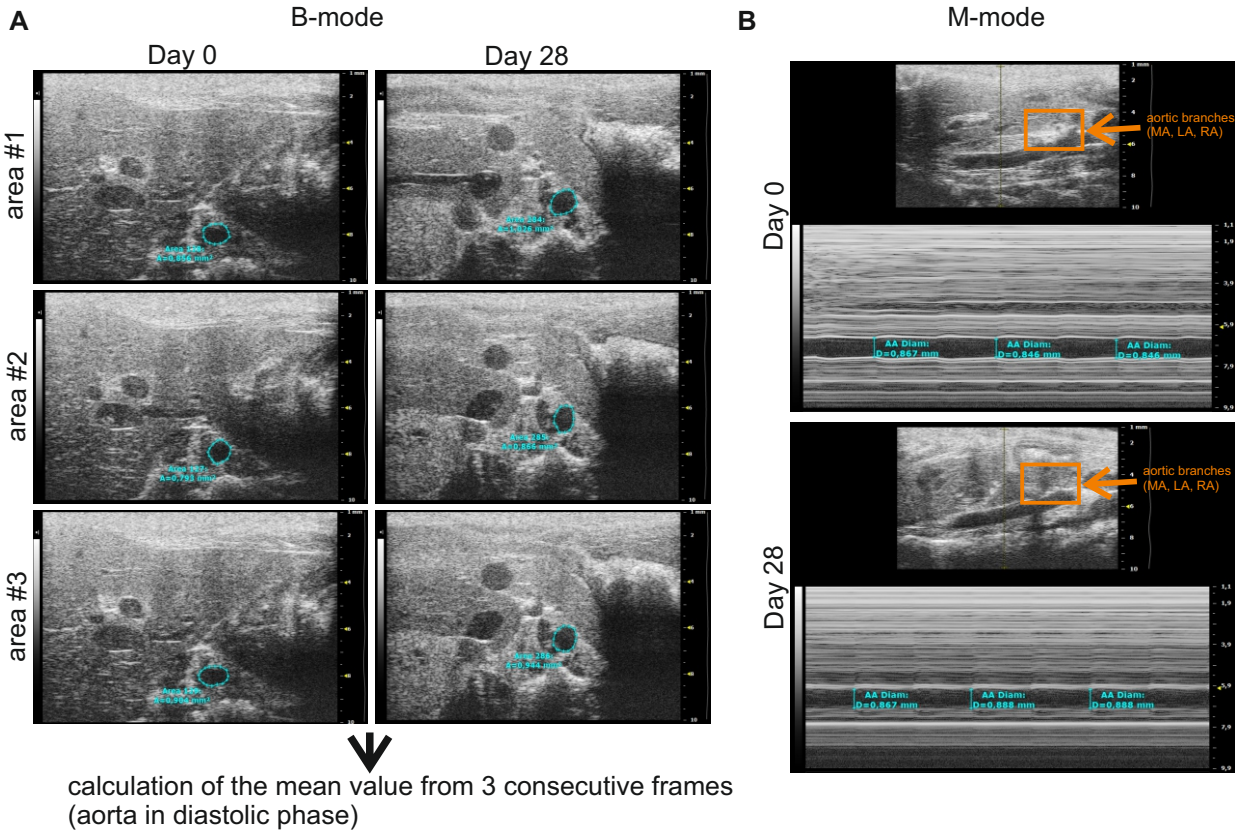
A
B-mode
Day 0
Day 28
area #1
area #2
area #3
B
M-mode
Day 0
Day 28
aortic branches
(MA, LA, RA)
AA Diam:
D=0,867 mm
AA Diam:
D=0,846 mm
AA Diam:
D=0,846 mm
aortic branches
(MA, LA, RA)
AA Diam:
D=0,867 mm
AA Diam:
D=0,888 mm
AA Diam:
D=0,888 mm
calculation of the mean value from 3 consecutive frames
(aorta in diastolic phase)

Fig. S3

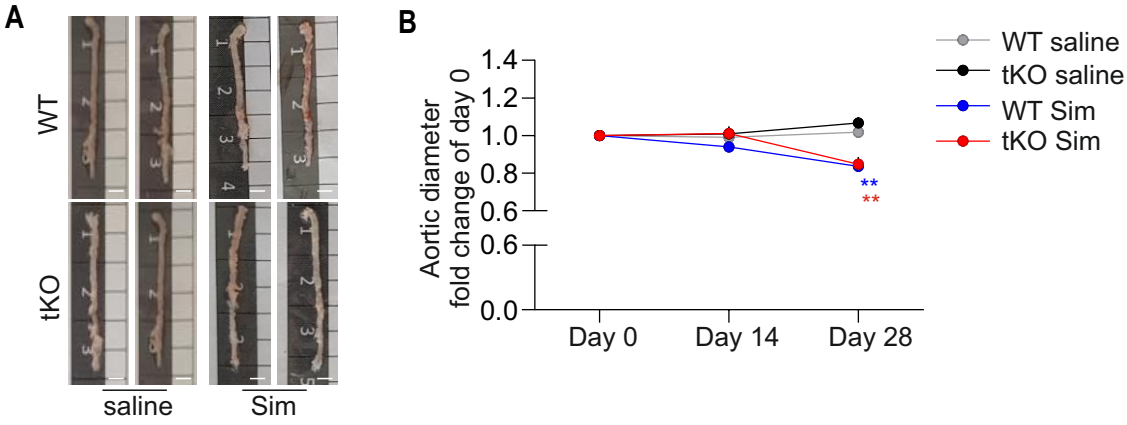
A
WT
tKO
saline
Sim
B
Aortic diameter
fold change of day 0
1.4
1.2
1.0
0.8
0.6
0.6
0.0
Day 0
Day 14
Day 28
**
**
WT saline
tKO saline
WT Sim
tKO Sim

Fig. S4

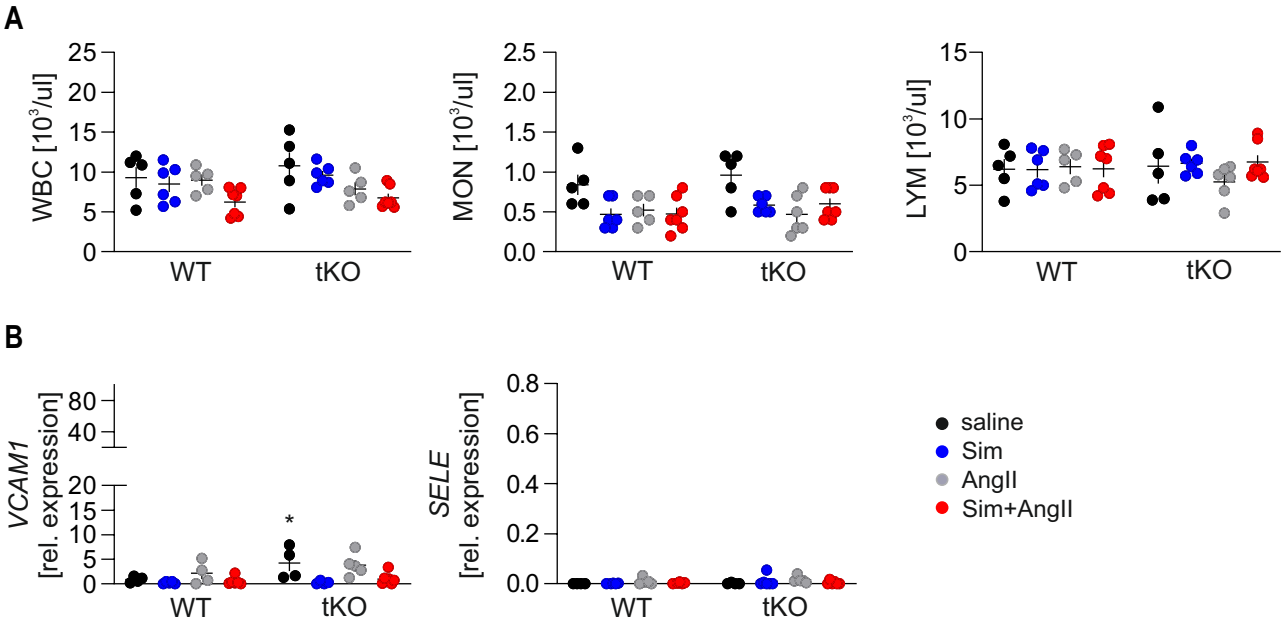
A
WBC [10^3/ul]
MON [10^3/ul]
LYM [10^3/ul]
WT
tKO
B
VCAM1 [rel. expression]
SELE [rel. expression]
*
saline
Sim
AngII
Sim+AngII

Fig. S5

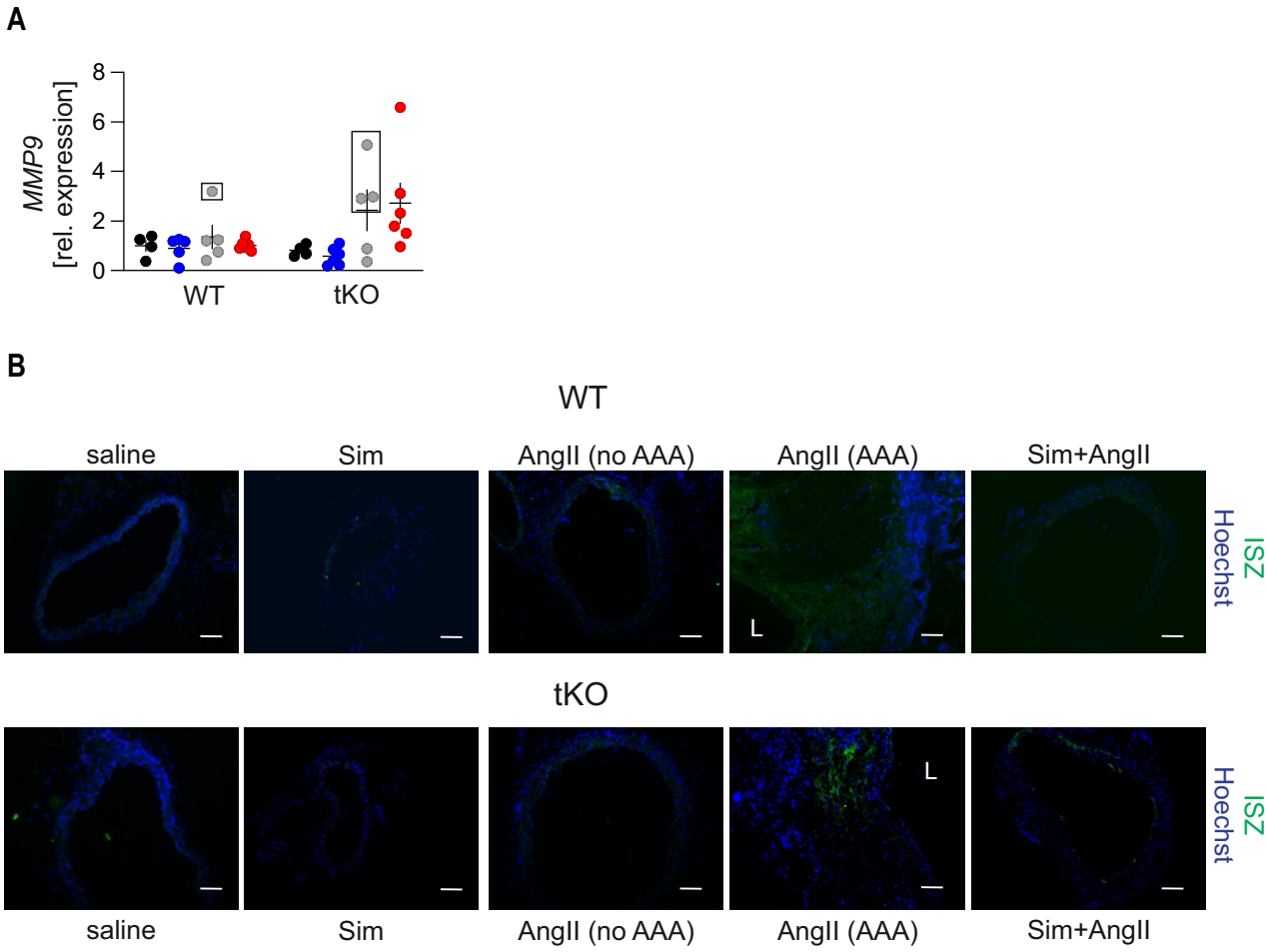
A
MMP9
[rel. expression]
8
6
4
2
0
WT
tKO
B
WT
saline
Sim
AngII (no AAA)
AngII (AAA)
Sim+AngII
L
Hoechst
ISL
tKO
L
Hoechst
ISL
saline
Sim
AngII (no AAA)
AngII (AAA)
Sim+AngII

Fig. S6

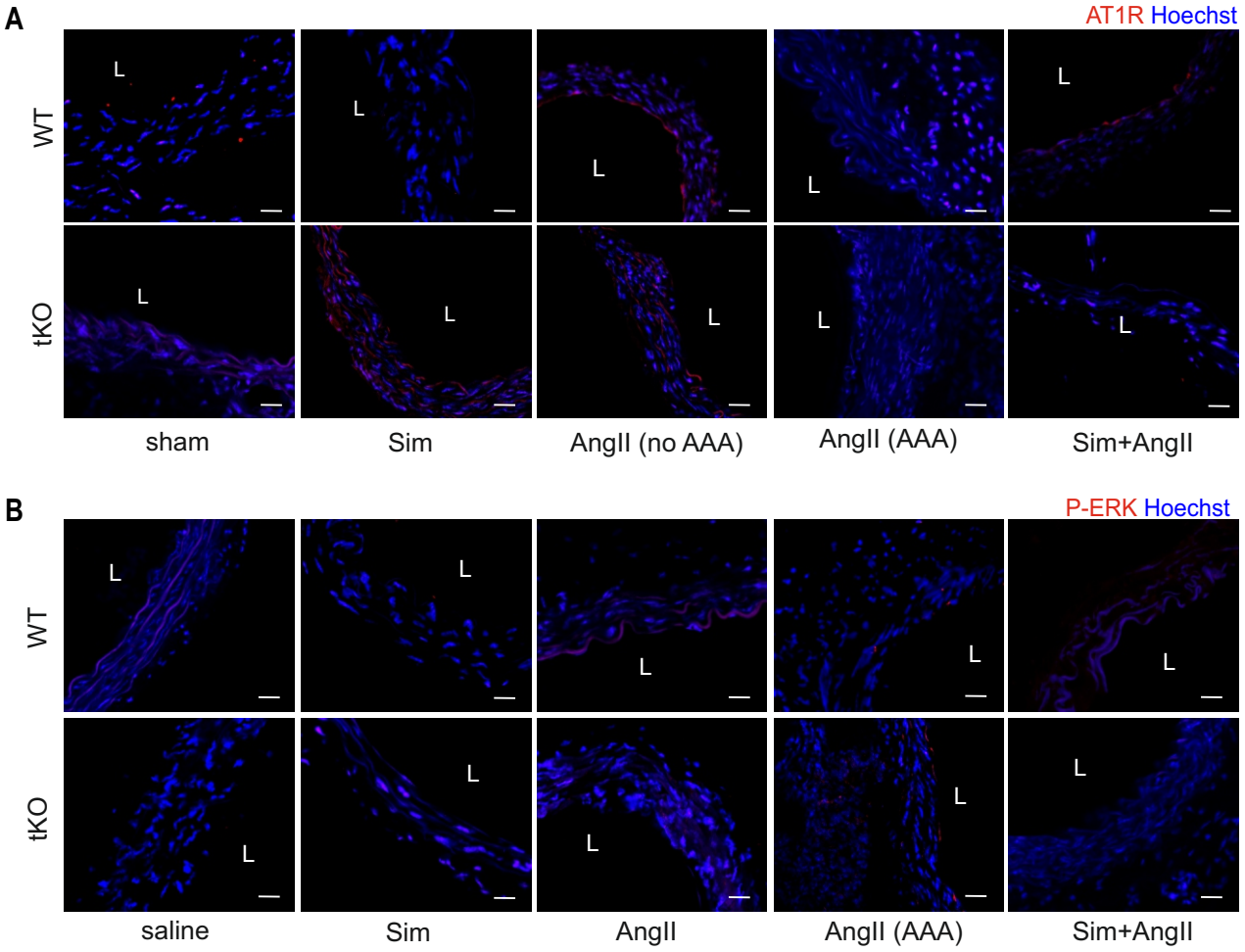
A
AT1R Hoechst
WT
tKO
L
sham
Sim
AngII (no AAA)
AngII (AAA)
Sim+AngII
B
P-ERK Hoechst
WT
tKO
L
saline
Sim
AngII
AngII (AAA)
Sim+AngII

Fig. S7