

## **Supplementary Information**

### **Vascular smooth muscle RhoA counteracts abdominal aortic aneurysm formation by modulating MAP4K4 activity**

Md Rasel Molla, Akio Shimizu, Masahiro Komeno, Nor Idayu A. Rahman, Joanne Ern Chi Soh, Le Kim Chi Nguyen, Mahbubur Rahman Khan, Wondwossen Wale Tesega, Si Chen, Xiaoling Pang, Miki Tanaka-Okamoto, Noriyuki Takashima, Akira Sato, Tomoaki Suzuki, Hisakazu Ogita

Supplementary Tables 1–3

Supplementary Figures 1–14

**Supplementary Table 1** Clinical characteristics of patients analyzed in this study.

Total (n)	30
Age (year)	72.7±1.7
Male/Female (n)	25/5
BMI (kg/m <sup>2</sup> )	23.0±0.7
Heart rate (bpm)	77±3
Systolic BP (mmHg)	128±3
Red blood cell (×10 <sup>4</sup> /μL)	427±10
ALT (IU/L)	18±2
LDL (mg/dL)	112±6
HbA1c	6.0±0.1

BMI: body mass index, BP: blood pressure, ALT: alanine aminotransferase,

LDL: low density lipoprotein cholesterol, Hb: hemoglobin

**Supplementary Table 2** Information of the primary antibodies used in this study.

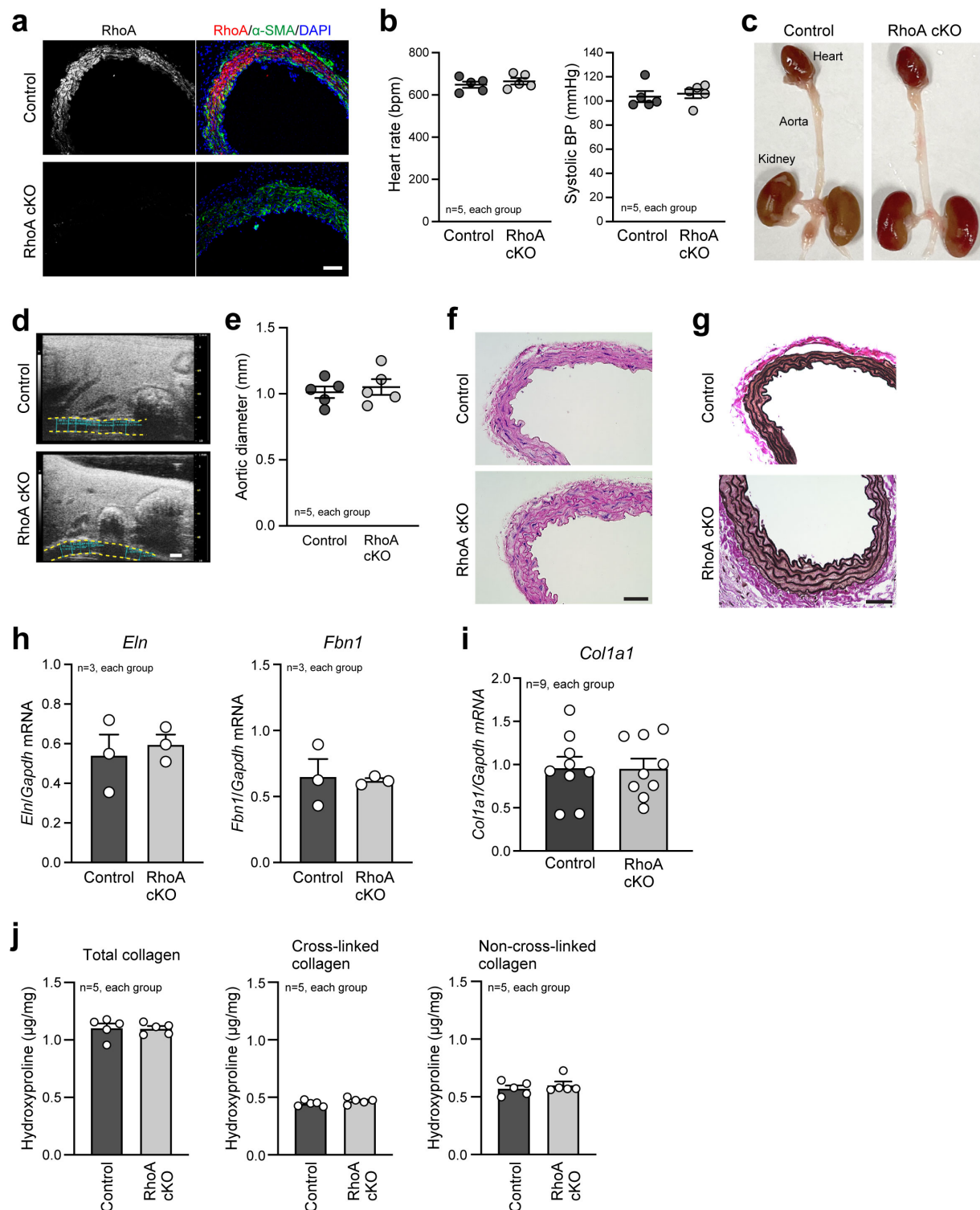
Name (Clone No.)	Source	Type	Company	Cat. No.	Dilution ratio
RhoA (67B9)	Rabbit	Monoclonal	Cell Signaling Technology	2117	WB (1:1000), IF and IHC (1:200)
RhoB (C-5)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-8084	IF (1:200)
GAPDH (3H12)	Mouse	Monoclonal	Medical & Biological Laboratories	M171-3	WB (1:1000)
$\alpha$ -Actin (1A4)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-32251	WB (1:1000), IF (1:200)
$\alpha$ -MHC (MF20)	Mouse	Monoclonal	R&D Systems	MAB4470	IF (1:500)
Aggrecan (4F4)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-33695	IF (1:200)
CD31 (SZ31)	Rat	Monoclonal	Dianova	DIA-310	IF (1:200)
CD31	Rabbit	Polyclonal	Abcam	ab28364	WB (1:1000)
P-p38 MAPK [Thr180/Tyr182] (D3F9)	Rabbit	Monoclonal	Cell Signaling Technology	4511	WB (1:1000), IF and IHC (1:200)
p38 MAPK (D13E1)	Rabbit	Monoclonal	Cell Signaling Technology	8690	WB (1:1000)
P-p44/42 MAPK [Thr202/Tyr204] (D13.14.4E)	Rabbit	Monoclonal	Cell Signaling Technology	4370	WB (1:1000), IF and IHC (1:200)
p44/42 MAPK (137F5)	Rabbit	Monoclonal	Cell Signaling Technology	4695	WB (1:1000)
P-MAP4K4	Rabbit	Polyclonal	Bioss	bs-5491R	WB (1:1000), IF and IHC (1:200)
MAP4K4	Rabbit	Polyclonal	Proteintech	55247-1-AP	WB (1:1000), IP (1:100)
CD68	Rabbit	Polyclonal	Abcam	ab125212	IF (1:200)
F4/80 (C-7)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-377009	IF (1:200)
MMP2 (8B4)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-13595	IF (1:200)
MMP9 (7-11C)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-13520	IF (1:200)
TIMP1 (G-6)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-365905	IF (1:200)
TIMP2 (3A4)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-21735	IF (1:200)
GFP (mFX75)	Mouse	Monoclonal	Wako Pure Chemical Industries	012-22541	WB (1:1000)
GFP (mFX73)	Mouse	Monoclonal	Wako Pure Chemical Industries	012-20461	IP (1:100)
P-MLC2 [Thr18/Ser19]	Rabbit	Polyclonal	Cell Signaling Technology	3674	WB (1:1000)
MLC2 (E-4)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-28329	WB (1:1000)
Phospho-MYLK [Ser1760]	Rabbit	Polyclonal	Invitrogen	44-1085G	WB (1:1000)
MYLK (A-8)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-365352	WB (1:1000), IP (1:100)
PP2A-C $\alpha$ / $\beta$ (1D6)	Mouse	Monoclonal	Santa Cruz Biotechnology	sc-80665	WB (1:1000), IP (1:100), IF (1:200)
Set	Rabbit	Polyclonal	Novusbio	NBP1-33713	WB (1:2000), IF (1:200)
HA	Rabbit	Polyclonal	Medical & Biological Laboratories	561	WB (1:1000)
Vimentin (D21H3)	Rabbit	Monoclonal	Cell Signaling Technology	5741	WB (1:1000)

IF: immunofluorescence, IHC: immunohistochemistry, IP: immunoprecipitation, WB: western blotting

**Supplementary Table 3** List of primer sets used in qPCR of this study.

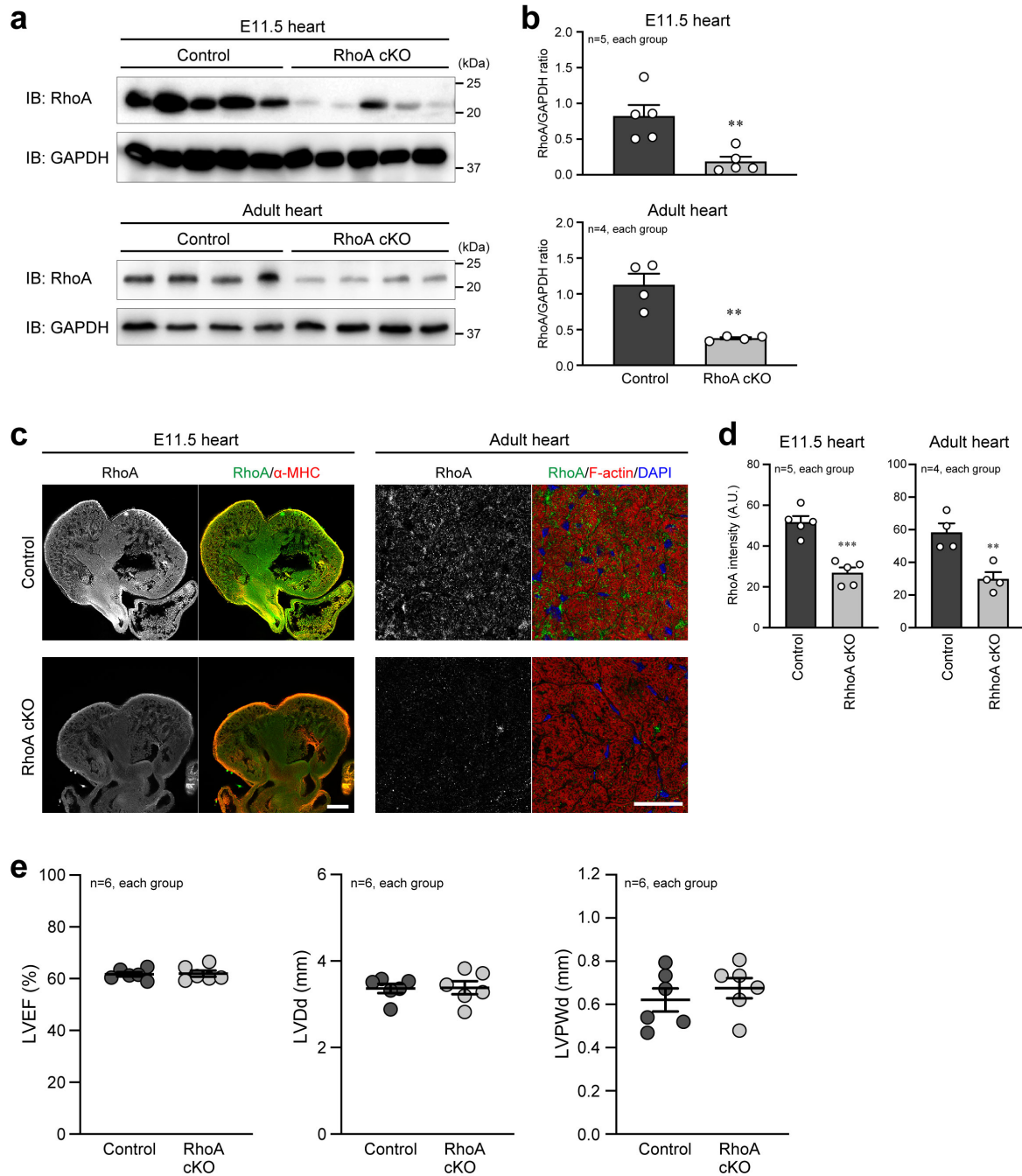
Human genes	Forward primer (5'-3')	Reverse primer (5'-3')
<i>RHOA</i>	AGCCTGTGGAAAGACATGCTT	TCAAACACTGTGGGCACATAC
<i>GAPDH</i>	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
Mouse genes	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Gapdh</i>	CCAAAAGGGTCATCATCTCC	GTCATGAGCCCTTCCACAAT
<i>Eln</i>	CGGGTCTGACAGCGGTAGT	CTCCAAGTCCTCCAGGACCT
<i>Fbn1</i>	GGACGCCAATTTGGAGGCT	CTTTCAGCGCATCGTGTCTCT
<i>Colla1</i>	GTCTGGTTTGGAGAGAGCAT	CAGTGATAGGTGATGTTCTGGG
<i>Acta2</i>	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
<i>Mylk</i>	TGGGGGACGTGAAACTGTTTG	GGGGCAGAATGAAAGCTGG
<i>Myh11</i>	GAGCAAACCTCAGGAGAGGAAAC	GTCCCGAGCGTCCATTTCTTC
<i>Il1b</i>	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
<i>Il6</i>	CTCTGGGAAATCGTGGAAT	CCAGTTTGGTAGCATCCATC
<i>Tnf</i>	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
<i>Ccl2</i>	GCCCCACTCACCTGCTGCTACT	CCTGCTGCTGGTCATCCTCTTGT
<i>Mmp2</i>	AGAGAGAGGCCCTCAGTTGCT	ACTCCAGTTAAAGGCAGCATCTAC
<i>Mmp9</i>	CTTCTGGCGTGTGAGTTTCCA	ACTGCACGGTTGAAGCAAAGA
<i>Timp1</i>	GCAACTCGGACCTGGTCATAA	CGGCCCCGTGATGAGAAACT
<i>Timp2</i>	TCAGAGCCAAAGCAGTGAGC	GCCGTGTAGATAAACTCGATGTC



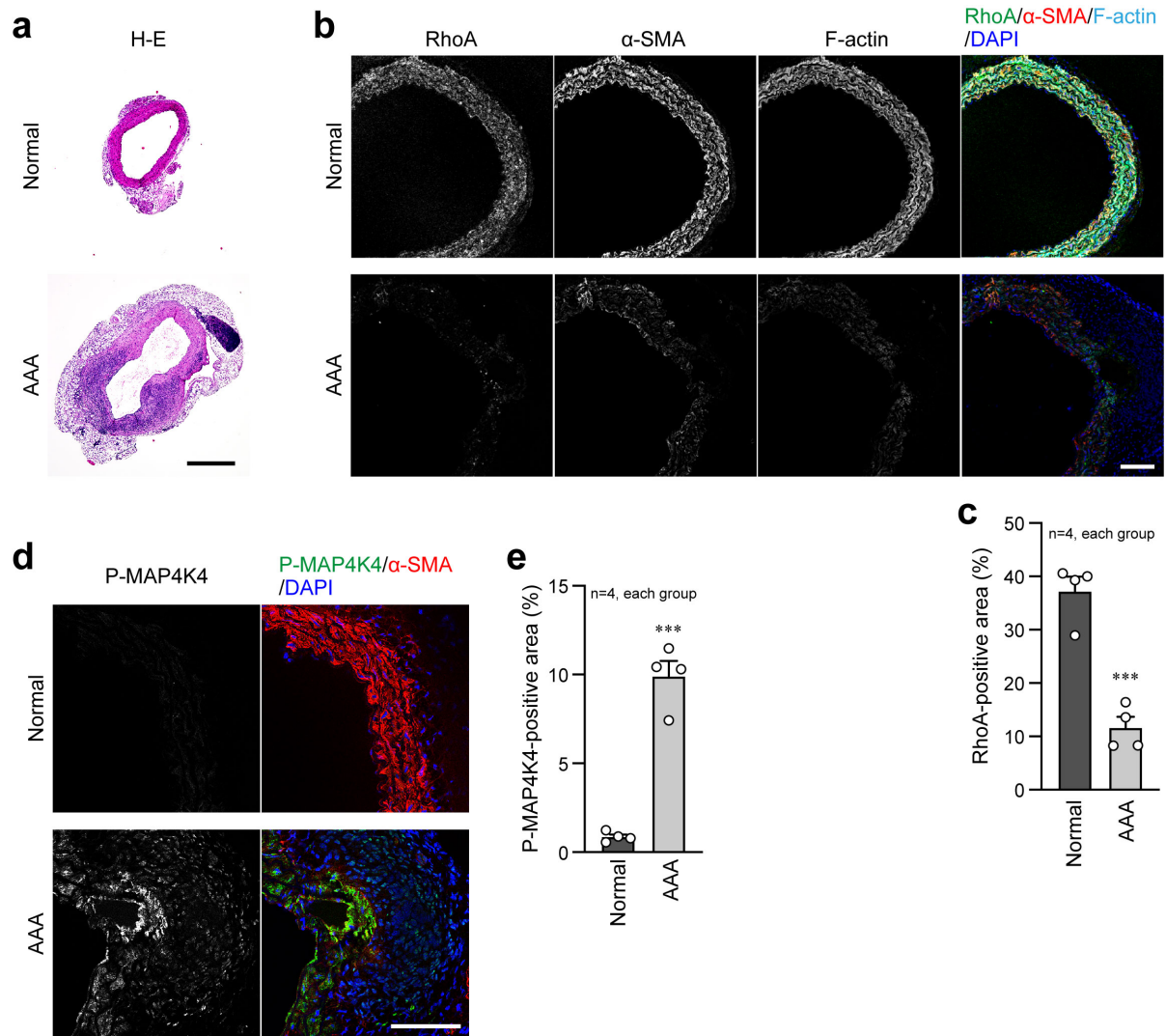


**Supplementary Fig. 1 Basal characteristics of the aorta in control and RhoA cKO mice.** **a** Co-immunostaining of RhoA and  $\alpha$ -SMA in the mouse aorta. Nuclei were counterstained with DAPI. **b** Hemodynamics in control and RhoA cKO mice. BP: blood pressure. n=5, each group. **c** External appearance of the extracted aorta with the heart and kidneys. **d** Ultrasonographic observation of the abdominal aorta, which is outlined by yellow dotted lines. **e** Summary graph of the abdominal aortic diameter. n=5, each group. **f** H-E staining of the aorta. **g** Verhoeff van Gieson staining of the aorta. **h** qPCR analysis of genes related to aortic elasticity. *Gapdh* mRNA was used as a control. n=3, each

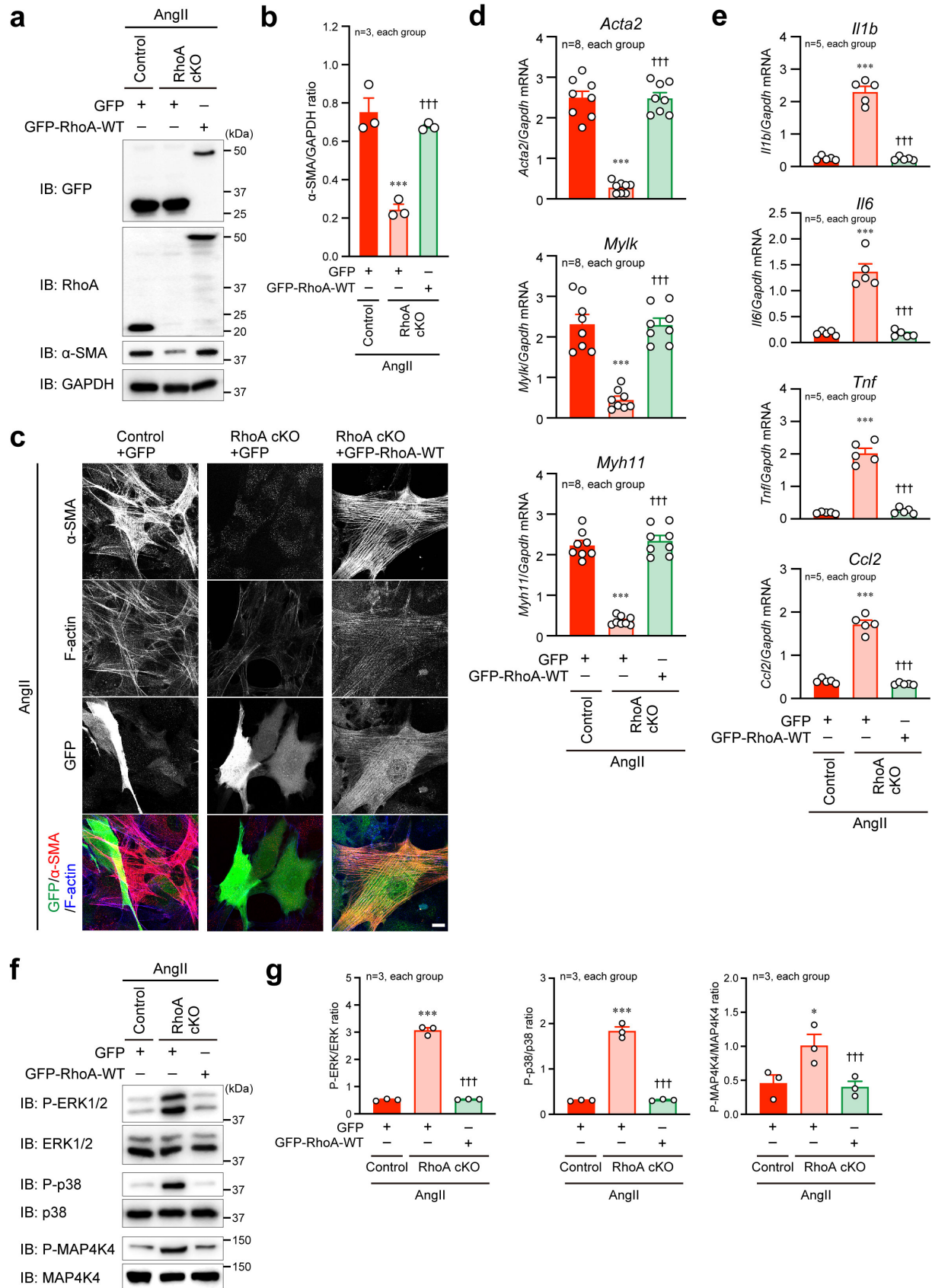
group. **i** qPCR analysis of collagen type I  $\alpha 1$  (*Col1a1*). *Gapdh* mRNA was used as a control. n=9, each group. **j** Contents of total, cross-linked and non-cross-linked collagens in the aorta, which were analyzed by the hydroxyproline assay. Scale bars: 50  $\mu\text{m}$  (**a**, **f**, **g**) and 1 mm (**d**). In (**b**, **e**, **h**, **i**, **j**), the data between the two groups were analyzed by *t*-test.



**Supplementary Fig. 2 Myocardial RhoA expression and cardiac function in VSMC-specific RhoA cKO mice.** **a** Western blotting for RhoA in the heart samples from embryonic day 11.5 (E11.5) and adult (12 weeks old) mice. GAPDH served as the loading control. **b** Summary graphs of the RhoA/GAPDH ratio examined in (a). n=5, each group in E11.5, and n=4, each group in Adult. **c** Immunostaining of RhoA and  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) in the mouse heart. F-actin and nuclei were visualized with phalloidin and DAPI, respectively. Scale bars: 200  $\mu$ m in the left panel and 20  $\mu$ m in the right panel. **d** Summary graphs of the RhoA intensity in (c). A.U.: arbitrary unit. n=5, each group in E11.5, and n=4, each group in Adult. **e** Echocardiographic measurements of parameters related to cardiac function in the adult mice. LVEF: left ventricular ejection fraction, LVDd: left ventricular diastolic diameter, LVPWd: left ventricular posterior wall thickness in diastole. n=6, each group. In (b, d, e), the data between the two groups were analyzed by *t*-test. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs. Control.



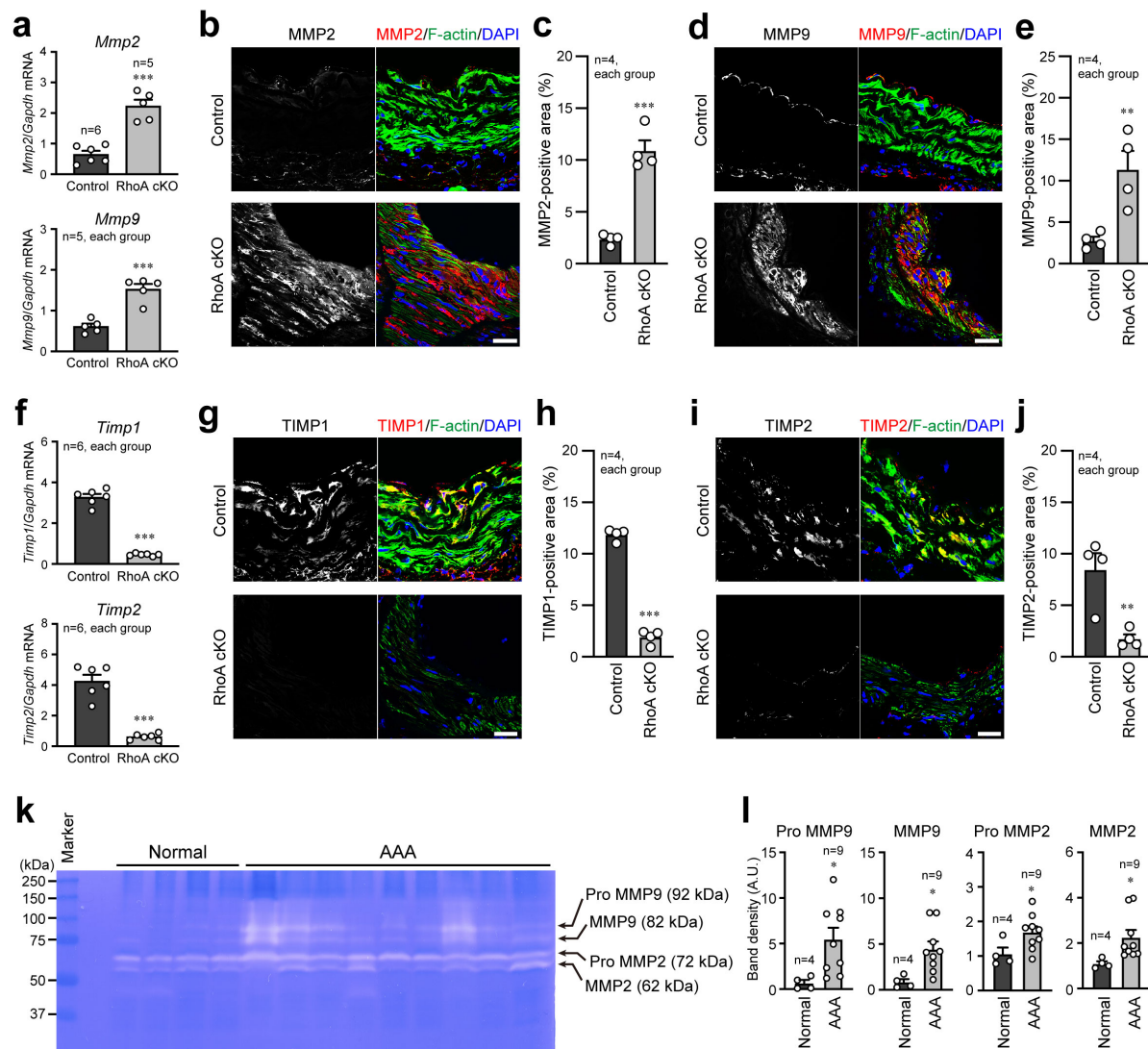
**Supplementary Fig. 3 AAA formation in control mice treated with AngII and BAPN.** **a** H-E staining of normal and AAA lesions in the control mice aorta after treatment with AngII and BAPN for 4 weeks. **b, d** Co-immunostaining of RhoA and  $\alpha$ -SMA (**b**) or P-MAP4K4 and  $\alpha$ -SMA (**d**) in the mouse aorta after the treatment. F-actin and nuclei were visualized with phalloidin and DAPI, respectively. **c, e** Summary graph of the percentage of RhoA- or P-MAP4K4-positive area. n=4, each group. Scale bars: 500  $\mu$ m (**a**) and 100  $\mu$ m (**b, d**). The data between the two groups were analyzed by *t*-test. \*\*\*  $p < 0.001$  vs. Normal.



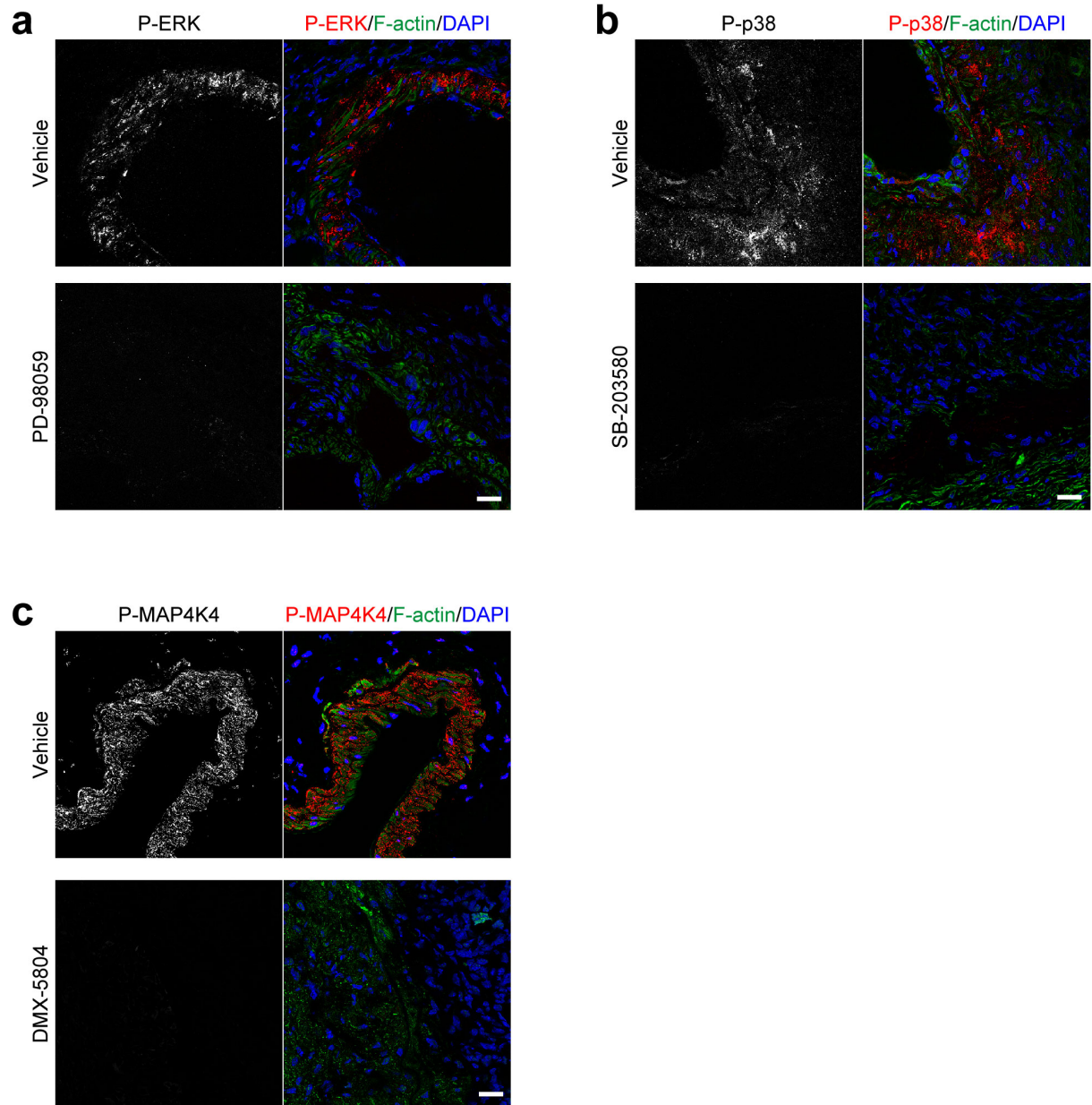
**Supplementary Fig. 4 Remedy experiments by re-expression of RhoA in RhoA cKO aortic VSMCs. a** After transfection of GFP or GFP-RhoA-WT expression plasmid, VSMCs were stimulated

with AngII for 24 h. The cell extracts were immunoblotted with the indicated antibodies. **b** Summary graph of the  $\alpha$ -SMA/GAPDH ratio in **(a)**.  $n=3$ , each group. **c** Immunofluorescence images of aortic VSMCs stained with the  $\alpha$ -SMA antibody after treatment with AngII for 24 h. F-actin was visualized with phalloidin. Scale bar: 20  $\mu$ m. **d, e** qPCR analyses of genes related to VSMC contractile phenotype (**d**) and inflammation (**e**). Aortic VSMCs were treated with AngII for 24 h.  $n=8$ , each group. **f** Western blotting with the indicated antibodies using aortic VSMCs after treatment with AngII for 24 h. GAPDH served as the loading control. **g** Summary graphs of the phosphorylated/total MAP kinase signaling molecule ratios examined in **(f)**.  $n=3$ , each group. In **(b, d, e, g)**, one-way ANOVA was applied to compare the data between groups. \*  $p<0.05$  and \*\*\*  $p<0.001$  vs. Control; †††  $p<0.001$  vs. GFP-transfected RhoA cKO VSMCs.



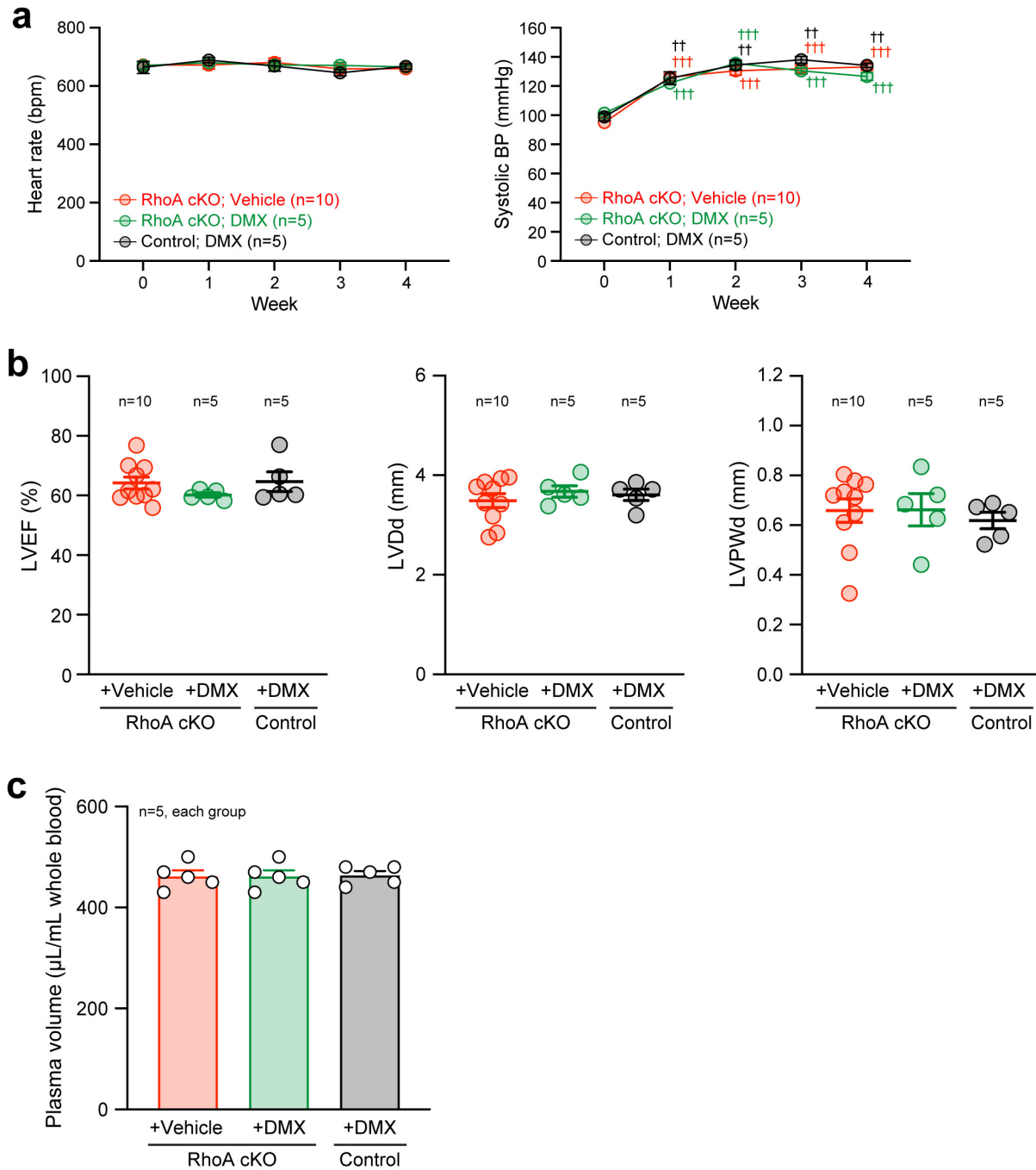


**Supplementary Fig. 5 Expression of molecules related to extracellular matrix degradation of the aorta.** **a, f** qPCR for MMP or TIMP mRNAs in aortic VSMCs after treatment with AngII for 24 h. n=5–6, each group. **b, d, g, i** Immunofluorescence images of MMPs or TIMPs in the control and RhoA cKO mouse aortas after the 4-week treatment with AngII+BAPN. F-actin and nuclei were visualized with phalloidin and DAPI, respectively. **c, e, h, j** Summary graph of the percentage of the positive area detected by antibody for each MMP or TIMP. n=4, each group. **k** Zymography using human aortic tissue samples. **l** Summary graphs of band densities observed in (**k**). A.U.: arbitrary unit. n=4 in Normal and n=9 in AAA. Scale bars: 30  $\mu$ m (**b, d, g, i**). In (**a, c, e, f, h, j, l**), the data between the two groups were analyzed by *t*-test. \*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs. Control or Normal.

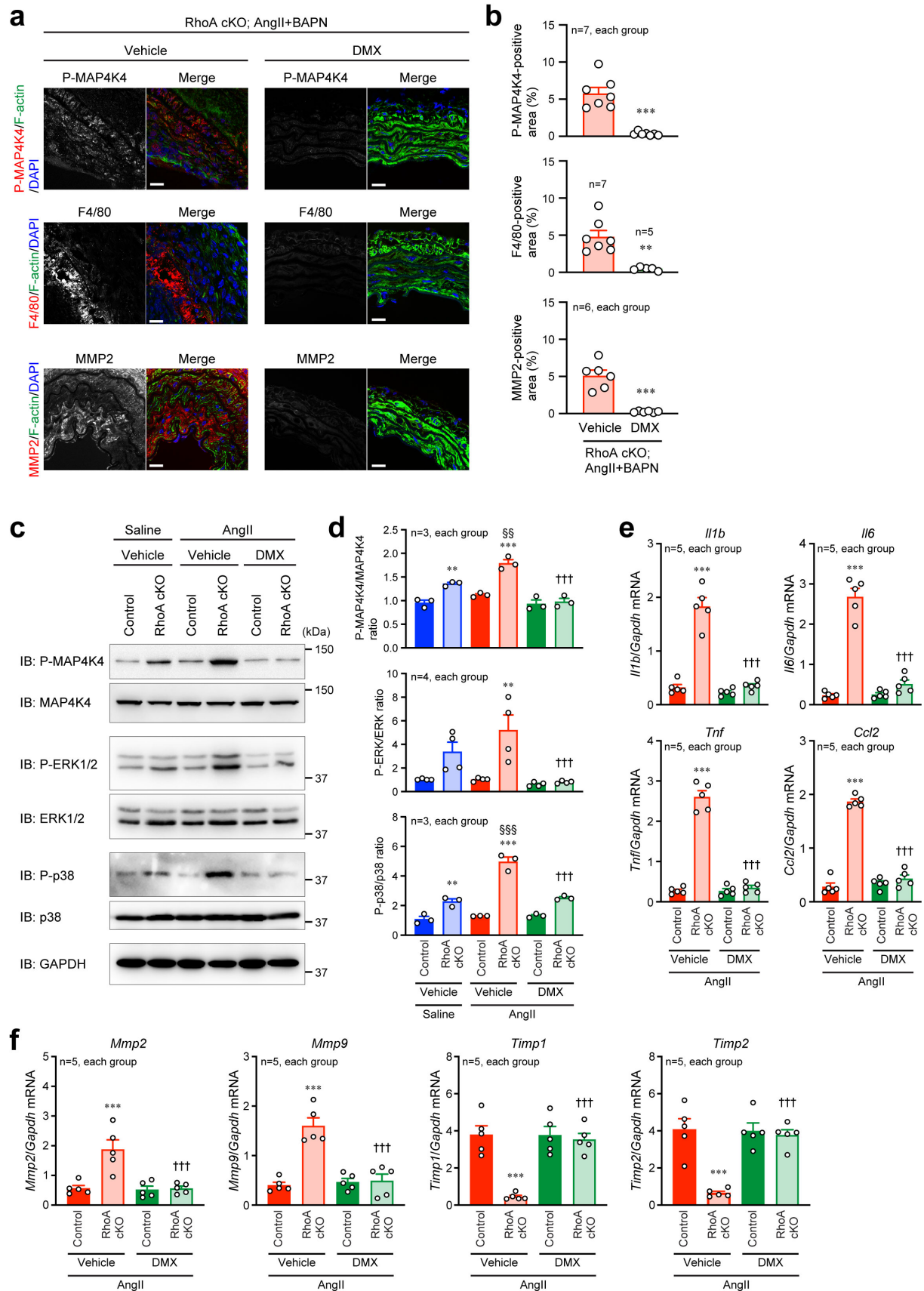


**Supplementary Fig. 6 Specificity of the antibodies used in immunofluorescence microscopy to detect each phosphorylated MAP kinase signaling molecule.** After RhoA cKO mice were treated with AngII and BAPN for 4 weeks, vehicle (0.5% DMSO) or the inhibitor for ERK (PD-98059, **a**), p38 (SB-203580, **b**) or MAP4K4 (DMX-5804, **c**) was intravenously injected into the mice. At 30 min after the injection, the aorta was extracted and stained with the indicated antibodies. F-actin and nuclei were visualized with phalloidin and DAPI, respectively. Scale bars: 20  $\mu$ m.



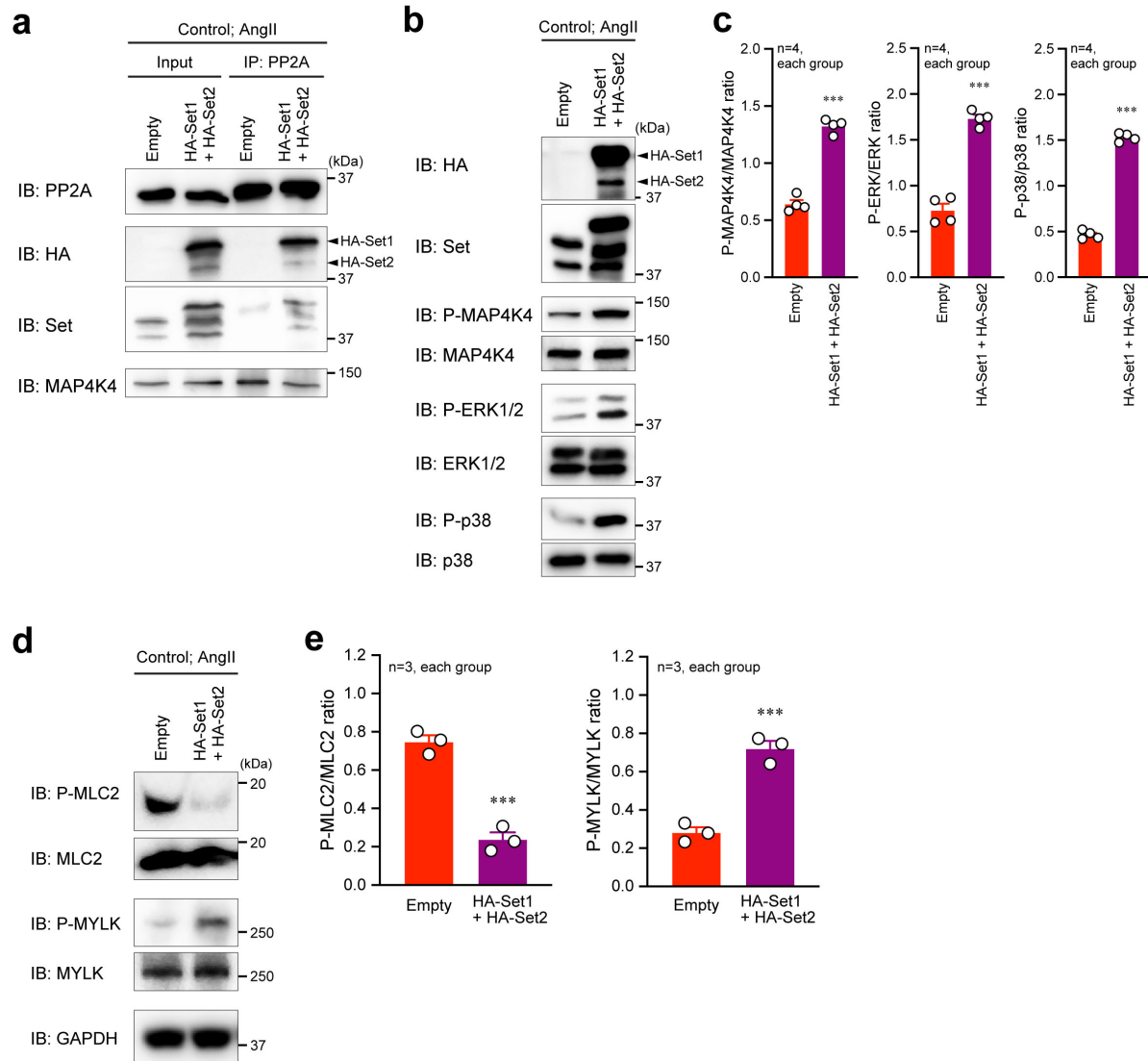


**Supplementary Fig. 7 Hemodynamics and cardiac functions after DMX-5804 treatment in addition to AngII and BAPN infusion.** **a** Changes in heart rate and systolic BP before (Week 0) and after treatment with AngII+BAPN in the presence of vehicle or DMX-5804. n=5–10, each group. **b** Echocardiographic measurement of parameters related to cardiac function at the end of treatment. LVEF: left ventricular ejection fraction, LVDD: left ventricular diastolic diameter, LVPWd: left ventricular posterior wall thickness in diastole. n=5–10, each group. **c** Plasma volume in 1 mL of whole blood was measured at the end of treatment. n=5, each group. Two-way (a) or one-way (b, c) ANOVA was applied to compare the data between groups. ††  $p < 0.01$  and †††  $p < 0.001$  vs. Week 0.

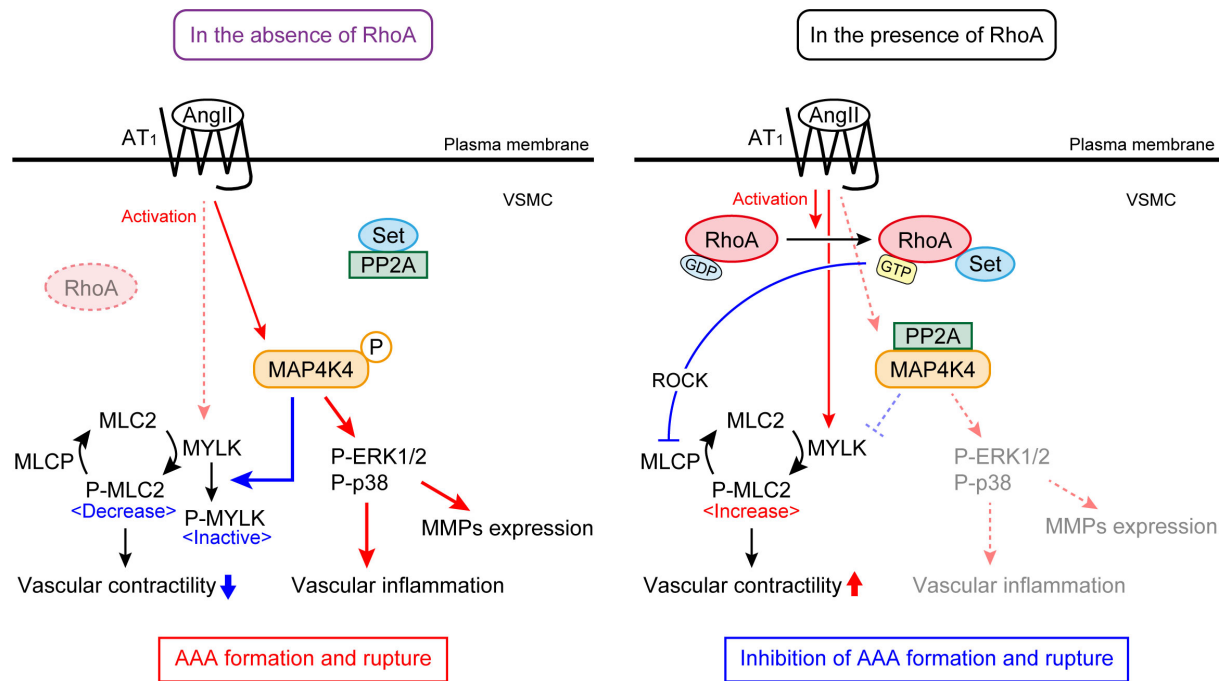


**Supplementary Fig. 8 Rescue of RhoA knockout-mediated excessive inflammatory response and MAP kinase signaling activation by DMX-5804 treatment.** a Immunofluorescence images of

the control and RhoA cKO mouse aortas stained with the indicated antibodies. The aorta was extracted after the 4-week treatment with AngII+BAPN in the presence of vehicle or DMX-5804. F-actin and nuclei were visualized with phalloidin and DAPI, respectively. Scale bars: 20  $\mu$ m. **b** Summary graphs of the percentage of the positive area detected by each antibody in the aorta. n=5–7, each group. **c** Western blotting with the indicated antibodies using cultured aortic VSMCs after treatment with saline or AngII in the presence of vehicle or DMX-5804 for 24 h. GAPDH served as the loading control. **d** Summary graphs of the phosphorylated/total protein ratios in each group. n=3–4, each group. **e, f** Graphs of qPCR results for inflammatory cytokines, MMPs and TIMPs in aortic VSMCs treated with AngII in the presence of vehicle or DMX-5804 for 24 h. n=5, each group. In (**b**), the data between the two groups were analyzed by *t*-test. One-way (**d, e, f**) ANOVA was applied to compare the data between groups. \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs. Vehicle or Control; †††  $p<0.001$  vs. AngII treatment in the presence of vehicle; §§  $p<0.01$  and §§§  $p<0.001$  vs. Saline.



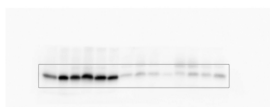
**Supplementary Fig. 9 Effect of Set overexpression on aortic VSMCs isolated from control mice.** **a** After control VSMCs transfected with both HA-tagged Set1 and Set2 expression plasmids or an empty plasmid were stimulated with AngII for 24 h, cell extracts were immunoprecipitated with the anti-PP2A antibody, followed by western blotting with the indicated antibodies. **b, d** Cell extracts of control VSMCs treated as described in (a) were immunoblotted with the indicated antibodies. **c, e** Summary graphs of the phosphorylated/total MAP kinase signaling molecule ratios (**c**) and the P-MLC2/MLC2 and P-MYLK/MYLK ratios (**e**). n=4 (**c**) and n=3 (**e**), each group. The data were analyzed by *t*-test. \*\*\*  $p < 0.001$  vs. Empty.



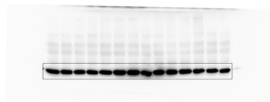
**Supplementary Fig. 10 Schematic representation of the protective effect of RhoA on AAA formation.** Signal transduction in aortic VSMCs and subsequent feature of the aorta are illustrated in the absence (Left panel) and presence (Right panel) of RhoA in VSMCs.

**Fig.1**  
**h**

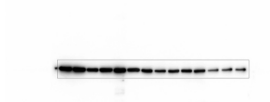
IB: RhoA



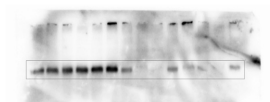
IB: GAPDH



IB: α-SMA



IB: CD31



IB: Vimentin



**Fig.3**  
**f**

IB: α-SMA



IB: GAPDH

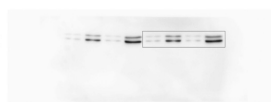


**Fig.5**  
**e**

IB: RhoA



IB: P-ERK1/2



IB: ERK1/2



IB: P-p38

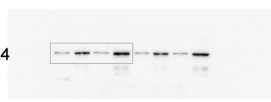


IB: p38

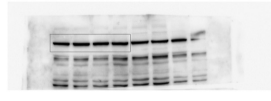


**i**

IB: P-MAP4K4

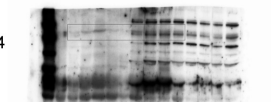


IB: MAP4K4

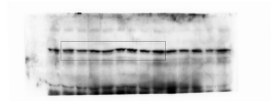


**m**

IB: P-MAP4K4

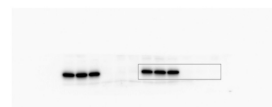


IB: MAP4K4

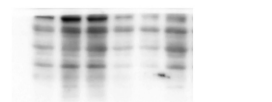


**Fig.6**  
**h**

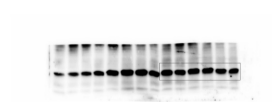
IB: RhoA



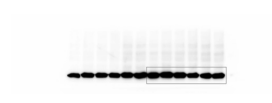
IB: P-MLC2



IB: MLC2



IB: GAPDH

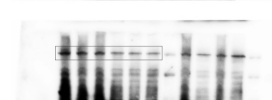


**j**

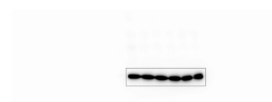
IB: P-MYLK



IB: MYLK

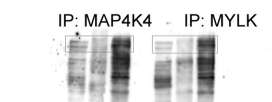


IB: GAPDH

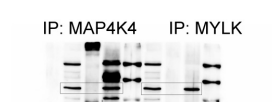


**l**

IB: MYLK

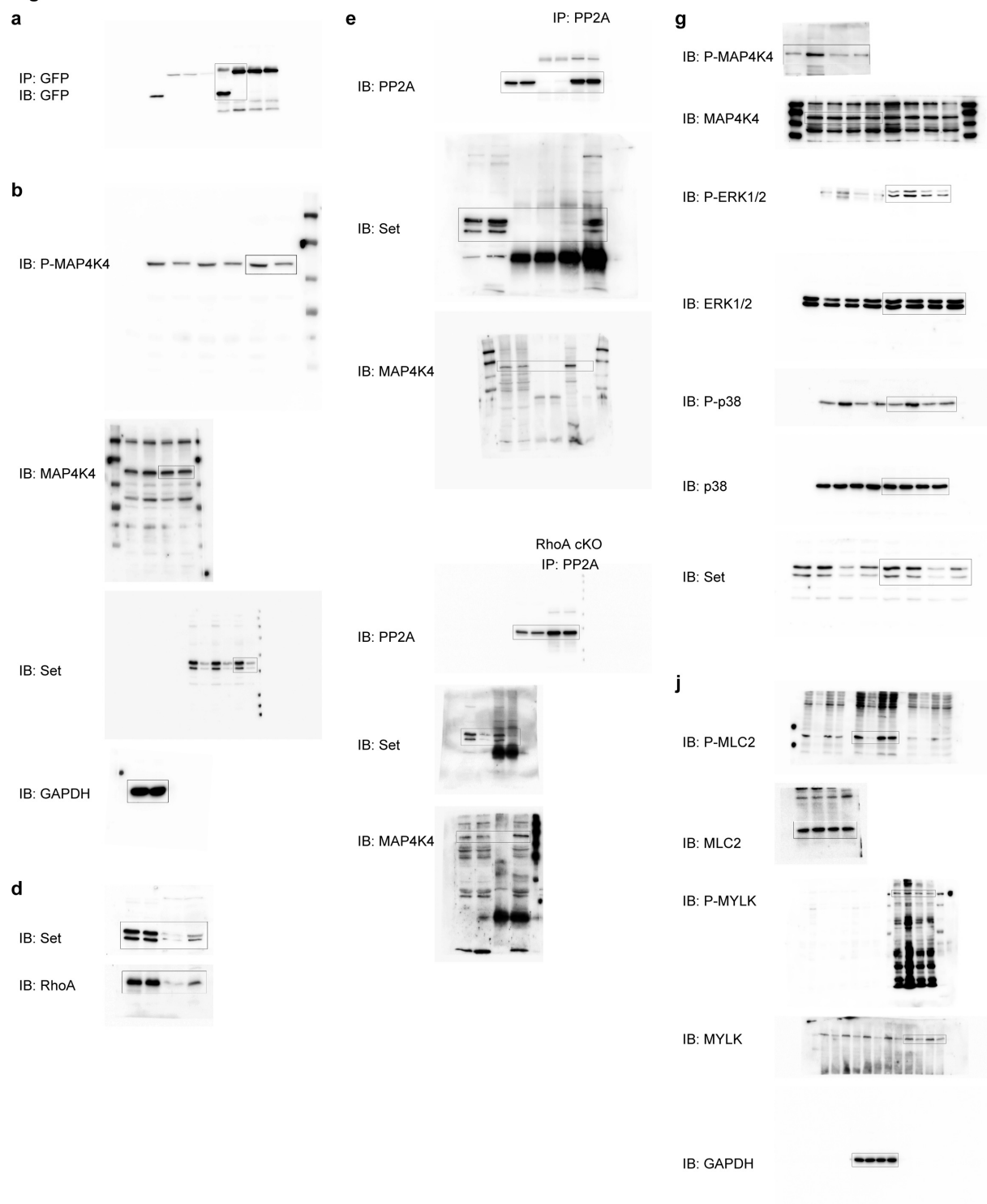


IB: MAP4K4



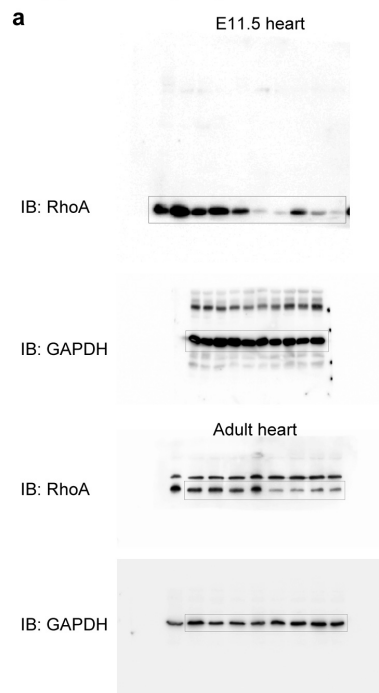
**Supplementary Fig. 11 Uncropped western blot images used in Fig. 1, 3, 5 and 6. Rectangles indicate the area shown in each figure panel.**

**Fig.7**

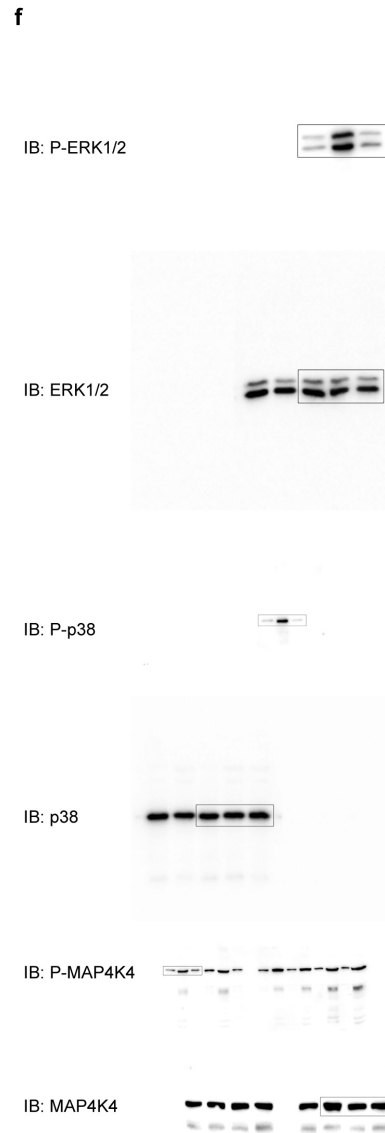


**Supplementary Fig. 12 Uncropped western blot images used in Fig. 7. Rectangles indicate the area shown in each figure panel.**

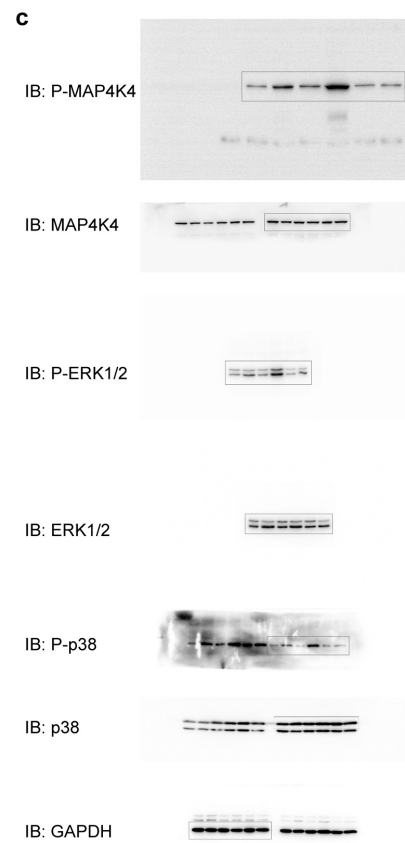
**Supplementary Fig. 2**



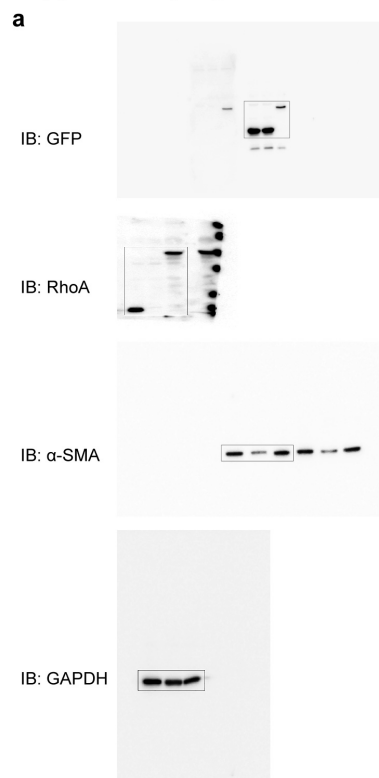
**Supplementary Fig. 4**



**Supplementary Fig. 8**



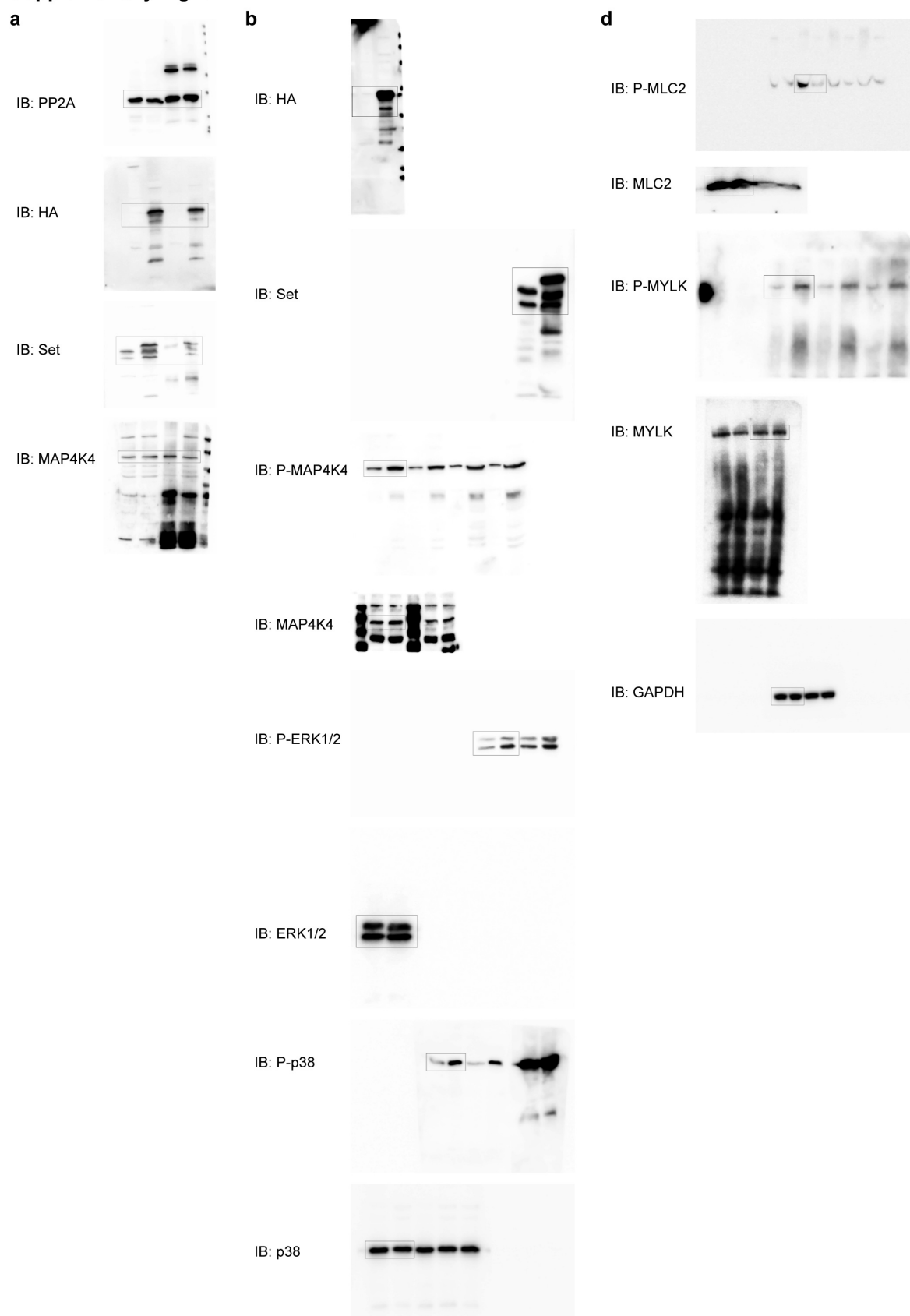
**Supplementary Fig. 4**



**Supplementary Fig. 13 Uncropped western blot images used in Supplementary Fig. 2, 4 and 8. Rectangles indicate the area shown in each figure panel.**



# Supplementary Fig. 9



**Supplementary Fig. 14** Uncropped western blot images used in Supplementary Fig. 9. Rectangles indicate the area shown in each figure panel.