Supplementary information

Cyclophilin A enhances vascular oxidative stress and development of angiotensin II-induced aortic aneurysms
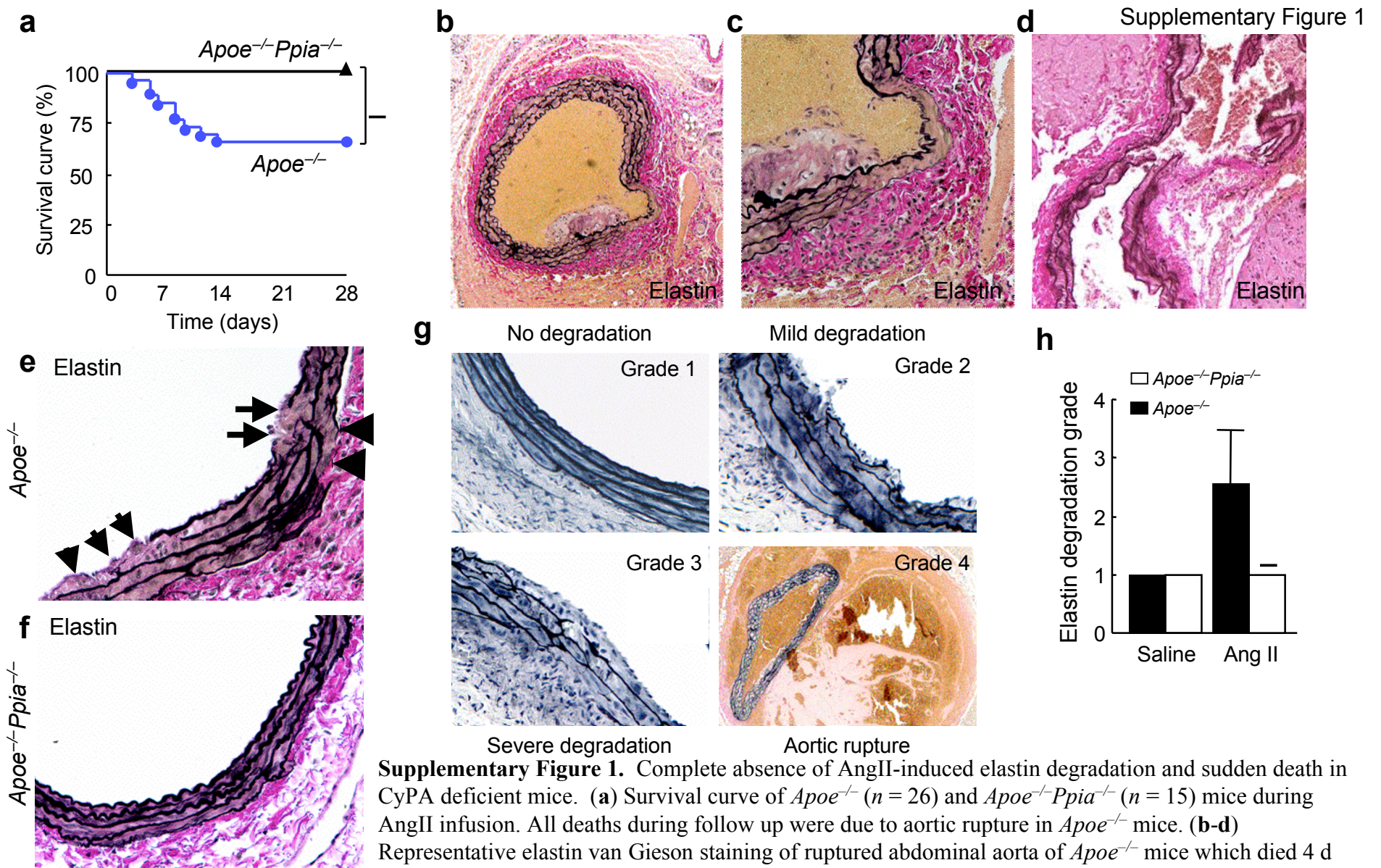
Kimio Satoh, Patrizia Nigro, Tetsuya Matoba, Michael R. O'Dell, Zhaoqiang Cui, Xi Shi, Amy Mohan, Chen Yan, Jun-ichi Abe, Karl A. Illig, and Bradford C. Berk

Supplementary Figures 1-7 and legends

Supplementary Tables 1
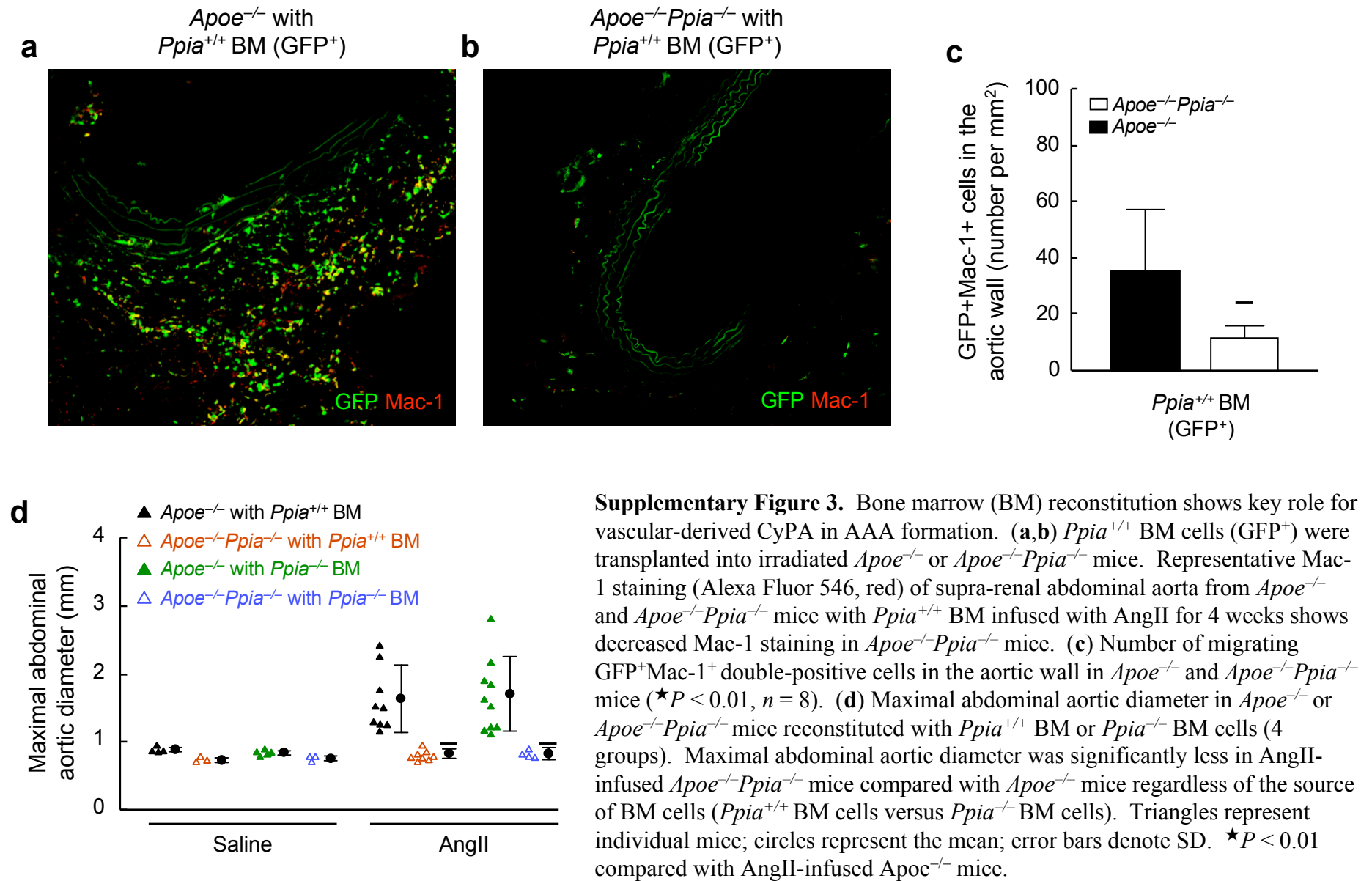
Supplementary Methods
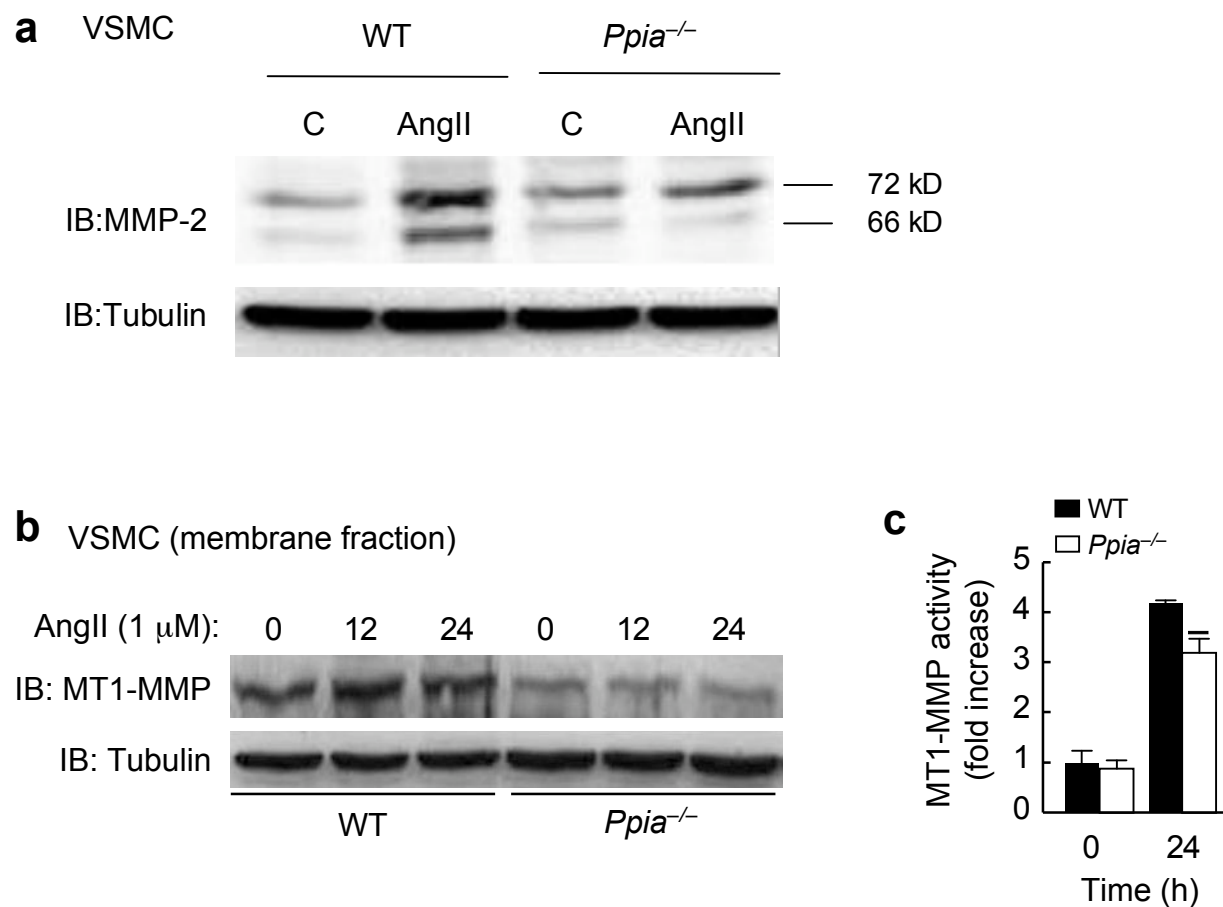
Supplementary References

Supplementary Figure 1. Complete absence of AngII-induced elastin degradation and sudden death in CyPA deficient mice. (**a**) Survival curve of $Apoe^{-/-}$ ($n = 26$) and $Apoe^{-/-}Ppia^{-/-}$ ($n = 15$) mice during AngII infusion. All deaths during follow up were due to aortic rupture in $Apoe^{-/-}$ mice. (**b-d**) Representative elastin van Gieson staining of ruptured abdominal aorta of $Apoe^{-/-}$ mice which died 4 d after AngII infusion. The orange colored cells are red blood cells that filled the aortic lumen as well as outside the aorta. (**e,f**) Elastin van Gieson staining of aorta from $Apoe^{-/-}$ and $Apoe^{-/-}Ppia^{-/-}$ mice 4 weeks after AngII infusion. The arrows indicate degradation of elastic lamina in $Apoe^{-/-}$ mice. In contrast, there was no degradation in $Apoe^{-/-}Ppia^{-/-}$ mice. (**g**) Based on elastin degradation-grading (4 grades) keys, statistical analysis on medial elastic lamina degradation showed a significant difference between $Apoe^{-/-}$ and $Apoe^{-/-}Ppia^{-/-}$ mice (**h**). ★$P < 0.01$ compared with $Apoe^{-/-}$ mice.
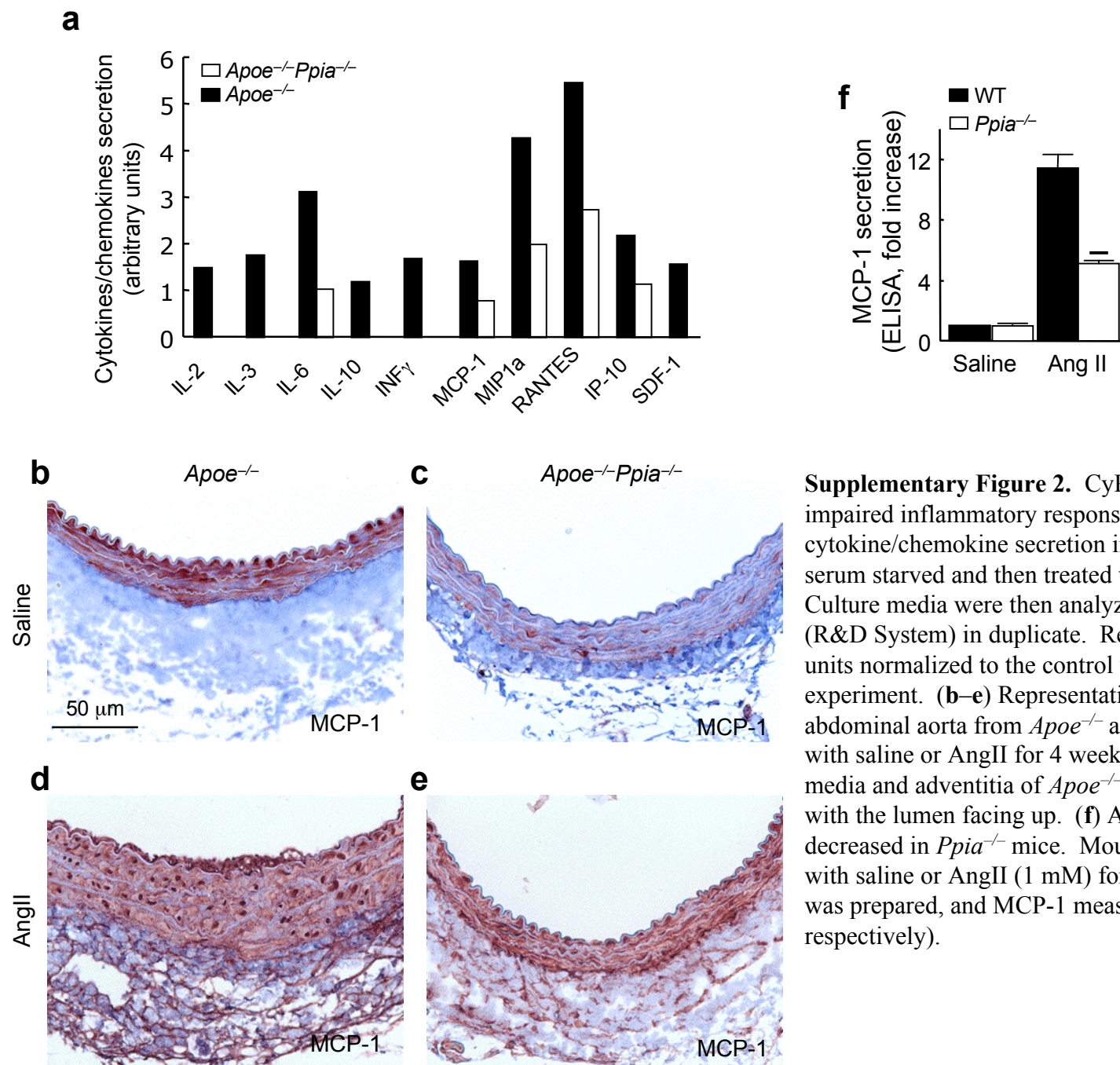
Supplementary Figure 2. CyPA deficiency contributes to the impaired inflammatory response. (**a**) Analysis of cytokine/chemokine secretion induced by AngII. VSMC were serum starved and then treated with 1 μM AngII for 12 h. Culture media were then analyzed by Mouse Cytokine Arrays (R&D System) in duplicate. Results are expressed in arbitrary units normalized to the control which was set to 1.0 for each experiment. (**b**–**e**) Representative MCP-1 staining of supra-renal abdominal aorta from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with saline or AngII for 4 weeks shows increased MCP-1 in media and adventitia of *Apoe*$^{-/-}$ mice. All sections are shown with the lumen facing up. (**f**) AngII induced MCP-1 secretion is decreased in *Ppia*$^{-/-}$ mice. Mouse aortic VSMC were treated with saline or AngII (1 mM) for 6 hours, conditioned medium was prepared, and MCP-1 measured by ELISA ($n = 4$, respectively).
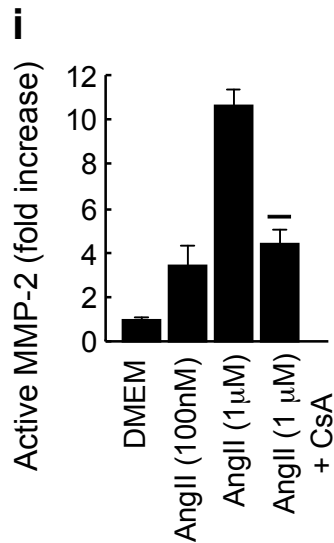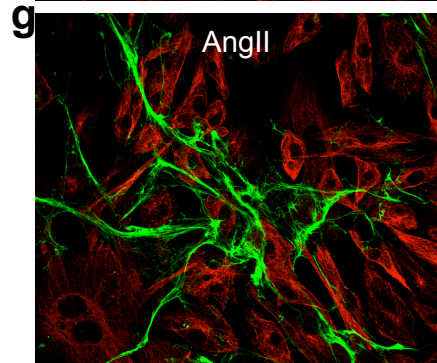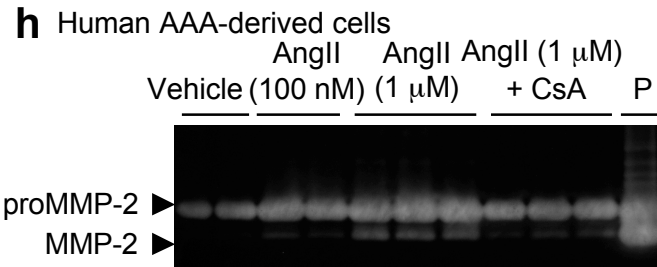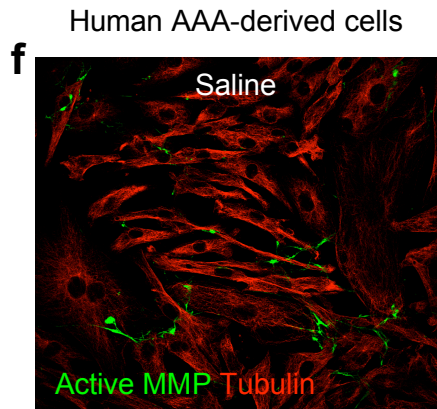
a *Apoe*−/− with *Ppia*+/+ BM (GFP+)

b *Apoe*−/−*Ppia*−/− with *Ppia*+/+ BM (GFP+)

GFP Mac-1

GFP Mac-1

c

GFP+Mac-1+ cells in the aortic wall (number per mm^2)

□ *Apoe*−/−*Ppia*−/−
■ *Apoe*−/−

Ppia+/+ BM (GFP+)

d

Maximal abdominal aortic diameter (mm)

▲ *Apoe*−/− with *Ppia*+/+ BM
△ *Apoe*−/−*Ppia*−/− with *Ppia*+/+ BM
▲ *Apoe*−/− with *Ppia*−/− BM
△ *Apoe*−/−*Ppia*−/− with *Ppia*−/− BM

Saline   AngII

Supplementary Figure 3. Bone marrow (BM) reconstitution shows key role for vascular-derived CyPA in AAA formation. (**a**,**b**) *Ppia*+/+ BM cells (GFP+) were transplanted into irradiated *Apoe*−/− or *Apoe*−/−*Ppia*−/− mice. Representative Mac-1 staining (Alexa Fluor 546, red) of supra-renal abdominal aorta from *Apoe*−/− and *Apoe*−/−*Ppia*−/− mice with *Ppia*+/+ BM infused with AngII for 4 weeks shows decreased Mac-1 staining in *Apoe*−/−*Ppia*−/− mice. (**c**) Number of migrating GFP+Mac-1+ double-positive cells in the aortic wall in *Apoe*−/− and *Apoe*−/−*Ppia*−/− mice (★$P < 0.01$, $n = 8$). (**d**) Maximal abdominal aortic diameter in *Apoe*−/− or *Apoe*−/−*Ppia*−/− mice reconstituted with *Ppia*+/+ BM or *Ppia*−/− BM cells (4 groups). Maximal abdominal aortic diameter was significantly less in AngII-infused *Apoe*−/−*Ppia*−/− mice compared with *Apoe*−/− mice regardless of the source of BM cells (*Ppia*+/+ BM cells versus *Ppia*−/− BM cells). Triangles represent individual mice; circles represent the mean; error bars denote SD. ★$P < 0.01$ compared with AngII-infused Apoe−/− mice.

a VSMC

WT   *Ppia*$^{-/-}$

C   AngII   C   AngII

IB:MMP-2

72 kD
66 kD

IB:Tubulin

b VSMC (membrane fraction)

AngII (1 μM):   0   12   24   0   12   24

IB: MT1-MMP

IB: Tubulin

WT   *Ppia*$^{-/-}$

c

■ WT
□ *Ppia*$^{-/-}$

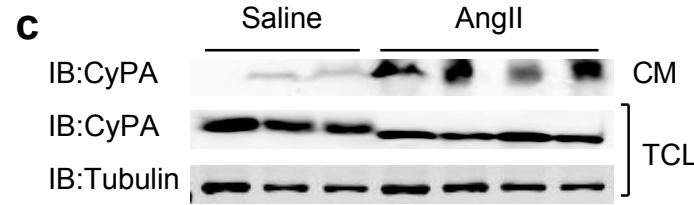MT1-MMP activity (fold increase)

5
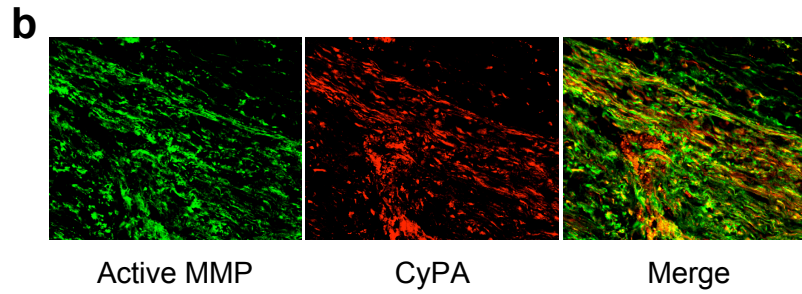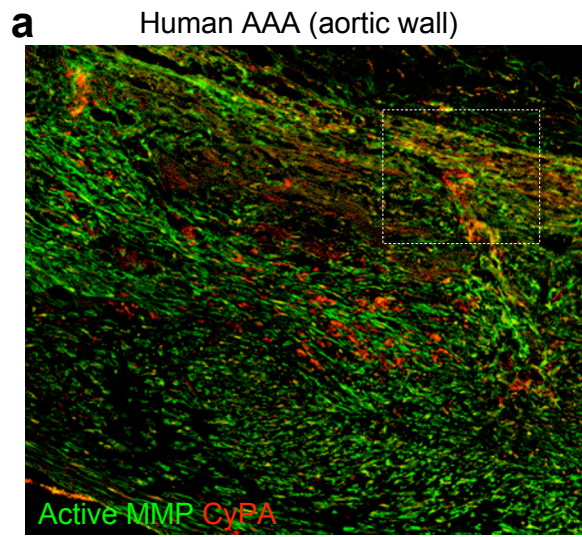4
3
2
1
0

0   24
Time (h)

Supplementary Figure 4. CyPA plays a crucial role in AngII-induced MMP-2 and MT1-MMP expression and translocation to the cell membrane. (**a**) Western blot of MMP-2 expression in WT and *Ppia*$^{-/-}$ VSMC after 1 μM AngII for 24 h. (**b**) Western blot of MT1-MMP expression in VSMC membrane fraction from WT and *Ppia*$^{-/-}$ VSMC after stimulation with 1 μM AngII for 0, 12, 24 h. (**c**) MMP-specific substrate assay for MT1-MMP in VSMC shows decreased MT1-MMP activity in response to 1 μM AngII.
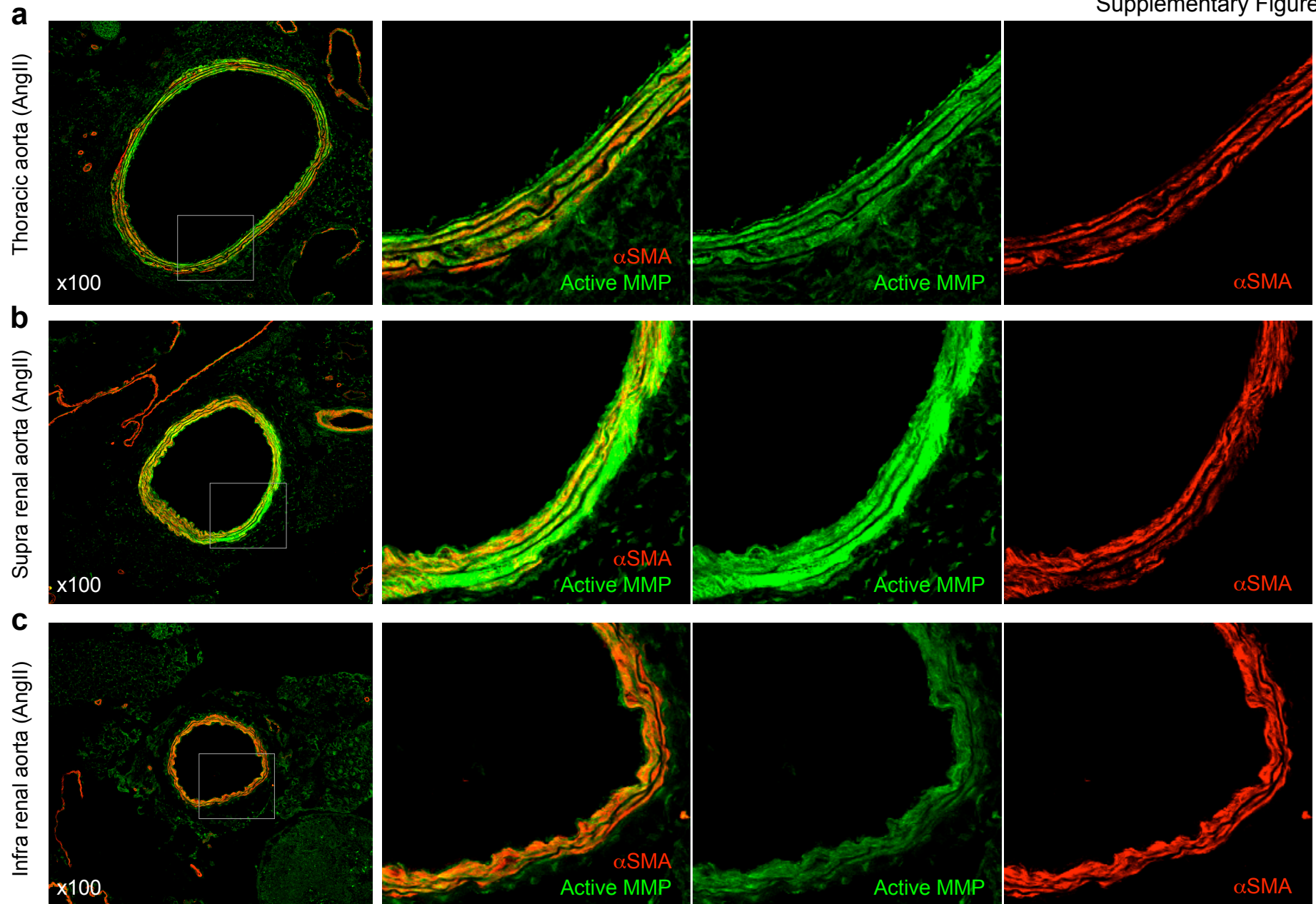
Supplementary Figure 5. Representative in situ zymography of aortic sections from the different portions of aorta in *Apoe*^{−/−} mice after 7 d of AngII-infusion. Active form of MMP was especially prominent in the media of aortic sections and co-localized with VSMC. Active MMP was more prominent in supra-renal (**b**) aorta than infra-renal (**c**) or thoracic (**a**) aorta.

a Human AAA (aortic wall)
Active MMP CyPA

b Active MMP | CyPA | Merge

c Saline | AngII
IB:CyPA — CM
IB:CyPA ⎤
IB:Tubulin ⎦ TCL

d Human AAA-derived cells
αSMA ToTo3

e CyPA Tubulin ToTo3

f Saline
Active MMP Tubulin

g AngII

h Human AAA-derived cells
Vehicle | AngII (100 nM) | AngII (1 μM) | AngII (1 μM) + CsA | P
proMMP-2 ▶
MMP-2 ▶

i Active MMP-2 (fold increase)
DMEM | AngII (100nM) | AngII (1μM) | AngII (1 μM) + CsA
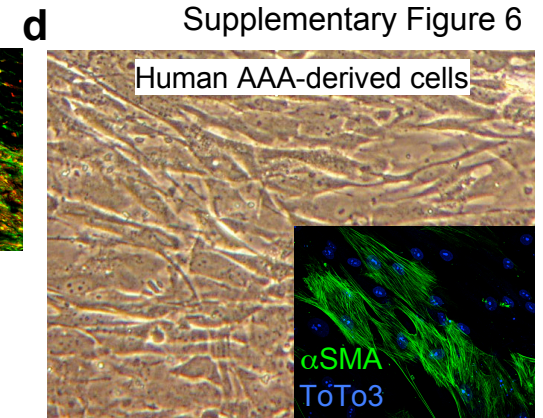
Supplementary Figure 6. CyPA plays a crucial role for MMP activation in the aorta of patients with AAA. (**a**,**b**) CyPA expression was high in the aortic wall of AAA lesions, especially in areas that express active MMP. (**c**) AngII treatment significantly increased secretion of CyPA from AAA tissue specimens. Each lane is from a separate piece of aorta. CM, conditioned medium; TCL, total cell lysate. (**d**) Representative photomicrograph of cells harvested from AAA samples. Inset shows immunostaining for α-smooth muscle actin (α-SMA) and ToTo3 (nucleus). (**e**) Representative photomicrograph of immunostaining for CyPA (green), α-tubulin (red), and ToTo3 (nucleus). (**f**,**g**) Representative in situ zymography (DQ gelatin) of human AAA-derived cells and immunostaining with α-tubulin after stimulation with 1 μM AngII. (**h**) Gelatin gel zymography of human AAA-derived cells showed MMP-2 activation in response to AngII, which was significantly reduced by treatment with CyPA inhibitor (CsA), suggesting the crucial role of CyPA for MMP activation in human AAA-derived cells. (**i**) Densitometric analysis of active MMP-2 changes relative to control (DMEM). ★$P < 0.01$ vs. AngII (1 mM). Results are mean ± SD.
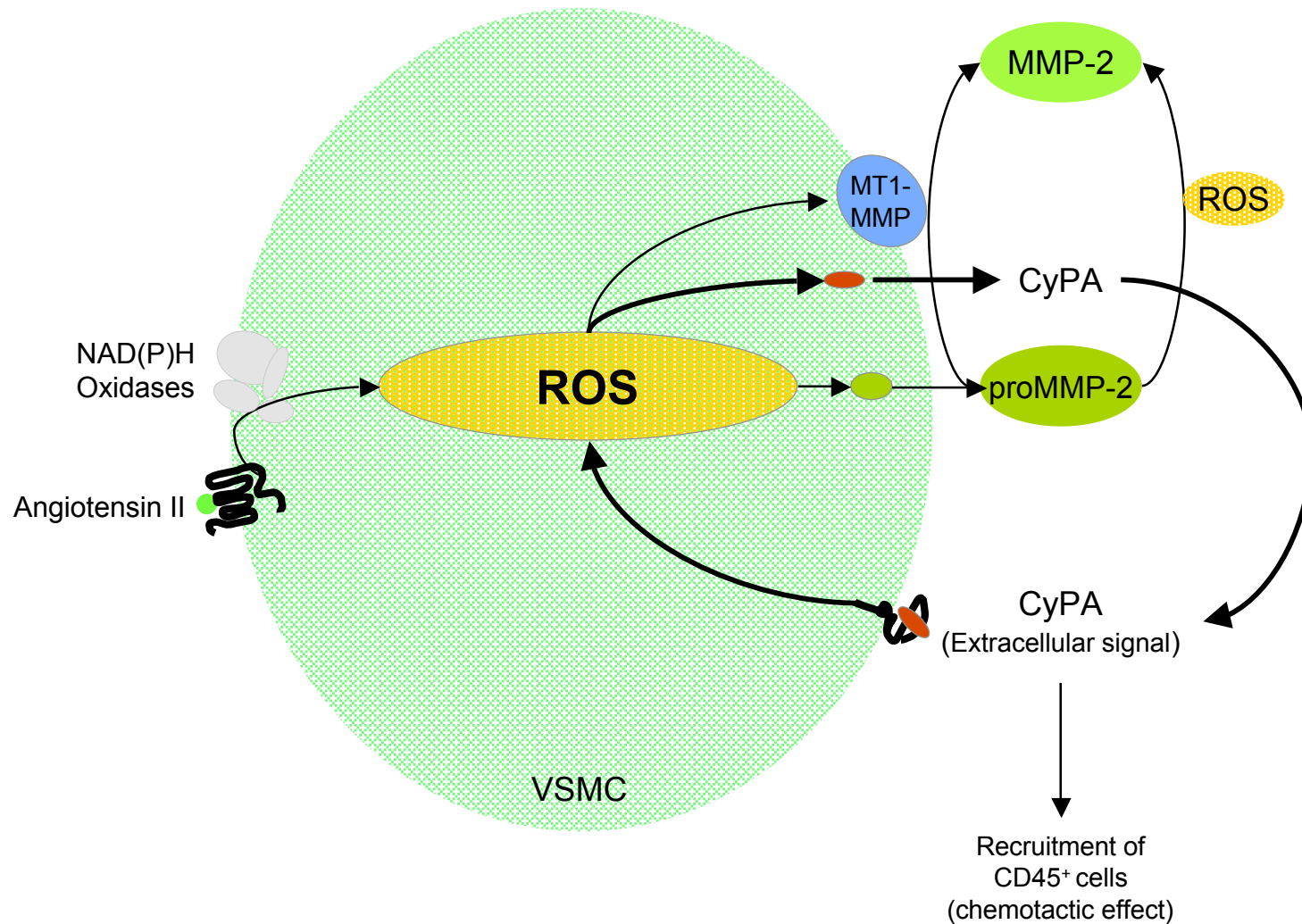
Supplementary Figure 7. CyPA plays a crucial role for ROS generation and MMP activation. AngII signaling increases reactive oxygen species (ROS) in a NAD(P)H oxidase-dependent manner, which induces secretion of CyPA and proMMP-2, as well as translocation of MT1-MMP to the cell membrane. Extracellular CyPA promotes the production of ROS and augments MMP-2 activation through extracellular signaling.

Table 1a. Blood pressure and plasma cholesterol levels of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice

	Apoe$^{-/-}$	*Apoe*$^{-/-}$*Ppia*$^{-/-}$	*p*
Blood pressure (mmHg)			
Before treatment	107.2 $\pm$ 15.6	108.1 $\pm$ 19.1	NS
Ang II infusion	136.4 $\pm$ 8.7 †	138.4 $\pm$ 8.7 †	NS
Total cholesterol (mg/dL)	646.3 $\pm$ 91.3	687.4 $\pm$ 106.4	NS
Total aorta weight per BW (ratio)			
Saline infusion	0.30 $\pm$ 0.04	0.28 $\pm$ 0.01	NS
Ang II infusion	1.64 $\pm$ 0.42 †	0.71 $\pm$ 0.29	*p* < 0.01

† *p* < 0.01 vs. saline infusion or before treatment. NS, no significant difference. Results are mean ± SD.

Table 1b. Blood pressure and total aortic weight of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice with *Ppia*$^{+/+}$ bone marrow

	Apoe$^{-/-}$	*Apoe*$^{-/-}$*Ppia*$^{-/-}$	*p*
Blood pressure (mmHg)			
Before treatment	102.6 $\pm$ 14.3	103.7 $\pm$ 19.8	NS
Ang II infusion	120.1 $\pm$ 17.4	123.6 $\pm$ 38.0	NS
Total aorta weight per BW (ratio)			
Saline infusion	0.47 $\pm$ 0.08	0.38 $\pm$ 0.07	NS
Ang II infusion	1.66 $\pm$ 0.59 †	0.68 $\pm$ 0.17	*p* < 0.01

† *p* < 0.01 vs. saline infusion or before treatment. NS, no significant difference. Results are mean ± SD.

Supplementary Methods

Generation of mice

All animal experiments were conducted in accordance with experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Rochester. $Ppia^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and were backcrossed to C57BL/6J mice for 10 generations. The $Apoe^{-/-}$ mice on a C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Double knockout $Apoe^{-/-}Ppia^{-/-}$ mice were generated by crossing $Ppia^{-/-}$ mice with $Apoe^{-/-}$ mice. The F1 generation was backcrossed with $Apoe^{-/-}$ mice to fix the $Apoe^{-/-}$ genotype, and littermates were crossed. All mice were genotyped by PCR on tail clip samples, and all experiments were performed with generations F4–F6 using littermate $Apoe^{-/-}Ppia^{+/+}$ as wild-type controls. We utilized a Cre/LoxP strategy to prepare VSMC-specific CyPA overexpressing transgenic mice (VSMC-Tg). In brief, a LacZ^{flox}-CyPA construct was prepared using the Z/EG vector kindly provided by Dr. Andras Nagy. This vector contains LacZ floxed by two loxP sites, driven by the chicken β-actin promoter and a cytomegalovirus (CMV) enhancer with enhanced green fluorescent protein (EGFP) downstream. We replaced EGFP with full-length wild type mouse CyPA carrying a Flag tag to make the LacZ^{flox}-Flag-CyPA construct. After appropriate ES cell screening and implantation, germline transmission was confirmed. Transgenic mice were backcrossed to C57BL/6J mice for 7 generations to establish experimental lines. For VSMC-specific overexpression of CyPA in transgenic mice, the LacZ^{flox}-CyPA transgenic mouse and SM22α-Cre mouse (C57BL/6J background) were

crossed. Breeding the LacZ^{flox}-CyPA mice to SM22α-Cre mice resulted in excision of LacZ and expression of CyPA in VSMC. Animals were housed under a 12-h light and 12-h dark regimen and placed on a normal chow diet.

Blood pressure and metabolic measurements

Blood pressures were obtained from the mice using a noninvasive tail-cuff system (BP-2000 Blood Pressure Analysis System; Visitech Systems, Apex, North Carolina, USA) as described previously[Kintscher, 2002 #11659]. Blood samples were obtained from the mice before sacrifice. The blood was collected from the abdominal vena cava at sacrifice. Serum cholesterol concentrations were determined by a commercially available enzymatic assay kit (Wako Chemicals).

Histological Analysis

After hemodynamic measurements, animals were anesthetized with an intraperitoneal injection of ketamine (80 mg kg^{-1}) and xylazine (5 mg kg^{-1}). For morphological analysis, aortas were perfused with normal saline and fixed with 10% phosphate-buffered formalin at physiological pressure for 5 minutes[Korshunov, #12833]. The whole aortas were harvested, fixed for 24 h, embedded in paraffin, and cross-sections (5 μm) were prepared. Paraffin sections were stained with elastin van Gieson staining or used for immunostaining.

Immunohistochemistry

Formaldehyde-fixed paraffin sections were incubated with primary antibody overnight at 4°C. The primary antibodies used were CyPA polyclonal (1:1000 dilution; BIOMOL Research Laboratories, Inc.), α-smooth muscle actin (clone 1A4, 1:400 dilution; Sigma-Aldrich, A5691), MCP-1 (sc-1784, 1:400 dilution; Santa Cruz), and leukocyte common antigen, CD45 (clone Ly-5, 1:100 dilution; BD Pharmingen). As a negative control, species- and isotype-matched IgG were used in place of the primary antibody. Slides were viewed with a microscope (BX41, Olympus) and with digital camera (Spot Insight 2, Diagnostic Instruments, Inc.). Because AAA lesion varies longitudinally, the supra-renal aorta was sectioned and 3 sections located at 500 μm intervals were examined. Mean number of CD45$^+$ and PECAM stained cells was calculated. Vessel areas were measured with Image Pro Plus software (Media Cybernetics Inc.).

Bone marrow transplantation

Bone marrow transplantation was performed as described[Satoh, 2006 #16759]. Briefly, recipient mice were lethally irradiated and received an intravenous injection of 5 x 10^6 donor bone marrow cells suspended in 100 μl calcium- and magnesium-free phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS). After transplantation, the mice were placed on a regular chow diet for 6 weeks followed by infusion of 1000 ng kg^{-1} min AngII for 4 weeks. Transgenic mice ubiquitously expressing green fluorescent protein (GFP) were obtained from Jackson Laboratory. The chimeric rate assessed by reconstitution with GFP$^+$ bone marrow cells was more than 99% by fluorescence-activated cell sorter analysis (FACSCanto II, Becton Dickinson).

Bone marrow-derived cell recruitment assays

Leukocyte counts were performed 4 weeks after infusion with AngII or vehicle.

Qualitative numbers or percentages of the migrating GFP$^+$CD45$^+$ cells was analyzed by

labeling with primary mAb's against CD45 (hematopoietic cells) and using a Confocal

microscopy (Olympus, FLUOVIEW).

Harvest of mouse aortic VSMC

Mouse aortic VSMC were isolated from 20-25 g male mice and maintained in

Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37°C in a

humidified atmosphere of 5% CO_2 and 95% air as described[Ishida, #7787]. Passage 4

to 6 VSMC at 70-80% confluence was used for experiments.

Preparation of conditioned medium

Conditioned medium from AngII-stimulated VSMC or control medium from DMEM-

incubated cells was collected and filtered to remove cell debris. Likewise, aortas of mice

infused with AngII for 7 d were incubated for 20 h in culture medium. Thereafter

medium was collected and concentrated to yield concentrated conditioned medium (CM).

The medium was concentrated 100-fold with a Centricon Plus-20 filter (Millipore

Corporation) to yield concentrated conditioned medium[Jin, 2000 #9552].

Human aortic samples

All protocols using human specimens were approved by the Institutional Review Board at

the University of Rochester. Informed consent was obtained from all subjects. We

obtained surgical specimens from individuals underlying elective repair of AAA. For ex vivo culture, we obtained fresh AAA samples during surgery and minced them approximately 1 mm thick. We maintained equal wet weight of the minced tissue in each well of 6-well plates cultured with DMEM. We collected the conditioned medium from 24 h after treatment with 1 μM AngII as described before[Yoshimura, 2005 #17503;Liao, 2000 #8487]. For in situ zymography (DQ gelatin), fresh AAA samples were snap-frozen into OCT.

Expression and Purification of His-tagged CyPA from High Five™ cells

The recombinant baculovirus encoding N-terminal 6 x His tagged rat CyPA protein was constructed using the Invitrogen Bac-to-Bac® Baculovirus expression system (Invitrogen) as described below. Briefly, a 508bp cDNA fragment containing the full-length CDS of rat *PPIA* was obtained by excising from BamHI/EcoRI sites of plasmid pGEX-2TK-CyPA. After gel purification, it was inserted in the *Bam*HI/*Eco*RI sites of pFastBac™HT B vector. The resulting plasmid was termed pFastBac His-CyPA. After transformation and amplification in *E. coli*, purified pFastBac His-CyPA was sequenced and was verified that CyPA sequence was fused at its N-terminal to 6 x His tag. This plasmid was further transformed into DH10Bac™ *E.coli* for transposition into the bacmid. After package, amplification, purification from Sf9 cells, the resulting baculovirus was titred and was further used to infect High Five™ cells. Finally, His-CyPA was purified from High Five™ cells by using Ni-NTA Agarose (Qiagen, Germandtown, MD, USA) according to the manufacturer's instructions.

Cytokine and chemokine array

Cytokine and chemokine secretion from mouse whole aorta was measured by array kit from R&D. Concentrated conditioned medium from WT and $Ppia^{-/-}$ mice were prepared 12 h after Ang II treatment and analyzed using Mouse Cytokine Array kit (ARY006, R&D).

MCP-1 ELISA

Monocyte chemoattractant protein-1 (MCP-1) secretion from mouse aortic VSMC was measured by ELISA ($n = 4$ for each analyzed group). Conditioned medium from WT and $Ppia^{-/-}$ VSMC were prepared 6 h after AngII treatment and analyzed using ELISA kit (88-7391, eBioscience).

Preparation of Membrane Fractions

Preparation of membrane fraction from VSMC was performed as described[Cavet, 2003 #12515]. Briefly, the VSMC monolayers were rinsed and scraped in 1 ml of cold PBS. After a brief centrifugation the cells were resuspended in 750 μl of membrane fractionation buffer (25 mM Tris-HCl, pH 7.4, 5 mM EGTA, 5 mM EDTA, 100 mM NaF, 5 mM dithiothreitol, and 1:1000 protease inhibitor mixture). The lysates were prepared by needle homogenization and nuclei, and unbroken cells were pelleted at 850 x g for 10 min. Equal amounts of protein in postnuclear supernatants were centrifuged at 100,000 x g for 1 h to obtain soluble fractions and total membrane pellets. The soluble fraction was removed, and the membrane pellets were solubilized in 250 μl of buffer containing 1% Triton X-100, sonicated, and rocked for 1 h at 4 °C and analyzed by western blotting.

Western blot analysis

VSMC were washed twice with PBS and harvested on ice in Cell Lysis Buffer (Cell signaling technology) with protease inhibitor cocktail (Sigma). Aortic tissue samples were frozen with liquid nitrogen, crushed and lysed in Cell Lysis Buffer. Total cell lysates were loaded on SDS-Page, electrotransfered into nitrocellulose membrane followed by blocking 1 h at room temperature in Odyssey blocking buffer (Licor Odyssey). The primary antibodies were CyPA (1:1000 dilution; BIOMOL Research Laboratories, Inc.), MMP-14 (1:100 dilution; Millipore, AB8221), p47phox (1:1000 dilution; Upstate), and α-tubulin (1:5000 dilution; Sigma-Aldrich, T5168). The Licor scanner (Odyssey) was used for detection and signal density evaluation.

ROS analysis

The evaluation of ROS production in response to AngII was performed as described before[Griendling, 2003 #12922]. After treatment with AngII (1 μM), VSMC were washed with PBS and loaded with 2,7-dichlorofluoresceine diacetate (H2DCF-DA) (5 μM; Molecular Probes) for 30 min. This cell-permeable compound is converted into a non-fluorescent product (H2DCF) after deacetylation by intracellular esterases and oxidized to the highly fluorescent dichlorofluoresceine (DCF). Cells were trypsinized, washed with PBS and analyzed on a FACSCanto flow cytometer (BD Bioscience, San Jose, CA). Experiments were performed in triplicate and were repeated at least five times.

Aortas were perfused with PBS (pH 7.4) at 100 mmHg for 5 min at 4°C. Aortic tissue was harvested, and the abdominal aorta were embedded in OCT (Tissue-Tek; Miles Inc., Elkhart, Illinois, USA) and snap-frozen. Dihydroethidine hydrochloride (5 µM, Molecular Probes) was topically applied to the freshly cut frozen aortic sections (10 µm) for 30 min at 37°C to reveal the presence of ROS as red fluorescence (585 nm) by confocal microscopy (Olympus, FLUOVIEW)[Castier, 2005 #17565]. All sections are shown with the luminal aspect facing upwards and the adventitia facing downwards.

MMP activity.

The evaluation of MMP activities in response to AngII was performed as described before[Manning, 2003 #13693;Valentin, 2005 #17532;Sun, 2007 #17586]. To verify the role of CyPA in AngII-induced MMPs activation, VSMC was treated with AngII (1 µM) in culture medium. Likewise, aortas of mice infused with AngII for 7 d were incubated during 20 h in culture medium. Thereafter medium was collected and filtered to remove cell debris. The medium was concentrated 100-fold with a Centricon Plus-20 filter (Millipore Corporation, Bedford, MA) to yield concentrated conditioned medium (CM). CM was eletrophoresed in SDS-PAGE gels containing 0.8mg ml^{-1} gelatin (Sigma-Aldrich). Gels were washed twice in 2.5% triton and incubated overnight in zymography buffer. Subsequently, SDS was removed from the gels by two washes (15 minutes) with 2.5% Triton X-100 (Sigma-Aldrich). Gels were incubated for 12 h (37°C) in zymography buffer (50 mmol l^{-1} Tris (pH 8.0), 10 mmol l^{-1} CaCl$_2$, 0.05% Brij 35), and stained with Coomassie brilliant blue. MMP-2 protein expression was analyzed by Western immunoblotting. Aortic tissue was homogenized in cell lysis buffer (Cell

Signalling Technology). Twenty-five micrograms of protein was separated by SDS-

PAGE under reducing conditions, blotted on nitrocellulose membranes, and incubated

with a Ab to detect MT1-MMP (1:100 dilution; Calbiochem, IM39). Recombinant

MMP-2 (1 ng) were used as positive control. For in situ zymography, freshly cut frozen

aortic sections (10 μm) were incubated with a fluorogenic gelatin substrate (DQ gelatin,

Molecular Probes) dissolved to 25 mg ml^{-1} in zymography buffer (50 mmol l^{-1} Tris-HCl

pH 7.4 and 15 mmol l^{-1} CaCl$_2$) according to the manufacturer's protocol. Proteolytic

activity was detected as green fluorescence (530 nm) by confocal microscopy (Olympus,

FLUOVIEW). Negative control zymograms were incubated in the presence of 5 mM

EDTA. The specific removal of essential divalent cations resulted in no detectable

gelatinolytic activity. All sections are shown with the luminal aspect facing upwards and

the adventitia facing downwards.

Generation of VSMC-Specific CyPA Overexpressing Transgenic Mouse

We utilized a Cre/LoxP strategy to prepare CyPA transgenic mice. In brief, a LacZ^{flox}-

CyPA construct was prepared using the Z/EG vector kindly provided by Dr. Andras

Nagy. This vector contains LacZ floxed by two loxP sites, driven by the chicken β-actin

promoter and a cytomegalovirus (CMV) enhancer with enhanced green fluorescent

protein (EGFP) downstream. We replaced EGFP with full-length wild type mouse CyPA

carrying a Flag tag to make the LacZ^{flox}-Flag-CyPA construct. ES cells transfected by

electroporation with linearized LacZ^{flox}-Flag-CyPA cDNA were screened by neomycin

resistance and LacZ expression. ES clones with a single copy by Southern blotting were

used to generate chimeric mice by ES cell - embryo aggregation. The chimeric mice

were bred to C57BL/6J mice to produce hemizygous transgenic offspring. Hemizygous offspring with germline transmission were identified by PCR of DNA harvested from tail snippets of weaned offspring. We obtained 9 germline mice from the 2A3 ES cell clone and 8 from the 3H9 ES cell clone. Transgenic mice were backcrossed to C57BL/6J mice for 7 generations to establish experimental lines. For VSMC-specific overexpression of CyPA in transgenic mice, the LacZ^{flox}-CyPA transgenic mouse and SM22α-Cre mouse (C57BL/6J background) were crossed. Breeding the LacZ^{flox}-CyPA mice to SM22α-Cre mice resulted in excision of LacZ and expression of CyPA in VSMC.

Enzymatic assay by MT1-MMP-specific substrate

The enzymatic activity of MT1-MMP in VSMC was assayed using specific fluorogenic substrate as described previously with minor modification. Briefly, 10 μl of samples from VSMC was added to 90 μl of 50 mM HEPES buffer (pH 7.5) containing 150 mM NaCl, 5 mM $CaCl_2$ and DANSYL-Pro-Leu-Ala-Cys(p-OMeBz)-Trp-Ala-Arg-NH2 (MT1-MMP substrate, Calbiochem) substrate. After 1 h of incubation at 37°C in the dark, the rate of substrate hydrolysis that mainly reflects gelatinolytic activity was evaluated by monitoring the increase in the fluorescence at Excitation 280 nm/Emission 340 nm for MT1-MMP and Excitation 325 nm/Emission 393 nm for MMP-2.

Supplementary References

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