# Age-Associated Sirtuin 1 Reduction in Vascular Smooth Muscle Links Vascular Senescence and Inflammation to Abdominal Aortic Aneurysm

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<u>Rationale:</u> Uncontrolled growth of abdominal aortic aneurysms (AAAs) is a life-threatening vascular disease without an effective pharmaceutical treatment. AAA incidence dramatically increases with advancing age in men. However, the molecular mechanisms by which aging predisposes individuals to AAAs remain unknown.

<u>Objective</u>: In this study, we investigated the role of SIRT1 (Sirtuin 1), a class III histone deacetylase, in AAA formation and the underlying mechanisms linking vascular senescence and inflammation.

- <u>Methods and Results</u>: The expression and activity of SIRT1 were significantly decreased in human AAA samples. SIRT1 in vascular smooth muscle cells was remarkably downregulated in the suprarenal aortas of aged mice, in which AAAs induced by angiotensin II infusion were significantly elevated. Moreover, vascular smooth muscle cell-specific knockout of SIRT1 accelerated angiotensin II-induced formation and rupture of AAAs and AAArelated pathological changes, whereas vascular smooth muscle cell-specific overexpression of SIRT1 suppressed angiotensin II-induced AAA formation and progression in *Apoe<sup>-/-</sup>* mice. Furthermore, the inhibitory effect of SIRT1 on AAA formation was also proved in a calcium chloride (CaCl<sub>2</sub>)-induced AAA model. Mechanistically, the reduction of SIRT1 was shown to increase vascular cell senescence and upregulate p21 expression, as well as enhance vascular inflammation. Notably, inhibition of p21-dependent vascular cell senescence by SIRT1 blocked angiotensin II-induced nuclear factor-κB binding on the promoter of monocyte chemoattractant protein-1 and inhibited its expression.
- <u>Conclusions</u>: These findings provide evidence that SIRT1 reduction links vascular senescence and inflammation to AAAs and that SIRT1 in vascular smooth muscle cells provides a therapeutic target for the prevention of AAA formation. (*Circ Res.* 2016;119:1076-1088. DOI: 10.1161/CIRCRESAHA.116.308895.)

Key Words: aging ■ angiotensin II ■ inflammation ■ aortic aneurysm, abdominal ■ SIRT1 protein, human

Abdominal aortic aneurysms (AAAs), characterized by a permanent, localized dilatation (ballooning) of the abdominal aorta that exceeds the normal diameter by >50%, are the most common form of aortic aneurysm. AAA rupture and the associated catastrophic physiological insult carry an overall mortality rate in excess of 80%; ruptured AAAs are the 13th leading cause of death in the United States.<sup>1,2</sup> Pathologically, AAAs are characterized by increased inflammatory cell infiltration, aberrant oxidant stress, medial elastin degradation, and medial collagen deposition. Apart from surgery, few medical treatments have been shown to prevent AAA development and growth,<sup>3,4</sup> primarily as a result of the limited understanding of its pathogenic mechanisms.

AAAs are found in up to 8% of men aged >65 years. AAA incidence increases steeply by 40% every 5 years in men who

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Nonstandard Abbr	Nonstandard Abbreviations and Acronyms				
AA	abdominal aortas				
AAA	abdominal aortic aneurysm				
Ang II	angiotensin II				
KO	knockout				
MCP-1/CCL2	monocyte chemoattractant protein-1				
MMP	matrix metalloproteinase				
MMP2	matrix metalloproteinase 2				
NF- κB	nuclear factor-ĸB				
RNAi	RNA interference				
SA-β-gal	senescence-associated $\beta$ -galactosidase				
SIRT1	Sirtuin 1				
SV-KO	SIRT1-VSMC-specific knockout				
Tg	transgenic				
VSMC	vascular smooth muscle cell				
WT	wild-type				

are >65 years old, indicating that age is a major risk factor for AAAs.<sup>2</sup> Although age-related alterations such as enhanced inflammatory responses, vascular stiffening, and oxidative stress make aged arteries more susceptible to vascular diseases, such as atherosclerosis,<sup>5-7</sup> the reasons why AAAs are often observed in patients with advanced age (>65 years) and how advanced age dramatically accelerates the development and progression of aneurysms in abdominal aortas remain unknown. Furthermore, little is known about the contributions of vascular aging in AAAs and whether an alteration of age-related molecules is required for AAA initiation and progression.

Sir2 (silent information regulator 2) proteins (sirtuins), a conserved nicotinamide adenine dinucleotide-dependent protein deacetylase, play critical roles in improving metabolism and healthspan.<sup>8-10</sup> SIRT1 (Sirtuin 1), the best characterized mammalian sirtuin, is highly expressed in the vasculature<sup>11</sup> and is an important modulator of cardiovascular functions in health and disease. Several studies from our laboratory and others indicate that SIRT1 protects against stress-induced vascular remodeling,<sup>12,13</sup> aortic stiffness and dissection,<sup>14,15</sup> and atherosclerosis in mice,16-18 suggesting a critical role of SIRT1 in vascular diseases. The activation of SIRT1 confers protective effects on atherosclerosis involving vascular endothelial and smooth muscle cell senescence.<sup>18-20</sup> Despite that advanced age is a known risk factor for AAAs, whether advanced age accelerates AAA formation in mice and the molecular mechanisms that link vascular cell senescence and AAA formation remain elusive. Here, we report that the expression and activity of SIRT1 were significantly decreased in human AAA samples. Age-associated SIRT1 reduction in vascular smooth muscle cells (VSMCs) of abdominal aortas increases their susceptibility to AAAs in mice. The reduction of SIRT1 was shown to increase vascular cell senescence, upregulate the expression of p21, and enhance inflammatory cell recruitment for vascular inflammation, which predisposes aortas to AAAs.

### Methods

An expanded Materials and Methods section is available in the Online Data Supplement.

### **Human Aortic Samples**

All protocols using human aortic samples were approved by the Ethical Committee of Chinese Academy of Medical Sciences and Peking Union Medical College. We obtained human AAA samples from 6 patients undergoing open surgical repair. AAA was diagnosed by ultrasound scanning before the operation. The control samples were trimmed from the adjacent nonaneurysmal aortic segments from the same patients. Information on the enrolled patients is provided in Online Table I.

### **Animal Experiments**

All animal protocols were approved by the Animal Care and Use Committee at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, and Peking Union Medical College. We used 2- to 3-month-old C57BL/6J male mice as young mice and 18- to 20-month-old C57BL/6J male mice as old mice. SIRT1-VSMC-specific transgenic (SV-Tg) *Apoe<sup>-/-</sup>* mice were generated by crossing SV-Tg mice<sup>12</sup> with *Apoe<sup>-/-</sup>* mice. We utilized a Cre/LoxP strategy to yield SIRT1-VSMC-specific knockout (SV-KO) mice. Age-matched male mice (young and old) and 4- to 6-month-olm mice (*Apoe<sup>-/-</sup>*, SV-Tg *Apoe<sup>-/-</sup>*; SV-KO, wild-type [WT]; *Apoe<sup>-/-</sup>*, *p2 I<sup>-/-</sup>Apoe<sup>-/-</sup>*) were infused with angiotensin II (Ang II) at a dosage of 1.44 mg/kg-per day or saline for 4 weeks as previously described.<sup>21</sup> In the CaCl<sub>2</sub>-induced AAA model, we induced AAA in both SV-KO (3 weeks) and SV-Tg (6 weeks) mice by periaortic application of 0.5 mol/L CaCl, as described previously.<sup>22</sup>

### SIRT1 Deacetylase Activity Assay

SIRT1 activity was assayed using a SIRT1 deacetylase activity assay kit (Sigma) according to the manufacturer's instructions using microplate reader (Synergy 4; BioTek).

### Senescence-Associated β-Galactosidase Activity Assay

Senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) activity was quantitatively measured according to the rate of conversion of 4-methylumbelliferyl- $\beta$ -d-galactopyranoside to the fluorescent hydrolysis product 4-methylumbelliferone at pH 6.0, as described previously.<sup>23</sup> The whole-aorta tissues were stained to determine SA- $\beta$ -gal activity using a commercial kit (ab65351; Abcam) according to the manufacturer's instructions, as previously reported.<sup>24</sup>

#### **Statistical Analyses**

For all statistical tests, a P<0.05 was considered statistically significant, and all tests were 2 tailed. Normality tests were assessed via the Shapiro–Wilk statistics with SPSS software package (version 19.0). All statistical analyses were performed using GraphPad Prism (version 6.01) software.

### Results

# Reduction of SIRT1 Expression and Activity in Human AAA Formation

To establish the impact of AAAs on SIRT1, we first examined the expression of SIRT1 in human AAA samples. Human AAA tissues and their control adjacent aortic sections without aneurysm were obtained from patients undergoing open surgery (Online Table I). As expected, proaneurysmal molecules, such as matrix metalloproteinase 2 (MMP2), human membrane type 1 MMP, and monocyte chemoattractant protein-1 (MCP-1/CCL2), were significantly elevated in human AAA sections (Figure 1A and 1B; Online Figure IA) compared with adjacent nonaneurysmal aortic sections. The activity of SIRT1 in human AAA samples was also significantly lower than that in adjacent nonaneurysmal aortic sections (Figure 1C). These results suggest that SIRT1 reduction is involved in the development of human AAA formation.



Figure 1. SIRT1 (Sirtuin 1) is decreased in human abdominal aortic aneurysm (AAA) samples and aged mouse abdominal aortas. A, Representative Western blots of SIRT1, membrane type 1-matrix metalloproteinase (MT1-MMP), monocyte chemoattractant protein-1 (MCP-1/CCL2), and MMP2 in human AAA samples and adjacent control aortas. **B**, Densitometric analysis of the protein levels of SIRT1, MT1-MMP, MCP-1/CCL2, and MMP2 in human AAA samples and adjacent control aortas. **B**, Densitometric analysis of the protein levels of SIRT1, MT1-MMP, MCP-1/CCL2, and MMP2 in human AAA samples and adjacent control aortas (n=6). **C**, Quantitative analysis of SIRT1 activity in homogenates of human AAA samples and adjacent nonaneurysmal aortic sections (n=6). **D**, Fluorescence intensity indicating SIRT1 activity in homogenates of young mouse compared with that of the aged mouse (n=6). **E**, Representative Western blots of SIRT1 and p21 in abdominal aortas (AA) from aged and young mice. Adventitial tissue was removed from the aorta as much as possible, and each sample was pooled from 3 abdominal aortas together for immunoblot analysis. **F**, Representative immunofluorescent staining of SIRT1,  $\alpha$ -smooth muscle actin (SMA), and 4',6-diamidino-2-phenylindole (DAPI) in suprarenal aortas of young and aged mice (scale bars, 50 µm). **G**, Densitometric analysis of the protein level of SIRT1, which is normalized to  $\alpha$ -SMA, in suprarenal aortas of young and aged mice (n=9). Three different visual fields were captured in each slide.

# Reduced SIRT1 Expression and Activity in the Aged Mouse Aortas

Because advanced age is a known risk factor for AAAs, we examined the effects of age on SIRT1 expression and activity in mouse aortas. SIRT1 activity was significantly lower in the whole aortas of aged mice than that of their young counterparts (Figure 1D). SIRT1 expression was decreased in whole aortas and substantially reduced in the abdominal aortas of aged mice compared with aortas from young mice (Figure 1E; Online Figure IB and IC). Moreover, the decrease in SIRT1 expression was mainly observed in the medial VSMCs of the suprarenal aortas (Figure 1F and 1G), where AAAs most often develop in mice with Ang II infusion.<sup>25</sup> Therefore, the reduction of SIRT1 activity and

expression in aged aortas may be linked to their susceptibility to AAAs.

# Aging Increases Ang II–Induced AAA Formation in Mice

Acute infusion of Ang II recapitulates many aspects of AAAs and is widely used to study AAAs.<sup>25,26</sup> To clarify the effects of advanced age on AAA formation, 22 C57BL/6J male mice aged 18 to 20 months (aged) and 23 C57BL/6J male mice aged 2 to 3 months (young) were infused with the same dose of Ang II for 4 weeks. In saline-infused groups, 1 in 16 aged mice displayed expanded abdominal aorta to the extent of AAA, whereas none of the young mice displayed AAA formation (Figure 2A and 2B). In Ang II–infused groups, the AAA



Figure 2. Angiotensin II (Ang II) increases abdominal aortic aneurysm (AAA) formation in aged mice. All mice were infused with saline or Ang II for 4 wk. A, Representative photographs showing mouse aortas infused with saline or Ang II. The arrow shows an AAA (scale bars, 5 mm). B and C, The incidence (B) and survival curve (C) of AAA in Ang II–infused young mice (n=23) compared with that in aged mice (n=22). There was no AAA formation in saline-infused young mice (n=16), and 1 of 16 saline-infused aged mice developed AAA. D and E, Maximal abdominal aortic diameter (D) and the ratio of aorta weight to body weight (E) in saline- and Ang II–infused young and aged mice. F and G, mRNA levels of monocyte chemoattractant protein-1 (MCP-1/CCL2; F) and matrix metalloproteinase 2 (MMP-2; G) detected by real-time polymerase chain reaction in aorta homogenates from young and aged mice infused with saline or Ang II for 4 wk. The arrows show representative staining with CD45 antibody. I, The number of CD45-positive cells accumulating in the suprarenal aortic wall of saline- and Ang II–infused mice (n=8–23). SV-KO indicates SIRT1 (Sirtuin 1)-vascular smooth muscle cell (VSMC)–specific knockout; and WT, wild-type.

incidence in aged mice was 86.4% (19/22), much higher than the 13.0% (3/23) observed in young mice (Figure 2A and 2B). Approximately 22.7% (5/22) of Ang II–infused aged mice died from aortic rupture, whereas only 4.3% (1/23) of Ang II– infused young mice died (Figure 2C). Additionally, Ang II infusion caused a greater increase in maximal abdominal aortic diameter, the ratio of total aortic weight to body weight in aged mice than in young mice (Figure 2D and 2E), accompanied with increased expression levels of MCP-1/CCL2, a critical chemokine in inflammatory cell recruitment for vascular inflammation and AAAs,<sup>27,28</sup> and MMP-2 as well (Figure 2F and 2G). Accordingly, inflammatory cell infiltration as determined by CD45 immunostaining was increased in the aortas of Ang II–infused aged mice compared with those of Ang II–infused young mice (Figure 2H and 2I). These results indicate that aging remarkably increases Ang II–induced AAA formation in mice.

# VSMC-Specific Ablation of SIRT1 Exacerbates Ang II–Induced AAA Formation and Related Pathological Changes In Vivo

To establish a causative link between the reduction of SIRT1 and AAA formation, we crossbred a conditional allele of Sirt1 ( $Sirt1^{flox}$ ) mice with mice with Cre recombinase driven by the



**Figure 3. Vascular smooth muscle cell (VSMC)**-specific SIRT1 (Sirtuin 1) ablation promotes abdominal aortic aneurysm (AAA) formation and vascular pathophysiological mechanisms induced by angiotensin II (Ang II) infusion. All mice were infused with saline or Ang II for 4 wk. **A**, Representative photographs showing macroscopic features of aneurysms induced by Ang II. The arrow shows a typical AAA (scale bars, 5 mm). **B** and **C**, The incidence (**B**) and survival curve (**C**) of Ang II-induced AAA in SIRT1-VSMC-specific knockout (SV-KO) mice (n=53) compared with that in wild-type (WT) mice (n=46). There was no AAA formation in saline-infused mice (n=12), and the number of mice that developed AAA included the deaths caused by abdominal aortic rupture. **D**, The maximal abdominal aortic diameter in saline- and Ang II-infused mice. **E**, Representative staining with elastin and elastin degradation score in suprarenal aortas from saline- and Ang II-infused mice. The magnified photographs). **F**, Aorta homogenates were obtained from WT and SV-KO mice infused with saline or Ang II for 4 wk. Western blot and densitometric analysis of the protein levels of monocyte chemoattractant protein-1 (MCP-1/CCL2) and matrix metalloproteinase 2 (MMP2) in aorta homogenates (n=4–6).

SM22α promoter to obtain SV-KO (*SM22-Cre<sup>+/-</sup>*; *Sirt1*<sup>flox/flox</sup>) mice (Online Figure IIA–IID), and their *Sirt1*<sup>flox/flox</sup> littermates were used as WT controls. Although SIRT1 activity and expression in the aortas of SV-KO mice were markedly reduced compared with their littermates (Online Figure IIE and IIF), there was no difference in the gross morphology of aortas between saline-infused SV-KO and WT mice (Figure 3A). However, 4 weeks of Ang II infusion caused a 64.2% (34/53) incidence of AAA in SV-KO mice compared with 19.6% (9/46) in WT mice (Figure 3B). Approximately 34.0% (18/53) of Ang II–infused SV-KO mice died because of aortic rupture, whereas only 8.7% (4/46) of Ang II–infused WT mice died (Figure 3C; Online Table II). To exclude the effect of SM22 deficiency after *Cre* knockin, we treated 10 *SM22-Cre<sup>+/-</sup>; Sirt1*<sup>+/+</sup> mice with Ang II for 4 weeks and found that only 2 developed AAAs, suggesting that SM22 deficiency in 1 allele does not influence Ang II–induced AAA formation. In addition, compared with Ang II–treated WT mice, the maximal abdominal aortic diameter, the ratio of total aortic weight to body weight, and the elastin degradation score were remarkably higher, whereas the elastic fiber content was significantly lower in Ang II–treated SV-KO mice (Figure 3D–3F; Online Figure IIIA and IIIB). The systolic blood pressure, heart rate, and serum lipid levels after Ang II infusion did not differ between SV-KO mice and WT mice (Online Tables III and IV).

Aberrant levels of oxidative stress play critical roles in AAA initiation and progression.<sup>21</sup> Therefore, we analyzed medial oxidative stress by immunostaining for 3-nitrotyrosine and 8-hydroxydeoxyguanosine (8-OH-dG), 2 oxidative stress



Figure 4. Vascular smooth muscle cell (VSMC)-specific SIRT1 (Sirtuin 1) overexpression prevents abdominal aortic aneurysm (AAA) formation and vascular pathophysiological mechanisms induced by angiotensin II (Ang II) infusion. All mice were infused with saline or Ang II for 4 wk. A, Representative photographs showing macroscopic features of aneurysms induced by Ang II. The arrow shows a typical AAA (scale bars, 5 mm). B and C, The incidence (B) and survival curve (C) of Ang II-induced AAA in SIRT1-VSMC-specific transgenic (SV-Tg)  $Apoe^{-/-}$  mice (n=29) compared with those in  $Apoe^{-/-}$  mice (n=33). There was no AAA formation in saline-infused mice (n=10), and the number of mice that developed AAA included the deaths caused by abdominal aortic rupture. D, The maximal abdominal aortic diameter in saline- and Ang II-infused mice. E, Representative staining with elastin and elastin degradation score in suprarenal aortas from saline- and Ang II-infused mice. The magnified photographs were taken at the location where the most severe elastin degradation occurred (scale bars, 150 and 50 µm; magnified photographs). F, Aorta homogenates were obtained from  $Apoe^{-/-}$  and SV-Tg  $Apoe^{-/-}$  mice infused with saline or Ang II for 4 wk. Western blot and densitometric analysis of the protein levels of monocyte chemoattractant protein-1 (MCP-1/CCL2) and matrix metalloproteinase 2 (MMP2) in aorta homogenates (n=4-6).

biomarkers. Aortas from Ang II–infused SV-KO mice exhibited a significant increase in the levels of both 3-nitrotyrosine and 8-OH-dG compared with those of Ang II–infused WT mice (Online Figure IIIC and IIID). In saline-infused mice, few inflammatory cells were found in the suprarenal aortic wall (Online Figure IVA). After Ang II infusion, inflammatory cell infiltration was increased in the aortas of Ang II–infused SV-KO mice compared with those of Ang II–infused WT mice (Online Figure IVA) and was accompanied by a significantly higher expression of MCP-1/CCL2 (Figure 3F; Online Figure IVB). SV-KO mice also displayed increased MMP2 protein expression and activity after Ang II infusion (Figure 3F; Online Figure IVC), with an upregulation of membrane type 1-MMP (Online Figure IVD) and increased TIMP1 repression (Online Figure IVD). These results indicate that the VSMCspecific ablation of SIRT1 exacerbates AAA formation and related pathological changes.

# VSMC-Specific Overexpression of SIRT1 Suppresses Ang II–Induced AAA Formation and Related Pathological Changes in *Apoe<sup>-/-</sup>* Mice

*Apoe*<sup>-/-</sup> mice with Ang II infusion is a widely used animal model for AAA formation,<sup>25,26</sup> and the incidence of AAAs in *Apoe*<sup>-/-</sup> mice in response to Ang II is much greater than that in age-matched WT mice.<sup>21,25,26,29</sup> Therefore, we studied whether SIRT1 VSMC-specific overexpression could reduce AAA development in vivo by examining the above-mentioned index in aortas obtained from VSMC-specific SIRT1 transgenic





mice (SV-Tg mice) in the Apoe-/- background (SV-Tg Apoe-/mice) with or without Ang II infusion for 4 weeks. In salineinfused groups, no AAAs were found among Apoe-/- and SV-Tg Apoe-/- mice. Ang II infusion for 4 weeks caused AAAs in 81.8% (27/33) of  $Apoe^{-/-}$  mice (Figure 4A and 4B), which was similar to the incidence of Ang II-induced AAAs in aged mice. Only 27.6% (8/29) of SV-Tg Apoe-/- mice developed AAAs after Ang II infusion. Approximately 33.3% (11/33) of Apoe-/- mice died because of aortic rupture during Ang II treatment, but the death rate of Ang II-infused SV-Tg Apoe<sup>-/-</sup> mice was only 10.3% (3/29) (Figure 4A through 4C; Online Table II). Compared with Ang II-treated Apoe<sup>-/-</sup> mice, the maximal abdominal aortic diameter, the ratio of total aortic weight to body weight, and the elastin degradation score were remarkably lower, whereas the elastic fiber content was significantly higher in Ang II-treated SV-Tg Apoe<sup>-/-</sup> mice (Figure 4D and 4E; Online Figure VA and VB) without noticeable effects on hemodynamic and lipid metabolic indices (Online Tables III and IV). Moreover, the VSMC-specific SIRT1 transgene remarkably attenuated the increased levels of oxidative stress (Online Figure VC and VD), vascular inflammation, MCP-1/CCL2 expression (Figure 4F; Online Figure VIA and VIB) and MMP2 activation (Figure 4F; Online Figure VIC and VID) caused by Ang II infusion in *Apoe<sup>-/-</sup>* mice. These results indicate that SIRT1 overexpression in VSMCs ameliorates the increased AAA formation and related pathological changes.

# SIRT1 in VSMCs Inhibits Calcium Chloride– Induced AAA Formation

To further investigate whether the inhibitory effects of SIRT1 in VSMCs on AAA formation are independent of Ang II, another type of mouse AAA model, calcium chloride (CaCl<sub>2</sub>)– induced AAA<sup>22</sup> was applied in both SV-KO and SV-Tg mice. We found that 3 weeks after CaCl<sub>2</sub> treatment, the ratio of total aortic weight to body weight and the maximal external and internal abdominal aortic diameter were significantly increased in SV-KO mice compared with those of their littermates (Figure 5A through 5D), and a substantial increase in the elastin degradation score was also observed (Figure 5E and 5F). Moreover, in situ immunochemical staining showed that compared with WT mice, SV-KO mice displayed significantly increased MCP-1/CCL2 and MMP-2 protein expression after CaCl<sub>2</sub> treatment (Online Figure VIIA through VIID). To determine the protective role of SIRT1 transgene



Figure 6. Vascular smooth muscle cell (VSMC)–derived SIRT1 (Sirtuin 1) is crucial for suppression of vascular cell senescence in angiotensin II (Ang II)–induced abdominal aortic aneurysms (AAAs). All mice were infused with saline or Ang II for 4 wk. A, C, and E, Representative photographs and densitometric analysis of senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal)–stained aortas from wild-type (WT) and SIRT1-VSMC–specific knockout (SV-KO; A), *Apoe<sup>-/-</sup>* and SIRT1-VSMC–specific transgenic (SV-Tg) *Apoe<sup>-/-</sup>* (C), young and aged (E) mice infused with saline or Ang II (scale bars, 5 mm; n=6 per group). B, D, and F, Representative images of SA- $\beta$ -gal–stained transverse sections of suprarenal abdominal aortas from WT and SV-KO (B), *Apoe<sup>-/-</sup>* and SV-Tg *Apoe<sup>-/-</sup>* (D), and young and aged (F) mice infused with saline or Ang II. The blue region was positively stained, and nuclei were counterstained using Nuclear Fast Red (scale bars, 50 µm).

gal-stained transverse sections of suprarenal abdominal aortas f aged (F) mice infused with saline or Ang II. The blue region was p Red (scale bars, 50 μm).
in AAAs, we treated SV-Tg and their WT controls with CaCl<sub>2</sub> for 6 weeks. In contrast, we found that the ratio of total aortic weight to body weight and the maximal external and internal abdominal aortic diameter were significantly decreased in SV-Tg mice compared with those of their littermates after CaCl<sub>2</sub> treatment (Online Figure VIIIA through VIIIF). SIRT1 overexpression in VSMCs was also found to significantly inhibit CaCl<sub>2</sub>-induced MCP-1/CCL2 and MMP-2 protein expression (Online Figure VIIIG and VIIIH). These results indicate that

AAA formation.

# SIRT1 Is Crucial for Suppression of Vascular Cell Senescence and p21 Expression in AAAs

SIRT1 in VSMCs plays a protective role in CaCl<sub>2</sub>-induced

Ang II infusion accelerates vascular cell senescence in vivo, which can be characterized by SA- $\beta$ -gal staining, as reported in the previous study,<sup>30</sup> and we found that SA- $\beta$ -gal–positive staining was mainly located in the media of the aortas (Online Figure IXA), suggesting a role of medial VSMC senescence in Ang II-induced AAAs. To investigate the role of SIRT1 in vascular cell senescence, SA-β-gal staining was performed in the aortas of Ang II- or saline-infused SV-KO mice and SV-Tg Apoe-/- mice and their respective controls. In salineinfused groups, few obvious SA-β-gal-positive areas were detected in both SV-KO mice and SV-Tg Apoe-/- mice. Ang II infusion led to the enlargement of SA-β-gal-positive regions in the aortas of WT mice, whereas the SIRT1-specific loss of function in VSMCs further increased the areas of SA- $\beta$ -gal-positive staining (Figure 6A). The increase of SA- $\beta$ gal-positive staining in the aortas of SV-KO mice mainly occurred at medial VSMCs (Figure 6B). Moreover, Ang IIinduced vascular cell senescence was further supported by the results of assays for SA-β-gal activity in the homogenates of whole aortas (Online Figure IXB). In contrast, SA-β-gal staining was suppressed in SV-Tg Apoe-/- mice (Figure 6C). Notably, SA-β-gal-positive staining in medial VSMCs was



**Figure 7. Vascular smooth muscle cell (VSMC)-derived SIRT1 (Sirtuin 1) is crucial for p53-p21 axis in angiotensin II (Ang II)induced abdominal aortic aneurysms (AAAs).** All mice were infused with saline or Ang II for 4 wk. **A** and **B**, Representative Western blots and densitometric analysis of Ac-p53, p53, and p21 proteins in aorta homogenates from wild-type (WT) and SIRT1-VSMC-specific knockout (SV-KO; **A**), *Apoe<sup>-/-</sup>* and SIRT1-VSMC-specific transgenic (SV-Tg) *Apoe<sup>-/-</sup>* (**B**) mice infused with Ang II (n=6). **C**, Representative Western blots and densitometric analysis of p21 protein expression in aorta homogenates from young and aged mice infused with Ang II (n=4–6). **D**, Western blots of p21 protein expression in human AAA samples and control non-AAA adjacent aortas. The values of p21, p53, and Ac-p53 for Ang II-infused control group were set to 1.0 in each graph.

markedly inhibited in SV-Tg  $Apoe^{-/-}$  mice (Figure 6D). As expected, the SA- $\beta$ -gal activity assay also confirmed the antiaging effect of SIRT1 overexpression (Online Figure IXC). Furthermore, vascular aging was demonstrated by increased pulse wave velocity in the left common carotid artery in vivo.<sup>31,32</sup> As expected, Ang II caused a modest increase in pulse wave velocity in WT mice, whereas it robustly increased the value in SV-KO mice (Online Figure IXD), and this trend was reversed in SV-Tg  $Apoe^{-/-}$  mice (Online Figure IXE). These results indicate that the manipulation of SIRT1 expression or activity affects Ang II–induced vascular cell senescence in AAAs.

To further investigate the role of vascular cell senescence in aging-increased AAA formation, we examined SA-β-galpositive areas in the aortas of young and aged mice with or without Ang II infusion. As shown in Figure 6E, the levels of SA-β-gal-positive areas in the aortas of saline-infused young and aged mice were low, and there was no difference between aged and young aortas; similar results were observed for SA-β-gal activity (Online Figure IXF), suggesting that vascular cell senescence was low and unaltered in aortas with advanced age. However, Ang II infusion significantly increased SA-β-gal-positive areas and SA-β-gal activity in the aortas of aged mice compared with those of young mice (Figure 6E; Online Figure IXF). The increase of SA-β-gal staining and SA-β-gal activity caused by Ang II was significantly greater in aged mice than that in young mice, indicating that aged aortas were more sensitive to Ang II-induced vascular senescence (Figure 6E; Online Figure IXF). Correspondingly, the increase of SA-β-gal-positive staining in the suprarenal aortas of aged mice mainly occurred at medial VSMCs (Figure 6F).

Cellular senescence marker p21 can be suppressed by SIRT1 in a p53-dependent and p53-independent manner.<sup>33,34</sup> We found that acetylated p53 (Ac-p53) and p21 expression levels were higher in the whole aorta homogenates from Ang II-infused SV-KO mice (Figure 7A), and total p53 was also significantly increased (Figure 7A), whereas the SIRT1 transgene significantly reversed the increased protein levels of Ac-p53, total p53 and p21 in Ang II-infused Apoe<sup>-/-</sup> mice (Figure 7B). These results suggest that SIRT1 inhibited Ang II-induced p21 expression by deacetylation of p53 in AAAs. The increased p21 expression caused by Ang II was significantly greater in the aortas of aged mice than that in young mice (Figure 7C). Similarly, increased p21 expression was found in human AAA samples (Figure 7D). These results provide evidence that the increased vascular senescence and deregulated p21 expression caused by the reduction of SIRT1 participates in the promotional effect of aging on AAAs.

# Suppression of p21 in VSMCs Abolishes Enhanced Nuclear Factor-ĸB Activation and MCP-1/CCL2 Expression by SIRT1 Inhibition

To further explore how SIRT1 reduction in VSMCs promotes Ang II–induced AAAs, gene expression microarray analysis was performed using RNA isolated from the aortas of Ang II–treated SV-KO mice and their *Sirt1<sup>flox/flox</sup>* littermates. Interestingly, analysis using ingenuity pathway analysis software revealed that the inflammatory response pathway was the pathway most affected by SIRT1 deficiency (Online Figure XA and XB and Online Table V), which was consistent with the aforementioned result of Online Figure IVA. Accordingly, nuclear factor (NF)- $\kappa$ B signaling was significantly activated



Figure 8. Suppression of p21 expression inhibits the promotional effect of SIRT1 (Sirtuin 1) reduction on angiotensin II (Ang II)-increased vascular smooth muscle cell (VSMC) senescence and transcriptional activation of monocyte chemoattractant protein-1 (MCP-1/CCL2). A, Senescence was evaluated through the senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) staining of saline or Ang II-treated wild-type (WT) and SIRT1-VSMC-specific knockout (SV-KO) VSMCs infected with Ad-U6 (a control RNAi vector) or Ad-p21 RNAi (vectors for adenovirus-mediated knockdown of p21) for 24 h. Blue-stained cells were considered senescent. The bar represents 150 µm. **B**, Statistical analysis of the percentage of SA- $\beta$ -gal-positive cells. Five random fields of view were analyzed for 1 group (n=7-10). **C**, Relative MCP-1/CCL2 mRNA expression level detected by real-time polymerase chain reaction in saline or Ang II-treated WT and SV-KO VSMCs infected with Ad-U6 or Ad-p21 RNAi. **D**–**F**, Relative binding level of RelA/p65 to input on indicated region of human MCP-1/CCL2 promoter. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

and the mRNA levels of a series of NF-kB target genes, including MCP-1/CCL2,<sup>27,28</sup> were significantly increased in the aortas of SV-KO mice compared with the WT mice after 4 weeks of Ang II infusion (Online Figure XIA through XID) in parallel with their protein levels as shown in Figures 3F and Online Figure IVB. Moreover, regulator effects in ingenuity pathway analysis further showed that CCR2, the receptor for MCP-1/CCL2, has the highest Consistency Score of all the regulators for cell recruitment, infiltration, and activation (Online Figure XII and Online Table VI). Furthermore, chromatin immunoprecipitation (ChIP) assays confirmed that VSMC-specific ablation of SIRT1 increased the binding activity of NF-kB on MCP-1/CCL2 promoter after Ang II infusion (Online Figure XIII), whereas a significantly lower binding level of NF-kB on MCP-1/CCL2 promoter was observed in the aortas of Ang II-infused SV-Tg Apoe--- mice compared with their Apoe--- littermates (Online Figure XIV), suggesting that SIRT1 reduction facilitates NF-kB-mediated transcriptional activation of MCP-1/CCL2 in AAAs. Moreover, p21 knockout mice were introduced to investigate its role in Ang II-induced transcriptional activation of MCP-1/CCL2 in AAAs. The results indicated that p21 knockout not only protects *Apoe<sup>-/-</sup>* mice against Ang II–induced AAA formation (Online Figure XVA through XVC) as previously reported<sup>30</sup> but also almost completely blocked the expression of MCP-1/CCL2 and MMP-2 in Ang II–induced AAAs (Online Figure XVD and XVE).

Thus, we suspected that the promotional effect of SIRT1 reduction on Ang II–induced MCP-1/CCL2 expression might rely on the existence of p21 and VSMC cell senescence. To verify this hypothesis, adenovirus-mediated p21 RNA interference (RNAi) was introduced in VSMCs isolated from the aortas of SV-KO mice or control WT mice before treated with saline or Ang II. As expected, p21 mRNA level in both saline- and Ang II–treated VSMCs was significantly repressed by transfection of Ad-p21 RNAi (vectors for adenovirus-mediated knockdown of p21; Online Figure XVI). The results of SA- $\beta$ -gal staining indicated that p21 knockdown not only blocked Ang II–induced VSMC senescence but also eradicated the promotional effect of SIRT1 knockout (Figure 8A and 8B; Online Table VII). Accordingly, Ang II–increased expression of MCP-1/CCL2 in SV-KO

VSMCs was also significantly reduced by p21 knockdown (Figure 8C). Finally, we also performed ChIP assay to detect the binding level of NF-kB on MCP-1/CCL2 promoter in saline- or Ang II-treated human VSMCs infected by either Ad-SIRT1 RNAi or Ad-p21 RNAi or both of them. Three binding sites of NF-κB on human MCP-1/CCL2 promoter by ChIP assays were selected out from the 5 potential binding sites to assess the impact of p21 knockdown to the effect of SIRT1 RNAi on transcriptional activation of MCP-1/CCL2 (Online Figure XVII). The results indicated that p21 knockdown by RNAi almost completely blocked the promotional effect of SIRT1 knockdown on Ang II-increased RelA/p65 binding on the 3 potential NF-kB binding sites of MCP-1/ CCL2 promoter (Figure 8D through 8F). These results support that p21-mediated VSMC cell senescence by SIRT1 reduction facilitates the transcriptional activation of MCP-1/ CCL2, which may promote inflammatory cell recruitment and vascular inflammation in AAAs.

### Discussion

In the present study, we demonstrated that SIRT1 acts as a novel molecular link that retards vascular senescence and inflammation to prevent AAA initiation and development. There are several major findings in this study. First, the expression and activity of SIRT1 were significantly decreased in human AAA samples. Second, SIRT1 in VSMCs was substantially downregulated in the suprarenal aortas of aged mice, in which AAAs induced by Ang II infusion were significantly elevated. Third, SIRT1 reduction in VSMCs amplified Ang II-induced vascular aging and AAA formation in mice in vivo, whereas genetic activation of SIRT1 in VSMCs displayed the opposite effect. The inhibitory effect of SIRT1 on AAA formation was also confirmed in the CaCl2-induced AAA model. Fourth, the reduction of SIRT1 was shown to increase vascular cell senescence and upregulate p21 expression and enhance vascular inflammation. Moreover, suppression of p21-dependent vascular cell senescence by SIRT1 inhibited Ang II-induced inflammation.

AAA incidence increases steeply by 40% every 5 years in men aged >65 years, indicating that age is a major risk factor for AAAs.<sup>2</sup> Why advanced age (>65 years) precipitates AAA development and progression is unknown. In the present study, we found that SIRT1 protein and activity were significantly suppressed in the aortic VSMCs of aged mice, and vascular cell senescence in the aortas of aged mice was low and comparable to that of young mice under natural conditions. Concomitantly, only a few incidences of spontaneous AAAs were observed in the same conditions. In contrast, AAA formation and vascular cell senescence in SV-KO mice and aged mice were significantly increased by Ang II infusion, indicating that the SIRT1 reduction in advanced age creates an environment that facilitates AAA initiation and progression caused by Ang II, but does not directly instigate AAAs. This conclusion is supported by human epidemiological evidence indicating that aging is a risk factor for AAA and only a small number of men with advanced age develop AAAs. Accumulating evidence has shown that SIRT1 plays an important role in healthy aging and age-related diseases,<sup>8,9</sup> which suggests that the SIRT1 reduction in advanced age may also predispose individuals to the aging of other tissues and to agerelated cardiovascular diseases.

Systemic inflammation has been linked to multiple chronic diseases of aging and may even contribute to their causation.<sup>35–37</sup> Although increases in chronic inflammation have been detected in the vasculature with age,<sup>5-7</sup> the molecular mechanisms that link vascular aging and inflammation to AAA formation remain elusive. Previous studies have identified important roles for p53 in promoting aging and NFκB in mediating inflammatory response, both of which can be targeted by SIRT1.<sup>34,38–41</sup> In the present study, we found that SIRT1 reduction increased Ang II-induced VSMC senescence and upregulated p53 acetylation and p21 protein. Inflammatory NF-KB activation and MCP-1/CCL2 were significantly increased in parallel with increased vascular cell senescence. Accordingly, AAA formation was significantly enhanced by SIRT1 reduction in both SV-KO mice and in aged mice (Online Figure XVIIIA). Moreover, Ang II-activated NF-KB transcription of MCP-1/CCL2 expression was significantly decreased by suppression of p21 in SIRT1 knockout VSMCs. These results suggest that the deregulated SIRT1-p53-p21 axis in VSMCs in response to Ang II accelerates NF-kB-induced vascular inflammation and renders aortas susceptible to AAAs. To the best of our knowledge, this is the first time that a direct link between vascular cell senescence and vascular inflammation to AAA formation by SIRT1 reduction has been reported. Importantly, AAA formation and the related pathological and molecular changes could be effectively inhibited by VSMC-specific overexpression of SIRT1, which provides a potential therapeutic target for AAAs (Online Figure XVIIIB). However, whether SIRT1 reduction in VSMCs induces NF-kB-mediated vascular inflammation and accelerates AAA formation by downregulation of p53/p21 in vivo requires further investigation. In addition, our results highlight the importance of VSMC senescence in the development of vascular inflammation and AAAs, which is also consistent with the well-known roles of VSMCs in AAA formation.<sup>21,29,42,43</sup>

In summary, our findings indicate that the age-related reduction of SIRT1 in VSMCs predisposes aortas to AAAs by facilitating p21-dependent vascular cell senescence, secretion of inflammatory cell recruitment molecules, and vascular inflammation. These findings indicate a direct link between vascular cell senescence and AAAs through vascular inflammation that provides a deeper understanding of the relationship between aging and age-related vascular diseases.

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# Disclosures

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# **Novelty and Significance**

### What Is Known?

- In humans, the incidence of abdominal aortic aneurysm (AAA) increases dramatically with age.
- SIRT1 is highly expressed in the vasculature and plays a protective role in vascular remodeling, aortic stiffness and dissection, and atherosclerosis in mice.

#### What New Information Does This Article Contribute?

- The expression and activity of SIRT1 are significantly decreased in human AAA samples.
- SIRT1 in VSMCs is downregulated in the suprarenal aortas of aged mice, in which AAAs induced by Ang II infusion were significantly elevated.
- VSMC-specific knockout of SIRT1 accelerates Ang II- or CaCl<sub>2</sub>-induced AAA formation, whereas VSMC-specific overexpression of SIRT1 displays the opposite effect.

 SIRT1 reduction increases Ang II-induced vascular cell senescence, upregulates the expression of p21 and enhances inflammatory cell recruitment for vascular inflammation in AAAs.

Age is a major risk factor for AAAs. Uncontrolled growth of AAAs is a life-threatening vascular disease without an effective pharmaceutical treatment. Our study shows that age-associated SIRT1 reduction in VSMCs accelerates the formation and rupture of AAAs, whereas VSMC-specific overexpression of SIRT1 suppresses AAA formation and progression. SIRT1-mediated inhibition of vascular cell senescence suppresses vascular inflammation in AAAs. These findings suggest that the manipulation of SIRT1 activation may effectively inhibit vascular cell senescence, vascular inflammation, and delay or prevent AAA initiation and progression.





Age-Associated Sirtuin 1 Reduction in Vascular Smooth Muscle Links Vascular Senescence and Inflammation to Abdominal Aortic Aneurysm Hou-Zao Chen, Fang Wang, Peng Gao, Jian-Fei Pei, Yue Liu, Ting-Ting Xu, Xiaoqiang Tang, Wen-Yan Fu, Jie Lu, Yun-Fei Yan, Xiao-Man Wang, Lei Han, Zhu-Qin Zhang, Ran Zhang, Ming-Hui Zou and De-Pei Liu

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# **ONLINE MATERIAL**

# Age-associated Sirtuin 1 reduction in vascular smooth muscle links vascular senescence and inflammation to abdominal aortic aneurysm

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# **Online methods**

# Animal experiments

All animal protocols were approved by the Animal Care and Use Committee at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College.

We used 2- to 3-month-old C57BL/6J male mice as young mice and 18- to 20-month-old C57BL/6J male mice as aged mice. We established SV-Tg mice as previously described<sup>1</sup>. *Apoe<sup>-/-</sup>* mice in the C57BL/6J background were obtained from Peking University (Beijing, China). SIRT1-VSMC-specific transgenic (SV-Tg) *Apoe<sup>-/-</sup>* mice were generated by crossing SV-Tg mice with *Apoe<sup>-/-</sup>* mice. The F1 generation was backcrossed with *Apoe<sup>-/-</sup>* mice to produce the *Apoe<sup>-/-</sup>* genotype. *Apoe<sup>-/-</sup>* mice served as controls.

We utilized a Cre/LoxP strategy to yield SIRT1-VSMC-specific knockout (SV-KO) mice (Online Figure II). *Sirt1<sup>flox/flox</sup>* mice in the 129 background were purchased from the Jackson Laboratory (Bar Harbor, Maine; Stock Number: 008041). A LoxP-flanked neomycin cassette immediately upstream of exon 4 and a third LoxP site downstream of exon 4 were inserted to create the targeted mutant Sirt1 allele. *Sirt1<sup>flox/flox</sup>* mice in the 129 background were backcrossed with mice in the C57BL/6J background for at least 10 generations to yield *Sirt1<sup>flox/flox</sup>* mice in the C57BL/6J background. *SM22a-Cre<sup>+/+</sup>* mice in the 129 background were backcrossed with mice in the C57BL/6J background for at least 10 generations to yield from the Jackson Laboratory (Bar Harbor, Maine; Stock Number: 006878). These mice have a Cre-recombinase gene inserted into the endogenous transgelin (*SM22a*) locus. *SM22a-Cre<sup>+/+</sup>* mice in the 129 background were backcrossed with mice in the C57BL/6J background. *Sint1<sup>flox/flox</sup>* mice were crossed with *SM22a-Cre<sup>+/+</sup>* mice, both in the C57BL/6J background, to generate SV-KO mice. The F1 generation (*SM22a-Cre<sup>+/-</sup>*; *Sint1<sup>flox/flox</sup>* mice were obtained, they were backcrossed with *Sint1<sup>flox/flox</sup>* mice to fix the *Sint1<sup>flox/flox</sup>* mice to generate SV-KO (*SM22a-Cre<sup>+/-</sup>*; *Sint1<sup>flox/flox</sup>*) mice and WT (*Sint1<sup>flox/flox</sup>*) littermates as controls.

 $p21^{-/-}$  mice in the C57BL/6J background<sup>2</sup> were purchased from The Jackson Laboratory (stock number 016565).  $p21^{-/-}$  Apoe<sup>-/-</sup> double knockout mice were generated by crossing  $p21^{-/-}$  mice with Apoe<sup>-/-</sup> mice. The F1 generation was backcrossed with Apoe<sup>-/-</sup> mice to produce the F2  $p21^{+/-}Apoe^{-/-}$  genotype. Then  $p21^{+/-}Apoe^{-/-}$  mice were mated with themselves to produce three genotypes of F3 generation including 1/4 ratio of  $p21^{-/-}Apoe^{-/-}$  double knockout mice and 1/4 ratio of  $Apoe^{-/-}$  mice. Apoe<sup>-/-</sup> mice were served as controls.

All mice were genotyped by PCR conducted on toe clip samples; primers are listed in Online Table VIII.

# Analyses and quantification of Ang II-induced AAAs

We used 4- to 6-month-old male mice on a normal chow diet. All age-matched mice (*Apoe<sup>-/-</sup>*, SV-Tg *Apoe<sup>-/-</sup>*, *p21<sup>-/-</sup> Apoe<sup>-/-</sup>*, SV-KO, and WT) were infused with Ang II (Sigma-Aldrich, St Louis, MO, USA, Cat. No. A9525) at a dosage of 1.44 mg·kg<sup>-1</sup>·d<sup>-1</sup> or saline for 4 weeks using Alzet osmotic pumps (model

2004; DURECT Corp.). The detailed manipulation was performed as previously described<sup>3</sup>. In the last week of the experiment, the mice underwent all physiologic assessments and then were sacrificed. The direct method was used to measure the outer diameter of the suprarenal aorta. After the aorta was dissected free from the surrounding connective tissue, a ruler was set aside and a picture was taken with a digital camera. The image of the aorta was used to measure the outer diameter of the suprarenal aorta by a researcher blind to group assignment. Suprarenal regions of abdominal aorta were identified between the last pair of intercostal arteries and the right renal branch. The adventitial circumferences at the maximal expanded portion of the suprarenal aorta were quantified as the maximal abdominal aortic diameter. The maximum width of the abdominal aorta was analyzed using Image Pro Plus software (Media Cybernetics) after adjusting the scale according to the ruler in aorta pictures. At least 3 measurements of the mean of each experimental group. Aneurysm formation was defined as the increase in the external width of the suprarenal aorta by 50% or greater compared with that in saline-infused mice as previously described.<sup>3</sup>

## Analyses and quantification of CaCl2-induced AAAs

We also induced AAA in mice (WT, SV-Tg or SV-KO) by periaortic application of 0.5 mol/l CaCl<sub>2</sub> as previously described with little modification<sup>4</sup>. Briefly, mice aged 8-12 weeks were anesthetized and underwent laparotomy. The abdominal aorta between the renal arteries and bifurcation of the iliac arteries was dissociated from the surrounding retroperitoneal structures under a stereo microscope (Nikon). Then, 0.5 mol/l CaCl<sub>2</sub>-treated cotton gauze was applied to the external surface of the aorta. NaCl (0.9%) was used in the sham control mice instead of CaCl<sub>2</sub>. After 15 minutes the incision was closed after aorta was rinsed with saline and the mice were allowed to recover. Three (SV-KO) or six (SV-Tg) weeks later, the mice were sacrificed and underwent laparotomy. Infrarenal regions of abdominal aorta were identified between the right renal branch and bifurcation of the iliac arteries. The methods used for quantification of CaCl<sub>2</sub>-induced AAAs are described in Methods section of Ang II-induced AAAs on infrarenal regions instead of suprarenal regions. The aortic internal diameter of mice was assessed in a blinded manner using a VisualSonics Vevo770 ultrasound biomicroscope (VisualSonics Inc, Toronto, ON, Canada) with a 30-MHz linear array ultrasound transducer and the inner lumen diameter.

### Blood pressure and serum lipid measurements

The heart rate and systolic blood pressure of the animals were measured using tail-cuff plethysmography (BP-2000 System; Visitech Systems, Apex, NC) as described previously<sup>1</sup>. Blood samples were obtained from the mice before sacrifice. Serum cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were measured at the clinical laboratory of Peking Union Medical College Hospital.

### Transit-time (TT) method for performing pulse-wave velocity (PWV) measurements

The TT method estimates the PWV from the pressure wave transit time between two measurement locations separated by a known distance as previously described<sup>5</sup>. Pulse-wave Doppler ultrasound velocity measurements were consecutively performed at the distal and proximal locations while

simultaneously recording the electrocardiogram (ECG) signal over a short period, during which the mouse was observed to have stable heart and breathing rates. TT was determined by subtracting the distal arrival time between the ECG R-wave peak and the foot of the velocity upstroke from the similarly determined proximal arrival time. As a threshold for consistent detection of the upstroke, arrival times were calculated relative to the time at which 20% of the peak value was attained. The left common carotid artery (LCCA) was chosen as a measurement target because it was easily accessible and offered a uniform and branch-free pathway of over approximately 10 mm between the aortic arch and the bifurcation. The distance between measurement sites was determined from a B-mode image encompassing both the distal and proximal locations. Proximal velocity measurements were taken 1 mm downstream from the aortic arch. Distal velocity measurements were taken 1.5 mm upstream from the bifurcation.

TT measurements were performed using a VisualSonics Vevo770 ultrasound biomicroscope (VisualSonics Inc., Toronto, ON, Canada). The mechanically scanned, single-element transducer had a 6-mm focal length and an f-number of 2. B-mode data were acquired at 40 MHz with a 30 frame-persecond (fps) frame rate. Doppler data were acquired at 30 MHz with a 50 kHz pulse-repetition frequency (PRF). The ECG signal was detected using a heart rate monitoring system (VisualSonics Inc., Toronto, ON, Canada) smoothed with a 1-kHz low-pass filter and digitized by the Vevo770 system at 8 kHz. The results were processed using VisualSonics analysis software.

## SIRT1 deacetylase activity assay

SIRT1 activity was assayed using a SIRT1 deacetylase activity assay kit (Sigma) according to the manufacturer's instructions. Briefly, SIRT1 was immunoprecipitated using a SIRT1 antibody (Millipore) from whole-aorta homogenates (200  $\mu$ g of protein) in immunoprecipitation (IP) buffer. The SIRT1 substrate reagent and NAD<sup>+</sup> were added to the SIRT1-conjugated beads (sc-2002; Santa Cruz Biotechnology) and incubated at 37 °C for 80 minutes after final washing. The substrate-SIRT1 mixture was placed on a 96-well plate, and the developer reagent was added to the wells at 37 °C for 20 minutes. The plate was read using a spectrophotometer with an excitation wavelength of 360 nm and an emission wavelength of 445 nm (Synergy 4 microplate reader; BioTek).

# SA-β-gal activity assay

SA- $\beta$ -gal activity was quantitatively measured according to the rate of conversion of 4methylumbelliferyl- $\beta$ -d-galactopyranoside (MUG) to the fluorescent hydrolysis product 4methylumbelliferone (4-MU) at pH 6.0, as described previously<sup>6</sup>. Briefly, aorta tissues were homogenized in the lysis buffer (5 mM CHAPS, 40 mM citric acid, 40 mM sodium phosphate, 0.5 mM benzamidine, and 0.25 mM PMSF, pH 6.0) and kept on ice for 1 h. The lysates were centrifuged at 12,000 × *g* for 5 mins, and the supernatant was mixed with 2× reaction buffer (40 mM citric acid, 40 mM sodium phosphate, 300 mM NaCl, 10 mM  $\beta$ -mercaptoethanol, and 4 mM MgCl<sub>2</sub> [pH 6.0] with 1.7 mM MUG), which was placed into a 37 °C water bath for 3 h. We added 50 µL reaction mix to 500 µL 400 mM sodium carbonate stop solution (pH 11.0), which was read at 150 µL/well in a 96-well plate using a Synergy 4 plate reader (BioTek) with excitation at 360 nm and emission at 465 nm. Normalized SA- $\beta$ -gal activity was expressed as the observed fluorescence divided by the mg of total protein in the assay. The whole-aorta tissues were stained to determine SA- $\beta$ -gal activity using a commercial kit (ab65351; Abcam) according to the manufacturer's instructions, as previously reported<sup>7</sup>. Briefly, the aortas were fixed in 2% formaldehyde containing 0.2% glutaraldehyde for 15 minutes and washed with PBS twice; subsequently, samples were incubated at 37 °C for 24 h in the staining solution (1 mg/ml X-gal in dimethylformamide, 5 nM potassium ferrocyanide, 5 nM potassium ferricyanide, 40 mM citric acid/sodium phosphate, 0.15 M NaCl, and 2 mM MgCl<sub>2</sub>, pH 5.9). The cells with blue color were considered SA- $\beta$ -gal positive. In primary vascular smooth muscle cells (VSMCs), SA-b-gal activity was also determined as previously described.<sup>8</sup> VSMCs were counterstained with 2.5 µg mL<sup>-1</sup> DAPI, and the proportion of SA-b-gal activity-positive cells was quantified using light and fluorescence microscopy.

### **Histological analyses**

After hemodynamic measurements, the animals were sacrificed. After the mice were sacrificed, aortas from the ascending aorta to the bifurcation of the common iliac artery were isolated. After macroscopic analysis, suprarenal abdominal aortas were subjected to histology analysis. For morphological analysis, the aortas were perfused with normal saline and fixed with 4% paraformaldehyde-PBS at physiological pressure for 5 minutes. Whole aortas were harvested, fixed for 24 h, and embedded in paraffin. For characterization of cross sections, aortic sections were collected serially from the proximal to the distal aorta. Histology was determined in sections (5 µm each) that were taken from suprarenal regions of abdominal aorta at intervals of 500 µm. At least 10 sections were analyzed per mouse. Paraffin sections were stained with elastin van Gieson staining or used for immunostaining. For the semi-quantification of elastin degradation, we used a standard for the elastin degradation score as previously described<sup>3</sup>. The grades were as follows: score 1, no degradation; score 2, mild elastin degradation; score 3, severe elastin degradation; score 4, aortic rupture. Elastic fiber content was quantified as described previously.<sup>9</sup> Briefly, after the cross sections were stained with elastin van Gieson staining, elastic fiber content was quantified in five separate representative images of each section by a single blinded observer using Image Pro Plus software (Media Cybernetics) after adjusting the scale. The areas of aortic media and the elastic fibers were calculated after they were individually outlined. The respective areas were averaged from all the images of a given aortic section and the ratio of elastic fiber content to total aortic media was determined.

### Immunohistochemistry

Slides were deparaffinized, and endogenous peroxidase activity was quenched with 3% (vol./vol.) hydrogen peroxide in 10% PBS for 10 mins. Nonspecific binding sites were blocked with 10% bovine serum in PBS at room temperature for 1 h. The slides were incubated at 4 °C overnight with diluted primary antibodies and with biotinylated secondary antibody at 37 °C for 30 mins and subsequently with horseradish peroxidase-labeled streptavidin solution at 37 °C for 20 mins. The slides were then stained with diaminobenzidine and counterstained with hematoxylin. The primary antibodies used were human SIRT1 (1104-1, 1:250 dilution; EPITOMICS),  $\alpha$ -smooth muscle actin (A5691, 1:400 dilution; Sigma-Aldrich), p21 (ab2961, 1:80 dilution; Abcam), MCP-1 (sc-1784, 1:400 dilution; Santa Cruz), leukocyte common antigen, CD45 (clone Ly-5, 1:100 dilution; BD Pharmingen), 8-OH-dG (MOG-020P, 1:100 dilution; Japan Institute for the Control of Aging), and nitrotyrosine (06-284, 1:500 dilution;

Millipore). The medial 8-OH-dG or nitrotyrosine content in the suprarenal aortic wall of mice was analyzed by calculating the integration optical density value of positive staining within the media using Image-Pro Plus software (Media Cybernetics). A mean value was determined from at least four sections in each animal.

# Immunofluorescent staining

Suprarenal abdominal aortas from young and aged mice were subjected to immunofluorescent analysis. The aortas were fixed with 4% paraformaldehyde-PBS for 24 hours, embedded in paraffin, and serial sections (5  $\mu$ m each) at 500  $\mu$ m intervals were made using suprarenal abdominal aortas as previously described.<sup>3</sup> After slides were deparaffinized, nonspecific binding sites were blocked in 10% bovine serum in PBS at room temperature for 1 h. The slides were then incubated at 4 °C overnight with diluted primary antibodies and with fluorescent-coupled secondary antibodies at 37 °C for 45 mins in the dark followed by DAPI for 5 mins at room temperature in the dark. The slides were then rinsed with PBS and evaluated under a fluorescence microscope. The primary antibodies used were human SIRT1 (LS-B4520, 1:100 dilution; LifeSpan Biosciences), mouse SIRT1 (07-131, 1:100 dilution; Millipore), and  $\alpha$ -smooth muscle actin (A5691, 1:100 dilution; Sigma-Aldrich).

### Cell culture, adenovirus generation and infection

Primary VSMCs were isolated from 2- to 3-month-old male mice and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% FBS at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air as previously described<sup>9</sup>. Passages 3 to 6 of VSMCs at 70-80% confluence were used for experiments. Human aortic smooth muscle cells (HASMCs) were purchased from ScienCell (Cat No: 6110, ScienCell) and cultured in Smooth Muscle Cell Medium (SMCM, Cat No: 1101, ScienCell) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, smooth muscle cell growth supplement (SMCGS, Cat. No. 1152, ScienCell), and 10% FBS. The adenovirus-mediated knockdown of SIRT1 (Ad-SIRT1 RNAi), p21 (Ad-p21 RNAi) and control vector (Ad-U6) were generated using the AdEasy vector kit (Quantum Biotechnologies) as described in the supplier's protocol. The RNAi sequences were reported previously<sup>10, 11</sup>. The VSMCs were infected for 24 hours with the above adenovirus at an MOI of 100, washed and incubated in 10% FBS-medium without virus and treated with saline, 100 nM angiotensin II (Sigma) or 10 mM CaCl<sub>2</sub> (Sigma) for 24 hours.

### **Real-time PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of total RNA was used for the first-strand synthesis with cDNA M-MuLV Reverse Transcriptase (New England Biolabs) using random primers. The QuantiTect SYBR Green RT-PCR Kit (QIAGEN) was used for the amplification reactions using the 1-step protocol described by the manufacturer with the Applied Biosystems Real-time PCR Detection System. The fluorescence curves

were analyzed using StepOne Software (Version 2.1). The primers are listed in Online Table VIII.

# Western blot analyses

Western blots were performed as described previously<sup>12</sup>. The primary antibodies used were human SIRT1 (1104-1, 1:1000 dilution; EPITOMICS), human MCP-1 (ab9669, 1:1000 dilution; Abcam), human and mouse MMP2 (sc-13595, 1:1000 dilution; Santa Cruz), human and mouse GAPDH (5174, 1:3000 dilution; Cell Signaling Technology),  $\beta$ -actin (4970, 1:3000 dilution; Cell Signaling Technology), mouse SIRT1 (07-131, 1:1000 dilution; Millipore), mouse p53 (Sc-73566, 1:1000 dilution; Santa Cruz), mouse Ac-p53 (06-758, 1:500 dilution; Millipore), mouse p21 (05-345, 1:1000 dilution; Millipore), mouse MCP-1 (ab7202, 1:2000 dilution; Abcam), mouse MT1-MMP (ab51074, 1:2000 dilution; Abcam), mouse TIMP2 (ab1828, 1:2000 dilution; Abcam), mouse TIMP1 (sc-5538, 1:1500 dilution; Santa Cruz), mouse p65 (8242, 1:1000 dilution; Cell Signaling Technology), mouse Phospho-NF- $\kappa$ B p65 (Ser536) (3033, 1:1000 dilution; Cell Signaling Technology). Western blots were quantified densitometrically using Quantity One software (Bio-Rad), and the intensity values were normalized to GAPDH or  $\beta$ -actin.

### Matrix metalloproteinase (MMP) activity

The detection of MMP activity was performed as previously reported<sup>13</sup>. Five micrograms of protein in aorta homogenates was electrophoresed in SDS-PAGE gels containing gelatin. The gels were washed with 2.5% Triton X-100 for 30 mins and incubated for 12-40 h in zymography development buffer at 37 °C. The gel was subsequently stained with Coomassie brilliant blue.

# Chromatin immunoprecipitation

ChIP assays were carried out as previously described.<sup>14</sup> Briefly, cells or tiny tissue pieces were crosslinked with 1% formaldehyde at room temperature for 10 min. The reaction was quenched with 125 mM glycine. Cells were collected, washed twice with cold PBS and resuspended in cell collection buffer (100 mM Tris-HCl, pH 9.4, with 10 mM DTT and complete protease inhibitor cocktail (Sigma) at room temperature for 30 min and 30 °C for 15 min. Nucleus/Chromatin preparation buffers I and II (buffer I: 10 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5, with 0.25% Triton X-100; buffer II:1 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5, 200 mM NaCl; Both are freshly added with complete protease inhibitor cocktail (Sigma)) were used to isolate nuclei. The pellet of nuclei was washed with cold PBS and resuspended in SDS lysis buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 0.5% Empigen BB (Sigma), 1% SDS and Complete Protease Inhibitor (Sigma)). Nuclear extracts were sonicated to generate chromatin fragment with an average size of 0.3 kb on a Bioruptor Plus sonication system (Diagenode). Chromatin lysate was cleared by centrifugation, diluted with 10 volumes of ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1, with 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl and Complete Protease Inhibitor; precleared with Dynabead protein A (Invitrogen) and subjected to immunoprecipitation with ChIP-grade antibody against normal rabbit IgG (ab46540, Abcam)or RelA/p65 (ab7970, Abcam). DNA-protein complex was precipitated with Dynabead protein A (Invitrogen), eluted in washing buffers I-IV. (buffer I: 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton X-100, 150 mM NaCl; buffer II: 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton X-100, 500 mM NaCl; buffer III: 10 mM Tris-HCl, pH 8.1, 1 mM EDTA, 250 mM LiCl, 1% Deoxycholate (Sigma), 1% NP-40; buffer IV: 10 mM Tris-HCl, pH 8.1, 1 mM EDTA) and treated with Proteinase K and RNase A in turn to reverse cross-links. DNA was purified with the QIAquick PCR purification kit or the Mini-Elute PCR purification kit (Qiagen) and analyzed by quantitative RT-PCR (LightCycler 96, Roche) with primers that targeted interesting DNA sequences. We analyzed mouse or human MCP-1 promoters using AliBaba2.1 (www.generegulation.com/pub/programs/alibaba2) to find the potential binding sites of interested transcription factors. Primer sequences were listed in Online Table IX.

### Statistical analyses

For all statistical tests, a P value of <0.05 was considered statistically significant, and all tests were 2tailed. Fisher's exact test was applied to the comparisons of AAA incidence and log-rank (Mantel-Cox) test was used for survival comparison between groups. Normality tests were assessed via the Shapiro-Wilk statistics with SPSS software package (version 19.0). Normally distributed datasets were analyzed with the unpaired Student's *t* test for 2 independent groups or paired *t* test for 2 dependent groups, and the one-way analysis of variance (ANOVA) followed by the post Bonferroni's multiple comparisons test for  $\geq$ 3 groups. Quantitative results are expressed as the mean ± standard deviation (SD) for normally distributed datasets. Where a normal distribution could not be confirmed, nonparametric tests were used (Mann-Whitney U test for 2 independent group, Wilcoxon signed rank test for 2 paired groups and the Kruskal-Wallis test with post Dunn's multiple comparisons test for  $\geq$ 3 groups) and quantitative results are expressed as the median with interquartile range. All statistical analyses were performed using GraphPad Prism (version 6.01) software and the detailed statistical methods were listed in Online Table X.



## **Online Figures and legends**

**Online Figure I** SIRT1 expression in human AAAs and in aged mouse aortas. (A) The expression of SIRT1 and several VSMC-related molecules in human AAA samples (5, 6). (B) Western blots of SIRT1 and p21 proteins in the different sections of aorta homogenates from young and aged mice. (C) Densitometric analysis of the protein level of SIRT1 in the different regions of aortas from young and aged mice. The numbers on the top indicate the SIRT1 reduction in aged aorta compared with that in young aorta. The whole aorta is divided into three parts as follows: the proximal thoracic aorta (the ascending aorta and transverse aortic arch), the distal thoracic aorta (the descending thoracic aorta) and the abdominal aorta. Adventitial tissue was removed from the aorta as much as possible and each vascular section from five aortas was pooled together for immunoblot. TA, thoracic aorta. AA, abdominal aorta.



**Online Figure II** Generation and assessment of a VSMC-specific *Sirt1* KO mouse model. (A) Strategy applied to delete *Sirt1* in mouse VSMCs. Sirt1 exon 4 is flanked by LoxP sites. (**B-C**) PCR genotyping on genomic DNA obtained from a WT (*Sirt1*<sup>+/+</sup> and *SM22a-Cre*<sup>-/-</sup>), a heterozygous (*Sirt1*<sup>flox/+</sup> and *SM22a-Cre*<sup>+/-</sup>), and a homozygous (*Sirt1*<sup>flox/flox</sup> and *SM22a-Cre*<sup>+/+</sup>) mouse. (**D**) Western blotting for assessing the expression of Sirt1 in mouse VSMCs obtained from the aortas of control mice (*SM22a-Cre*<sup>-/-</sup>; *Sirt1*<sup>flox/flox</sup>) and VSMC-specific *Sirt1* KO mice (*SM22a-Cre*<sup>+/-</sup>; *Sirt1*<sup>flox/flox</sup>). (**E**) Quantitative analysis of SIRT1 activity in aorta homogenates from WT and SV-KO mice (*n*=6 per group). (**F**) Representative Western blots of SIRT1 in the tissues of WT and SV-KO mice.



**Online Figure III** VSMC-specific SIRT1 ablation promotes AAA formation and ROS production induced by Ang II infusion. (**A**) The ratio of aorta weight to body weight in saline- and Ang II-infused WT or SV-KO mice. (**B**) The elastin fiber content in suprarenal aortas from saline- and Ang II-infused WT or SV-KO mice. (**C**) Representative immunostaining and densitometric analysis of the medial nitrotyrosine in the suprarenal aortic wall of WT and SV-KO mice infused with saline or Ang II for 4 weeks (n=5-6 per group). Scale bars, 50 µm. (**D**) Representative immunostaining and densitometric analysis of the medial 8-OH-dG content in the suprarenal aortic wall of WT and SV-KO mice infused with saline or Ang II for 4 with saline or Ang II for 4 weeks (n=5-6 per group). Scale bars, 50 µm.



**Online Figure IV** Increased inflammatory response and MMP activation in the aortas of Ang IIinfused SV-KO mice. (A) Representative immunostaining and densitometric analysis of CD45 in the suprarenal aortic wall of WT and SV-KO mice infused with saline or Ang II for 4 weeks. The arrows show representative staining with CD45 antibody (n=6-9 per group). Scale bars, 50  $\mu$ m. (B) Representative immunostaining of MCP-1/CCL2 in the suprarenal aortic wall of saline and Ang IIinfused mice (scale bars, 50  $\mu$ m). (C) Aorta homogenates were obtained from WT and SV-KO mice infused with saline or Ang II for 4 weeks and MMP activities were examined by zymography. Representative images and densitometric analysis of MMP activities in aorta homogenates (n=4-6 per group). (D) Aorta homogenates were obtained from WT and SV-KO mice infused with saline or Ang II for 4 weeks. Western blot and densitometric analysis of protein levels of the MT1-MMP, TIMP1, and TIMP2 in aorta homogenates (n=4-6 per group).



**Online Figure** V SIRT1 transgene attenuates AAA formation and ROS production in the aortas of Ang II-infused *Apoe<sup>-/-</sup>* mice. (A) The ratio of aorta weight to body weight in saline- and Ang II-infused *Apoe<sup>-/-</sup>* or SV-Tg *Apoe<sup>-/-</sup>* mice. (B) The elastin fiber content in suprarenal aortas from saline- and Ang II-infused *Apoe<sup>-/-</sup>* or SV-Tg *Apoe<sup>-/-</sup>* mice. (C) Representative immunostaining and densitometric analysis of the medial nitrotyrosine in the suprarenal aortic wall of *Apoe<sup>-/-</sup>* and SV-Tg *Apoe<sup>-/-</sup>* mice infused with saline or Ang II for 4 weeks (*n*=5-6 per group). Scale bars, 50 µm. (D) Representative immunostaining and densitometric analysis of the medial 8-OH-dG content in the suprarenal aortic wall of *Apoe<sup>-/-</sup>* mice infused with saline or Ang II for 4 weeks (*n*=5-6 per group). Scale bars, 50 µm. (D) Representative immunostaining and densitometric analysis of the medial 8-OH-dG content in the suprarenal aortic wall of *Apoe<sup>-/-</sup>* and SV-Tg *Apoe<sup>-/-</sup>* mice infused with saline or Ang II for 4 weeks (*n*=5-6 per group). Scale bars, 50 µm.



**Online Figure VI** SIRT1 transgene decreases inflammatory response and MMP activation in the aortas of Ang II-infused *Apoe<sup>-/-</sup>* mice. (A) Representative immunostaining and densitometric analysis of CD45 in the suprarenal aortic wall of *Apoe<sup>-/-</sup>* and SV-Tg *Apoe<sup>-/-</sup>* mice infused with saline or Ang II for 4 weeks. The arrows show representative staining with CD45 antibody (n=6-9 per group). Scale bars, 50 µm. (B) Representative immunostaining of MCP-1/CCL2 in the suprarenal aortic wall of saline and Ang II-infused mice (scale bars, 50 µm). (C) Aorta homogenates were obtained from *Apoe<sup>-/-</sup>* and SV-Tg *Apoe<sup>-/-</sup>* mice infused with saline or Ang II for 4 weeks and MMP activities were examined by zymography. Representative images and densitometric analysis of MMP activities in aorta homogenates (n=4-6 per group). (D) Aorta homogenates were obtained from *Apoe<sup>-/-</sup>* and SV-Tg *Apoe<sup>-/-</sup>* mice infused with saline or Ang II for 4 weeks. Western blot and densitometric analysis of protein levels of the MT1-MMP, TIMP1, and TIMP2 in aorta homogenates (n=4-6 per group).



**Online Figure VII** Increased MCP-1/CCL2 and MT1-MMP expression in the infrarenal aortas of CaCl<sub>2</sub>-induced SV-KO mice. (A) Representative immunostaining of MCP-1/CCL2 in the infrarenal aortic wall of WT and SV-KO mice 3 weeks after treated with saline or CaCl<sub>2</sub> (scale bars, 100  $\mu$ m). (B) Densitometric analysis of the medial MCP-1/CCL2 content in the infrarenal aortic wall of WT and SV-KO mice treated with saline or CaCl<sub>2</sub> (n=5 per group). (C) Representative immunostaining with MMP-2 in the infrarenal aortic wall of WT and SV-KO mice 3 weeks after treated with saline or CaCl<sub>2</sub> (scale bars, 100  $\mu$ m). (D) Densitometric analysis of the medial MMP2 content in the infrarenal aortic wall of WT and SV-KO mice treated with saline or CaCl<sub>2</sub> (n=5 per group).



**Online Figure VII** SIRT1 transgene inhibits CaCl<sub>2</sub>-induced AAA formation and related vascular pathological changes. (A) Representative photographs showing macroscopic features of aneurysms induced by CaCl<sub>2</sub> in WT and SV-Tg mice for 6 weeks (scale bars, 3 mm). (B) The ratio of aorta weight to body weight in saline- or CaCl<sub>2</sub>-treated mice. (C-D) The maximal internal (C) and external diameter (D) of infrarenal aortas in WT (n=5-8) and SV-Tg mice (n=5-8) six weeks after treatment with saline or CaCl<sub>2</sub>. (E) Representative staining with elastin in infrarenal aortas from saline- or CaCl<sub>2</sub>-treated mice (n=5-8 per group). (F) Elastin degradation score in infrarenal aortas from saline- or CaCl<sub>2</sub>-treated mice (n=5-8 per group). (G) Representative immunostaining and densitometric analysis of MCP-1/CCL2 in the infrarenal aortic wall of WT and SV-Tg mice 6 weeks after treated with saline or CaCl<sub>2</sub> (scale bars, 100  $\mu$ m) (n=5 per group). (H) Representative immunostaining and densitometric analysis of MMP-2 in the infrarenal aortic wall of WT and SV-Tg mice 6 weeks after treated with saline or CaCl<sub>2</sub> (scale bars, 100  $\mu$ m) (n=5 per group).



**Online Figure IX** VSMC-derived SIRT1 is involved in Ang II-induced vascular aging. (A) Representative staining of SA- $\beta$ -gal and  $\alpha$ -SMA in the abdominal aorta of saline and Ang II-infused *Apoe<sup>-/-</sup>* mice. Nuclei were counterstained by Nuclear Fast Red (scale bars, 50 µm). (**B**, **C**, **F**) SA- $\beta$ -gal activity assay results of aorta homogenates from WT and SV-KO (**B**), *Apoe<sup>-/-</sup>* and SV-Tg *Apoe<sup>-/-</sup>* (**C**), young and aged (**F**) mice infused with saline or Ang II (*n*=5-6 per group). (**D**-**E**) Quantitative analysis of PWV performed on the LCCA of indicated groups (*n*=6-10 per group).

# A

 $\lor$  Top Diseases and Bio Functions

$\vee$	Diseases	and	Disorders	
~	Diacuaca	unu	Diaoracia	

Name p-value range					
Inflammatory Response	€ 400 ÷ 2.77E-08 - 2.49E-46	329			
Connective Tissue Disorders		220			
Inflammatory Disease	9.56E-09 - 5.95E-40	258			
Skeletal and Muscular Disorders	+ 3.88E-08 - 5.95E-40	281			
Infectious Disease		142			
	1 2 3 4 5 6 7 8 9 >				

immune response regulati syste		tion of immune tem process	defense re	defense response		positive gulation of une system process	regulation of biological process	regulation of cytokine production	
response to external biotic stimulus	imm effe proc	une signal ctor transduction		regulation of response to stimulus	chemo	axis	taxis	regulation of	
leukocyte activation	respo ext stin	onse to ernal nulus	regulation of response immune res stimulus	ation positive regulation of response netresponse, c) ulus stimulus		se to ine adaptive immune response lar negative		multicellular organismal process negative regulation of biological	regulation of multicellular organismal process regulation of developmental
response to biotic stimulus	pos regu of res to st	itive organic lation ponse mulus positive regulation of cellular process		leukocyte migration positive regulation of s locomotion	respor chem stimu response to interferon-beta	nical ulus regulat intrace sign	regulation of cellular process tive tion of ellular hal chemotaxis	process	leukocyte cell-cell adhesion
cell surface receptor	resp	oonse	e cellular response to cytokine stimulus	response to chemical	cellular response to interferon-be	regulation of ERK1 regulation and ERK2 cascade negative		leukocyte aggregatio regulation	n positive regulation of leukocyte cell-cell
signaling pathway	to s	tress	leukocyte differentiatior	of response to stress	processing an presentation peptide antig	nd regul of of resp <sup>an</sup> to stir	ation ponse mulus	leukocyte cell-cell adhe	adhesion eSiON
immune system	1 proce	regi of c com mov	ellular of positive izati regulation of cellular	encom on mutualisi para	passing m throug sitism	gh C	ell activation	hiological adhesion	multi-organism process
		regul phage	ation of positive regulation of transpo	response n prt	response to stimulu		biological regulation	locomotion	gulation of cell killing
	0 5 10 15 20 25 30 35 40 45 50 The Degree of Up Regulation								

Up-regulated Biological Pathway

**Online Figure** X Results of IPA analysis. Gene expression microarray analysis was performed using RNA isolated from the aortas of Ang II-treated SV-KO mice and their *Sirt1*<sup>flox/flox</sup> littermates. Briefly, SV-KO mice and their *Sirt1*<sup>flox/flox</sup> littermates were subjected to Ang II infusion, and 4 arteries were collected at 4 weeks after Ang II infusion as one sample, and the samples from both SV-KO mice and their *Sirt1*<sup>flox/flox</sup> littermates were performed for the microarray assay. Microarray data analysis identified 1159 genes that were differentially expressed including 911 upregulated and 248 downregulated genes in the Ang II-treated SV-KO compared with those of Ang II-treated *Sirt1*<sup>flox/flox</sup> littermates. (A) Functional analysis of these genes using Ingenuity Pathway Analysis (IPA) software revealed that

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Inflammatory Response was the most affected functions. (**B**) The sub-categories of inflammatory response associated with inflammatory cell recruitment, infiltration and activation are significantly activated in aortas of Ang II-treated SV-KO mice compared with those of Ang II-treated *Sirt1*<sup>flox/flox</sup> littermates. The color represent the degree of up regulation and the size is determined by the  $-\log 10$  (p value).



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B

**Online Figure XI** VSMC-specific SIRT1 ablation activates NF-κB signaling pathway and NF-κB target genes including CCL2/MCP-1 in Ang II-induced AAAs (**A**) Canonical pathway analysis revealed that NF-κB signaling was strongly affected (z-score: 3.657, *P*-value: 4.03E-07) in aortas of Ang II-treated SV-KO mice compared with those of Ang II-treated Sirt1<sup>*flox/flox*</sup> littermates. (**B**) IPA upstream regulator analysis showed that NF-κB was significantly activated in aortas of Ang II-treated SV-KO mice compared with those of Ang II-treated Sirt1<sup>*flox/flox*</sup> littermates. (**B**) IPA upstream regulator analysis showed that NF-κB was significantly activated in aortas of Ang II-treated SV-KO mice compared with those of Ang II-treated Sirt1<sup>*flox/flox*</sup> littermates. It was the most important inflammatory transcription regulator (TR) altered. The target genes regulated by NF-κB in aortas of Ang II-treated SV-KO mice including MCP-1, also known as CCL2, a critical chemokine for inflammatory cell recruitment SV-KO mice. (**C**) The mRNA level of p65 in the aortas of SV-KO and WT mice infused with saline or Ang II for 4 weeks. (**D**) Representative Western blot of phosphorylation of p65 and total p65 in aorta homogenates from SV-KO and WT mice infused with saline or Ang II for 4 weeks.

6236	NATE NETBORES 400 TO MT PATH	BAT AND TO	RI LIST	CUSTONIZE TABLE			Consist	ency Sco	4.000 - 2.	646	(p1 of 5)	• 🖪 😥
ID	🗸 Consistency Score 🕱	Regulato	rs T 🗙	Iarget Molec	ules in F	)at aset			T	×	Diseases & Fu	unctions
1	4.000	+CCR2	all	*Cc12, *CCL5,	+Cc19, *	*CD40, *CD86, *	CXCL12, +CXCL:	2, *CXCL6, *	all	16	ectivation of	cells
2	3.873	IFNG	all	1+CCL17, +CCL1	, +Cc12,	+CCL5, +CCR2,	+CXCL12, +CXCI	L2, +CXCL6,	all	15	igration of	ayeloid cells
3	3.742	IFNG .	all	+CCL17, +CCL:	2, +Cc12,	+CCL5, +CXCL12	, +CXCL2, +CX	CL6, TCYBB,	all	14	igration of	granulocytes
4	3.606	Hbb-b1	all	1+Cel2, +Cel7,	+CCR2, *	*CCR5, *CD74, *	CIIIA, +CLEC7	A, +CXCL10,	all	131	eukocyte mig	ration
5	3.606	Hbb-b1	all	1+Cel2, +Cel7,	+CCR2, *	+CCR5, +CD74, +	CIIIA, +CLECT	A, +CXCL10,	all	13	ell novement	of blood c
6	3.606	Hbb-b2	all	*Cel2, *Cel7,	+CCR2, *	*CCR5, *CD74, *	CIIIA, +CLECT	A, +CXCL10,	all	13	eukocyte mig	ration
7	3.606	Hbb-b2	all	+Cc12, +Cc17,	+CCR2, 1	*CCR5, *CD74, *	CIIIA, *CLECT.	A, TCXCL10,	all	13	ell novement	of blood c
8	3.606	INFSF12	all	+CCL2, +Ce12,	+CCL5,	+CCR1, +CCR5, +	CXCL10, +CXCL:	2, +CXCL6, +	all	13	ell novement	of myeloid
9	3.606	Hbb-b2	all	+Cel2, +Cel7,	+CCR2, 4	+CCR5, +CD74, +	CIIIA, +CLEC7	A, TCXCL10,	all	13	igration of	cells
10	3.606	Hbb-b1	all	+Cc12, +Cc17,	+CCR2,	+CCR5, +CD74, +	CIIIA, +CLECT	A, +CXCL10,	all	13	igration of	cells
11	3.606	THE SE 12	all	+CCL2, +Ce12,	+CCL5,	+CCR1, +CCR5, +	CXCL10, +CXCL:	2, +CXCL6, +	all	13	ell novement	of leukocyte
12	3.606	Hbb-b2	all	+Cel2, +Cel7,	+CCR2,	*CCR5, *CD74, *	CIIIA, +CLEC7	A, TCXCL10,	all	13	ell movement	
13	3.606	Hbb-b1	all	+Cc12, +Cc17,	+CCR2,	+CCR5, +CD74, +	CIIIA, +CLECT	A, +CXCL10,	all	13	ell novement	
14	3.479	+CCR2	all	+Ce12, +CCL5,	+CD40, *	CD86, +CXCL6,	+IL18, +IL1B,	+IL6, +IIGA	L,all	10	ectivation of	blood cells
15	3.479	+CCR2	all	1+Cel2, +CCL5,	+CD40, 1	*CDS6, *CXCL6, *	*IL18, *IL1B,	+IL6, +IIGA	L,all	10	ectivation of	leukocytes
16	3.464	Nrth	all	+Ce12, +CCL5,	+CCR2, +	+CCR5, +CCR7, +	CD40, +CXCL10,	+IL18, +IL	6,all	12	ell novement	of lymphoc
17	3. 464	INFSF12	all	+CCL2, +Ce12,	+CCL5, *	+CCR1, +CXCL10,	+CXCL2, +CXCI	L6, +CICR5,	all	12	ell novement	of granulo
18	3.464	INFSF12	all	+CCL2, +Cc12,	+CCL5,	+CCR1, +CCR5, +	CXCL10, +CXCL:	2, +CXCL6, +	all	12	ell novement	of phagocyte
19	3.441	INF	all	+ALB, +CCL17,	+CCL2, *	*Cel2, *CCL5, *	CCR1, +CXCL12,	+CICL2, +.	all	19	igration of	myeloid cells
20	3. 333	TIL 1B	all	+CCL17, +CCL	2, +Cc12,	+CCL5, +CXCL2,	+CXCL6, +IL6,	+IIGAM, +S	AA1 all	1 9	igration of	granulocytes
21	3. 333	+Cc12	all	+CCL5, +Ce17,	+Cc19, *	*CXCL10, *CXCL2	, +EIS1, +IGF	1, +IL1B, +I	16all	1 9	quantity of 1	eukocytes
22	3. 333	+Cel2	all	+CCL5, +Cel7,	+Cc19, *	+CXCL10, +CXCL2	, +EIS1, +IGF	1, +IL1B, +I	16all	1 99	quantity of c	ells
23	3. 333	+Cc12	all	+CCL5, +Ce17,	+Cc19, *	+CXCL10, +CXCL2	, +EIS1, +IGF	1, *IL18, *I	16all	1 9	unntity of b	lood cells
24	3. 328	Nrlh	all	+Cel2, +CCL5,	+CCR2, *	+CCR5, +CCR7, +	CD40, +CXCL10,	+HCK, +IL1	B,all	13	ell movement	of mononuc
25	3. 317	INSIG1	all	+CCL17, +CCL	, +CCR5,	+CCR7, +CXCL10	, +CXCL13, +C	CL2, +CXCL6	,all	11	ell novement	of phagocyte
26	3. 317	INFSF12	all	+CCL2, +Ce12,	+CCL5,	+CCR1, +CXCL10,	+CXCL2, +CXCI	L6, +IL1B, +	all	11	ell novenent	of neutrop
27	3, 207	P38 MAPK	all	+CCL2. +Ce12.	+CCLS.	+CD40, +CD86, +	CXCL10, +CXCL	12. +CYEE. +		14	ccumulation	of cells
28	3, 207	IFNG	all	+CCL2. +Ce12.	+CCLS.	+CCR2, +CISS, +	CXCL12, +CXCR	. +FPR2. +.		14	ell novenent	of monocytes
29	3, 182	+CCR2	all	+Ce12. +CCL5.	+CD40.	+CXCL6. +IL18.	+ IL 18. + IL 6. 1	SLPI	all	1 8	ctivation of	phagocytes
30	3 175	NULL /		+CC12. +Cc12	+0015	+CCR7 +CD40 +	CD86. +CYCI 12	+CTC1 2. +		12	rangeigratio	n of calls

**Online Figure XI** Most related Regulator Effects in IPA analysis. The Regulator Effects feature in IPA empowers to generate a hypothesis for how a phenotype, function or disease is regulated in the dataset by activated or inhibited upstream regulators. Regulator Effects in IPA analysis showed that the consistency Score of CCR2, the receptor of MCP-1/CCL2 was highest in all the regulators. NF- $\kappa$ B and MCP-1/CCL2 also showed high Consistency Scores for inflammatory cell recruitment, infiltration and activation.



**Online Figure XIII** Loss-of-function of SIRT1 in VSMCs increases NF- $\kappa$ B binding on MCP-1 promoter. Chromatin immunoprecipitation (ChIP) assays were performed with chromatin prepared from aortas of saline and Ang II-infused WT and SV-KO mice. Chromatin was immunoprecipitated with normal rabbit IgG or antibody against p65/RelA, and precipitated genomic DNA was analyzed by real-time polymerase chain reaction using different primers for the indicated areas of the MCP-1 promoters or 3' -UTR region. (A) Diagrammatic drawing shows that the putative binding sites on mouse MCP-1 promoters predicted by software. (B) ChIP-qPCR results of binding levels of IgG and p65/RelA on MCP-1 promoter and 3'-UTR. (n=3 per group)



**Online Figure XIV** SIRT1 overexpression inhibits Ang II-increased NF-κB binding on MCP-1 promoter. Chromatin immunoprecipitation (ChIP) assays were performed with chromatin prepared from aortas of saline and Ang II-infused *Apoe<sup>-/-</sup>* and SV-Tg *Apoe<sup>-/-</sup>* mice. Chromatin was immunoprecipitated with normal rabbit IgG or antibody against p65/RelA, and precipitated genomic DNA was analyzed by real-time polymerase chain reaction using different primers for the indicated areas of the MCP-1 promoters or 3' -UTR region. ChIP-qPCR results of binding levels of IgG and p65/RelA on MCP-1 promoter are showed. (n=3 per group)



**Online Figure XV** p21 knockout blocks AngII-induced AAAs and expression of MMP-2 and MCP-1 in *Apoe<sup>-/-</sup>* mice. (**A**) Relative mRNA level of p21 in *Apoe<sup>-/-</sup>* and *p21<sup>-/-</sup>Apoe<sup>-/-</sup>* mice (n=6). (**B**) The incidence of AngII-induced AAA in *p21<sup>-/-</sup>Apoe<sup>-/-</sup>* mice (*n*=11) compared with that in *Apoe<sup>-/-</sup>* mice (*n*=16). There was no AAA formation in saline-infused mice (*n*=6 per group), and all deaths were due to aortic rupture. (**C**) The ratio of aorta weight to body weight in saline- and AngII-infused mice. (**D-E**) mRNA levels of MMP-2 (D) and MCP-1 (E) detected by real-time PCR in aorta homogenates from *Apoe<sup>-/-</sup>* and *p21<sup>-/-</sup>Apoe<sup>-/-</sup>* mice infused with Ang II (*n*=11 for *Apoe<sup>-/-</sup>*; *n=*9 for *p21<sup>-/-</sup>Apoe<sup>-/-</sup> group*) or saline (*n*=6 per group).



**Online Figure XVI** p21 mRNA level in both saline and Ang II-treated VSMCs was significantly repressed by transfection of Ad-p21 RNAi. Relative p21 mRNA expression level detected by real-time PCR in saline or Ang II-treated WT and SV-KO VSMCs infected with Ad-U6 or Ad-p21 RNAi (n=3-6 per group).



**Online Figure XVII** Ang II treatment increases NF- $\kappa$ B binding on human MCP-1 promoter. Chromatin immunoprecipitation (ChIP) assays were performed with chromatin prepared from saline or Ang II-treated human VSMCs. Chromatin was immunoprecipitated with normal rabbit IgG or antibody against RelA/p65, and precipitated genomic DNA was analyzed by real-time polymerase chain reaction using different primers for the indicated areas of the MCP-1 promoters or 3' -UTR region. Three binding sites of NF- $\kappa$ B (-1092 to -1082; -593 to -583; -134 to -124) on human MCP-1/CCL2 promoter by ChIP assays were selected out from the 5 potential binding sites to assess the impact of p21 knockdown on the effect of SIRT1 RNAi on transcriptional activation of MCP-1/CCL2. (A) Diagrammatic drawing shows that the putative binding sites of NF- $\kappa$ B on human MCP-1 promoters predicted by software. (B) ChIP-qPCR results of binding levels of IgG, RelA/p65 on MCP-1 promoter are showed. (*n*=3 per group)



**Online Figure XVIII** Schematic summary. Our results show a direct link between vascular cell senescence and vascular inflammation to AAA formation by SIRT1 reduction and that effective SIRT1 activation in VSMCs might prevent AAA formation. (**A**) SIRT1 expression/activity is decreased in the medial VSMCs of the suprarenal aortas of aged mice. The reduction of SIRT1 increases vascular cell senescence and upregulates the expression of p21 in response to AngII infusion. Accordingly, NF- $\kappa$ B binding activity and MCP-1 expression are increased in VSMCs, which promotes inflammatory cell recruitment to the media, magnifies vascular inflammation and instigates AAA formation. (**B**) VSMC-specific overexpression of SIRT1 can effectively inhibit AAA formation and related pathological and molecular changes.

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