

# Angiotensin II Type 1 Receptor-Associated Protein Regulates Kidney Aging and Lifespan Independent of Angiotensin

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**Background**—The kidney is easily affected by aging-associated changes, including glomerulosclerosis, tubular atrophy, and interstitial fibrosis. Particularly, renal tubulointerstitial fibrosis is a final common pathway in most forms of progressive renal disease. Angiotensin II type 1 receptor (AT1R)-associated protein (ATRAP), which was originally identified as a molecule that binds to AT1R, is highly expressed in the kidney. Previously, we have shown that ATRAP suppresses hyperactivation of AT1R signaling, but does not affect physiological AT1R signaling.

**Methods and Results**—We hypothesized that ATRAP has a novel functional role in the physiological age-degenerative process, independent of modulation of AT1R signaling. ATRAP-knockout mice were used to study the functional involvement of ATRAP in the aging. ATRAP-knockout mice exhibit a normal age-associated appearance without any evident alterations in physiological parameters, including blood pressure and cardiovascular and metabolic phenotypes. However, in ATRAP-knockout mice compared with wild-type mice, the following takes place: (1) age-associated renal function decline and tubulointerstitial fibrosis are more enhanced; (2) renal tubular mitochondrial abnormalities and subsequent increases in the production of reactive oxygen species are more advanced; and (3) life span is 18.4% shorter (median life span, 100.4 versus 123.1 weeks). As a key mechanism, age-related pathological changes in the kidney of ATRAP-knockout mice correlated with decreased expression of the prosurvival gene, *Sirtuin1*. On the other hand, chronic angiotensin II infusion did not affect renal sirtuin1 expression in wild-type mice.

**Conclusions**—These results indicate that ATRAP plays an important role in inhibiting kidney aging, possibly through sirtuin1-mediated mechanism independent of blocking AT1R signaling, and further protecting normal life span. (*J Am Heart Assoc.* 2017;6:e006120. DOI: 10.1161/JAHA.117.006120.)

**Key Words:** aging • chronic kidney disease • fibrosis • renin angiotensin system

Aging is defined as the degeneration of physiological functions required for survival and reproductive ability as a result of increased age. Many factors, including genetic, temporal, and environmental aspects, are involved in this process.<sup>1,2</sup> In humans, incidence of aging-associated diseases, such as arteriosclerotic disease, osteoporosis,

dementia, and cancer, is increasing worldwide. It has been established that certain conditions, such as hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking, can hasten the aging process.<sup>3</sup> In contrast, aging can be slowed by improving lifestyle habits, such as engaging in moderate exercise and limiting caloric intake. Therefore, the

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## Clinical Perspective

### What Is New?

- Angiotensin II type 1 receptor-associated protein inhibits age-associated renal function decline and tubulointerstitial fibrosis through a novel mechanism other than inhibition of the angiotensin II/angiotensin II type 1 receptor signaling pathway.
- Angiotensin II type 1 receptor-associated protein inhibits age-associated decrease in renal sirtuin1 expression.

### What Are the Clinical Implications?

- The findings of the present study suggest that an angiotensin II type 1 receptor-associated protein activation therapy could ameliorate the progression of chronic kidney disease that accompanies aging and further lengthen life span.

pathophysiology of aging is extremely complex and can be influenced by a diverse range of factors.

On a molecular level, aging is regulated by both an attenuation of inhibitory factors and the potentiation of augmenting factors. Aging inhibitory factors include sirtuin1 (SIRT1), which was detected in studies examining the life-span-extending effects of caloric restriction, and Klotho, which was identified in mice that exhibited premature senility.<sup>4,5</sup> Furthermore, advanced glycation end products, transforming growth factor- $\beta$  (TGF- $\beta$ ), oxidative stress, and angiotensin II (Ang II) have been implicated as aging augmenting factors.<sup>3,6–8</sup>

Ang II type 1 receptor (AT1R)-associated protein (ATRAP) was originally identified as a functional molecule that binds to the carboxyl-terminal domain of AT1R.<sup>9,10</sup> Expression of ATRAP has been observed in the heart, kidneys, vascular smooth muscle cells, and adipose tissue, with the strongest expression observed in the kidneys. ATRAP has been suggested to selectively inhibit the excessive activation of AT1R signaling when various pathological stimuli (eg, exogenous Ang II administration, cuff vascular injury, and high-sodium diet) are applied.<sup>11,12</sup> To this end, ATRAP functions as an endogenous inhibitor that suppresses hypertension and organ dysfunction. However, ATRAP does not evidently affect the physiological AT1R signaling pathway under conditions without pathological stimuli.

It has been reported that ATRAP may directly interact with several molecules other than AT1R, and that ATRAP may exert distinct functions independent of modulation of AT1R signaling. These possible functions include regulation of sarcoplasmic/endoplasmic reticulum calcium ATPase 2a activation and inhibition of the calcineurin/nuclear factor of activated T cells (NFAT) pathway through binding to calcium-modulating cyclophilin ligand (CAML).<sup>13–15</sup> However, the physiological

and pathological significance of the interactions between ATRAP and additional molecules remains unclear.

In this study, we hypothesized that ATRAP has a functional role in the long-term process of aging, independent of modulation of AT1R signaling. In ATRAP-knockout (KO) mice, age-associated renal function decline and fibrosis were more enhanced than in wild-type control (WT) mice, and their life spans were also shortened. Furthermore, the age-associated exacerbation of renal function decline and fibrosis in ATRAP-KO mice significantly correlated with the decreased expression of the prosurvival gene, SIRT1, which appeared to be independent of the Ang II-AT1R signaling pathway.

## Materials and Methods

This study was performed in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal studies were reviewed and approved by the Animal Studies Committee of Yokohama City University (Yokohama, Japan).

### Animals

ATRAP-KO mice generated on a C57BL/6 background by targeted gene disruption were used in the study.<sup>16,17</sup> All experiments were performed with age-matched ATRAP-KO mice and WT mice (3–4, 11–12, and 22–25 months old; n=6–11 in each group). Mice were housed on a 12-hour light/dark cycle at a temperature of 25°C and allowed free access to a standard diet (0.3% NaCl, 3.6 kcal/g, 13.3% energy as fat; Oriental MF; Oriental Yeast Co, Ltd, Andover, MA). The study was performed in accord with the National Institute of Health guidelines for the use of experimental animals. All animal studies were reviewed and approved by the Animal Studies Committee of Yokohama City University. Gross appearance of both ATRAP-KO and WT mice was investigated in a blinded manner.

### Blood Pressure and Heart Rate Measurements

Systolic blood pressure and heart rate were measured using the tail-cuff method (BP-monitor MK-2000; Muromachi Kikai Co, Tokyo, Japan). The MK-2000 BP monitor made it possible to measure blood pressure without preheating animals and using anesthesia, thus avoiding this very stressful condition, as described previously.<sup>18</sup> At least 8 readings were taken for each measurement.

### Metabolic Cage Analysis

Metabolic cage analysis was performed as previously described (Techniplast, Paola, Malta).<sup>19,20</sup> Daily food and

water intake were measured. Mice were given free access to tap water and fed a standard diet.

### Chronic Ang II Infusion Analysis

Ang II infusion was performed as previously described.<sup>17</sup> Briefly, Ang II (200 ng/kg/min) was infused subcutaneously into mice by an osmotic minipump (Model 2002; ALZET, Palo Alto, CA) for 28 days.

### Histological and Immunohistochemical Analyses

Histological analysis was performed as described previously.<sup>17,18</sup> The heart, aorta, and kidney were fixed with 4% PFA and embedded in paraffin. Sections (4- $\mu$ m thick) were stained with hematoxylin and eosin, periodic acid-Schiff, Masson's trichrome, and Elastica van Gieson. For analysis of renal structures, glomerular sclerosis was semiquantitatively evaluated and expressed as the glomerular sclerosis index, as described previously.<sup>21</sup> Briefly, 30 glomeruli per sample were graded as 0, 1, 2, 3, or 4, indicating absent, <25, 25 to 50, 51 to 75, or >75% sclerosis, respectively. Cardiac and renal fibrotic areas and aortic medial thickness were measured digitally using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). Immunohistochemistry was performed as described previously.<sup>18,22</sup> Sections were incubated with either an anti-SIRT1 antibody (1:50; 07-131; Millipore, Billerica, MA) or anti-4-hydroxy-2-nonenal (4-HNE) antibody (1:100; MHN-100P; JALCA, Haruoka, Japan). The 4-HNE-stained area was measured digitally using a fluorescence microscope (BZ-9000; Keyence).

### Real-Time Quantitative Reverse Transcription PCR Analysis

Total RNA was extracted from renal tissues with ISOGEN (Nippon Gene Co, Ltd, Toyama, Japan), and cDNA was synthesized using the SuperScript III First-Strand System (Invitrogen). Time quantitative reverse-transcription polymerase chain reaction was performed with an ABI PRISM 7000 Sequence Detection System by incubating the reverse-transcription product with TaqMan PCR Master Mix and designed TaqMan probes (collagen-1 $\alpha$ , Mm00801666\_g1; collagen-3 $\alpha$ , Mm01254476\_m1; transforming growth factor- $\beta$ , Mm01178820\_m1; tumor necrosis factor- $\alpha$ , Mm00443258\_m1; uncoupling protein 2, Mm00627599\_m1; peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  [PGC-1 $\alpha$ ], Mm01208835\_m1; BCL2/adenovirus E1B 19-kDa interacting protein 3, Mm01275600\_g1; calcium-modulating cyclophilin ligand [CAML], Mm00500327\_m1; nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2 [NFATc2/NFAT1], Mm01240677\_m1; nuclear factor of

activated T cells, cytoplasmic, calcineurin-dependent 4 [NFATc4/NFAT3], Mm00452375\_m1; nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3 [NFATc3/NFAT4], Mm01249200\_m1 and interleukin-2, Mm00434256\_m1; Applied Biosystems, Foster City, CA), as described previously.<sup>17,19,23</sup> mRNA levels were normalized to those of the 18S rRNA control.

### Immunoblot Analysis

Immunoblot analysis was performed essentially as described previously.<sup>17,19,23</sup> Briefly, total protein extract was prepared from tissues with SDS-containing sample buffer, and the protein concentration of each sample was measured with a Detergent Compatible Protein Assay Kit (Bio-Rad, Hercules, CA) using BSA as the standard. Equal amounts of protein extract from tissue samples were fractionated on a 5% to 20% polyacrylamide gel (ATTO Technology, Inc., Amherst, NY), then transferred to a PVDF membrane using the iBlot Dry Blotting System (Invitrogen, Carlsbad, CA). Membranes were blocked for 1 hour at room temperature with PBS containing 5% skim milk powder, and probed overnight at 4°C with specific primary antibodies (SIRT1, 07-131; Millipore; Sirtuin3 [SIRT3], sc-99143; Santa Cruz Biotechnology, Santa Cruz, CA; nicotinamide phosphoribosyltransferase [Nampt], sc-67020; Abcam, Cambridge, MA; 4-HNE, MHN-100P; JALCA). Membranes were washed and further incubated with secondary antibodies for 15 minutes at room temperature. Sites of the antibody-antigen reaction were visualized by enhanced chemiluminescence substrate (GE Healthcare, Little Chalfont, UK). The  $\beta$ -actin band recognized by a specific antibody (A5441; Sigma-Aldrich, St. Louis, MO) was used as a loading control. Images were analyzed quantitatively using a Fuji LAS3000 Image Analyzer (FujiFilm, Tokyo, Japan).

### Biochemical Analysis

Arterial blood was collected by cardiac puncture after mice had been killed in the fed state. Whole blood was centrifuged at 500g at 4°C for 10 minutes to separate plasma. An enzymatic assay was used for determination of glycoalbumin (Asahi Kasei Pharma, Tokyo, Japan), and a direct assay was used for the determination of low-density lipoprotein cholesterol and high-density lipoprotein cholesterol (Sekisui Medical, Tokyo, Japan).

### Electron Microscopy Analysis

Electron microscopy analysis was performed as described previously.<sup>24</sup> Briefly, aged ATRAP-KO and WT mice were anesthetized with isoflurane and perfused through the right aortic arch with heparinized (5 U/mL) physiological saline and

2.5% glutaraldehyde in 0.1 mol/L of phosphate buffer at pH 7.4. Specimens for transmission electron microscopy were immersed in 1% osmium tetroxide for 2 hours, dehydrated in an ethanol series, and embedded in an Epon mixture. Ultrathin sections were stained for electron microscopy with uranyl acetate and lead citrate and examined using a Hitachi H-7500 transmission electron microscope operated at 80 kV (Hitachi, Ltd, Tokyo, Japan). Observations were at  $\times 5000$  and photographed with a charge-coupled device camera ( $n=4$  in each group).

### Statistical Analysis

Data are expressed as mean  $\pm$  SE. Normal distribution of all variables was checked using the Shapiro–Wilk test. Differences were analyzed using the Student unpaired *t* test or 2-way ANOVA with Bonferroni post-test. Life span of mice was assessed using the Kaplan–Meier method, and survival distribution between groups was compared using the log-rank test.  $P<0.05$  was considered statistically significant.

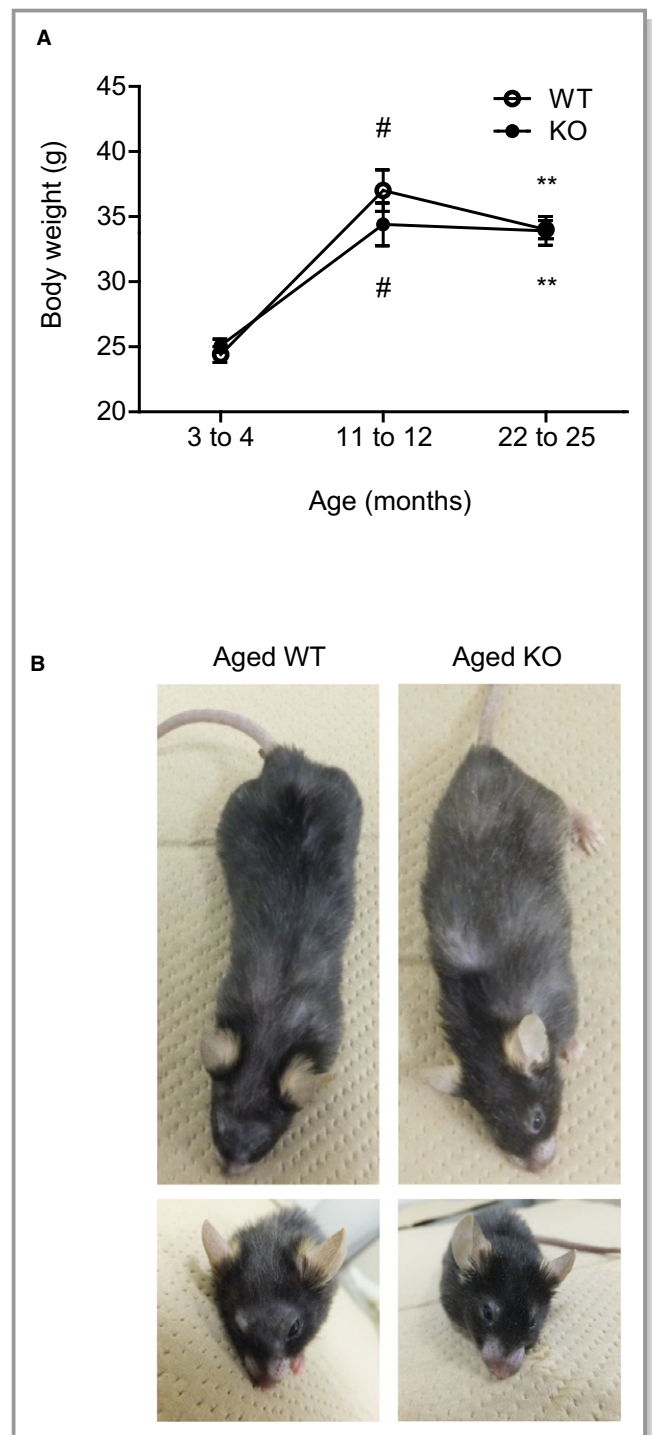
### Results

#### ATRAP Deficiency Does Not Affect Aging in Terms of Outward Appearance

ATRAP-KO mice grew normally and were indistinguishable from WT mice at 3 to 4 months of age, as reported previously.<sup>16,17</sup> ATRAP deficiency did not have any apparent effects on body weight from 3- to 4- to 22- to 25-month-old mice (hereafter, 3- to 4-month-old mice are referred to as “young” mice and 22- to 25-month-old mice are referred to as “aged” mice; Figure 1A). Aged ATRAP-KO mice exhibited no evident difference in gross appearance, including body size, compared with aged WT mice and displayed similar signs of aging, such as gray and white hair and some hair loss (Figure 1B).

#### ATRAP Deficiency Exerts No Evident Effects on the Physiological Parameters of Senescence

We next examined the physiological parameters of aged ATRAP-KO mice (Table). There was no significant difference in systolic blood pressure or heart rate between aged ATRAP-KO and WT mice (Table). To examine whether caloric intake was affected by ATRAP deficiency, metabolic cage analysis was performed on aged ATRAP-KO mice. In this study, daily food intake and water intake were comparable between aged ATRAP-KO and WT mice (Table), which was consistent with similar body weight increases in the 2 groups. In addition, epididymal adipose tissue and liver weights as well as glucose and lipid metabolism were comparable between aged ATRAP-KO and WT mice (Table).



**Figure 1.** ATRAP deficiency does not affect external signs of aging. A, Senescence-related changes in body weight were similar in ATRAP-KO and WT mice. Values are expressed as mean  $\pm$  SE ( $n=6-8$ ). # $P<0.01$ , 3 to 4 months old vs 11 to 12 months old, \*\* $P<0.01$ , 3 to 4 months old vs 22 to 25 months old. B, There was no difference in gross appearance between aged ATRAP-KO and WT mice. Mice exhibited similar signs of senescence, such as gray and white hair as well as some hair loss. ATRAP indicates angiotensin II type 1 receptor-associated protein; KO mice, angiotensin II type 1 receptor-associated protein-knockout mice; WT mice, wild-type mice.



**Table.** Blood Pressure, Heart Rate, Food Intake, Water Intake, and Metabolic Parameters of Aged WT and ATRAP-KO Mice

| Variable                            | WT (n=6–8) | ATRAP-KO (n=6–8) |
|-------------------------------------|------------|------------------|
| Systolic blood pressure, mm Hg      | 115.3±0.8  | 116.3±1.6        |
| Heart rate, bpm                     | 684.6±10.6 | 697.0±6.2        |
| Food intake, g/d                    | 4.3±0.1    | 4.3±0.2          |
| Water intake, g/d                   | 6.6±0.7    | 7.9±0.5          |
| Liver weight, g                     | 1.5±0.1    | 1.6±0.1          |
| Epididymal adipose tissue weight, g | 0.7±0.1    | 0.7±0.2          |
| Glycoalbumin, %                     | 1.4±0.2    | 1.4±0.3          |
| LDL cholesterol, mg/dL              | 8.3±1.7    | 7.0±0.9          |
| HDL cholesterol, mg/dL              | 38.3±8.1   | 44.8±5.4         |

All values are mean±SE. ATRAP indicates angiotensin II type 1 receptor-associated protein; ATRAP-KO mice, angiotensin II type 1 receptor-associated protein-knockout mice; HDL, high-density lipoprotein; LDL, low-density lipoprotein; WT mice, wild-type mice.

### ATRAP Deficiency Does Not Affect the Cardiovascular Aging Phenotype

We then examined cardiac and vascular injury in aged ATRAP-KO and WT mice. With respect to age-related changes in cardiac tissue, there were no significant differences in myocardial hypertrophy and cardiac fibrosis between aged ATRAP-KO and WT mice (Figure 2A through 2C). mRNA expression of cardiac brain natriuretic peptide, a sensitive marker of cardiac overload and function, was also comparable in aged ATRAP-KO and WT mice (Figure 2D). With regard to age-related atherosclerotic lesion development, there were no significant differences in aortic fibrosis or aortic wall thickness between aged ATRAP-KO and WT mice (Figure 2E and 2F). These results indicated that age-related cardiovascular damage was similar in ATRAP-KO and WT mice.

### ATRAP Deficiency Exacerbates Aging-Associated Renal Fibrotic Lesions

There were no evident differences in gross morphology or weight between kidneys of aged ATRAP-KO and WT mice (Figure 3A and 3B), and age-related glomerular sclerotic lesions developed similarly in aged ATRAP-KO and WT mice (Figure 3C and 3D). However, aged ATRAP-KO mice exhibited approximately a 2-fold larger tubulointerstitial fibrosis area compared with aged WT mice (renal tubulointerstitial fibrosis area: ATRAP-KO versus WT, 43.8±9.0% versus 21.1±2.9%;  $P<0.01$ , by ANOVA; Figure 3C and 3E). In aged ATRAP-KO mice, creatinine clearance, an index of renal function, was reduced by 58.7% compared with aged WT mice (creatinine clearance: ATRAP-KO versus WT, 249.7±34.2 versus 425.5±82.2  $\mu\text{L}/\text{min}$ ;  $P<0.05$ , by unpaired *t* test).

### ATRAP Deficiency Enhances Renal Expression of Transforming Growth Factor- $\beta$ and Collagen Genes

Because histological analysis revealed exacerbated tubulointerstitial fibrosis in the aged ATRAP-KO mouse kidney, we examined renal expression of fibrosis-related genes (collagen-1 $\alpha$  and 3 $\alpha$ , transforming growth factor- $\beta$ , and tumor necrosis factor- $\alpha$ ) in age-matched ATRAP-KO and WT mice. Renal expression levels of collagen-1 $\alpha$  and tumor necrosis factor- $\alpha$  mRNA were comparable (Figure 4A and 4D). However, renal expression of collagen-3 $\alpha$  mRNA was markedly increased in aged ATRAP-KO mice compared with aged WT mice (Figure 4B). Furthermore, renal expression of TGF- $\beta$  mRNA was significantly increased in aged ATRAP-KO mice (Figure 4C).

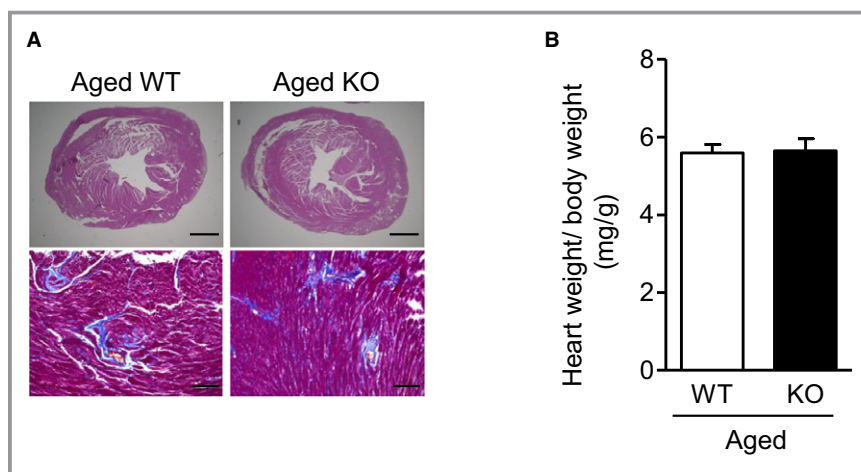
### ATRAP Deficiency Exacerbates Mitochondrial Dysfunction by Senescence in Renal Epithelial Cells

The kidney is an organ with a high energy demand, rich in mitochondria, and mitochondrial dysfunction in the kidney plays a critical role in the pathogenesis of kidney diseases. Therefore, we investigated relevant mechanisms contributing to tubulointerstitial damage in aged ATRAP-KO mice using electron microscopy analysis of renal proximal tubular cells. Proximal tubular cells from aged ATRAP-KO mice exhibited a clearly evident decrease in normal morphology of mitochondria as well as a marked increase in abnormal mitochondria with swelling and disintegration of the cristae, resulting in an accumulation of senescent mitochondria compared with aged WT mice (Figure 5A).

We next investigated renal mitochondrial functions in aged ATRAP-KO mice and WT mice by analyzing the expression of molecules critically involved in regulation of mitochondrial function and autophagy. Uncoupling protein 2 is a mitochondrial membrane protein expressed in various organs, including the kidney, and regulates mitochondrial ATP production.<sup>25</sup> Renal uncoupling protein 2 mRNA expression level was significantly decreased in aged ATRAP-KO mice compared with aged WT mice (Figure 5B).

PGC-1 $\alpha$ , one of the key modulators regulating mitochondrial biogenesis, and B-cell lymphoma 2/adenovirus E1B 19-kDa interacting protein 3 have been reported to be important initiators of mitochondrial autophagy.<sup>26,27</sup> We found that renal mRNA expression of PGC-1 $\alpha$  and B-cell lymphoma 2/adenovirus E1B 19-kDa interacting protein 3 was significantly decreased in aged ATRAP-KO mice compared with aged WT mice (Figure 5B).

Mitochondria are the main cellular source of reactive oxygen species (ROS). To assess the effects of the



**Figure 2.** ATRAP deficiency does not affect cardiovascular aging phenotype. A, Representative images of hematoxylin and eosin–stained sections of hearts of aged ATRAP-KO and WT mice (top: original magnification,  $\times 40$ ; bar, 1 mm), and representative images of Masson’s trichrome–stained sections of hearts of aged ATRAP-KO and WT mice (bottom: original magnification,  $\times 200$ ; bar, 100  $\mu\text{m}$ ). B, Ratio of heart weight/body weight was similar in aged ATRAP-KO and WT mice. Values are expressed as mean $\pm$ SE (n=6–8). C, Cardiac fibrosis was comparable between aged ATRAP-KO and WT mice. Values are expressed as mean $\pm$ SE (n=6–8). D, Cardiac BNP mRNA expression was similar in aged ATRAP-KO and WT mice. Values are expressed as mean $\pm$ SE (n=6–8). E, Representative images of hematoxylin and eosin–stained sections of aortas of aged ATRAP-KO and WT mice (top: original magnification,  $\times 100$ ; bar, 100  $\mu\text{m}$ ), representative images of Masson’s trichrome–stained sections of aortas of aged ATRAP-KO and WT mice (middle: original magnification,  $\times 200$ ; bar, 100  $\mu\text{m}$ ), and representative images of Elastica van Gieson–stained sections of aortas of aged ATRAP-KO and WT mice (bottom: original magnification,  $\times 200$ ; bar, 100  $\mu\text{m}$ ). F, Aortic wall thickness was comparable between ATRAP-KO and WT mice. Values are expressed as mean $\pm$ SE (n=6–8). ATRAP indicates angiotensin II type 1 receptor-associated protein; BNP, brain natriuretic peptide; KO mice, angiotensin II type 1 receptor-associated protein-knockout mice; WT mice, wild-type mice.

accumulation of abnormal mitochondria on renal ROS status, we examined renal accumulation of 4-HNE by immunoblot of 4-HNE-modified proteins and immunohistochemical analysis. Renal 4-HNE generation is mainly derived from mitochondria, and 4-HNE level reflects ROS generation in the kidney.<sup>28–30</sup> We found that renal 4-HNE level was increased in aged ATRAP-KO mice compared with WT mice, and a relatively high level of 4-HNE immunostaining was observed in the inner cortex and outer medulla of aged ATRAP-KO mice (Figure 5C and 5D).

### ATRAP Deficiency Downregulates Renal Expression of Prosurvival Gene SIRT1

To further investigate the mechanism exacerbating mitochondrial abnormality in aged ATRAP-KO mice, we evaluated renal expression of sirtuins in age-matched ATRAP-KO and WT mice. Among sirtuins, SIRT1 and SIRT3 are reported as key factors for the maintenance of mitochondrial biogenesis and functions, and SIRT1 in particular plays an important role to improve mitochondrial function through activation of PGC-1 $\alpha$

by deacetylation.<sup>31–33</sup> In the current study, renal expression level of SIRT1 protein was specifically and significantly decreased in aged ATRAP-KO mice compared with WT mice, whereas there was no significant difference in SIRT3 protein expression (Figure 6A). Nampt is the rate-limiting enzyme for biosynthesizing nicotinamide adenine dinucleotide in mammals and the Nampt/nicotinamide adenine dinucleotide axis regulates sirtuin signaling.<sup>34</sup> We found no significant difference in renal expression level of Nampt protein between aged ATRAP-KO mice and WT mice.

We next examined the effect of ATRAP deficiency on the intrarenal distribution of SIRT1 protein by immunohistochemistry. Although the results of immunohistochemical analysis revealed a relatively high level of SIRT1 immunoreactivity in the inner cortex and outer medulla of young ATRAP-KO and WT mice, SIRT1 immunoreactivity was decreased in these regions in aged ATRAP-KO mice to a far greater degree than aged WT mice (Figure 6B). These results indicated that the exacerbated renal fibrosis in aged ATRAP-KO mice was associated with the specific downregulation of renal SIRT1 expression.

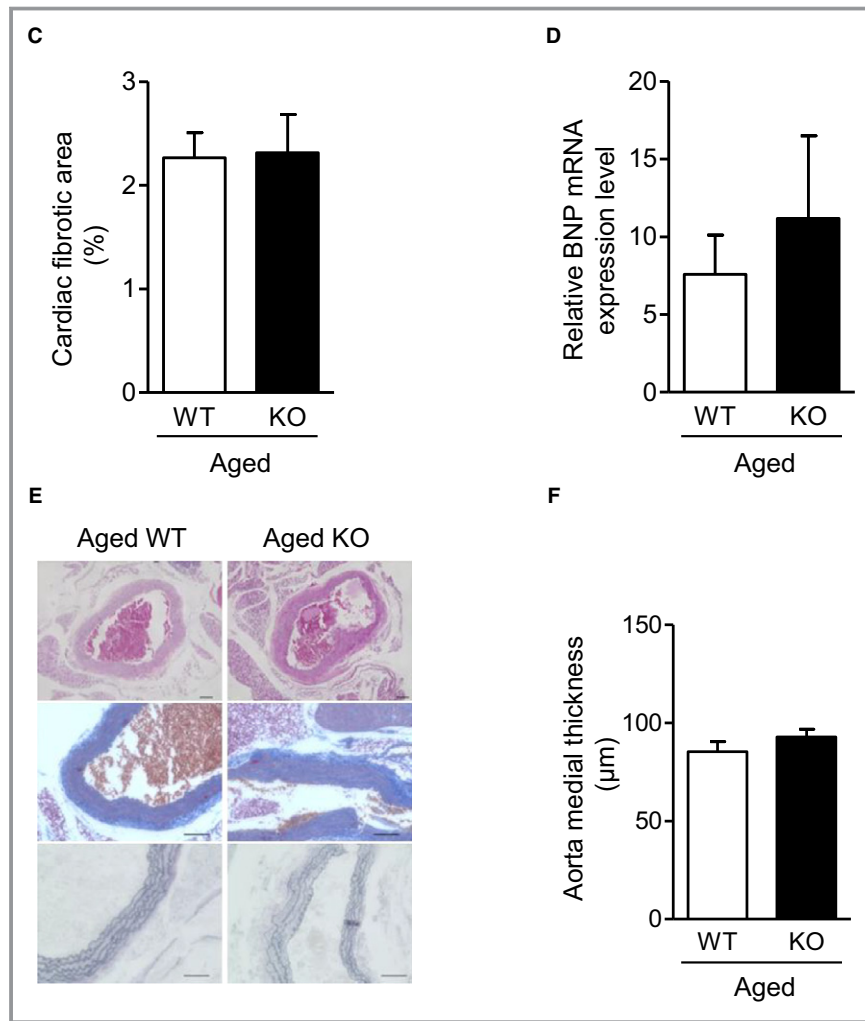


Figure 2. Continued

### Chronic Stimulation of the Ang II-AT1R Axis Does Not Affect Renal SIRT1 Expression

To examine whether activation of Ang II-AT1R signaling is involved in the specific downregulation of renal SIRT1 expression in aged ATRAP-KO mice, we examined the effects of Ang II stimulation on renal prosurvival gene expression in young WT mice. Chronic Ang II infusion did not affect renal SIRT1 expression, but significantly decreased renal SIRT3 and Nampt expression (Figure 7). These results indicated that downregulation of renal SIRT1 expression in aged ATRAP-KO mice was not mediated by activation of the Ang II-AT1R signaling pathway.

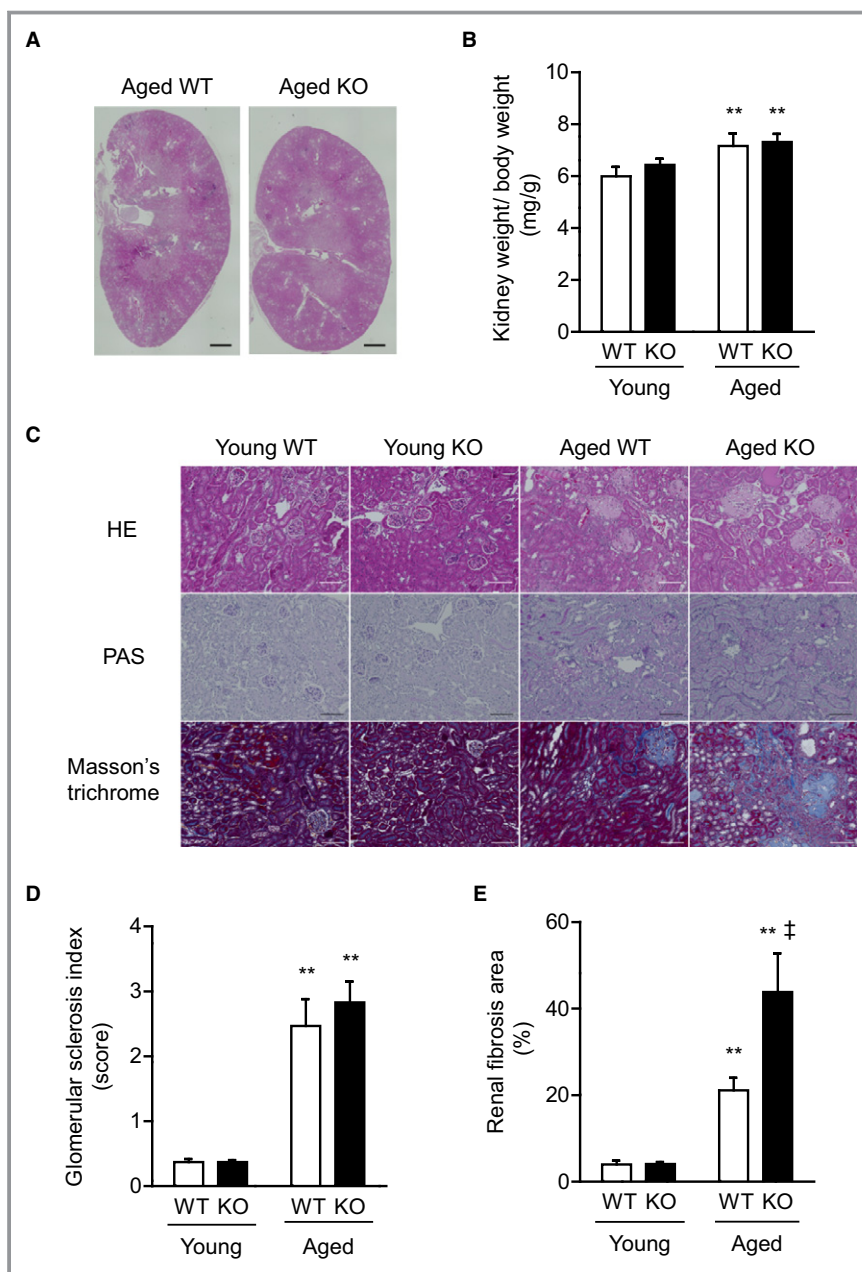
### ATRAP Deficiency Does Not Promote the CAML-NFAT Signaling Pathway in Kidney Aging

CAML is reportedly another direct interacting partner of ATRAP.<sup>13,15</sup> ATRAP inhibits the calcineurin/NFAT pathway by binding to CAML. Therefore, we examined renal expression

levels of CAML and NFATs mRNA in ATRAP-KO and WT mice. Results showed that there were no significant differences in renal expressions of CAML, NFAT 3, and NFAT 4 between age-matched ATRAP-KO and WT mice (Figure 8A, 8C, and 8D). On the other hand, renal expression levels of NFAT1 and interleukin-2, an essential transcript of NFATs, mRNA were decreased in aged ATRAP-KO mice rather than aged WT mice (Figure 8B and 8E). These results indicate that activation of the CAML-NFAT signaling pathway is not involved in the exacerbation of age-associated renal function decline and fibrosis in ATRAP-KO mice.

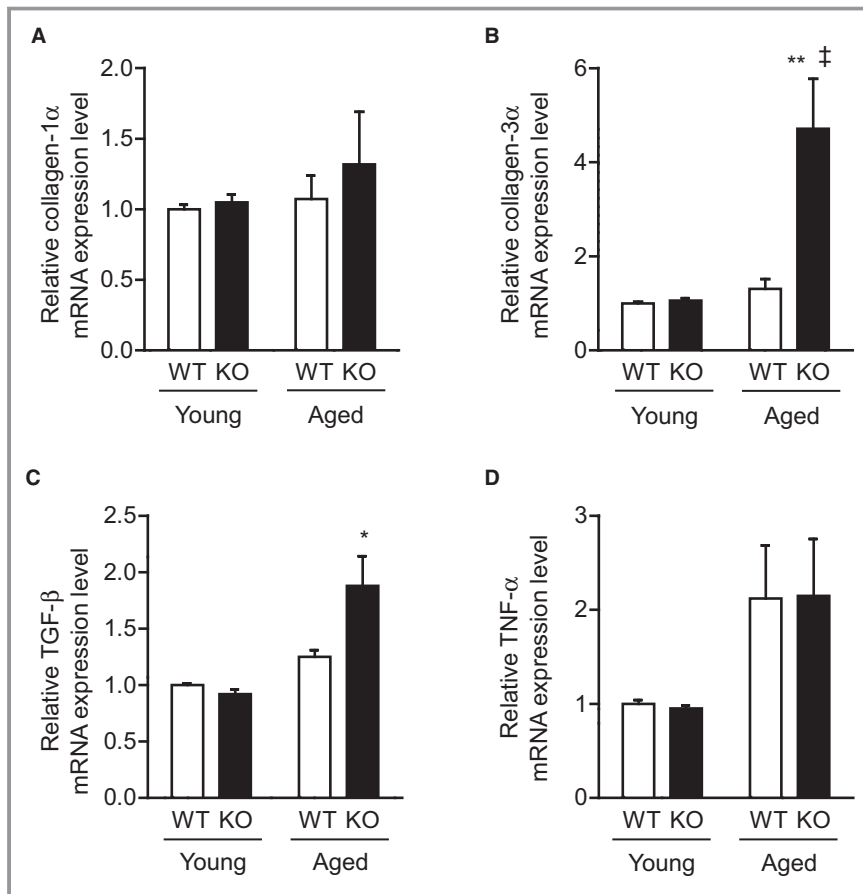
### ATRAP Deficiency Shortens Life Span

Finally, we analyzed the life span of ATRAP-KO mice. Median life spans of ATRAP-KO and WT mice were 100.4 and 123.1 weeks, respectively, indicating that ATRAP-KO mice exhibited a significantly shortened longevity compared with WT mice ( $P=0.0002$ , by log-rank test; Figure 9).



**Figure 3.** ATRAP deficiency exacerbates renal fibrosis by senescence. A, Representative images of hematoxylin and eosin–stained sections of kidneys of young and aged ATRAP-KO and WT mice (original magnification,  $\times 40$ ; bar, 1 mm). B, Ratio of kidney weight/body weight was comparable between age-matched ATRAP-KO and WT mice. Values are expressed as mean $\pm$ SE (n=6–8). \*\* $P < 0.01$ , young vs aged mice. C, Representative images of hematoxylin and eosin–, PAS–, and Masson’s trichrome–stained sections of kidneys of young and aged ATRAP-KO and WT mice (original magnification,  $\times 200$ ; bar, 100  $\mu$ m). D, Glomerular sclerosis index was significantly and similarly increased in both aged ATRAP-KO and WT mice compared with young groups. Values are expressed as mean $\pm$ SE (n=6–8). \*\* $P < 0.01$  vs young. E, Renal fibrotic area was significantly increased in aged ATRAP-KO mice compared with aged WT mice. Values are expressed as mean $\pm$ SE (n=6–8). \*\* $P < 0.01$  vs young; ‡ $P < 0.01$  vs WT mice. ATRAP indicates angiotensin II type 1 receptor-associated protein; HE, hematoxylin and eosin; KO mice, angiotensin II type 1 receptor-associated protein-knockout mice; PAS, periodic acid–Schiff; WT mice, wild-type mice.





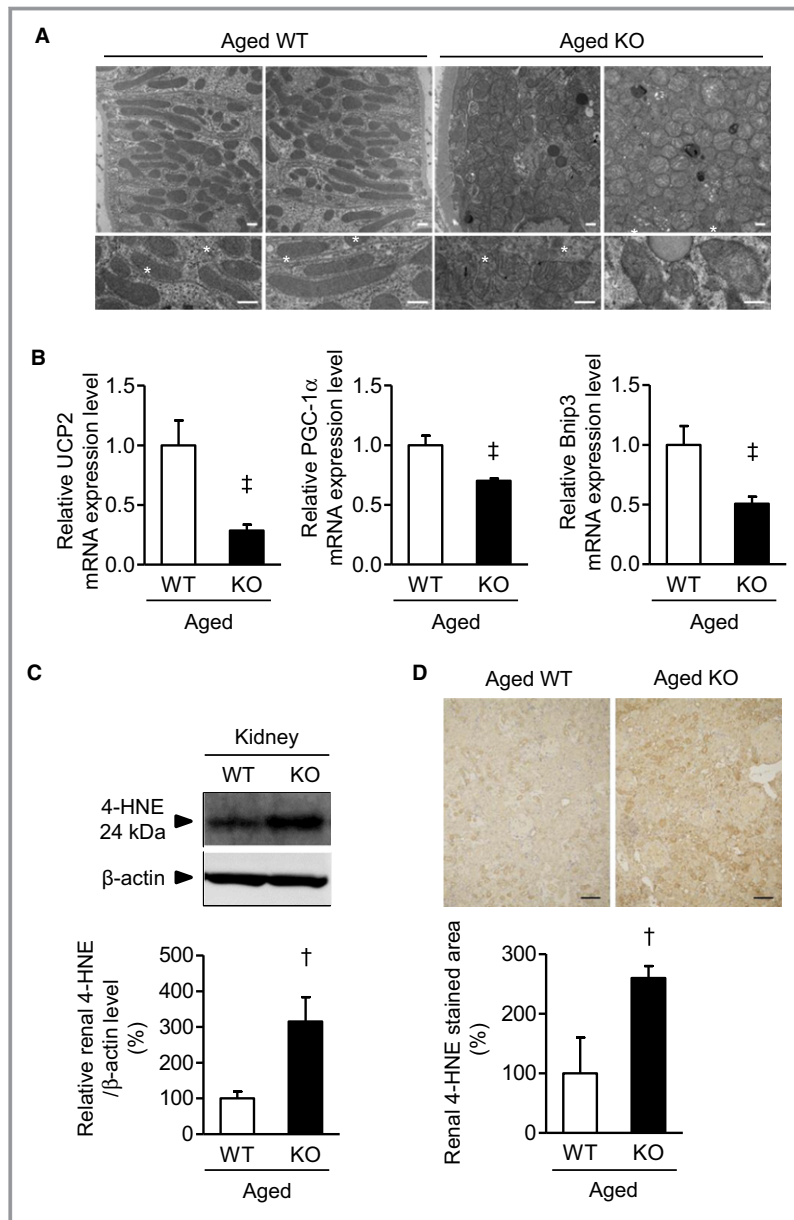
**Figure 4.** ATRAP deficiency enhances upregulation of renal collagen-3α and transforming growth factor-β (TGF-β) mRNA expression by senescence. Renal mRNA expression of fibrosis-related factors (A, collagen-1α; B, collagen-3α; C, TGF-β; D, TNF-α) in young and aged ATRAP-KO and WT mice. Values are expressed as mean±SE (n=6–11). \**P*<0.05 vs young; \*\**P*<0.01 vs young; ‡*P*<0.01 vs WT mice. ATRAP indicates angiotensin II type 1 receptor-associated protein; KO mice, angiotensin II type 1 receptor-associated protein-knockout mice; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; WT mice, wild-type mice.

## Discussion

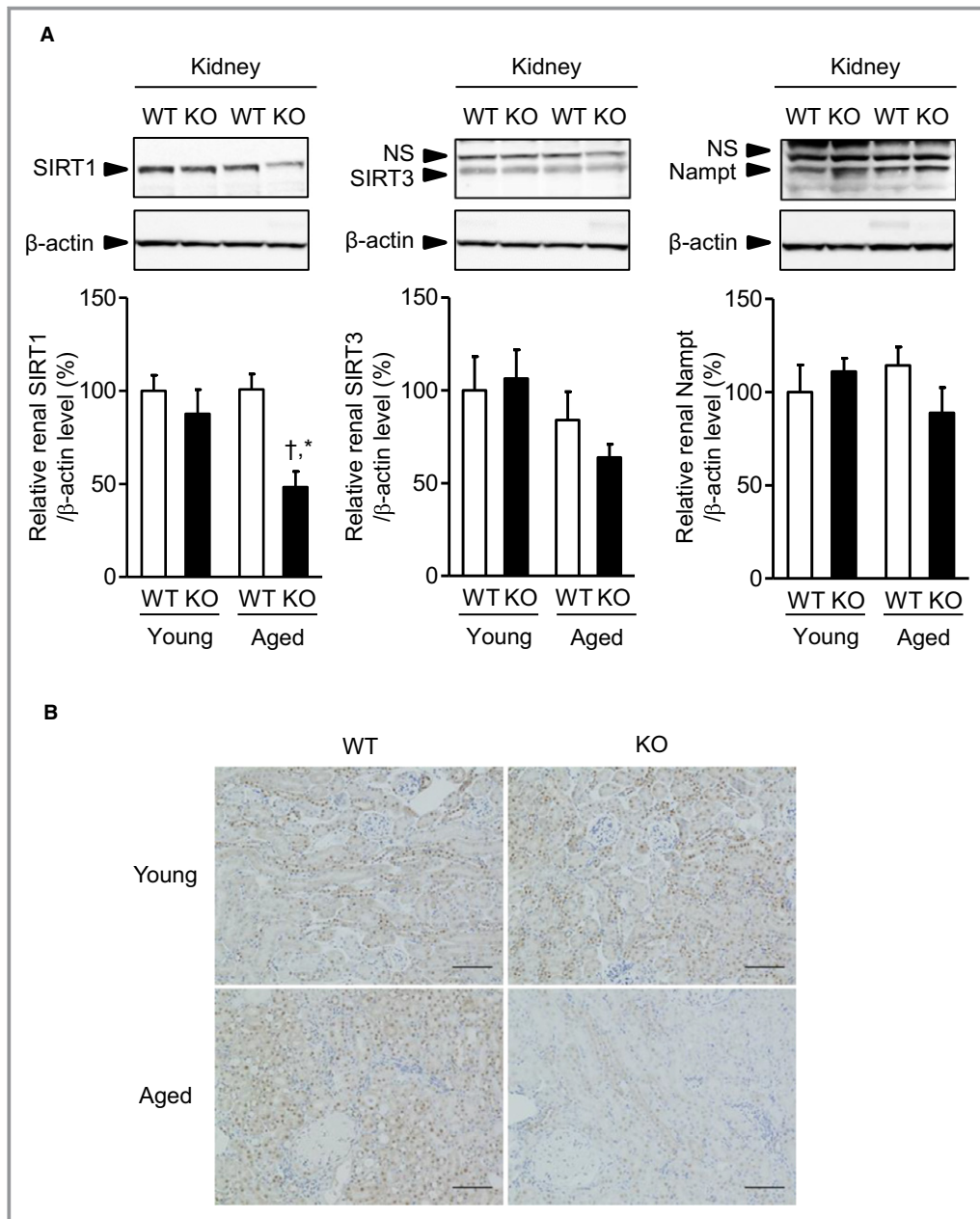
The activation of AT1R signaling is a form of stimulation that promotes aging.<sup>8,35</sup> In the current study, we found that although chronic Ang II infusion in vivo significantly decreased the expression of SIRT3 and Nampt in the kidney, it did not affect renal SIRT1 expression (Figure 7). This finding is consistent with the results of a previous study that reported that renal expression levels of SIRT3 and Nampt are significantly increased in systemic AT1R-deficient mice compared with WT mice, but without any change in SIRT1 expression.<sup>36</sup> AT1R-deficient mice also exhibited a prolongation of life span, together with increased abundance of kidney mitochondria and suppressed oxidative stress.<sup>36</sup> Therefore, activation of Ang II-AT1R signaling is likely to selectively decrease expression of the prosurvival genes, SIRT3 and Nampt, but not SIRT1, in the kidney, thereby promoting renal

aging.<sup>37</sup> Whereas there were no significant differences in renal SIRT3 and Nampt levels between aged ATRAP-KO and WT mice, renal SIRT1 expression was significantly decreased in aged ATRAP-KO mice compared with aged WT mice (Figure 6). Thus, the mechanism for the promotion of renal aging by an ATRAP deficiency appears to be independent of the stimulation of the Ang II-AT1R signaling pathway.

ATRAP in cardiovascular and adipose tissue can inhibit hyperactivation of tissue AT1R signaling caused by certain pathological stimuli, such as chronic Ang II infusion or dietary loading, and improve cardiovascular disturbances and insulin resistance.<sup>16,23,38–40</sup> However, ATRAP exerts no evident influence on the physiological AT1R signaling pathway. For example, the phenotype of ATRAP transgenic mice with overexpression of ATRAP in cardiovascular tissue is similar to that of their littermate WT control mice under nonstimulating conditions.<sup>19,20,23,38,40,41</sup> In the current study, there were no



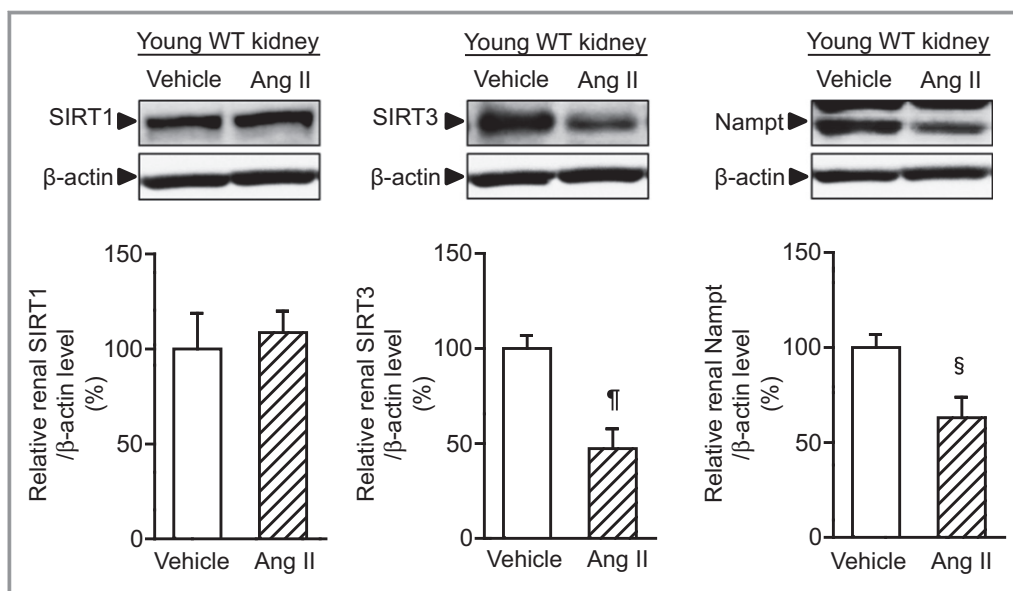
**Figure 5.** ATRAP deficiency provokes renal mitochondrial abnormalities and exacerbates oxidative stress. **A**, Representative electron microscopy images of renal proximal tubules in aged ATRAP-KO and WT mice (original magnification,  $\times 5000$ ; bar, 500 nm). Proximal tubular cells of aged ATRAP-KO mice exhibited an evident decrease in normal mitochondria morphology as well as an increase in abnormal mitochondria, with swelling and disintegration of the cristae. **B**, Renal mRNA expression of mitochondrial functions (UCP2, PGC-1 $\alpha$ , and Bnip3) in aged ATRAP-KO and WT mice. Values are expressed as mean $\pm$ SE (n=6–8).  $^{\ddagger}P < 0.01$  vs WT mice. **C**, Renal protein expression of 4-HNE in aged ATRAP-KO and WT mice. Values are expressed as mean $\pm$ SE (n=6–8).  $^{\ddagger}P < 0.05$  vs WT mice. **D**, Representative immunohistochemistry for 4-HNE (top) and quantitative analysis (bottom) in kidney sections of aged ATRAP-KO and WT mice. Areas positive for 4-HNE are evident as brown dots in sections (original magnification,  $\times 100$ ; bar, 100  $\mu$ m). Values are expressed as mean $\pm$ SE (n=6–8).  $^{\ddagger}P < 0.05$  vs WT mice. 4-HNE indicates 4-hydroxy-2-nonenal; ATRAP, angiotensin II type 1 receptor-associated protein; Bnip3, B-cell lymphoma 2/adenovirus E1B 19-kDa interacting protein 3; KO mice, angiotensin II type 1 receptor-associated protein-knockout mice; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$ ; UCP2, uncoupling protein 2; WT mice, wild-type mice.



**Figure 6.** ATRAP deficiency provokes downregulation of renal SIRT1 protein expression. A, Renal protein expression of prosurvival factors (SIRT1, SIRT3, and Nampt) in young and aged ATRAP-KO and WT mice. Values are expressed as mean±SE (n=6–8). \* $P<0.05$  vs young; <sup>†</sup> $P<0.05$  vs WT mice. B, Immunohistochemistry for SIRT1 protein in kidney sections of ATRAP-KO and WT mice. Areas positive for SIRT1 are evident as brown dots in sections (original magnification, ×200; bar, 100 μm). ATRAP indicates angiotensin II type 1 receptor-associated protein; KO mice, angiotensin II type 1 receptor-associated protein-knockout mice; Nampt, nicotinamide phosphoribosyltransferase; NS, nonspecific band; SIRT1, sirtuin1; SIRT3, sirtuin3; WT mice, wild-type mice.

significant differences in blood pressure, cardiovascular injury, and glucose/fat metabolism profiles between aged ATRAP-KO and WT mice (Figure 2; Table). These results also support that during the physiological degenerative process of aging, inhibition of the AT1R signaling pathway is not central to the functional role of ATRAP.

The kidney is an organ that is affected strongly by aging. Age-related histological changes in the kidney include glomerulosclerosis, tubular atrophy, and interstitial fibrosis, which are considered features of chronic kidney disease (CKD).<sup>42–44</sup> In the current study, we found kidney-dominant exaggeration of the aging phenotype in ATRAP-KO mice.



**Figure 7.** Activation of the Ang II–AT1R axis by chronic Ang II infusion does not reduce renal SIRT1 in young WT mice. Renal protein expression of prosurvival factors (SIRT1, SIRT3, and Nampt) in Ang II– or vehicle-infused young WT mice. Values are expressed as mean±SE (n=6–8). <sup>§</sup>P<0.05 vs vehicle; <sup>†</sup>P<0.01 vs vehicle. Ang II indicates angiotensin II; ATRAP, angiotensin II type 1 receptor-associated protein; Nampt, nicotinamide phosphoribosyltransferase; SIRT1, sirtuin1; SIRT3, sirtuin3; WT mice, wild-type mice.

Additionally, although aged ATRAP-KO mice exhibited the same extent of glomerular injury as aged WT mice, tubulointerstitial fibrosis was markedly more exacerbated (Figure 3).

The endogenous ATRAP protein is most abundantly expressed in the kidney among the tissues examined to date, and in the kidney, it is highly expressed in tubular epithelial cells in the proximal and distal tubules, but is faintly expressed in glomeruli.<sup>18</sup> Therefore, abundant expression of endogenous ATRAP could be a reason for the kidney-dominant promotion of aging-related changes in ATRAP-KO mice. Furthermore, intrarenal distribution of ATRAP would be a plausible explanation for the differential effects of ATRAP deficiency on glomerular sclerosis and interstitial fibrosis, and deletion of endogenous ATRAP from renal tubular epithelial cells is suggested as an important contributor to the exaggerated renal fibrosis. Importantly, tubulointerstitial fibrosis is a major determinant of the extent of renal damage and is a central event in progression of CKD, which is a clinical feature of premature aging as well as a common end point for various types of renal disease.<sup>45</sup>

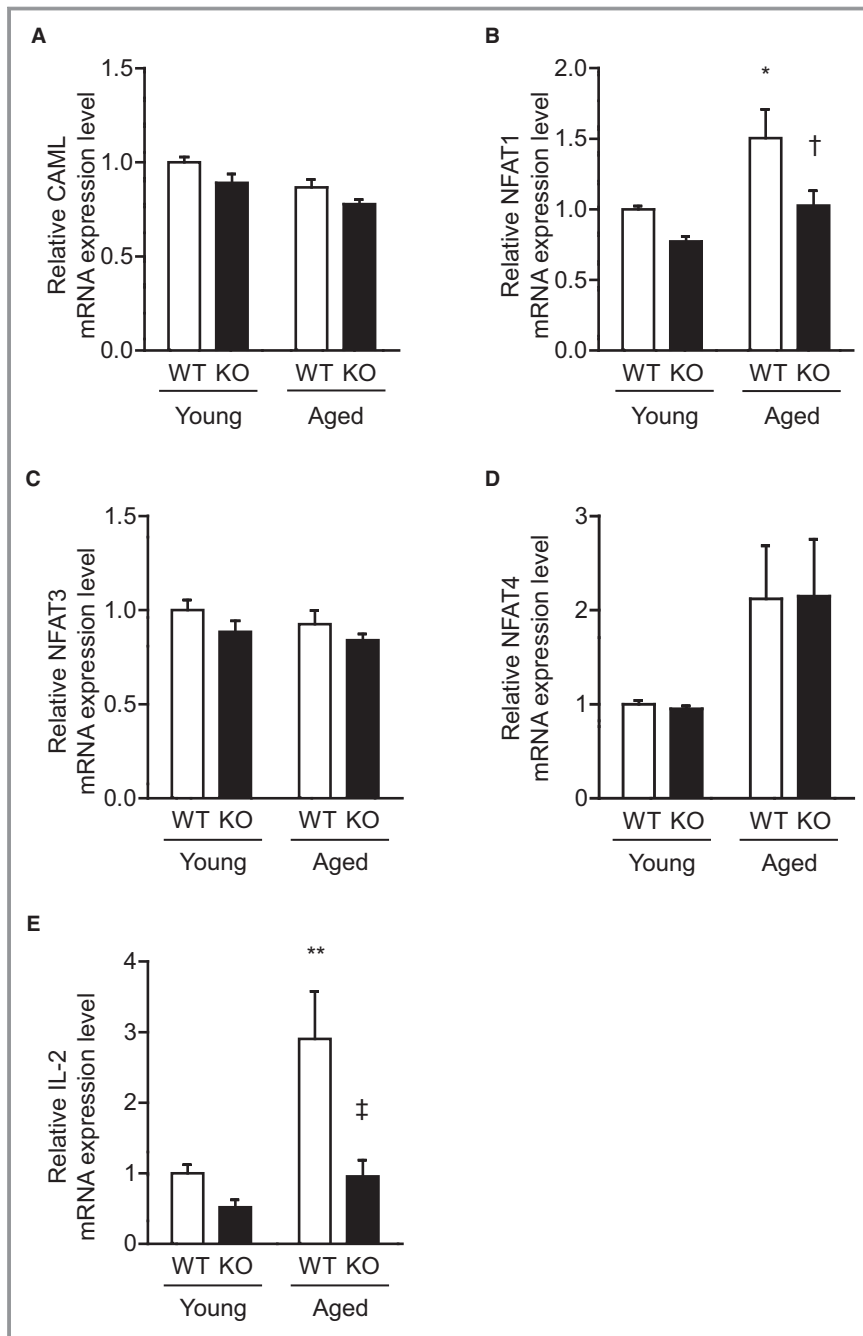
We found that mitochondrial abnormalities in proximal tubules of the kidney were more advanced in aged ATRAP-KO mice than in aged WT mice (Figure 5). Mitochondria are a site for aerobic respiration as well as for production of ROS.<sup>46</sup> We also found that expression of 4-HNE was markedly increased in kidney of aged ATRAP-KO mice compared with aged WT mice. Increased ROS production augments fibrosis in the

kidney.<sup>28–30</sup> These results suggest that functional mitochondrial abnormalities and subsequent increases in ROS production could be involved in the exacerbation of tubulointerstitial fibrosis in aged ATRAP-KO mice.

As to the significance of the specific downregulation of renal SIRT1 expression in aged ATRAP-KO mice (Figure 6), SIRT1, which is primarily expressed in the renal tubule, functions as a protein deacetylase to activate PGC-1 $\alpha$  and plays an important role in mitochondrial homeostasis through proper regulation of biogenesis and autophagy mechanisms.<sup>31,47,48</sup> Therefore, decreased renal expression of SIRT1, a longevity-promoting molecule, is often associated with mitochondrial abnormalities. These processes lead to increased oxidative stress, which may contribute to the promotion of renal fibrosis observed in aged ATRAP-KO mice. Furthermore, given that the functional interaction and deacetylation of PGC-1 $\alpha$  and SIRT1 plays a critical role in the maintenance of mitochondrial function,<sup>49</sup> the combined downregulation of renal PGC-1 $\alpha$  and SIRT1 is likely to be critically involved in exacerbation of mitochondrial abnormality and accumulation of oxidative stress in the kidney of ATRAP-KO mice. Further studies are required to elucidate the molecular mechanism behind ATRAP-mediated regulation of the expression and function of PGC-1 $\alpha$  and SIRT1 in the kidney.

It has been reported that ATRAP directly interacts with CAML and suppresses senescence in vascular smooth muscle cells by inactivation of the calcineurin/NFAT pathway.<sup>13,15</sup>

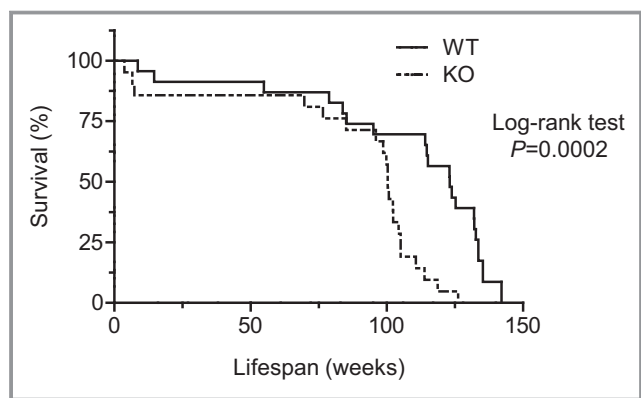




**Figure 8.** ATRAP deficiency does not enhance renal CAML and NFAT signaling pathway by senescence. Renal mRNA expression of factors in the CAML-NFAT signaling pathway (A, CAML; B, NFAT1; C, NFAT3; D, NFAT4; E, IL-2) in young and aged ATRAP-KO and WT mice. Values are expressed as mean±SE (n=6–8). \* $P<0.05$  vs young; \*\* $P<0.01$  vs young; † $P<0.05$  vs WT mice; ‡ $P<0.01$  vs WT mice. ATRAP indicates angiotensin II type 1 receptor-associated protein; CAML, calcium-modulating cyclophilin ligand; IL-2, interleukin-2; KO mice, angiotensin II type 1 receptor-associated protein-knockout mice; NFAT1, cytoplasmic, calcineurin-dependent 2 (NFATc2); NFAT3, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 4 (NFATc4); NFAT4, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3 (NFATc3); WT mice, wild-type mice.

Interleukin-2, which is regulated by NFATs, is known as a key factor to maintain and activate T cells.<sup>50</sup> In the present study, aged ATRAP-KO mice did not exhibit an enhancement of the

CAML/calcineurin/NFAT signaling pathway compared with aged WT mice (Figure 8). These results suggest that the inhibitory effects of ATRAP on kidney aging are not mediated



**Figure 9.** ATRAP deficiency shortens life span. Cumulative survival of ATRAP-KO mice (n=21) and WT mice (n=23) was analyzed using the Kaplan–Meier method.  $P=0.0002$  vs WT mice by the log-rank test. Median life spans of ATRAP-KO mice and WT mice were 100.4 and 123.1 weeks, respectively. ATRAP indicates angiotensin II type 1 receptor-associated protein; KO mice, angiotensin II type 1 receptor-associated protein-knockout mice; WT mice, wild-type mice.

by inactivation of calcineurin/NFAT pathway by binding to CAML.

It should be noted that ATRAP-KO mice have a significantly shorter life span than WT mice (Figure 9). Although exacerbation of CKD in the form of aging-associated renal function decline and increased renal fibrosis was noted in ATRAP-KO mice, other major factors that affect life span (eg, blood pressure, caloric intake, glycolipid metabolism, and cardiovascular injuries) were the same as in WT mice. In general, progression of CKD is known to eventually result in end-stage renal failure as well as increase the risk for cardiovascular disease, infections, and cancer.<sup>51</sup> In the current study, we were unable to identify any direct causes of death in ATRAP-KO mice. However, it is important to note that phenotypic changes in the kidney can strongly affect life span. Although the kidney is easily affected by aging-associated changes, the inhibition of renal aging could lengthen overall life span.

There are several limitations in the present study. First, we did not analyze the dephosphorylation and transcriptional activity of NFAT directly. Second, we did not perform rigorous assessment of mitochondrial activity, although the examination of mitochondrial dysfunction relies, to some extent, on relatively nonspecific examination of ROS.

In conclusion, the results of this study suggest the lack of inhibitory effects of ATRAP on physiological AT1R signaling under normal conditions, even on the long-term process of physiological aging, and further suggest that ATRAP is able to ameliorate the progression of CKD that accompanies aging through SIRT1-mediated mechanism other than inhibition of the Ang II-AT1R signaling pathway. It is possible that therapeutic strategies involving ATRAP activation could lengthen life span.

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## Disclosures

None.

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