Supplemental Material

Data S1.

Expanded Materials & Methods

Cell lines and culture methods

Human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (SMCs) were purchased from Lonza Group Ltd. (Basel, CHE). They were cultured in endothelial growth medium (EGM-2; EGM-2 SingleQuots, Lonza) or SMC growth medium (SmGM-2; SmGM-2 SingleQuots, Lonza), as described previously.[24] When the cells reached 80% confluency, the culture medium was replaced with phenol red-free Dulbecco's modified Eagle's Medium (DMEM; GIBCO, Invitrogen) with 10% charcoal-stripped fetal bovine serum (FBS; Biowest) and maintained for 24 h before E2 treatment, as phenol red is known to possess estrogenic properties.[22,23]

Cell treatment

Cells were treated with 10 nmol/L E2 in phenol red-free DMEM supplemented with 2% charcoal-stripped FBS. Control cells were exposed to the same volume of medium without E2. For the inhibition experiments, the culture medium was exchanged with DMEM containing 2% charcoal-stripped FBS and maintained for 30 min, 1 h, 3 h, 6 h, and 24 h before the addition of ICI 182780

(1 μmol/L; Sigma), sirtinol (50 μmol/L; Sigma), compound C (10 μmol/L; Sigma), or G15 (2 μmol/L; Cayman).

Animal models

All animal experiments were conducted in compliance with the protocol reviewed by the Institutional Animal Care and Use Committee and approved by the Faculty of Medicine, Kagoshima University, and followed the recommendations of the guidelines for animal experimentation at research institutes (Ministry of Education, Culture, Sports, Science and Technology, Japan and Ministry of Health, Labor and Welfare, Japan) and the guidelines for the proper conduct of animal experimentation (Science Council of Japan). 12-week-old female C57BL/6 mice or ApoE KO mice were anesthetized with a combination of 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol by intraperitoneal injection (i.p.) and subjected in a random and allocation concealment fashion to bilateral ovariectomy (OVX) or sham surgery. We used 6 to 7 animals per group in each experiments, and assigned 3 animals for immunohistochemical analysis and the other animals for immunoblot analysis. Bodyweight and food intake were measured every week after the surgical procedures as described previously.[24] In a different experiment, 12-week-old (Young) and 90-week-old (Old) female C57BL/6 mice were used.

Experimental procedures

The effects of E2 in OVX mice were investigated via the subcutaneous implantation of E2 pellets (0.5 mg per pellet releasing 8.3 μg/day; Innovative Research; OVX + E2), or control pellets (OVX) for 8 weeks, as previously described.[24] To inhibit conventional autophagy, OVX mice implanted with an E2 pellet were treated with either 3-MA (10 mg/kg) or with PBS as vehicle via i.p. injection five days per week for 2 weeks.[47]

Tissue preparation and estradiol analysis

All mice were killed with an overdose of sodium pentobarbital 8 weeks after surgery, and blood samples were collected from the left ventricle, as reported previously.[48] After blood was drawn, mice were perfused with phosphate-buffer saline (pH 7.4), and the vessels were harvested for use in the following experiments. The ascending aortas were immediately fixed with 4 % paraformaldehyde phosphate buffer solution for Masson's Trichrome staining and immunohistochemical analysis. For electron microscopy analysis, the removed vessels were immediately placed into electron microscopy fixative. For immunoblot analysis, the isolated vessels were rinsed in phosphate buffered saline and stored at -80℃ until use. For mitochondrial isolation, aorta samples from the ascending artery to the bifurcation of the common iliac artery were

harvested and immediately used in the experiment on ice. Serum was obtained by centrifugation of blood for 10 min at 850 *g* and 4°C and stored at -80°C. The concentration of 17β estradiol was measured enzymatically using a commercially available kit (VioVision).

Histological analysis

Histological analysis was performed as described elsewhere.[49] In brief, mice aortas were fixed with 4 % paraformaldehyde phosphate buffer solution, embedded in paraffin, and sectioned into 4 μm tissue sections. Interstitial fibrosis was evaluated using the Masson's trichrome staining.

Immunoblot analysis

Cell lysates from in vitro and in vivo samples were processed and used for immunoblot analysis using a NUPAGE Electrophoresis System (Invitrogen), as reported previously.[47] The primary antibodies used were as follows: p53 (Cell Signaling Technology, 9282, and Santa Cruz, sc99), , Acetyl-p53 (Cell Signaling Technology, 2570), AMPKα (Cell Signaling Technology, 2532), phospho-AMPKα (Th172; Cell Signaling Technology, 2535), LKB1 (Cell Signaling Technology, 3050), phospho-LKB1(Ser428; Cell Signaling Technology, 3482), Atg7 (Cell Signaling Technology, 2631), SIRT1 (Merck Millipore, 07-131), Ulk1 (Abcam, ab128859), phospho-Ulk1(Ser555; Merck Millipore,

ABC 124 and Cell Signaling Technology, 5869), p21 (Santa Cruz, sc6246), p16 (Santa Cruz, sc1207), PAI-1 (Santa Cruz, sc8979), p62 (SQSTM1; MBL, PM045), LC3 (MBL, M186-3), Rab9 (Sigma, R5404), Drp1 (DLP1; BD Biosciences, 611112), phosphor-Drp1 (Ser616; Cell Signaling Technology, 4494), phosphor-Drp1 (Ser637; Abcam, ab193216), PGC1-α (Santa Cruz, sc518025), Mfn1 (Abcam, ab57602), Mfn2 (Sigma, M6319), OPA1 (BD Biosciences, 612606), Rip1 (Cell Signaling Technology, 3493S), total OXPHOS (Abcam, ab110413), β-actin (Santa Cruz, sc47778), and α-tubulin (Sigma, T9026). Either horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, 1706515) or goat anti-mouse antibody (Santa Cruz, 516102) was then added. Densitometric analyses were performed using the ECL prime system (GE Healthcare UK Ltd., Little Chalfont, UK).

Immunohistochemistry

Immunohistochemistry staining of the tissue sections and cultured cells was performed, as described previously.[47] Briefly, tissue sections and cultured cells were stained with anti-Rab9 mouse monoclonal antibody (1:100; Abcam, ab2810), anti-LAMP2 rabbit polyclonal antibody (1:100; Sigma, L0668), anti-TOMM20 rabbit monoclonal antibody (1:100; Abcam, ab186734), anti-LC3B rabbit polyclonal antibody (1:100; Abcam, ab51520), Alexa Fluor 488-conjugated goat anti-

rabbit IgG (1:100; Abcam, ab150081), and Alexa Fluor 594-conjugated goat anti-mouse IgG (1:100; Abcam, ab150120). Analyses were performed using a confocal microscope (Zeiss, LSM700). The cells of each group were randomly selected from 3 independent experiments and the merged signals (yellow) were counted. Tissue sections were also incubated with primary anti-4 hydroxynonenal (4-HNE) antibody (1:50; Abcam, ab46545), then treated with an amino acid polymer conjugated to anti-rabbit IgG Fab′ labeled with horseradish peroxidase (Histofine Simple Stain Mouse MAX-PO, Nichirei) and then developed using the Histofine Simple Stain DAB solution (Nichirei). Analyses were performed using fluorescence microscopy (Keyence, BZ-X710). The number of merged yellow signals were counted in the aortic sections of mice for each sample.

Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase (SA-β gal) activity was measured using a SA-β gal staining kit (Cell Signaling Technology), according to the manufacturer's protocol. SA-β gal-positive cells were counted using a microscope (Keyence, BZ-X710), as reported previously.[24] Briefly, more than 200 cells in, at least 5 random fields were counted to determine the percentage of SA-β gal-positive cells.

Transmission electron microscopy analysis

The harvested vessels of mice and cultured HUVECs and VSMCs were fixed in 0.2 mol/L sodium cacodylate-buffered (pH 7.4), 5 % glutaraldehyde solution for 2 h, and then rinsed (2 × 10 min) in 0.1 mol/L sodium cacodylate-buffered (pH 7.4) 7.5 % saccharose and post-fixed in 2 % OsO_4 solution for 2 h. After dehydration in an ethanol gradient (50 % ethanol for 5 min, 70 % ethanol for 5 min, 90 % ethanol for 10 min, and 100 % ethanol for 3 × 10 min), samples were embedded in EPON812. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined using a HITACHI H-7650 transmission electron microscope (HITACHI, Tokyo, Japan) at 80 kV.

Mitochondrial isolation

The mitochondrial fraction of HUVECs and VSMCs was purified using a mitochondrial isolation kit (Thermo Scientific), according to the manufacturer's instructions. The mitochondrial fraction of the isolated mice aorta was purified using a different mitochondrial isolation kit (MITOISO1; Sigma), according to the manufacturer's instructions.

Evaluation of mitochondrial superoxide production

Intra-mitochondrial superoxide production was measured using the MitoSox Red mitochondrial superoxide indicator for live-cell imaging (Invitrogen), according to the manufacturer's protocol. Fluorescence was observed under a microscope (Keyence, BZ-X710) equipped with an 20× Plan Apochromat 20×/0.8 N.A objective.

H_2O_2 measurement

H_2O_2 production was measured using the Amplex Red H_2O_2 assay kit (Molecular Probes; Invitrogen), according to the manufacturer's instructions.

ATP production

ATP production was measured with the ATP Bioluminescent Assay kit (Sigma). Briefly, mitochondria (20 μg) were incubated in the ATP assay mix and MSH buffer containing 625 μmol/L ADP and substrate (10 mmol/L pyruvate and 10 mmol/L malate).

Mitochondrial membrane potential evaluation

The state and integrity of the mitochondrial membrane potential was visualized via the staining of cultured cells with 5,5′,6,6′-tetrachloro-1,1′3,3′-tetramethylbenzimidazolocarbocyanine iodide (JC-

1) and tetramethylrhodamine ethyl ester (TMRE), using the MitoPT®JC-1 and MitoPT®TMRE (ImmunoChemistry Technologies) kits, respectively, according to the manufacturer's instructions. The number of green fluorescent cells was determined within at least 100 cells, counted in no less than 3 random fields; the percentage of green-fluorescent cells was then calculated.

Mitotracker staining

Mitochondria were stained with MitoTracker Red FM (Invitrogen) according to the manufacturer's instructions. Fluorescence images of live cells were obtained using the LSM700 confocal laser scanning microscope (Zeiss, LSM700). To quantify mitochondrial morphology, a cell was judged to have fragmented mitochondria if > 50% of the mitochondria visible in the cell were punctate or circular. We randomly selected > 80 cells in independent 3 experiments and calculated the percentage of cells with fragmented mitochondria.

Short interfering RNA transfection

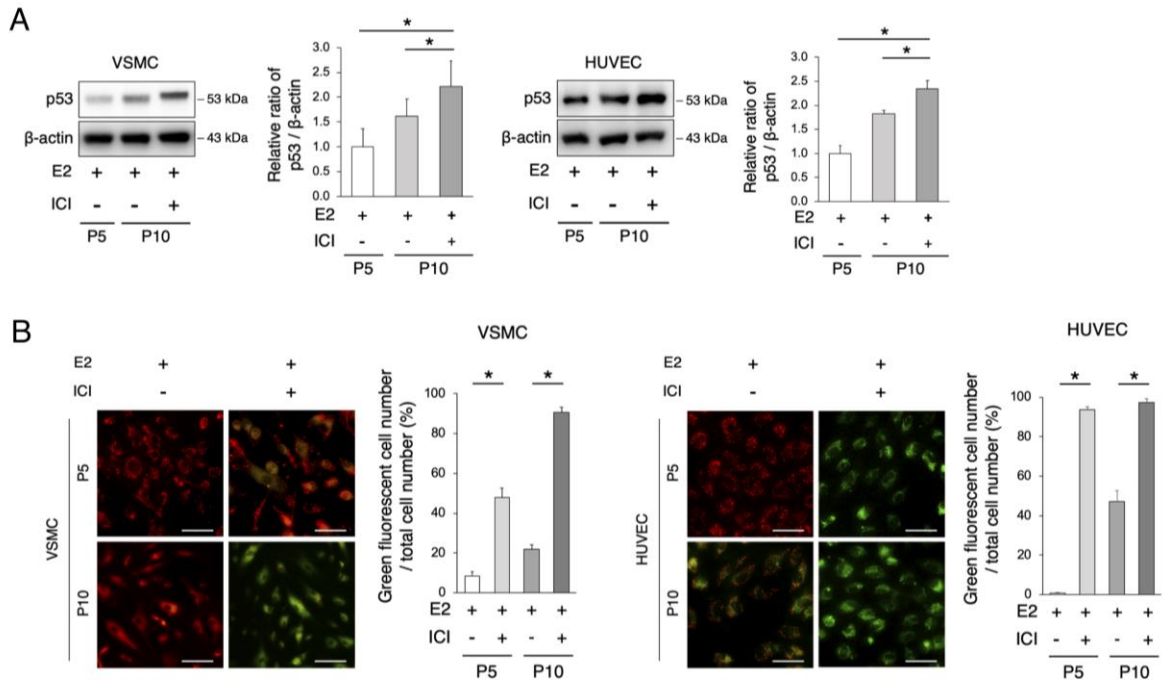
For Rab9 RNA knockdown, cells were transfected with Rab9 or control siRNA (Santa Cruz, sc-44065, sc-37007) using the siRNA Transfection Reagent (Santa Cruz, sc-29528), according to the manufacturer's instruction. For ATG7 RNA knockdown, cells were transfected with the

corresponding siRNA (Thermo Fisher Scientific) using the Lipofectamine RNAiMAX transfection reagent (13778150; Invitrogen), according to the manufacturer's protocol. Immunoblots of lysates were performed to confirm the knockdown efficiency.

Statistical analysis

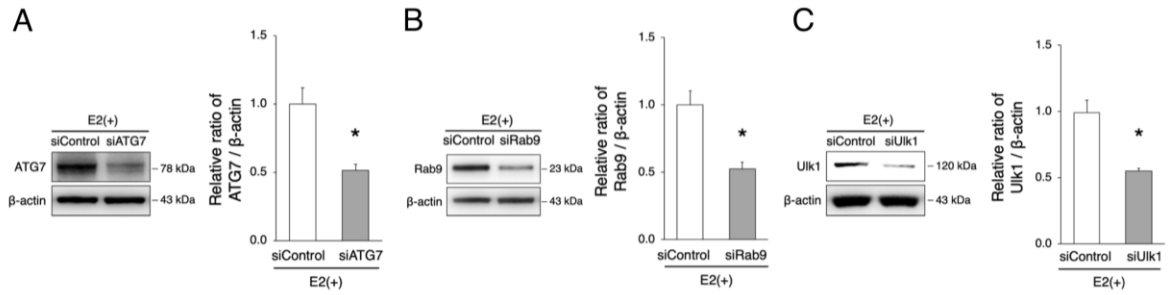
Data are expressed as the mean ± standard error of the mean (SEM). The student's *t*-test was used to determine the statistical significance of differences between two groups. For data sets with a skewed distribution or smaller sample size (n = 3 to 5 per group), the nonparametric statistical analysis was performed using the Wilcoxon rank sum test. Exceptions were the data in Figure 1A, 2E, 2F, and Figure S1B that were analyzed by two-way analysis of variance. Analyses were performed by JMP Pro 15 (SAS Institute, Cary, NC, USA). *P* values <0.05 were considered significant.

Figure S1. Estrogen delays cell aging via the maintenance of the mitochondrial function.



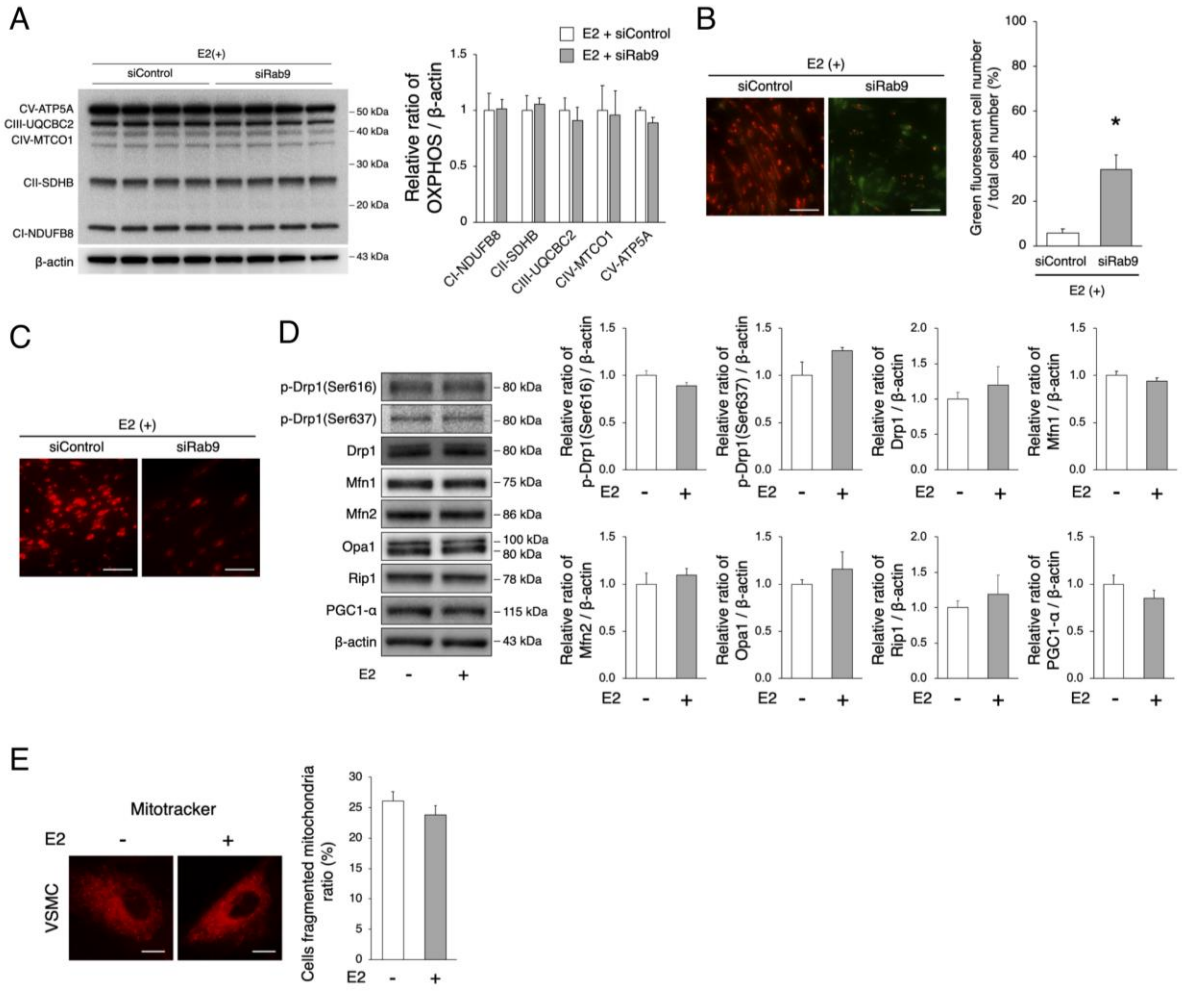
A, Immunoblots, and quantitative analysis results of p53 are shown. The protein expression of p53 was higher in E2-treated cells collected after 10 passages than those collected after 5 passages. The administration of ICI attenuated the E2-mediated delay of p53 expression in E2-treated cells collected after 10 passages. **P* < 0.05 vs. E2(-) (n = 6 per group). **B**, The mitochondrial membrane potential was evaluated using JC-1. Red indicates mitochondria in which the membrane potential is maintained, whereas green indicates depolarized mitochondria. The quantification of HUVECs and VSMCs with depolarized mitochondria is shown. Scale bar, 50 μm. **P* < 0.05 vs E2 (HUVECs, n = 5 per group; VSMCs, n = 5 per group). Statistical analysis was performed using 2-way analysis of variance. All data are shown as the mean ± SEM. E2 indicates 17β-estradiol; HUVECs, human umbilical vein endothelial cells; VSMCs, vascular smooth muscle cells.

Figure S2. Autophagy-related factors were knockdown via siRNA-transfection in E2 treated VSMCs.



A, Representative immunoblots of ATG7 are shown in E2-treated VSMCs transfected with siATG7 or siControl. **P* < 0.05 vs. siControl (n = 3 per group). **B**, Representative immunoblots of Rab9 are shown in E2-treated VSMCs transfected with siRab9 or siControl. **P* < 0.05 vs. siControl (n = 3 per group). **C**, Representative immunoblots of Ulk1 are shown in E2-treated VSMCs transfected with siUlk1 or siControl. **P* < 0.05 vs siControl (n = 3 per group). All data are shown as the mean ± SEM. E2 indicates 17β-estradiol; VSMCs, vascular smooth muscle cells.

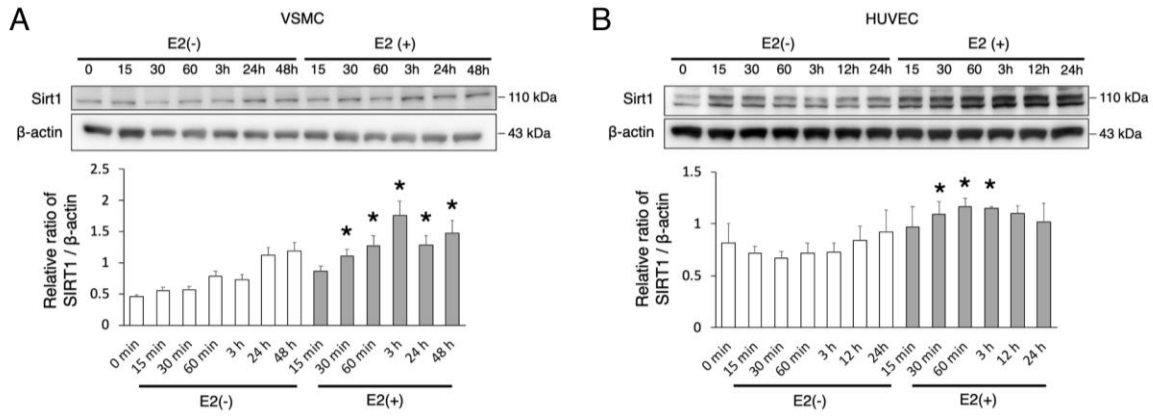
Figure S3. E2 is not involved in mitochondrial dynamics and maintains the mitochondrial function via Rab9 dependent alternative autophagy.



A, Representative immunoblots and quantitative analysis of total OXPHOS in E2-treated VSMCs transfected with siRab9 or siControl. The expression of total OXPHOS was not different between the two groups (n = 3 per group). **B**, The mitochondrial membrane potential was evaluated using JC-1. Red indicates mitochondria in which the membrane potential is maintained, whereas green indicates depolarized mitochondria. The quantification of VSMCs with depolarized mitochondria is shown. Scale bar, 50 μm. *P < 0.01 vs E2 (VSMCs, n = 3 per group). **C**, TMRE staining for the assessment of the mitochondrial membrane potential. Red indicates polarized mitochondria in

which the membrane potential is maintained. Scale bar, 50 μm. **D,** Representative immunoblots and quantitative analysis results of p-Drp1 (Ser616), p-Drp1 (Ser637), Drp1, Mfn1, Mfn2, Opa1, Rip1, and PGC-1α in VSMCs with or without E2 treatment are shown. Protein expression was not different between the two groups (n = 4 per group). **E**, Mitotracker Red was added into the medium to stain mitochondria in VSMCs with or without E2. The ratio of cells with fragmented mitochondria was not different between the two groups (n = 3 per group). Scale bar, 10 μm. All data are shown as the mean ± SEM. E2 indicates 17β-estradiol; OXPHOS, oxidative phosphorylation; VSMCs, vascular smooth muscle cells.

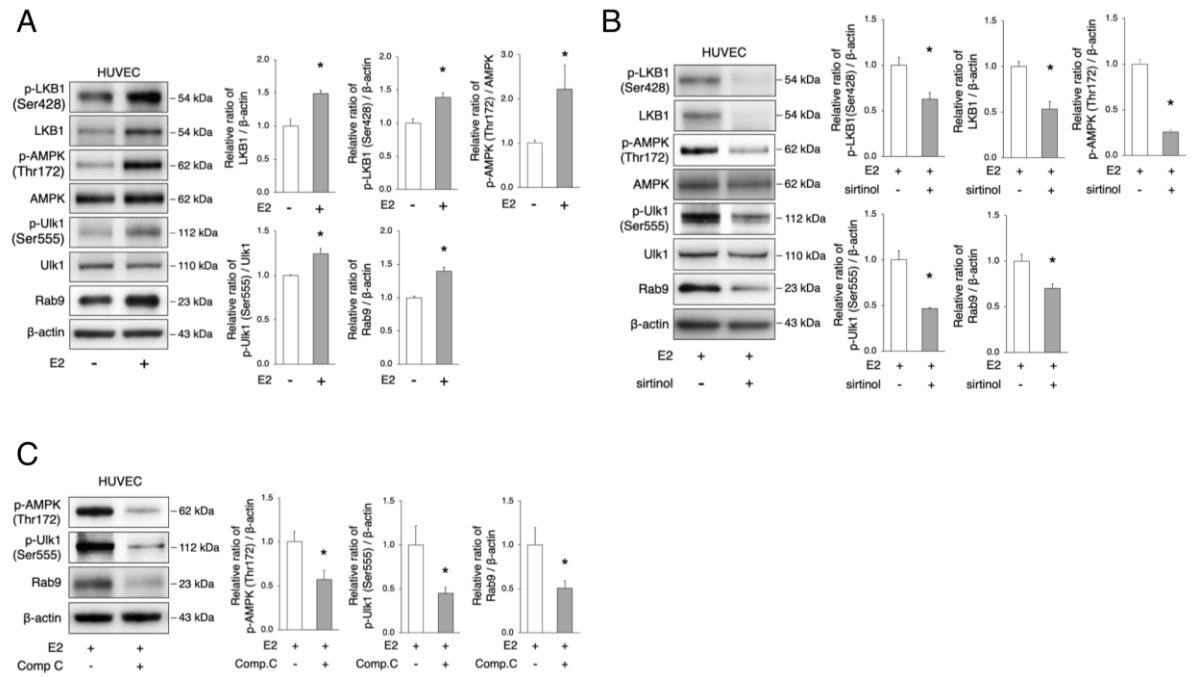
Figure S4. E2 upregulates SIRT1 in HUVECs and VSMCs.



A, Representative immunoblots and quantitative analysis of SIRT1 in VSMC with or without E2 treatment for 15 min, 30 min, 1 h, 3 h, 6 h, 24 h, and 48 h. **P* < 0.05 vs E2(-) (n = 3 per group). **B**, Representative immunoblots and quantitative analysis of SIRT1 in HUVEC with or without E2 treatment for 15 min, 30 min, 1 h, 3 h, 12 h, and 24 h. **P* < 0.05 vs E2(-) (n = 4 per group).

E2 indicates 17β-estradiol; HUVECs, human umbilical vein endothelial cells; VSMCs, vascular smooth muscle cells.

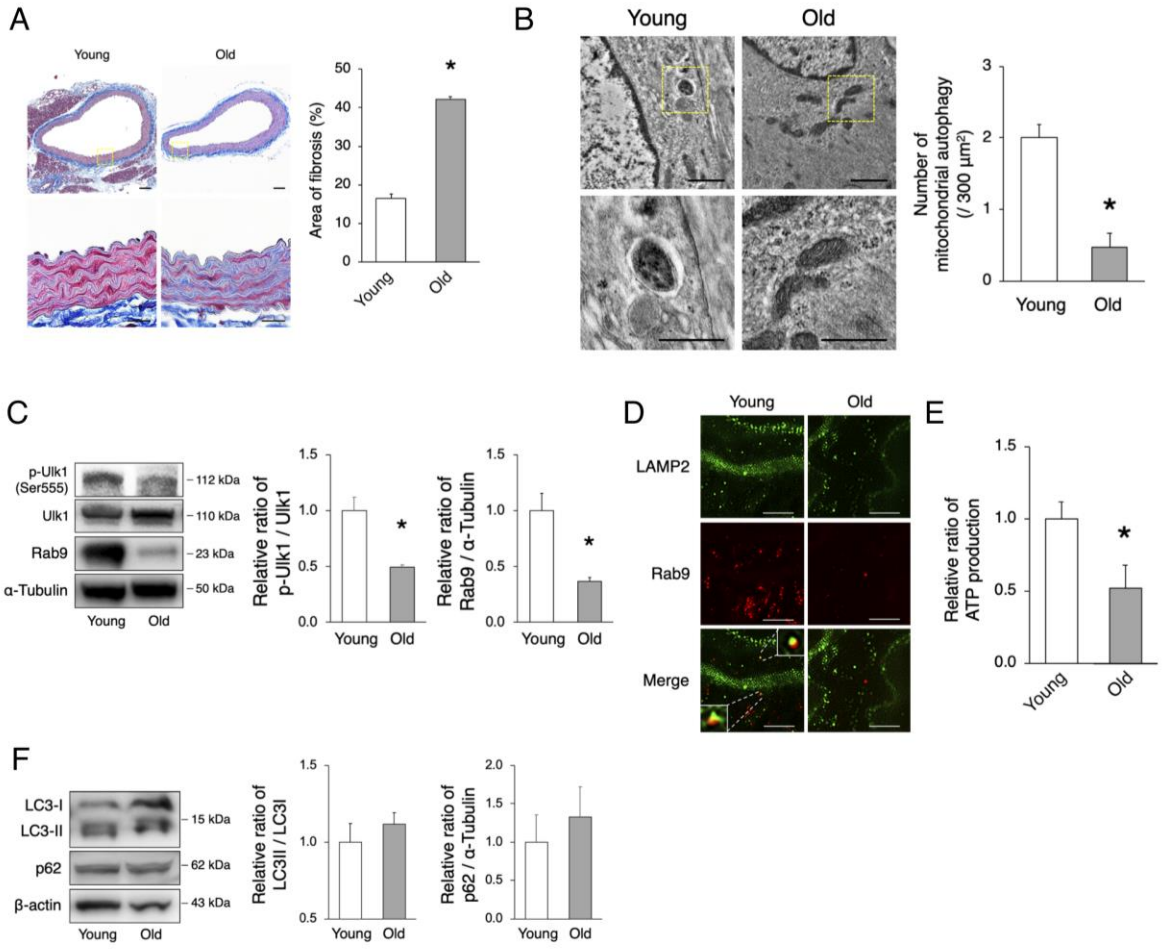
Figure S5. The SIRT1/LKB1/AMPK/Ulk1 axis is involved in the induction of alternative autophagy in HUVECs.



A, Representative immunoblots and quantitative analysis of LKB1, AMPK, Ulk1, and Rab9 in HUVECs with or without E2 treatment. E2 treatment activated LKB1, AMPK, Ulk1, and Rab9. **P* < 0.05 vs E2(-) (n = 3 per group). **B**, Representative immunoblots and quantitative analysis of LKB1, AMPK, Ulk1, and Rab9 in E2-treated HUVECs with or without sirtinol. The administration of sirtinol attenuated the activation of LKB1, AMPK, Ulk1, and Rab9 by E2. **P* < 0.05 vs E2(+) (n = 3 ,4 per group). **C**, Representative immunoblots and quantitative analysis of AMPK, Ulk1, and Rab9 in E2-treated HUVECs with or without Compound C. The administration of Compound C attenuated the activation of AMPK, Ulk1, and Rab9 by E2. **P* < 0.05 vs. E2(+) (n = 4 per group).

All data are shown as the mean ± SEM. HUVECs indicate human umbilical vein endothelial cells; E2, 17β-estradiol.

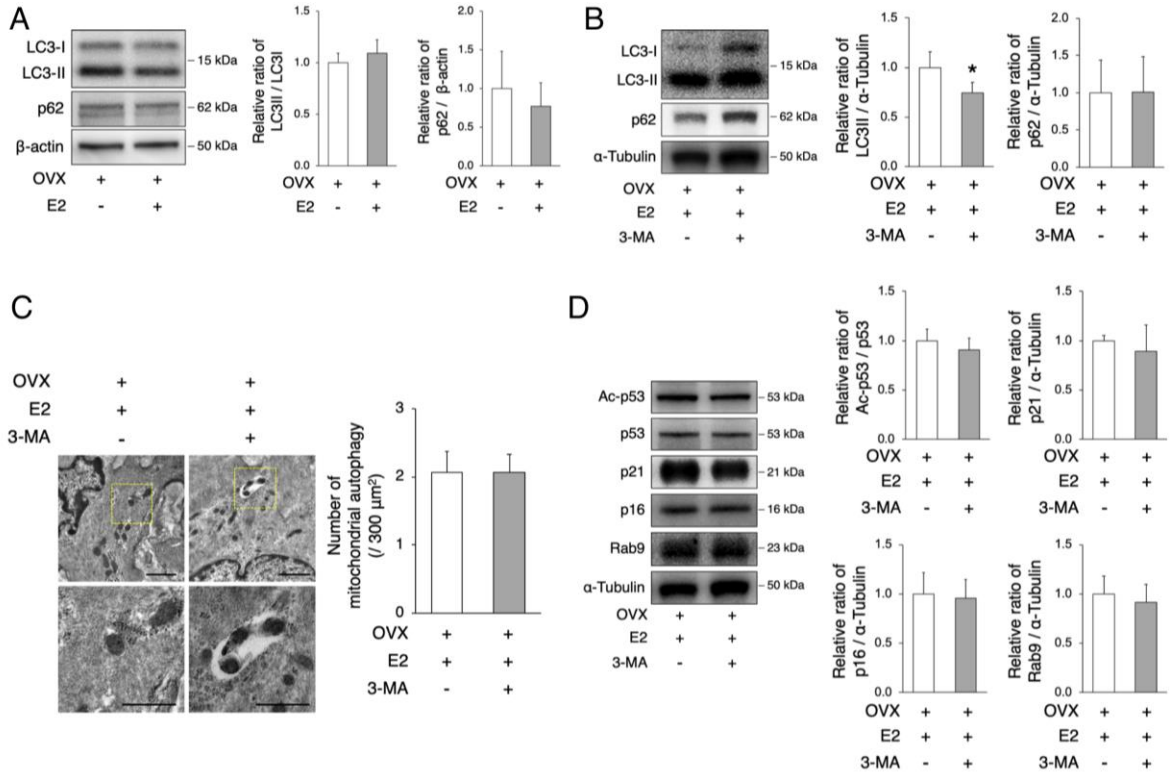
Figure S6. Old C57BL/6 mice show signals of arterial senescence.



A, Assessment of aortic fibrosis in Young and Old mice using the Masson's trichrome staining. Enlarged images of the areas delineated by the dashed rectangles are shown below. The area of fibrosis was greater in Old mice. Scale bar, 25 μm. **P* < 0.05 vs. Sham (n = 3 per group). **B**, Electron microscopy images of the aortas from Young and Old mice (upper panel, scale bar, 1 μm). Enlarged images of the areas delineated by the dashed rectangles are shown below (scale bar, 500 nm). The aorta of each mice was randomly selected (more 1000 μm^2) in 3 samples per group and the number of mitochondria engulfed by autophagosomes was counted. The

number of mitochondrial autophagy was lower in Old mice. *P < 0.05 vs. Young. **C**, Representative immunoblots and quantitative analysis of Ulk1 and Rab9 in Young and Old mice. The expression of activated Ulk1 and Rab9 in Old mice was lower than that in Young mice. *P < 0.05 vs. Young (n = 4 per group). **D**, Representative images of LAMP2 (green) and Rab9 (red) immunohistochemistry of the aorta in Young and Old mice. The number of Rab9 - LAMP2 co-localizing signals was lower in Old mice. **E**, Relative ATP production by isolated mitochondria from the aorta of Young and Old mice. ATP production was lower in Old versus Young mice. *P < 0.05 vs. Young (n = 3 per group). **F**, Representative immunoblots and quantitative analysis of LC3 and p62 in Young and Old mice. The expression of both LC3 and p62 was not different between the two groups (n = 4 per group). All data are shown as the mean ± SEM. LAMP2 indicates lysosome-associated membrane protein 2.

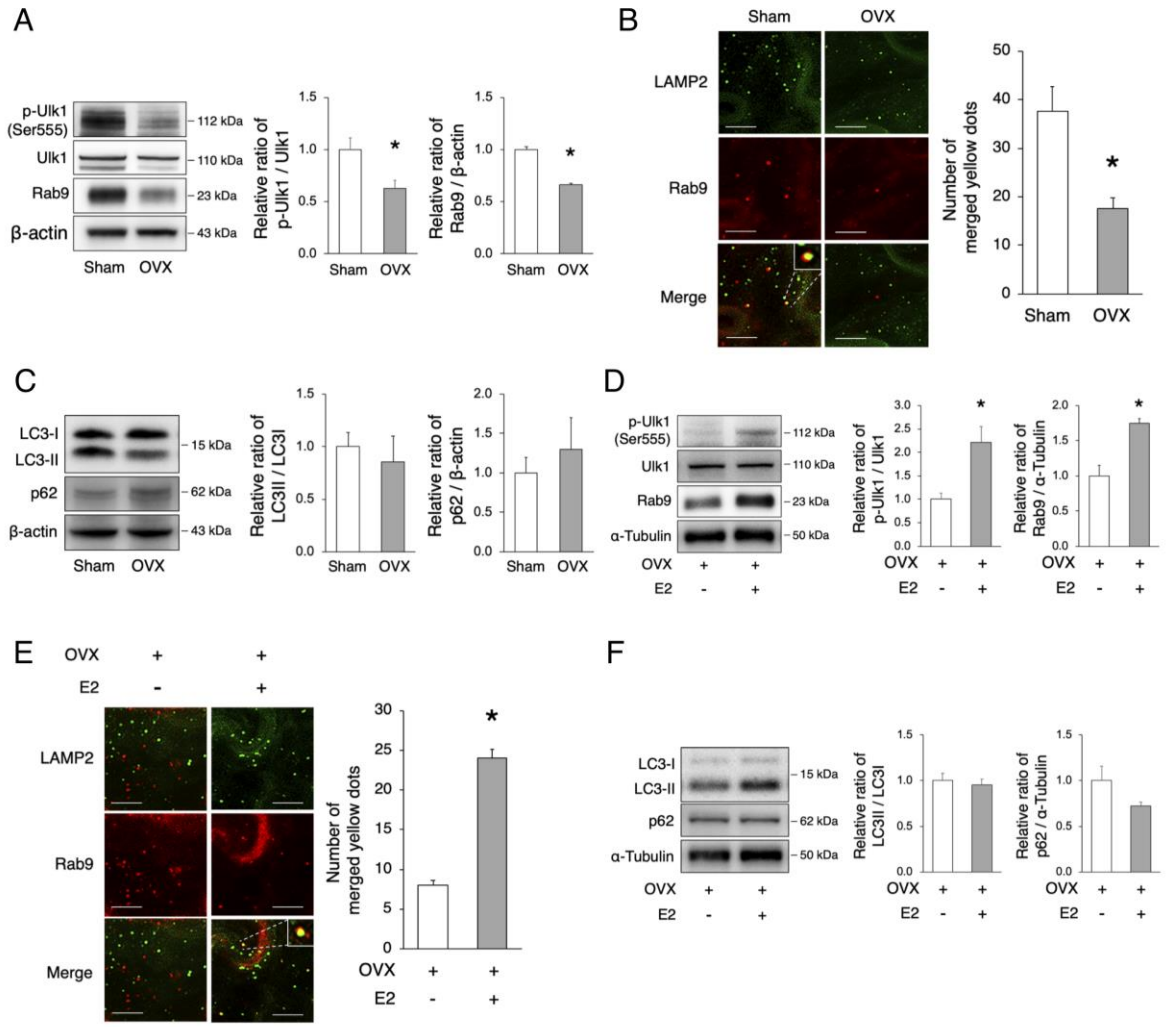
Figure S7. Effect of E2 supplementation in OVX mice.



A, OVX mice were implanted with either E2 or control pellets for 8 weeks. Representative immunoblots and quantitative analysis of LC3 and p62 in OVX and OVX + E2 mice. The expression of both LC3 and p62 was not different between the two groups (n = 3 per group). **B-D,** OVX mice were implanted with an E2 pellet were treated with or without 3-MA. **B**, Representative immunoblots and quantitative analysis of LC3 and p62 in OVX + E2 mice with or without 3-MA. The administration of 3-MA decreased the LC3II levels in OVX + E2 mice. **P* < 0.05 vs. OVX + E2 (n = 5 per group). **C**, Representative immunoblots and quantitative analysis of Ac-p53, p21, p16, and Rab9 in OVX + E2 mice with or without 3-MA. The expression of Ac-p53, p21, p16, and Rab9

was not significantly different between the two groups (n = 5 per group). **D**, Electron microscopy images of the aorta from OVX + E2 and OVX + E2 mice treated with 3-MA (upper panel, scale bar, 1 μm). Enlarged images of the areas delineated by the dashed rectangles are shown below (scale bar, 500 nm). The aorta of each mice was randomly selected (more 1000 μm^2) in 3 samples per group and the number of mitochondria engulfed by autophagosomes was counted. The number of mitochondrial autophagy was not different between the two groups. All data are shown as the mean ± SEM. E2 indicates 17β-estradiol; OVX, ovariectomy; 3-MA, 3-Methyladenine.

Figure S8. Mitochondrial autophagy in ApoE KO mice treated with E2.



A-C, Apolipoprotein E knockout (ApoE KO) mice were subjected to OVX or sham surgery. **A**, Representative immunoblots and quantitative analysis of Ulk1, and Rab9 in Sham-operated and OVX mice. The expression of activated Ulk1 and Rab9 in Sham mice was higher than that in OVX mice. *P < 0.05 vs. Sham (n = 3 per group). **B**, Representative images of LAMP2 (green) and Rab9 (red) immunohistochemistry of the aorta in Sham-operated and OVX mice. The number of yellow signals was lower in OVX mice. Scale bar, 5 μm. *P < 0.05 vs. Sham (n = 3 per group). **C**,

Representative immunoblots and quantitative analysis of LC3 and p62 in Sham-operated and OVX mice. The expression of both LC3 and p62 was not different between the two groups (n = 3 per group). **D-E**, ApoE KO OVX mice were implanted with either E2 or control pellets for 8 weeks. **D**, Representative immunoblots and quantitative analysis of Ulk1 and Rab9 in OVX and OVX + E2 mice. The expression of activated Ulk1 and Rab9 in OVX + E2 mice was higher than that in OVX mice. *P < 0.05 vs. Sham (n = 4 per group). **E**, Representative images of LAMP2 (green) and Rab9 (red) immunohistochemistry in OVX and OVX + E2 mice. The merged yellow signals were higher in OVX + E2 mice. Scale bar, 5 μm. *P < 0.05 vs OVX (n = 3 per group). **F**, Representative immunoblots and quantitative analysis of LC3 and p62 in OVX and OVX + E2 mice. The expression of both LC3 and p62 was not different between the two groups (n = 4 per group). All data are shown as the mean ± SEM. ApoE KO indicates Apolipoprotein E knockout; E2, 17β-estradiol; OVX, ovariectomy; LAMP2, lysosome-associated membrane protein 2.