SUPPORTING DOCUMENT FOR

ENDOTHELIAL SIRTUIN 1-DEFICIENCY PERPETRATES DOWN-REGULATION OF MMP-14 AND NEPHROSCLEROSIS: RELEVANCE TO FIBROSIS OF VASCULAR SENESCENCE

DETAILED METHODS

Mice with endothelial SIRT1 deletion. Endothelial SIRT1-deleted mouse model was created by cross-breeding of B6;129-Sirt1^{tm1Ygu}/J (homozygous for targeted allele *SIRT1*co/co, viable and fertile, containing a loxP-flanked neomycin cassette upstream and downstream of exon 4 of the targeted gene) with Tie2-Cre transgenic mice (B6.cg-tg(tek-cre)1ywa/J) expressing cre-recombinase in vascular endothelial cells (both from Jackson Lab). The resulting SIRT1$^{endo+/-}$ mice were mated with SIRT1$^{Flox/Flox}$ mice to obtain endothelial-deleted SIRT1 mutant mice. Endothelial SIRT1$^{endo-/-}$ knockout (and corresponding SIRT1$^{Flox/Flox}$ knockout-control) and SIRT1$^{endo+/-}$ heterozygote mice (and corresponding SIRT1$^{Flox/WT}$ heterozygote-control) were obtained at Mendelian ratios and used in the experiments. Animal protocols were conducted in accordance with the National Institutes of Health (NIH) 99 guidance and were approved by the Institutional Animal Care and Use Committee. Male mice between 12 and 16 weeks of age were used in the present study. These mice thrive normally and have normal blood pressure at the age of 3 months. Genotyping was performed by tail DNA PCR analysis**.** Tail DNA was isolated using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St Louis, USA). Primer sequences used for genotyping floxed SIRT1 allele were as follows SIRT1

forward: 5’GGT TGA CTT AGG TCT TGT CTG3’; SIRT1 reverse: 5’CGT CCC TTG TAA TGT TTC CC3’ and for SIRT1 null allele 5’AGG CGG ATT TCT GAG TTC GA3’. Tie2 transgene was detected using following primers: forward 5’GCG GTC TGG CAG TAA AAA CTA TC3’ and reverse 5’ GTG AAA CAG CAT TGC TGT CAC TT3’. The PCR products were analyzed on 1.5% TAE acrylamide gels.

Cell culture*.* Primary human umbilical vein endothelial cells (HUVEC) (ATCC, Manassas, USA), passages 2-5, were maintained in endothelial basal medium-2 (EBM-2, Lonza, Walkersville, USA) supplemented with 2% FBS and growth factors (hydrocortisone, hFGF-B, VEGF, hEGF, IGF-1, ascorbic acid and heparin), under conditions of 37°C and 5% CO_2.

Isolation of primary renal endothelial cells (EC). Mice were anesthetized and perfused with 20 ml PBS. Kidney was collected from $SIRT1^{endo-/-}$ and control $SIRT1^{Flox/Flox}$ mice. Tissue was minced manually and tissue fragments digested in DMEM medium containing 1 mg/ml Dispase (Roche) and 1 mg/ml collagenase H (Roche) at 37°C for 1 hour. The digested homogenate was passed through a 40 µm syringe filcon filter (BD Biosciences), and pellet was collected by centrifugation at 1500 rpm for 6 minutes. Primary ECs were magnetically separated, first using negative selection with CD16 coated magnetic beads followed by positive selection with CD31 coated magnetic beads (twice). Freshly isolated renal EC were cultured in Iscove's Modified Dulbecco's Medium (IMDM) medium containing 15% FBS, 1% BSA, 10^{-4} mM 2-mercaptoethanol, 0.2 mg/ml human transferrin and 0.01 mg/ml insulin (all from Gibco) supplemented with 100 ng/ml of recombinant mouse VEGF, EGF and basic FGF (Invitrogen). Isolated primary endothelial cells were positive for EC markers including

CD31 (BD Biosciences) and endoglin (R&D system), evaluated by immunofluorescence and quantitative real-time PCR with SIRT1exon4 primer (Forward: TGTCTCCTGTGGGATTCCTGACTTC, Reverse: TGGCTTGAGGGTCTGGGAGGT).

Acetylcholine-induced vasorelaxation of aortic rings. The descending thoracic aortas from mice were divided into cylindrical segments which were mounted on a wire-myograph and bathed in Krebs buffer gassed with 95% O_2-5% CO_2 for recording of isometric tension; the vessels were pre-constricted with phenylephrine to 70% of maximal response, and used for assessment of acetylcholine-induced vasorelaxation (0.001-100 μmol/L).

Staining of en face aortae for senescence-associated β-galactosidase (SA-β-gal). Senescence-associated-β galactosidase staining of aortas was performed as described previously. Images were obtained using a compound Nikon TE-2000U microscope equipped with a Spot Insight digital camera (Diagnostic instruments) at 60x to 600x magnifications.

Aortic outgrowth assay. Thoracic aortas were obtained from 12-week-old mice. Aortic rings cross-sectioned with 1 mm interval were embedded in 3D matrigel in 96-well plate and cultured at 37°C. Newly formed capillary cords in explant cultures were imaged every 24 hours using Nikon TE2000 microscope equipped with a CCD camera (Hamamatsu Photonics) at a magnification of 40x. Quantitative angiogenesis assays were performed according to previously published protocol.

MMP-14 expression and activity in endothelial cells. To inhibit SIRT1 activity, HUVEC were treated with 50 μM SIRT1 Inhibitor III for 48 h. MMP-14 protein expression

was examined by immunoblotting using primary anti-MMP-14 antibody (Abcam, Cambridge, USA). Beta-tubulin was used as loading control. To analyze the *in vitro* matrix-degrading activity of endothelial MMP and its dependence on SIRT1, HUVEC were cultured at low density in 3D matrigel for 2 weeks with or without addition of SIRT1 Inhibitor III. The medium was changed every 3 days with the inhibitor replenished. Cells were fixed with 4% PFA, stained with Coomassie Brilliant blue (3 g/l Coomassie Brilliant Blue R250, 45% methanol, 10% acetic acid) for 1 hour and destained in 10% acetic acid solution containing 20% methanol. The images were captured using Nikon TE2000 microscope at a magnification of 100x. Five random images were obtained in each group (control, 5 μM SIRT1 Inhibitor III, 10 μM SIRT1 Inhibitor III and 50 μM SIRT1 Inhibitor III), and the length (in pixels) of the formed capillary structures, as well as the integrated intensity and intensity (along the linescan) of the "halo" surrounding each cell were analyzed using MetaVue software (Molecular Device, USA).

MMP-14 immunofluorescence. Aortic segments were fixed with 4% PFA for 60 minutes, washed with PBS, permeabilized with 0.1% Triton in PBS for 10 min, and block with PBS/1%BSA for 1 hour at room temperature (RT). Next, the sections were incubated with primary anti-MMP-14 (1:100, Abcam, Cambridge, MA, USA) antibody overnight at 4°C, followed by incubation with secondary Alexa-Fluor 594 antibody (1:500, Invitrogen, Carlsbad, USA) at RT for 1 hour. The images were taken using a Nikon TE-2000U microscope and analyzed with ImageJ software.

Folate model of acute kidney injury and chronic progressive nephropathy. A single intraperitoneal injection of 250 mg/kg body weight folic acid (FA) in 0.3M $NaHCO_3$ vehicle or vehicle alone was administered in 12-week-old mice. The animals were

sacrificed after 48 hours (acute kidney injury phase), 28 and 90 days (chronic fibrotic phase). Kidneys, urine and blood samples were collected from each animal. Kidney sections were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Paraffin sections (4 µm thick) were stained with Masson's trichrome and the amount of collagen deposition (blue area) was then digitally quantified using with ImageJ. The spontaneous patchy fibrosis in untreated mice were assessed under high (600x) magnification using the grid method. All sections were examined in a blinded fashion, without knowledge of the origin of individual preparations.

Measurement of cytokines and chemokines levels. Profiling of cyto- and chemokines was accomplished using multiplex analysis of the plasma obtained from experimental animals using Luminex 2 IS100 automated system (Luminex Inc., TX, USA) and data analyzed using Luminex 100IS curve-fitting software (version 2.3).

In vivo induction of MMP-14 with concanavalin A. Prolonged SIRT1 inhibition in mice was achieved by administration of selective SIRT1 Inhibitor III (EMD Millipore, MA, USA) added to drinking water at a daily dose of 1 mg/kg body weight for four weeks. SIRT1 Inhibitor III (6-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamid) is a cell-permeable indole compound that acts as a potent and highly selective inhibitor of SIRT1. It has been shown to be well absorbed after oral administration with a serum half-life of 136 minutes in mice. Animals were treated with SIRT1 inhibitor with or without additional intravenous injections of concanavalin A (ConA, Sigma Aldrich, MO, USA) at a dose of 5 mg/kg body weight administered once a week. Daily water consumption did not differ between treated and control groups. Previous studies have provided strong evidence that ConA is a powerful inducer of MMP-14. The results of

toxicological studies clearly indicate that toxic effects of ConA are dependent on the dose and the route of administration. The dose used in our study has been shown to be safe and no signs of hepatic, hematologic or any overt toxicity were reported. In the assessment of aortic matrilytic activity, aortic rings isolated from the animals were cultured in 3D matrigel for up to 12 days. After sacrificing the animals, all rings were incubated under the same conditions, the treatment with SIRT1 inhibitor or ConA was not continued. The integrated intensity and intensity along the linescan of the “halo” surrounding each ring were analyzed with MetaVue software (Molecular Device, USA).

Zymography. HUVEC were pretreated with 10 μM SIRT1 inhibitor III for 5 days. On day 5, culture medium was replaced by serum-free medium and cells and culture media were harvested 24 hours later. Zymography was performed using electrophoresis of samples on 10% SDS gels impregnated with gelatin (Bio-Rad), followed by renaturation and developing buffers (both from Bio-Rad). Densitometric analysis of bands was performed to quantify the results.

Histologic studies. The kidneys were perfused in situ with cold PBS, removed, and mid-coronal sections were fixed in 4% PFA for 16 hours, followed by incubation in 30% sucrose. Paraffin sections (4 μm thick) were stained with hematoxylin and eosin and an average of 3 sections per animal were examined. The morphologic evaluation of folic acid- induced acute kidney injury was performed using well-established criteria in a blind manner.

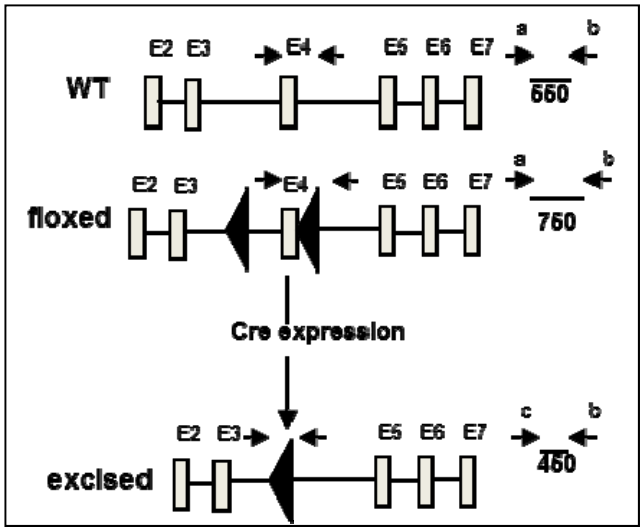
Microalbuminuria and creatinine measurement. Albuminuria and urinary creatinine excretion were determined in morning urine samples using Albuwell M and Creatinine

Companion assays respectively (Exocell, Philadelphia, USA). The results were presented as albumin-to-creatinine ratio (ACR) in mg/g. Serum creatinine concentration was measured using a commercially available kit (Abcam, MA, USA).

Statistical analysis. All experiments were repeated at least three times. Data are presented as means ± SE. For multiple comparisons between groups, ANOVA with Bonferroni's post-hoc analysis was performed. A P value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL FIGURE 1

A



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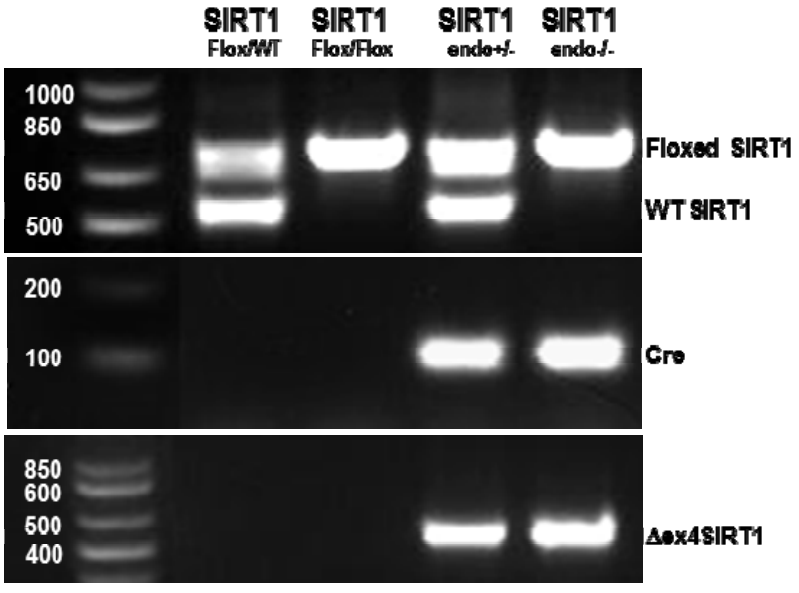
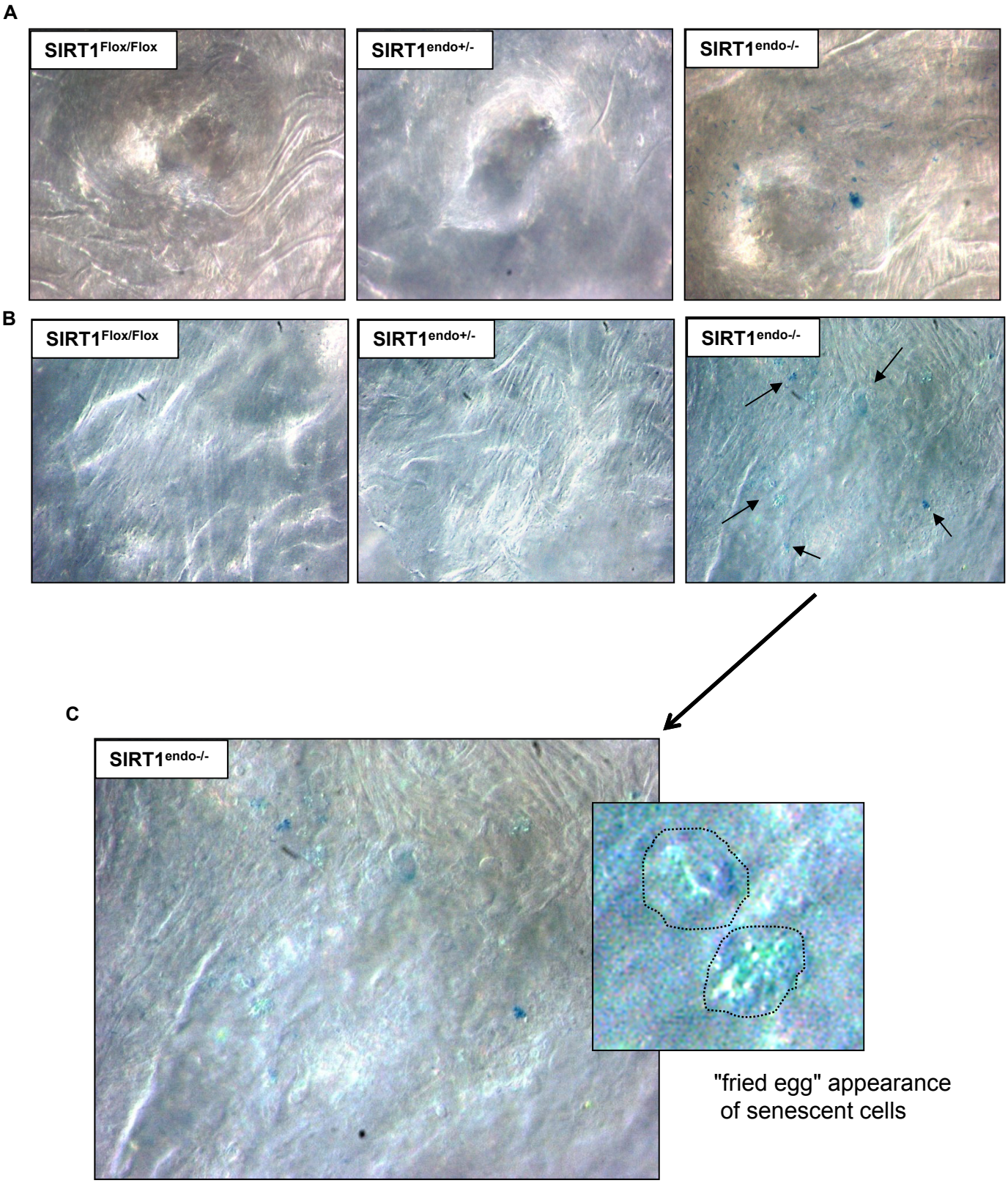
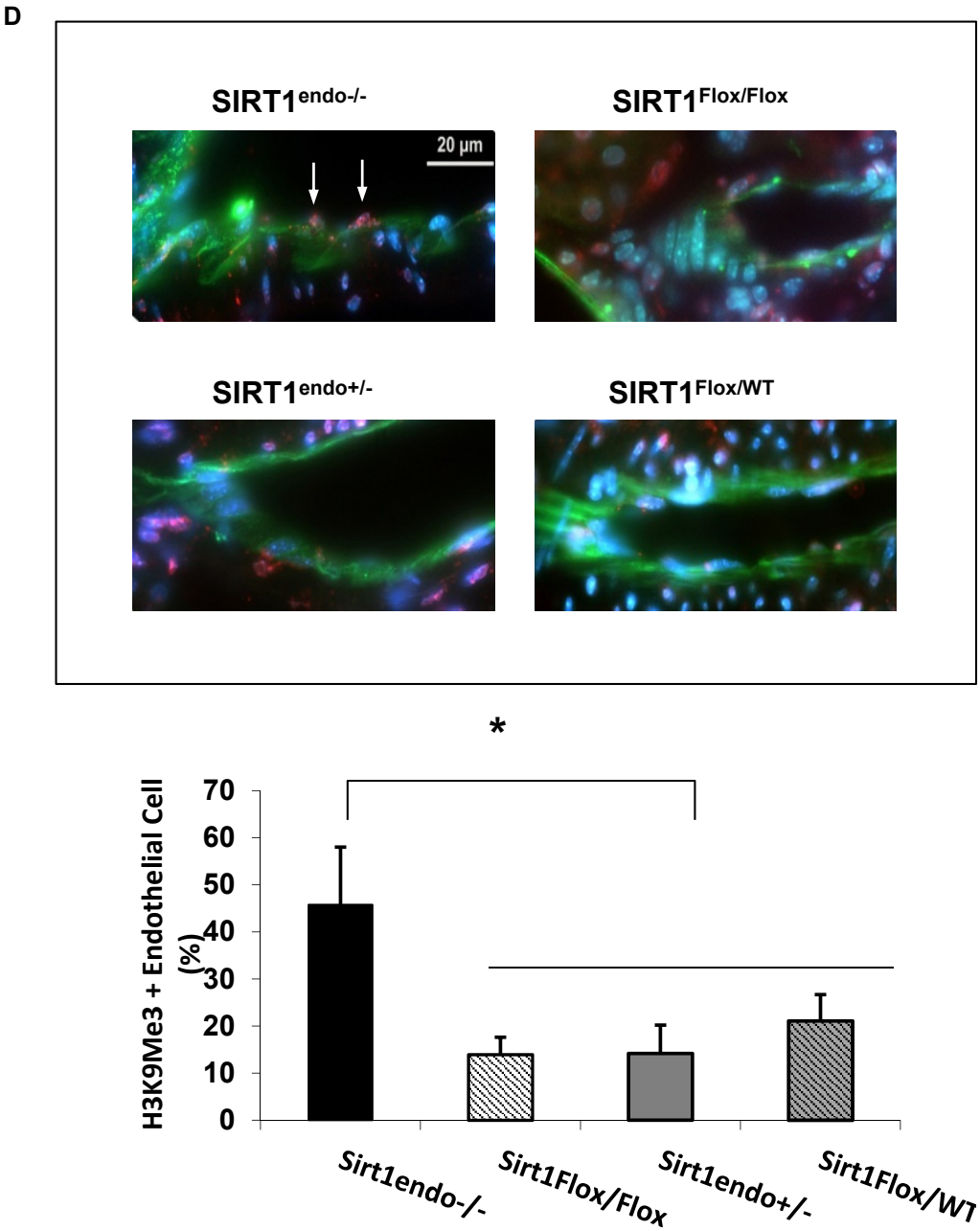


C

SUPPLEMENTAL FIGURE 2



SUPPLEMENTAL FIGURE 2



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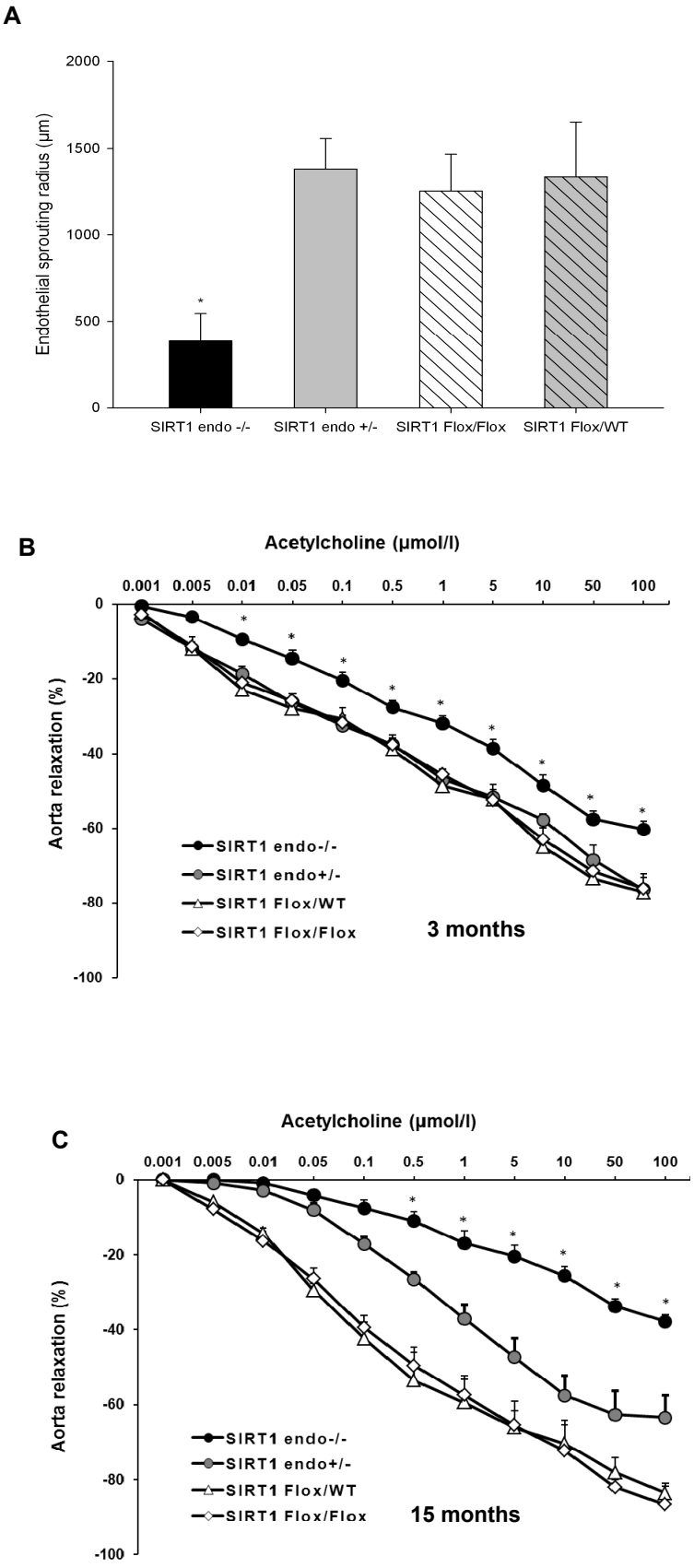

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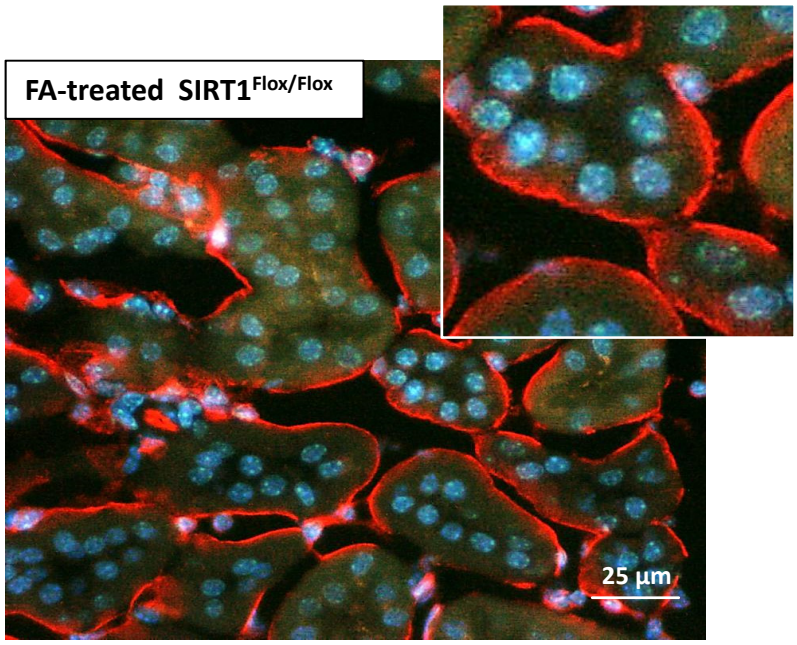

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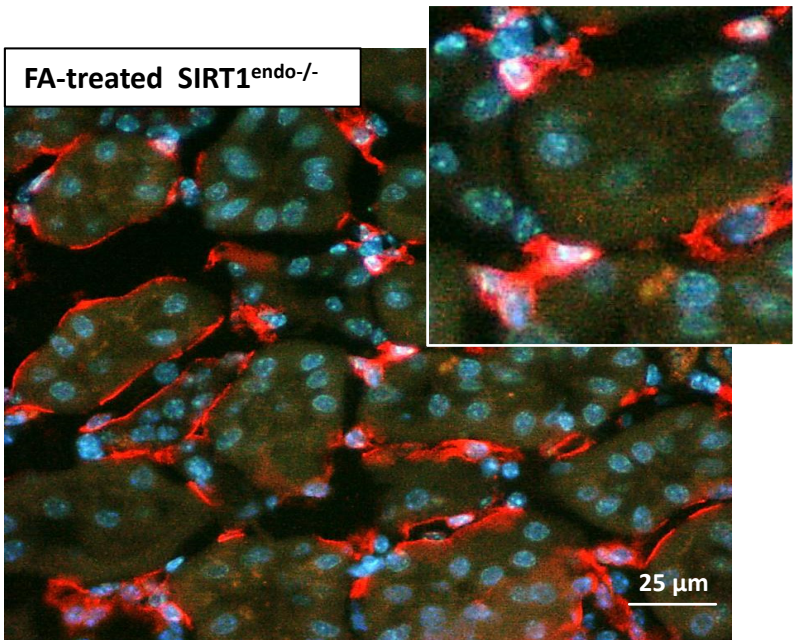

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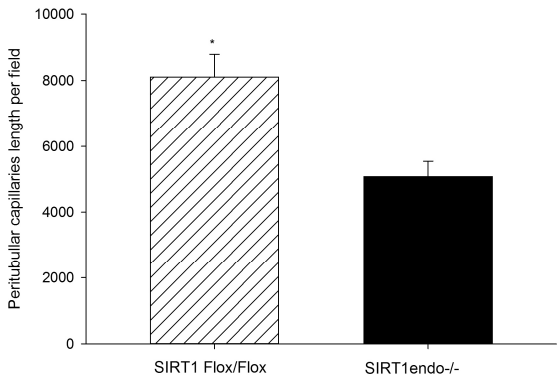

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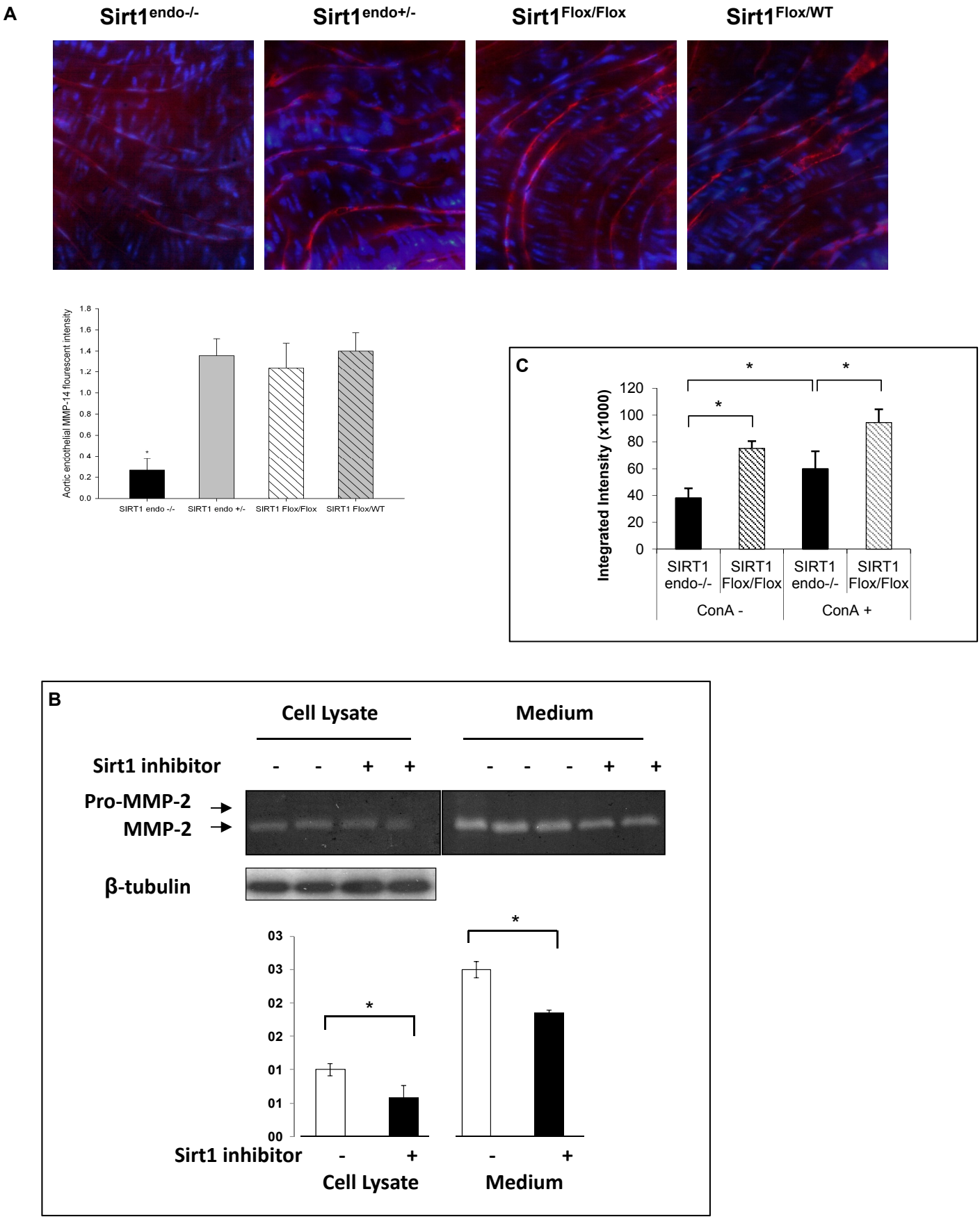

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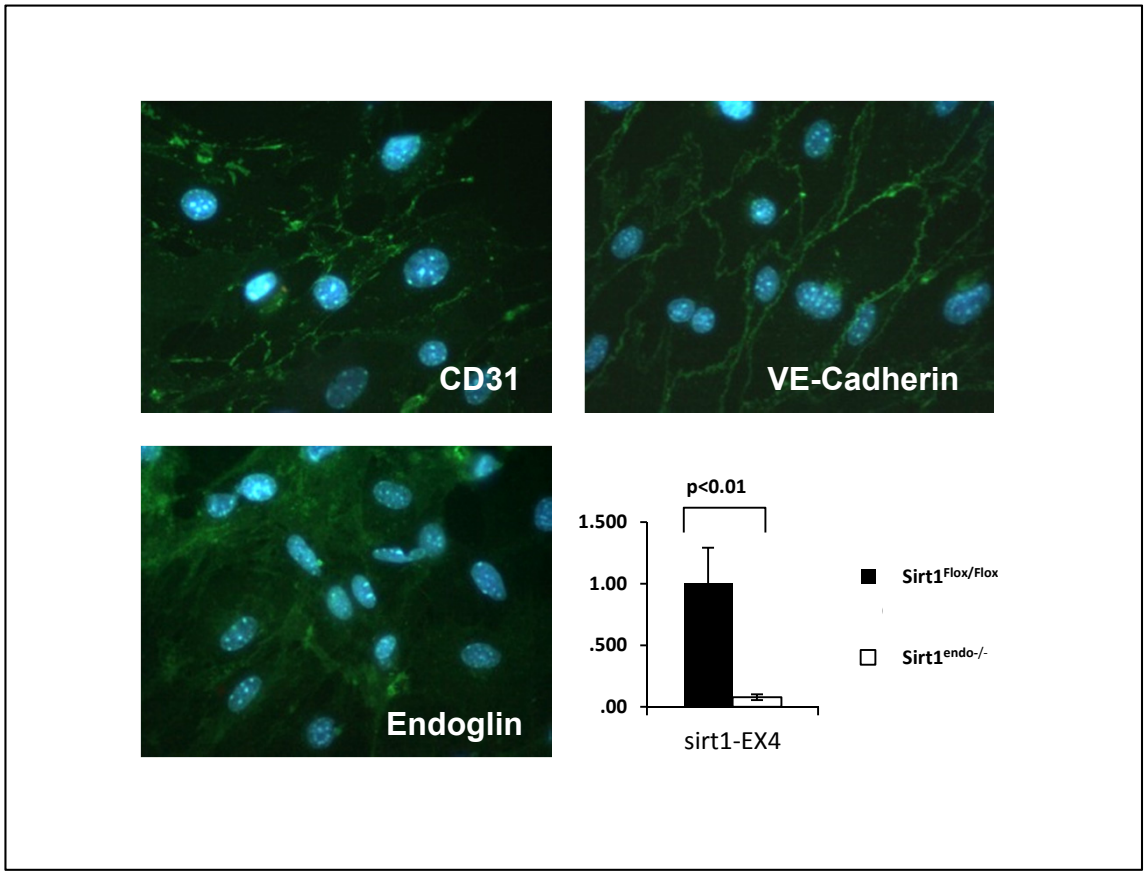

SUPPLEMENTAL FIGURE 7