Supplemental Materials

Methods

Antibodies, siRNA, and reagents. Short interfering RNA (siRNA) targeted to human p90RSK1 (RPS6KA1, #NM_001006665; 5'-GUGGGCACCUGUAUGCUAU-3') and p90RSK2 (RPS6KA2, #NM_001006932; 5'-CAAGCGAUGUGUGCAUAAA-3') were purchased from Thermo Scientific Dharmacon. Non-specific siRNA negative control was purchased from Invitrogen (#12935-112). Rabbit and mouse anti-p53 (#SC-6243 and #SC-126), rabbit and mouse anti-Bcl-2 (#SC-492 and #SC-7382), rabbit anti-VCAM-1 (#SC-8304), mouse anti-ICAM-1 (#SC-8439), goat anti-PECAM-1 (#SC-1506), rabbit anti-Myc (#SC-789), and goat anti-SENP2 (#SC-67075, epitope corresponding to amino acids 1-300) were purchased from Santa Cruz Biotechnology. Rabbit anti-ERK5 (#3372), rabbit anti-caspase3 (#9665), and rabbit anti-cleaved Caspase3 (#9664) were purchased from Cell Signaling Technology, Inc. The rabbit and mouse anti-SUMO2/3 were purchased from ABGENT (#AP1224a) and MBL (#M114-3), respectively. Rat anti-VE-cadherin (#555289) and rabbit anti-eNOS (#610299) were purchased from BD Biosciences (Pharmingen). Rabbit anti-E-selectin (# 3631-100) was purchased from Bio Vision. Anti-Xpress (#R910-25) antibody was purchased from Invitrogen.

Plasmid and adenovirus vector construction. The plasmids encoding human HA-SUMO3 and mouse pCS2-myc-SENP2 were kind gifts from Dr. Ronald T. Hay (University of Manchester, UK) (13) and Dr. Wei Hsu (University of Rochester, NY) (14), respectively. Human pcDNA-Flag-p53 and human Flag-SENP2 were obtained from the non-profit Addgene Plasmid Repository (Dr. Thomas Roberts, Addgene plasmid 10838 (15) and Dr. Edward Yeh, Addgene plasmid 18047 (16)). Truncated forms of SENP2 were constructed by inserting EcoRI-XhoI (Fr 1, 3, and 4) or EcoRV-XhoI (Fr. 2) fragment, generated by PCR, into the pCMV-Taq2B vector. The Gal4-wild type and p90RSK were created by inserting a KpnI-XbaI fragment, generated by PCR, into the pBIND vector (Promega). V16-SENP2 fragment mutants were created by inserting mouse truncated SENP2s isolated from pCMV-Taq2B-SENP2 fragments into EcoRV1 and NotI sites of the pACT vector. The phospho-mutants of mouse pCS2-myc-SENP2 and human Flag-SENP2-T35A, -S38A, and -T368A were created by using the QuikChange site-directed mutagenesis kit (Stratagene). Adenovirus vectors including SENP2-WT and SENP2-T368A were subcloned into the pENTR vector (Invitrogen) using specific enzyme sites, and then a recombinase reaction was performed to get a pDEST-based vector following manufacture's instruction (#K4930-00, ViraPower Adenoviral Expression System, Promega). All constructs were verified by DNA sequencing.

Generation of mouse anti-phospho-SENP2-T368. A peptide corresponding to the amino acids 358-378 of mouse SENP2 (Biotin-NPEGQ-DRRTEDLFEFT*EDMEKEISNA) was synthesized (Peptibody Inc.) with an amino-terminus biotin label in both phosphorylated and unphosphorylated forms. A mouse anti-phospho-SENP2-T368 single-chain Fv (scFv) was generated against the phospho-peptide by phage display (17) with a naïve human phage display library constructed from peripheral blood lymphocytes (18, 19). The phospho-peptide was bound to streptavidin-coated immunowells (#15500, Pierce) and the phage library was enriched for three rounds, switching to neutravidin-coated wells for the second round to avoid isolating anti-streptavidin scFvs. Individual clones from the second and third rounds were screened by

phage ELISA and a clone reactive with phosphorylated peptide, but not with un-phosphorylated peptide, was identified. The scFv was sequenced, re-amplified and cloned into a human immunoglobulin heavy chain expression (20) vector modified by deletion of the CH1 domain to produce an scFv-Fc fusion protein. This plasmid was transiently transfected into HEK293T cells and the medium was harvested every three days. The culture supernatants from three harvests were combined, and the scFv was purified on an Aspire protein G tip (Thermo-Fisher). The protein was eluted from the tip and dialyzed into PBS and used for Western blotting experiments. We used peroxidase-labeled affinity purified anti-human IgG (H+L; #474-1006, KPL) as the secondary antibody for immunoblotting.

Isolation of mouse ECs (MECs). MECs were isolated according to our previous publication (1). Briefly, lungs were harvested, minced finely with scissors, and then digested in 15 mL of 2 mg/ml collagenase (#4177; Worthington Biochemicals Corp.) at 37^0C for 45 min. The suspension was then triturated 15 to 20 times using a syringe attached to a cannula, avoiding frothing, and filtered through a 70 μm cell strainer. The crude cell preparation was centrifuged and the pellet resuspended in cold DPBS with Ca/Mg (# SH30264.01; HyClone Laboratories Inc.) and 0.1% BSA (#A9576; Sigma-Aldrich). The cell concentration was adjusted to $3x10^7$ cells/mL. Dynabeads were conjugated with sheep anti-rat IgG (#110.35; Invitrogen) and then incubated with rat anti-mouse PECAM-1 (#553370; Pharmingen) according to the manufacturer's instruction. The cell suspension was incubated with the anti-mouse PECAM-1-coated beads (35 μl beads/mL cell suspension) at room temperature for 30 min with end-over-end rotation. Using a magnetic separator stand (#Z5410; Promega), cells bound to the beads were recovered, washed with DMEM containing 20% FBS, resuspended in 6 mL complete culture medium (DMEM containing 20% FBS and supplemented with 100 μg/mL porcine heparin and 100 μg/mL EC growth supplement (ECGS; #BT-203, Biomedical Technologies Inc.), nonessential amino acids, sodium pyruvate, and antibiotics at standard concentrations), and then plated in a 0.2% gelatin-coated 6 cm dish. Once the cells reached 70-80% confluence, they were detached with trypsin-ethylenediaminetetra-acetate (EDTA) to generate single-cell suspension, pelleted, and then resuspended in 2 mL DPBS with Ca/Mg and 0.1% BSA and incubated with the anti-PECAM-1-coated beads (35 μl beads/mL cell suspension) at RT for 15 min as described previously (2). Cells bound to beads were washed and plated in the complete culture medium. Cells were used for experiments at passage 3. The purity of MECs was performed by Western blotting and immuno-fluorescence (data now shown) using an anti-PECAM-1 antibody.

Immunoprecipitation (SUMO assay) and Western blot analysis. Cells were collected in PBS containing 10 mM N-ethylmaleimide (NEM), and cell extracts were prepared in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1 mM dithiothreitol, 1:200-diluted protease inhibitor cocktail (Sigma), 1 mM PMSF and 10 mM NEM, and 0.1 mM iodoacetamide). SUMOylation was detected as previously decribed (7). Briefly, p53 and ERK5 were immunoprecipitated from cell lysates, and the immunoprecipitation products were dissolved in 2X SDS sample buffer, resolved by SDS-PAGE, electrotransferred onto a Hybond enhanced chemiluminescence nitrocellulose membrane. SUMOylated proteins were detected by Western blotting with mouse anti-SUMO2/3 and visualized by enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech.) according to the manufacturer's instruction. Results were normalized to the lowest SUMOylation level within each set of experiments and statistical significance was

determined by comparing the average level of the control group to each of the experimental data points.

To determine protein binding, HeLa cells (1 x 10^6 cells) were plated in 100 mm dishes and transfected in Opti-MEM (Invitrogen) with lipofectamine mixture containing flag-tagged SENP2 truncated mutants in the presence of Xpress-tagged RSK1 and Myc-tagged SENP2 for 4 hr. Cells were washed and cultured for 24 hr in fresh complete growth medium. Cell lysates were made by incubating cells in 0.5 ml of RIPA lysis buffer for 30 min at 4°C. After centrifugation for 20 min at 12,000 rpm, the whole cell lysates were immunoprecipitated with rabbit anti-Myc beads. Bound proteins were released in 2X SDS sample buffer and analyzed by Western blotting using mouse anti-Xpress (#R910-25, Invitrogen).

In vitro phosphorylation of SENP2 by activated p90RSK. Glutathione-*S*-transferase (GST)–SENP2–truncated mutant proteins were expressed in *Escherichia coli*, purified with glutathione-Sepharose 4B as described (#52-2303-00 AK, Pharmacia Biotech Inc.), and used in in vitro kinase assays by activated p90RSK as described previously (5). Briefly, each GST-SENP2 fragment (3 μg) was incubated for 30 minutes at 30°C in a reaction mixture (40 μL) containing 15 μmol/L ATP, 10mmol/L $MgCl_2$, 10mmol/L $MnCl_2$, 3 μCi (0.111 Bq) of [γ-32P]ATP, and 10 ng of active recombinant human p90RSK. The reaction was terminated by adding 6 μL of 6× electrophoresis sample buffer and boiling for 5 minutes. Samples were analyzed on 10% SDS-PAGE, followed by autoradiography. To confirm the SENP2 phosphorylation site by p90RSK, an *in vitro* kinase assay was performed using anti-phospho-SENP2-T368. Briefly, recombinant SENP2 protein prepared by immunoprecipitation using anti-SENP2 and 10 ng of active recombinant human p90RSK were incubated for 30 minutes at room temperature in the reaction mixture (upto 50 μL) containing 15 μmol/L ATP, 10mmol/L $MgCl_2$ followed by immunoblotting with anti-phospho-SENP2 (T368).

LC-MS/MS analysis of SENP2 phosphorylation by p90RSK. Kinase reactions were performed in 1.5 mL Protein LoBind tubes (#022431081, Eppendorf). GST-SENP2 fusion protein (1 μg) was attached to glutathione-agarose resin. The beads (30 μL packed volume) were washed with 50 mM ammonium bicarbonate to remove any protease inhibitors left after protein purification. The beads were then resuspended in 100 μL of the kinase assay solution, and the kinase reaction was initiated by the addition of 1 μg recombinant RSK1 and 400 μM/L ATP. After 30 min of kinase reaction at 30°C, the reaction buffer was removed from the beads, which were then washed with 50 mM ammonium bicarbonate three times. For LC-MS/MS analysis, the GST-ERK5 fusion protein on beads was resuspended in 20 μL of solution containing 50 mM ammonium bicarbonate and 188 ng trypsin and was digested overnight at 37°C. The sample was reduced by 2 mM DTT for 30 min, with shaking at 60°C, alkylated by 10 mM iodoacetamide (IAA) for 30 min in the dark at room temperature (RT), and finally quenched by 10 mM cysteine for 30 min at RT in the dark. A second aliquot of 188 ng of trypsin was then added and digested for additional 3 hr. Chymotrypsin digestion was performed in the identical manner. Digested samples were lyophilized and resuspended in 10 μL of 5% acetonitrile and 0.05% formic acid, and 15% of this peptide digest (1.5 μL) was loaded on a Magic C18 AQ (#PM3/61200/00, Michrom) nanospray tip, packed to 5 cm. This tip was loaded, using a pressure bomb and washed after installation on the HPLC with 5% methanol and 0.1% formic acid for 10 min with a flow rate of 600 nL/min (about 10 column volumes = 6.6 μL). The peptides were eluted and analyzed by an LC-MS/MS run, using a 5-15% methanol gradient over 2.5 min, followed by a

15-60% methanol gradient for 67 min, a 60% methanol isocratic step of 4 min, and ending with a 3 min 95% methanol step, with all solvents containing 0.1% formic acid. A full MS survey scan was performed every 3 seconds and the top 7 peaks were selected to produce an MS/MS fragmentation spectrum. The MS and fragmentation spectrum data were used in a Mascot search of the human proteome to identify peptide sequences modified by phosphate groups. The following search criteria were used for selecting fragmentation spectra that mapped to phosphorylated peptides: peptide tolerance = -0.8 to + 0.5, a minimum ion score of 15, and a fragmentation spectrum containing fragment ions that either included or flanked the phosphorylated amino acid position. Representative fragmentation spectra are included in Fig. 3A. Phosphorylation sites that mapped to the same SENP2 protein position in both the trypsin and chymotrypsin-treated samples were regarded as confirmed phosphorylation sites.

Mammalian two-hybrid analysis and transfection of cells. Cells were plated in 12-well plates at 5 x 10^4 cells/well. The mammalian two-hybrid assay was performed as described previously (6). Briefly, cells were transfected in Opti-MEM with lipofectamine mixture containing the pG5-luc vector and various pBIND and pACT plasmids (#E2440, Promega) for 4 hr. Cells were washed and fresh DMEM supplemented with 10% fetal bovine serum was added. pBIND and pACT containing Gal4 and VP16, respectively, were fused with p90RSK and SENP2-WT or fragment mutants as indicated. Since pBIND also contains the Renilla luciferase gene, the expression and transfection efficiencies were normalized with the Renilla luciferase activity. Cells were collected 36 hr after transfection, and the luciferase activity was assayed with the dual luciferase kit (#E1960, Promega) using a TD-20/20 Luminometer (Turner Designs). Transfections were performed in triplicate, and each experiment was repeated at least three times.

Real-time PCR assay. Total RNA was extracted using the TRIzol reagent according to the manufacturer's instruction. The reverse transcriptase reaction (PCR) was performed in 20 μl mixtures containing 1 mg of total RNA according to the manufacture's protocol (#170-8890; Bio-Rad,). For real-time PCR, the following specific primers were designed using Primer Express 3.0 software (Table 1).

Table 1. Primers for KLF2, eNOS, E-selectin, VCAM-1, ICAM-1 and GAPDH mRNAs.

Primers	Species	Sequence
KLF-2	Mouse	For 5'-ACC AAG AGC TCG CAC CTA AA-3'
		Rev 5'-GTG GCA CTG AAA GGG TCT GT-3'
eNOS	Mouse	For 5'-GGG AAA GCT GCA GGT ATT TGA T-3'
		Rev 5'-CAC TGT GAT GGC TGA ACG AAG A-3'
e-selectin	Mouse	For 5'-TGG TAC ATG AGC ACT GAG ATC G-3'
		Rev 5'-GTAGTCCCGCTGACAGTATG -3'
ICAM-1	Mouse	For 5'-GTGATGCTCAGGTATCCATCCA-3'
		Rev 5'-CACAGTTCTCAAAGCACAGCG-3'
VCAM-1	Mouse	For 5'-AGTTGGGGATTCGGTTGTTCT-3'
		Rev 5'-CCCCTCATTCCTTACCACCC-3'
GAPDH	Mouse	For 5'-TGGCAAAGTGGAGATTGTTGCC-3'
		Rev 5'-AAGATGGTGATGGGCTTCCCG-3'

Real-time PCR was performed using the MyiQ^{TM}2 Two-Color Real Time PCR System (Bio-Rad) and SYBR Green (#170-8882, Bio-Rad) to measure gene expression. The cycling program was set as follows: thermal activation for 10 min at 95°C and 40 cycles of PCR (melting for 15 s at 95°C, followed by annealing/extension for 1 min at 60°C). Specific gene expression data were normalized to GAPDH gene expression.

Analysis of apoptosis. In accordance with recently published guidelines, apoptosis was quantified by two methodologically unrelated criteria (7). Late stage apoptosis was quantified by counting cells with positive TUNEL staining (In Situ Cell Death Detection Kit; #11684795910, Roche) (8). TUNEL analysis was carried out according to the manufacturer's instruction, and the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; #D9564, Sigma) to identify nuclei. From each of the ten randomly selected fields, a total of 150 to 200 cells were counted and TUNEL positive cells were expressed as percent of total cells counted. Samples were examined using a 40X lens under an epi-fluorescence microscope (BX51, Olympus) equipped with a Spot CCD camera (#22.1 Spot). Apoptosis was also quantified by Western blotting to detect cleaved caspase-3. All measurements were performed blinded and at least three independent experiments were performed.

Animals. Tamoxifen (4-OHT: 4-hydroxytamoxifen)-inducible EC-specific transgenic mice, wild type *p90rsk* (WT-*p90rsk*-ETg) and dominant negative p90rsk (K94A/K447A; DN-*p90rsk*-ETg), were generated by using mice expressing tamoxifen-inducible *Cre*-recombinase *Cre*ER^{T2} under the regulation of the vascular endothelial cadherin (VE-*Cad)* promoter (21) (kindly provided by Dr. M. Luisa Iruela-Arispe, UCLA) (22). Rat WT-*p90rsk* and DN-*p90rsk* cDNA containing the Kozak and Flag sequences from pCMV-Taq2b were cloned into the NotI/XhoI site of the plasmid Z/EG, which consists of the pCAGGS promoter, directing expression of a *loxP* flanked (*lacZ*/neomycin-resistance) fusion gene and three SV40 polyadenylation sequences (23). We gave these constructs to the University of Rochester Transgenic Facility and obtained five founder mice. Genotyping of pups was confirmed by PCR analysis of tail clipping using the standard procedure. To induce overexpression of the WT- *or* DN-*p90rsk* gene, 4-week old heterozygous WT- or DN-*p90rsk*-ETg mice were injected peanut oil with or without 2 mg of 4-OHT for 5 consecutive days, and p90RSK protein expression was confirmed 2 weeks later in ECs isolated from lungs of non-transgenic litter control (NLC) or ETg mice using Western blotting. For atherosclerosis studies, *senp2*$^{+/-}$, WT-, or DN-*p90rsk*-ETg mice were crossed to *Ldlr*$^{-/-}$ (in the C57Bl/6 background) mice. We generated triple Tg/KO mice with the DN-*p90rsk*-ETg/*senp2*$^{+/-}$/*Ldlr*$^{-/-}$ genotype to test if *senp2*$^{+/-}$ could affect the atherogenic effect observed in DN-*p90rsk*-ETg/*Ldlr*$^{-/-}$ mice. All mice were maintained under pathogen-free conditions at the Aab Cardiovascular Research Institute at the University of Rochester.

Immunohistochemistry and confocal microscopy. To determine SENP2 localization, HUVECs were transduced with Ad-SENP2-WT or Ad-SENP2-T368A for 18 hr followed by stimulation with d-flow for 0, 2, or 4 hr. The cells were quickly washed two times with cold PBS, fixed with 4% paraformaldehyde in PBS for 15 min, and then permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 min. Cells were incubated with blocking buffer (5% goat serum, 0.1% NP-40 in PBS) for 60 min to block nonspecific binding and incubated with anti-Myc (1:500 dilution in 2% goat serum, 0.1% NP-40 in PBS) for overnight at

4°C. The cells were washed three times with PBS and incubated with Alexa Fluor 488 labeled anti-rabbit IgG (#A-11008, Molecular Probes; 1:1000 dilution) for 1 hr at room temperature. The cells were counterstained with DAPI to identify nuclei. The samples were analyzed using a FV1000 Olympus laser scanning confocal microscope equipped with a PLAPON 60X (N.A.1.42) objective lens. Quantification of the overlay image was done using the Photoshop CS program.

***En Face* immunostaining.** The s-flow and d-flow areas within the aorta were identified based on the published and generally accepted anatomical locations where such flow patterns are known to occur (2, 9). We performed *en face* staining as described in our previous reports (6, 9). Briefly, animals of 6-8 weeks of age were euthanized by CO_2 inhalation. The arterial tree was perfused via the left ventricle first with saline containing heparin (40 USPU/ml) and then with 4% paraformaldehyde in PBS for 10 min. Fixed aorta was dissected out and the adipose tissue around the vessel was removed. It was cut open longitudinally and permeabilized with PBS containing 0.1% Triton X-100 and blocked by TBS containing 10% goat serum and 2.5% Tween-20 for 30 min. Aortas were incubated with serum-free protein blocking buffer (#X0909, DAKO) for 30 min followed by incubation with rabbit anti-p90RSK, anti-phospho-p90RSK S380, anti-VCAM-1, anti-rabbit IgG as a control (10 μg/ml), anti-phospho-SENP2 T368, or anti-SENP2 in the presence of rat anti-VE-cadherin (7 μg/ml, an endothelial cell marker) in the antibody diluent buffer (#S0809, DAKO) for overnight. After a PBS rinse, anti-rabbit IgG and anti-rat IgG (1:1000 dilution, Alexa Fluor 546 and 488, respectively, Molecular Probes) were applied for 1 hr at room temperature. For the detection of apoptosis, we used two different methods. To do the in situ TUNEL assay, aortas immunostained with anti-VE-cadherin (with Alexa-546 anti-rabbit IgG) were incubated in the TUNEL reaction mixture (Roche Diagnostics) for 1 hr at 37°C according to the manufacturer's instructions (mixture without the enzyme TdT as a control). For annexin-V labeling, we injected annexin-V-Alexa 568 into aortas via the left ventricle after perfusion with saline containing heparin (40 USPU/ml), followed by fixation with 4% paraformaldehyde in PBS for 10 min. Then these aortas were immunostained with anti-VE-cadherin as described above. The samples were analyzed using a FV1000 Olympus laser scanning confocal microscope equipped with a PLAPON 60X (N.A.1.42) oil objective lens. For quantification of the E-selectin or VCAM-1 expression level, 10 to 15 optical sections were collected at 0.3-0.5 μm increments and then a z-stack of sections representing about 4 μm thick specimen from the luminal surface was obtained. For quantification of EC apoptosis, aortas of 7-week-old mice (n=4 each genotype) were used as controls.

Supplemental References

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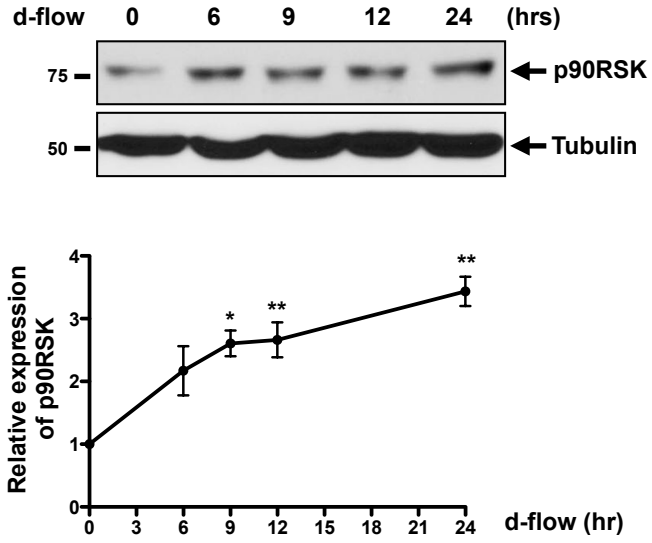
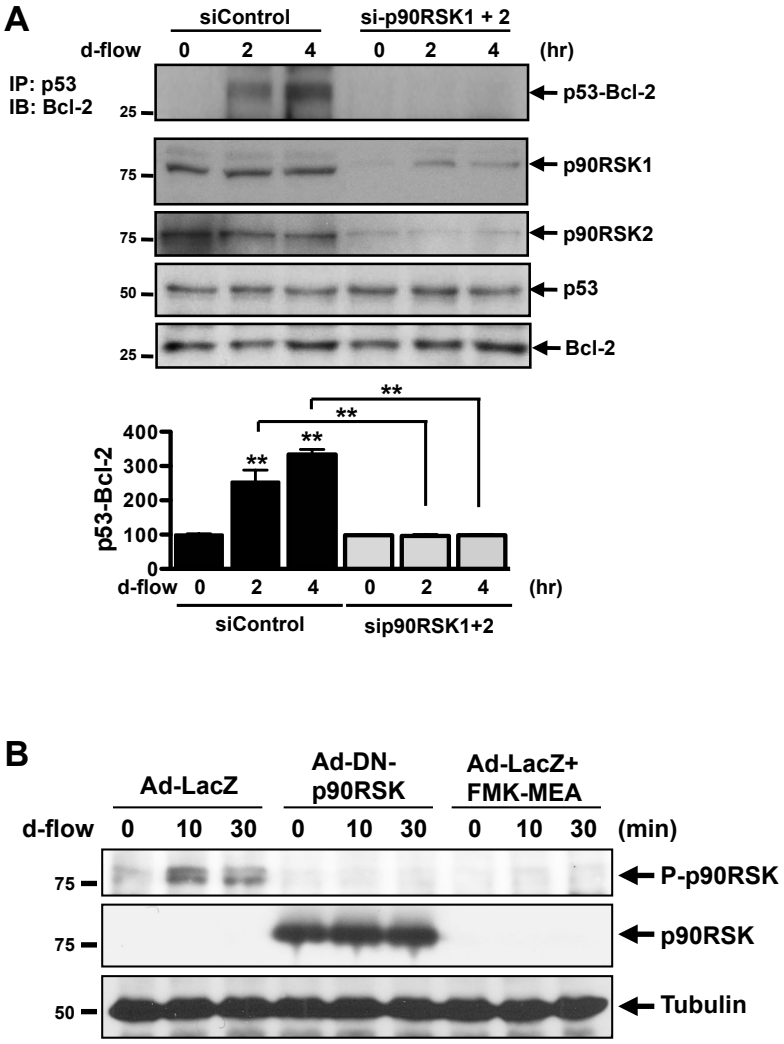
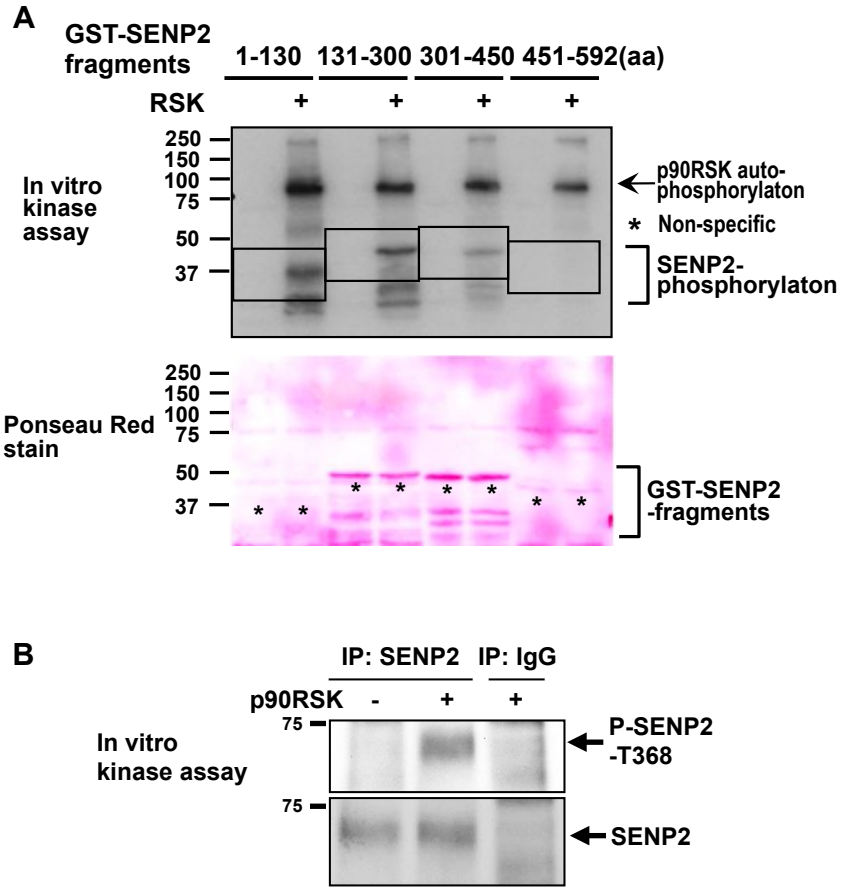


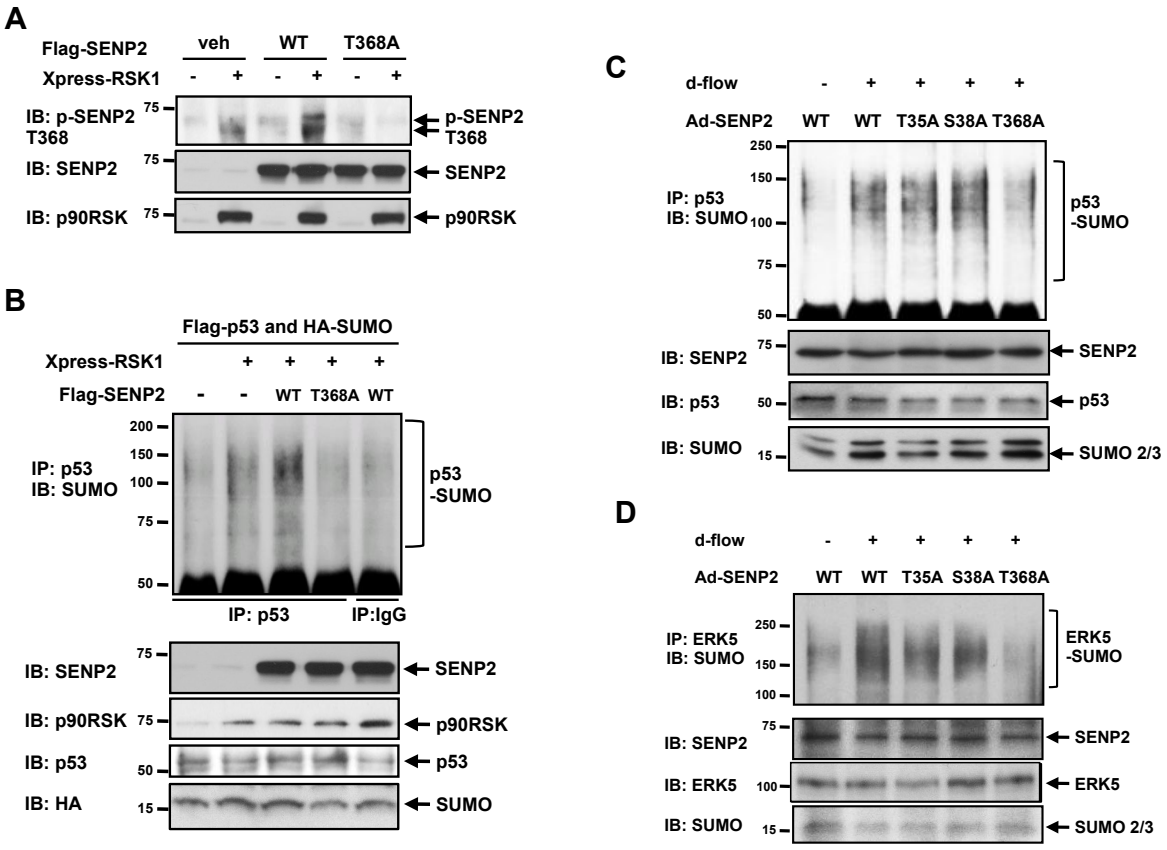
Supplemental Figure 1. D-flow increases p90RSK expression. HUVECs were stimulated by disturbed flow (d-flow, upper panel) for indicated times and p90RSK expression was examined by Western blotting using anti-p90RSK. Quantification of d-flow-induced p90RSK expression (lower panel) is shown after normalization by tubulin levels. Data represent mean ± SEM, (n =3), **P*<0.05 and ***P*<0.01 by One-way ANOVA followed by Bonferroni's post hoc test.



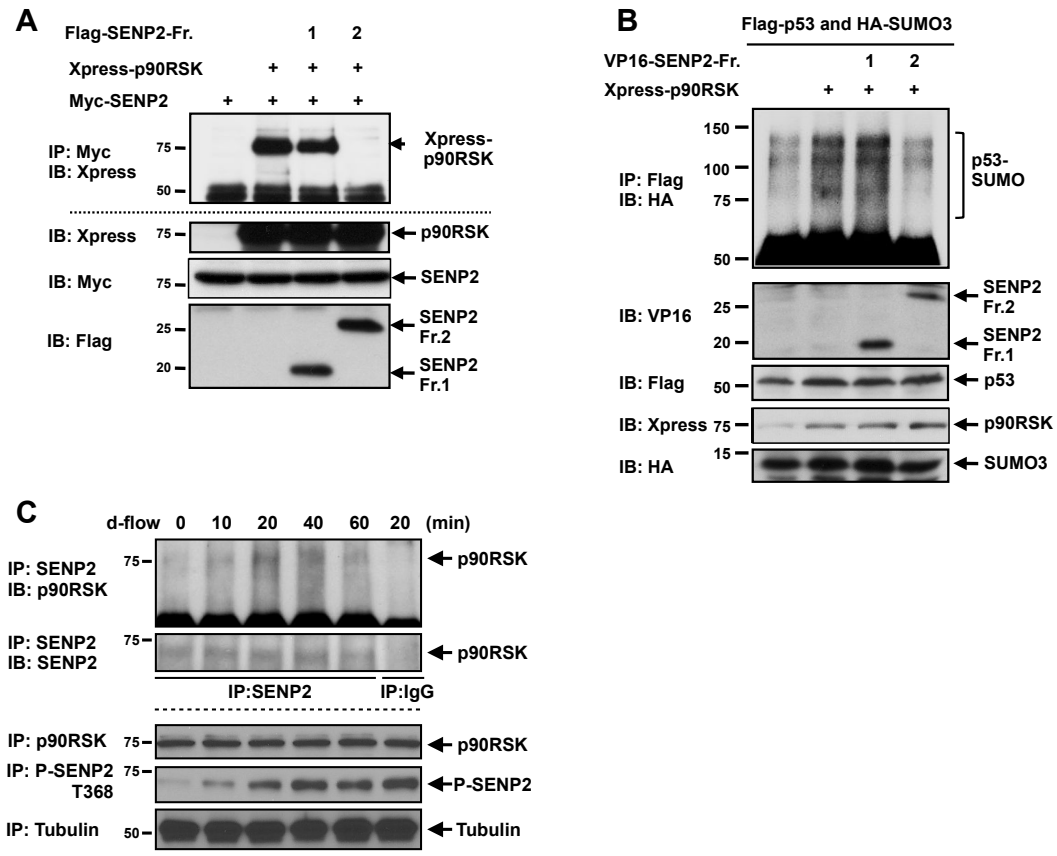
Supplemental Figure 2. Deactivation of p90RSK abolishes d-flow-induced p53-Bcl-2 binding and p90RSK phosphorylation at Ser380. (A) HUVECs were transfected with control or a mixture of siRNAs against p90RSK isoform 1 and 2 followed by d-flow stimulation for indicated times, and p53-Bcl-2 binding was detected. Quantified data are shown as mean ± SEM (n =3), $^{**}P<0.01$ by One-way ANOVA followed by Bonferroni's post hoc test. **(B)** HUVECs were pretreated with either vehicle or FMK-MEA and then transduced with Ad-LacZ or Ad-DN-p90RSK followed by d-flow stimulation. Activation (upper panel) and expression (middle panel) of p90RSK was detected using the anti-p90RSK or anti-phospho-p90RSK (Ser380).



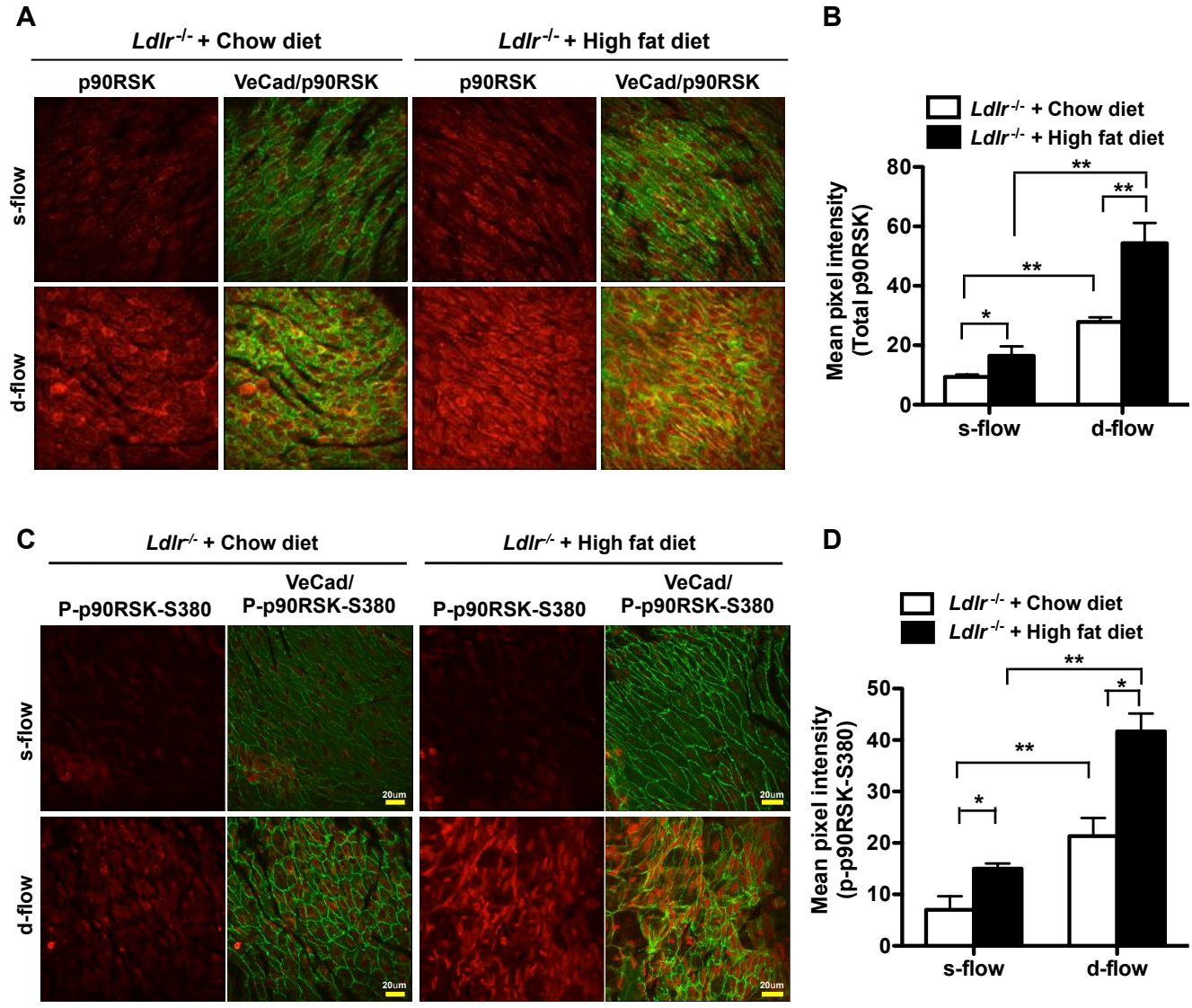
Supplemental Figure 3. p90RSK phosphorylates SENP2 *in vitro*. (A) *In vitro* kinase assay was performed using 4 different GST-SENP2 fragments as substrates to determine whether p90RSK-directly phosphorylates SENP2. The *in vitro* kinase assay (upper panel) demonstrates the ^{32}P incorporation into GST-SENP2 fragments. p90RSK is active as evidenced by its autophosphorylation as indicated. Ponseau Red staining demonstrates the presence of amount of each fragment in the gel (lower panel). **(B)** *In vitro* kinase assay was performed using recombinant SENP in the absence or presence of p90RSK followed by immunoblotting with anti-phospho-SENP2 (T368) antibody (upper panel). The same amount of recombinant SENP2 was immunoprecipitated by anti-SENP2 as indicated by anti-SENP2 immunoblotting (lower panel).



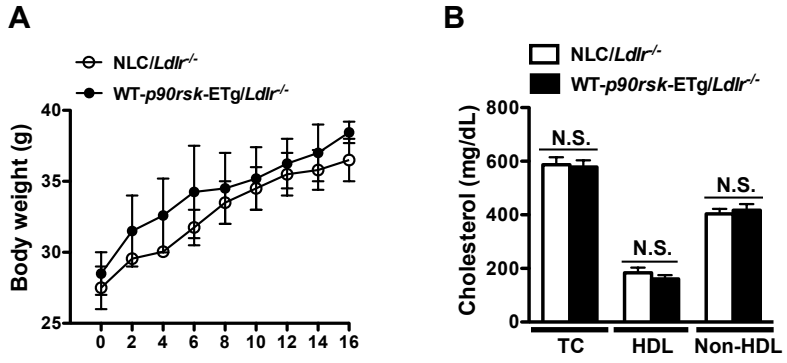
Supplemental Figure 4. p90RSK-induced SENP2 T368A has an important role on p53 and ERK5 SUMOylation. (A) HUVECs were transfected with human SENP2 wild type (WT), T368A mutant, or control vector (veh) with or without p90RSK, and SENP2 T368 phosphorylation was detected. **(B)** HUVECs were transfected with human SENP2 WT or T368A mutant with Flag-p53, HA-SUMO3, Xpress-p90RSK1, or empty vector as indicated, and p53 SUMOylation was detected. **(C-D)** HUVECs were transduced with Ad-SENP2 WT, T35A, S38A, and T368A, SENP2 phosphorylation mutants as indicated and stimulated by d-flow or a static condition (-) for 3 hr. SUMOylation of endogenous p53 **(C)** and ERK5 **(D)** was detected as described in Methods. All experiments were performed in triplicates.



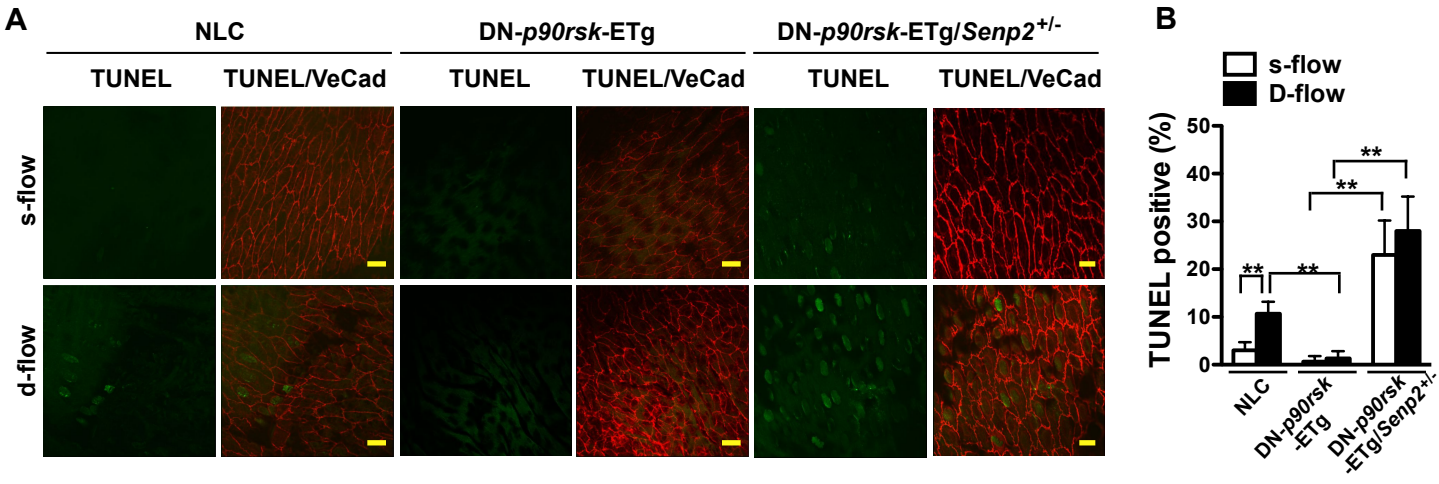
Supplemental Figure 5. p90RSK-SENP2 association is critical for p53 and ERK5 SUMOylation. (A) HeLa cells were transfected with Xpress-tagged p90RSK and Myc-tagged SENP2 WT in the presence of Flag-tagged SENP2 Fr.1 or 2. Myc-tagged SENP2 WT was immunoprecipitated with anti-Myc followed by Western blotting with anti-Xpress (top). The expression of SENP2 wild type and fragments were detected by Western blotting using antibodies against their tags. **(B)** Cell lysates were subjected to immunoprecipitation with anti-Flag for p53 followed by immunoblotting with anti-HA for SUMO3 (top). The expression of p90RSK, SENP2 fragments, p53, and SUMO3 was detected by Western blotting using antibodies against their tags. **(C)** HUVECs were stimulated by d-flow for indicated times. SENP2 was immunoprecipitated with anti-SENP2 or IgG followed by Western blotting with anti-p90RSK and -SENP2. SENP2 phosphorylation at T368 was examined using anti-phospho-SENP2 T368.



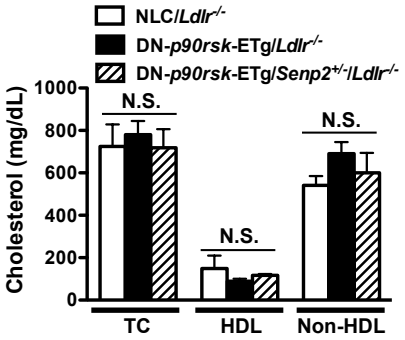
Supplemental Figure 6. *En face* immunohistochemistry for the expression and activation of p90RSK in the disturbed and steady laminar flow areas of *Ldlr*$^{-/-}$ mouse aorta. *En face* preparations were double-stained with anti-Ve-Cad and anti-p90RSK **(A)** or anti-phospho-p90RSK-S380 **(C)** in the aortic arch of 7-week-old *Ldlr*$^{-/-}$ mice fed normal chow or high fat diet for 2 weeks. Scale bars: 20 μm. Quantification of p90RSK expression **(B)** and phosphorylation at S380 **(D)** in the d- and s-flow areas of the aortic arch from *Ldlr*$^{-/-}$ mice (Right panel). Data represent mean ± SEM, (n =3). **P*<0.05, ***P*<0.01 by One-way ANOVA followed by Bonferroni's post hoc test.



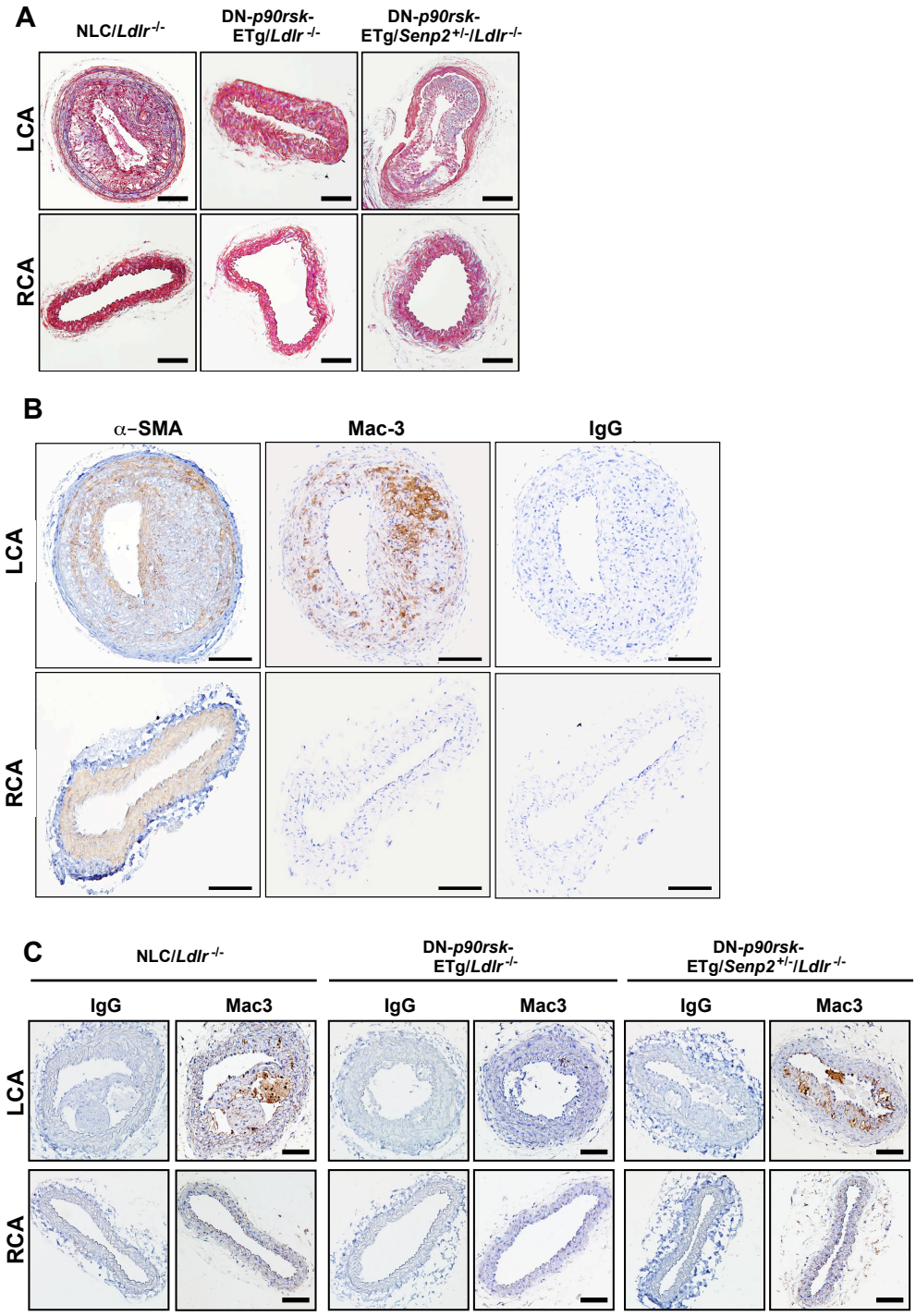
Supplemental Figure 7. Body weight and plasma cholesterol levels in mice. (A) NLC/*Ldlr*$^{-/-}$ and WT-*p90rsk*-ETg/*Ldlr*$^{-/-}$ mice (n=14 for each genotype) were fed high cholesterol diet for 4 weeks. Body weight was measured at indicated times. Data represent mean ± SEM. **(B)** Serum cholesterol profiles in these mice (n=14 for each genotype) were determined after 16 weeks of high cholesterol diet. Data represent mean ± SEM. One-way ANOVA followed by Bonferroni's post hoc tests were performed in statistical analyses.



Supplemental Figure 8. The role of p90RSK-SENP2 in EC apoptosis *in vivo*. (A) *En face* preparations of the aortic arch of 7-week-old mice from each genotype group were double-stained with TUNEL (Green) and anti-Ve-Cad (Red) to confirm EC apoptosis. Scale bars: 20 μm. **(B)** The graph shows percent of apoptotic cells in the d- and s-flow areas of the aortic arch. Data represent mean ± SEM, (n =3 for each genotype), $^{**}P<0.01$ by One-way ANOVA followed by Bonferroni's post hoc test.



Supplemental Figure 9. Plasma cholesterol levels in DN-*p90rsk*-ETg/*Ldlr*$^{-/-}$ and DN-*p90rsk*-ETg/*Senp2*$^{+/-}$/*Ldlr*$^{-/-}$ mice.
NLC/*Ldlr*$^{-/-}$ (n=9), DN-*p90rsk*-ETg/*Ldlr*$^{-/-}$ (n=10), or DN-*p90rsk*-ETg/*Senp2*$^{+/-}$/*Ldlr*$^{-/-}$ (n=7) mice were fed high fat diet for 4 weeks after partial ligation surgery. Cholesterol profiles were determined in the serum isolated from these mice at the end of high fat diet feeding. N.S., Non-significant, Data represent mean ± SEM. One-way ANOVA followed by Bonferroni's post hoc tests were performed in statistical analyses.



Supplemental Figure 10. Infiltration of Mac3-positive cells after partial ligation in NLC/ *Ldlr*$^{-/-}$, DN-*p90rsk*-ETg/*Ldlr*$^{-/-}$ and DN-*p90rsk*-ETg/*Senp2*$^{+/-}$/*Ldlr*$^{-/-}$ mice under high fat diet. (A) Representative images (Masson's trichrome staining) of atherosclerotic lesions 4 weeks after partial left carotid artery (LCA) ligation in NLC/*Ldlr*$^{-/-}$ (from n=4), DN-*p90rsk*-ETg/*Ldlr*$^{-/-}$ (n=5), and DN-*p90rsk*-ETg/*Senp2*$^{+/-}$/*Ldlr*$^{-/-}$ (n=5) mice are shown. Unligated right carotid artery (RCA) in each mouse genotype is used as control. Scale bars: 50 μm. **(B)** Representative images of alpha-SMA immunochemistry for smooth muscle cells and MAC3 immunohistochemistry for macrophages in the atherosclerotic lesions after LCA ligatioon. **(C)** Infiltration of Mac3-positive cells (macrophages) into the intimal lesions of LCA and RCA. B-C, Scale bars: 50 μm. Representative images from triplicates of each group.