SUPPLEMENTAL MATERIAL

Preventing cholesterol-induced Perk signaling in smooth muscle cells blocks atherosclerotic plaque formation

Abhijnan Chattopadhyay, PhD,[1,a], Pujun Guan (关蒲骏), MM,[1,2,a], Suravi Majumder, PhD[1], Kaveeta Kaw[1], BS, Zhen Zhou (周桢), MD[1], Chen Zhang, MD[3,4], Siddharth K. Prakash, MD, PhD[1], Anita Kaw, BA[1], L. Maximillian Buja, MD, PhD[5], Callie S. Kwartler, PhD[1], Dianna M. Milewicz, MD, PhD[1*].

Affiliations: Division of Medical Genetics, Department of Internal Medicine, The University of Texas Health Science Center at Houston, Houston, TX 77030. [2]Graduate School of Biomedical Sciences, University of Texas MD Anderson Cancer Center and UTHealth, Houston, TX, USA. [3]Division of Cardiothoracic Surgery, Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, Texas, USA. [4]Department of Cardiovascular Surgery, Texas Heart Institute, Houston, Texas, USA. [5]Department of Pathology and Laboratory Medicine, The University of Texas Health Science Center at Houston, Houston, TX 77030.

[a]These authors contributed equally to this work.

Short title: Perk signaling in SMCs drives atherosclerosis

Corresponding author: Dianna M. Milewicz, MD, PhD

Expanded Materials & Methods

Immunoblot

Immunoblot analyses were performed using standard methods. Cells underwent lysis in 50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Triton X-100 and 1mM EDTA, supplemented with protease inhibitor cocktail (Millipore Sigma) and phosphatase inhibitor cocktails (Millipore Sigma) for 15 minutes on ice and then sonicated on ice for 15 seconds. Following centrifugation at top speed for 10 minutes at 4^0C, protein concentrations in the cleared lysates were estimated by Bradford assay (Bio-Rad Laboratories). 10μg total protein per sample was used to resolve on 4-20% TGX gels (Bio-Rad Laboratories), transferred to PVDF membranes (Millipore Sigma), blocked with 5% dry milk in Tris-buffered saline (TBS), containing 0.05% Tween-20 (TBST) and probed with different antibodies. Bands were visualized by chemiluminescent substrate (GE Healthcare).

AAV-*PCSK9^{DY}* injection and high fat diet

At 6 weeks of age, both male and female *Perk$^{SMC-/-}$* and *Perk$^{SMC+/+}$* mice were injected with a single dose of AAV-*PCSK9^{DY}* containing $1.1X10^{11}$ viral particles into a retro-orbital vein. At 7 weeks of age, the mice were placed on HFD (#TD.88137, Envigo Teklad, Madison, WI) and maintained for 12 weeks. Induction of hyperlipidemia 2 weeks after injection with AAV-*PCSK9^{DY}* was initially confirmed by measuring total blood cholesterol levels using Accutrend Plus™ Meter (Roche Diagnostics). Total blood cholesterol was monitored every 4 weeks after starting the HFD.

Hematoxylin and eosin (H&E) staining

Mice were perfused with phosphate buffered saline (PBS) after euthanasia. Aortas (N=6 per genotype) were then isolated and fixed in 10% formalin overnight at 4°C. For hematoxylin and eosin staining, the deparaffinized sections were rehydrated, stained with hematoxylin, washed with water, dipped in acid alcohol, rinsed with tap water, counterstained with eosin, rinsed with water again, dehydrated and then mounted in Permount (ThermoFisher scientific).

Immunofluorescence staining of aortic tissue

Mice were perfused with PBS after euthanasia. Aortas (N=6 per genotype) were then isolated and fixed in 10% formalin overnight at 4°C. Deparaffinized sections were rehydrated, incubated in sodium citrate buffer (pH 6.0) at 98ºC for 20 minutes, permeabilized with Tris-buffered saline (TBS) containing 0.025% Triton X-100 and blocked for 1.5 hours with 5% bovine serum albumin (BSA) in TBS. Samples were incubated overnight at 4°C with goat anti-αSMA antibody (ab21027; Abcam) in combination with any one of the following: rabbit anti-Pai1 (ab66705, Abcam), rabbit anti-Vcam1 (ab134047, Abcam) and rat anti-F4/80 (MF48000, ThermoFisher Scientific, clone BM8). The slides were then washed with TBS containing 0.01% Tween 20 (TBST) and incubated with appropriate combinations of anti-goat Alexa Fluor™ 647 with anti-rabbit or anti-rat Alexa Fluor™ 594. Following this incubation, the slides were washed again with TBST and mounted with ProLong™ Diamond Antifade Mountant (Invitrogen). Slides were allowed to dry for at least 24 hours in the dark and imaged using a Nikon A1 Confocal Laser Microscope at the UTHealth Center for Advanced Microscopy. Negative control staining was performed using normal goat IgG in combination with normal rabbit or rat IgG.

Single cell RNA-sequencing

Aortic tissue from the aortic root to the distal aortic arch was isolated from age-matched male mice of each genotype at baseline and following AAV-*PCSK9*^{DY} injection and HFD. Tissues from 4 mice per genotype, per treatment were pooled together and digested to obtain single cell suspensions, as previously described.[1] Viable single cells were detected and collected using flow cytometry. Chromium Single Cell 3′ v2 Reagent Kit (10x Genomics) was used to generate barcoded cDNA, followed by cDNA amplification, truncation and library preparation. Sequencing was performed using a NovaSeq 6000 Next Generation Sequencing system (Illumina) at the Baylor College of Medicine Single Cell Genomics Core. Cells with extremely high number of genes were included in the analysis, since post-hoc analysis revealed that removal of cells with more than 6000 genes detected, would exclude 115 cells, accounting for 0.2% of all cells. All these cells were distributed sparsely across all cell types and did not form any unique cluster. So the influence of the doublets is insignificant. Data were analyzed after filtering the ambient RNA by Cell Ranger for downstream analysis. U-statistic in the UCell package was utilized to determine the gene signatures of mSMCs, contractile SMCs, endothelial cells, pericytes and cells undergoing endothelial to mesenchymal transition (EMT), based on previously established markers: modulation - *Spp1, Ibsp, Fn1, Col2a1, Lum, Lcn2, Timp1, Tnfrsf11b;* contractile - *Acta2, Cnn1, Myh11, Tagln, Tpm2, Sost, Flna;* pericytes - *Colec11, Ndufa4l2, Vtn, Higd1b, Ifitm1, Notch3, Nrip2, Olfr558;* EMT - *Cdh5, Pecam1, Tie1, Tek, Vwf, Tgfbr2, Fn1, Postn, Eln, Vim, Dcn.*[1, 27]

RNAscope

Formalin-fixed paraffin-embedded aortic tissue sections were de-paraffinized, baked at 60^0C, treated with hydrogen peroxide, subjected to target retrieval using citrate buffer and then treated with Protease Plus reagent. Probe pair sets against *Acta2* mRNA were combined with those against either *Vcam1* or *Serpine1* (gene that codes for Pai1) and hybridized to their targets. Probes were then hybridized to a cascade of three signal amplification molecules following which the signals were developed using Opal 650 dye (Akoya Biosciences, Marlborough, MA) for *Acta2* and Opal 570 for the other genes. The slides were mounted with ProLong™ Diamond Antifade Mountant (Invitrogen) and allowed to dry for at least 24 hours in the dark and imaged using a Nikon A1 Confocal Laser Microscope at the UTHealth Center for Advanced Microscopy.

RNA extraction and quantitative real time PCR

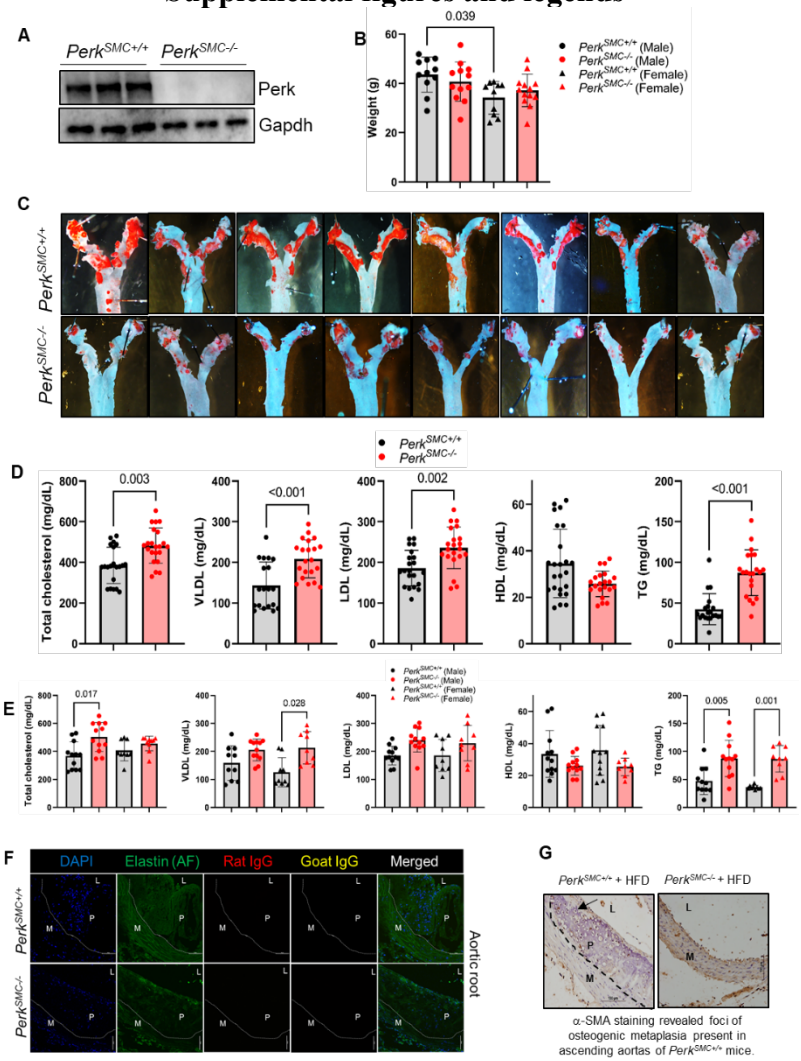
We isolated total RNA from explanted cells using a PureLink RNA Mini kit (Thermo Fisher Scientific), followed by quantification using Nanodrop (ThermoFisher Scientific). cDNA was synthesized using QScript reagent (Quantabio). Quantitative real time PCR (qRT-PCR) was performed using Taqman chemistry for contractile genes (Applied Biosciences) and SYBR Green (Millipore Sigma) for all other genes using master mixes obtained from Quantabio. *Gapdh* and *18S rRNA* were used as endogenous controls for Taqman and SYBR reactions respectively. qPCR primer sequences and antibody information are provided in the Major Resources Table.

Transwell migration assay

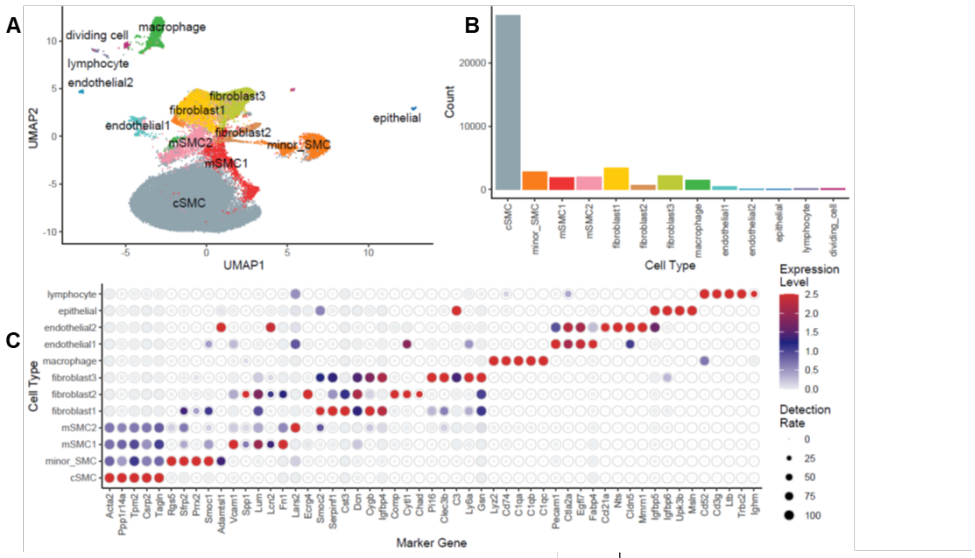
Perk$^{SMC-/-}$ and *Perk*$^{SMC+/+}$cells were plated on the upper layer of a cell culture insert containing a permeable membrane overnight and then treated with MBD-Chol for 72 hours. The cells were then washed with PBS, methanol and distilled water, followed by staining with NucBlue (Thermo Fisher Scientific). The permeable membranes containing the migrated cells were excised,

mounted on glass slides using Permount (Thermo Fisher Scientific), covered with cover slips and sealed with clear nail polish. The cells were then imaged using filters for DAPI on a Zoe Fluorescent Cell Imager (Bio-Rad Laboratories). The experiment was performed in triplicate using independent samples and four randomly chosen fields were imaged per sample each time. The migrated cells were counted using ImageJ software.

Supplemental figures and legends



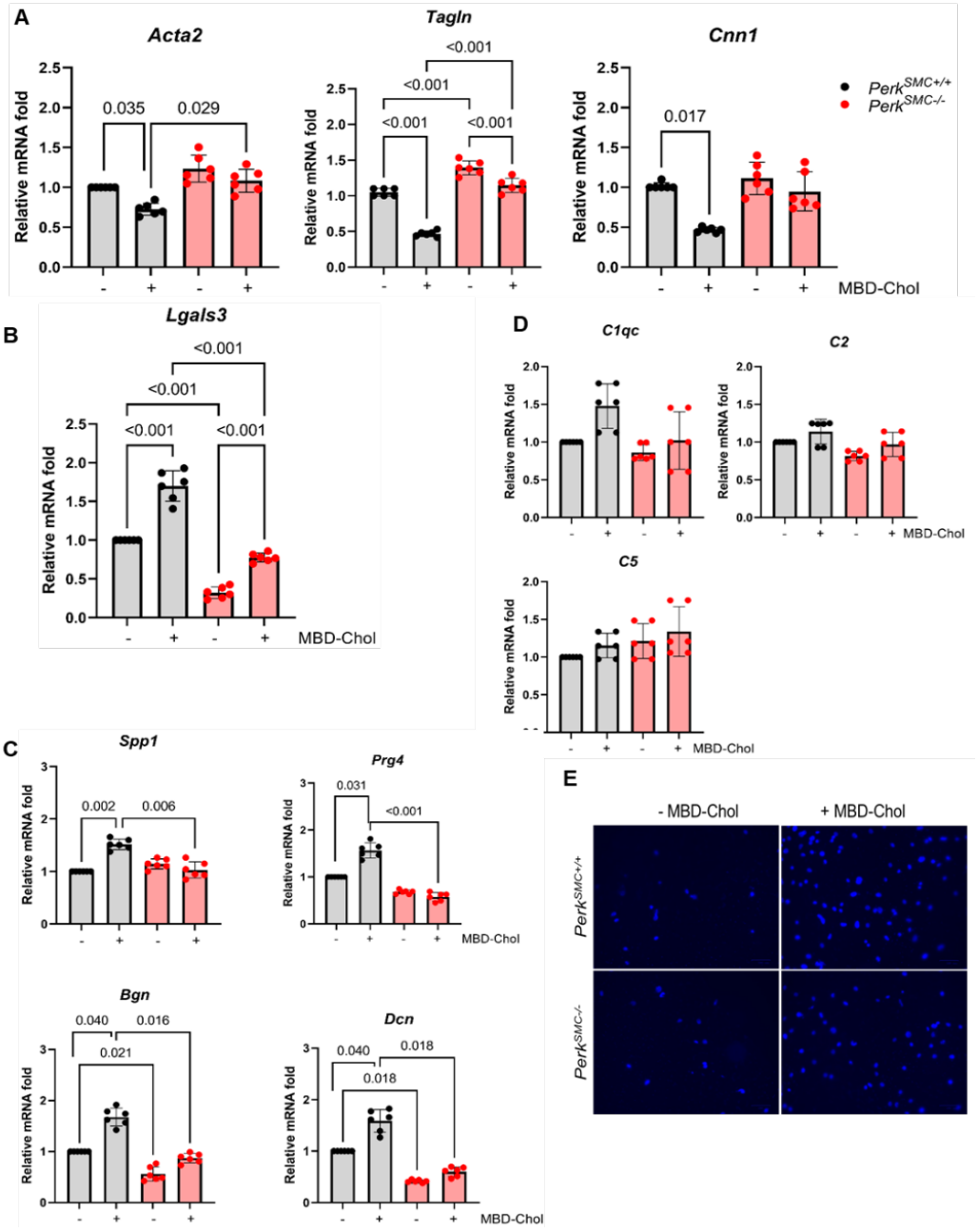
Supplemental Figure S1. SMC-specific deletion of Perk protects against atherosclerosis. **A**. Deletion of Perk from SMCs was verified using immunoblot analysis. **B**. Male *Perk*$^{SMC+/+}$ mice demonstrated higher weights than female *Perk*$^{SMC+/+}$ mice but there were no significant differences among weights of *Perk*$^{SMC+/+}$ mice and *Perk*$^{SMC-/-}$ mice in either sex, or between those of male and female *Perk*$^{SMC-/-}$ mice (analyzed by Kruskal-Wallis test, followed by Dunn's multiple comparisons test). **C.** Magnified views of the aortic roots and arches show extensive plaques in *Perk*$^{SMC+/+}$ in contrast to *Perk*$^{SMC-/-}$ aortas. Lipid profiles of control and *Perk*$^{SMC-/-}$ mice separated by only genotype (**D**) and separated by both genotype and sex, (**E**). Data were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons test for total cholesterol, VLDL, HDL and triglyceride levels (when genotypes were separated by sex) and by Mann Whitney U test when not separated by sex, while LDL levels were analyzed by 2-way ANOVA followed by Tukey's multiple comparisons test when separated by sex and by unpaired student's t-test followed by Welch's correction when not separated by sex. **F**. Negative control immunofluorescent staining of *Perk*$^{SMC+/+}$ and *Perk*$^{SMC-/-}$ aortic roots and ascending aortas using rat IgG (as isotype control for rat anti-F4/80) and goat IgG (as isotype control for goat anti-α-SMA). **G**. Immunostaining against α-SMA of *Perk*$^{SMC+/+}$ ascending aortas contain foci of osteogenic metaplasia (black arrow) which are absent in *Perk*$^{SMC-/-}$ aortas. M – medial layer, P – plaque, L – lumen; AF - autofluorescence.



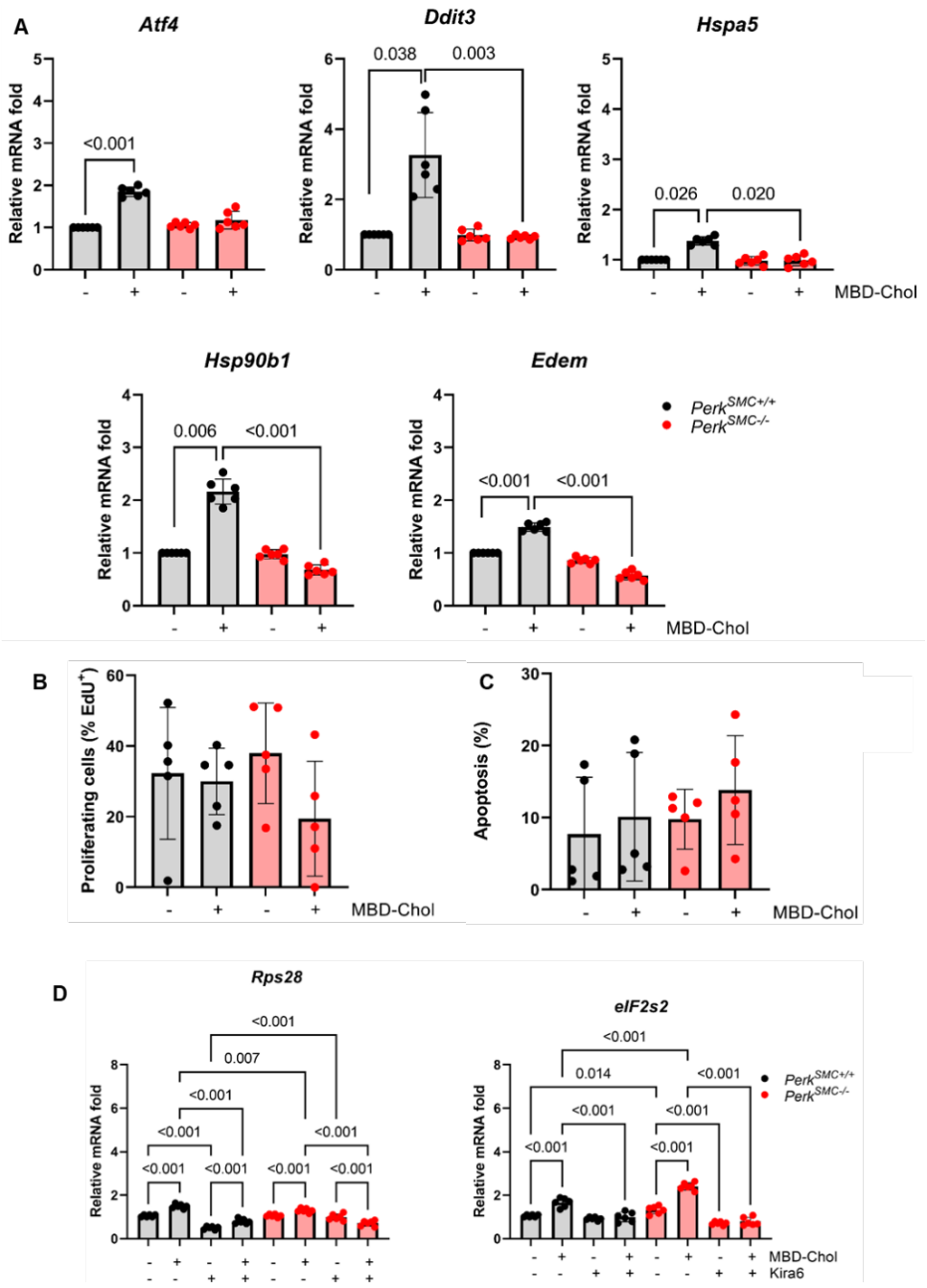
Group	Fraction reads in cells
$Perk^{SMC+/+}$ (baseline)	95.7%
$Perk^{SMC-/-}$ (baseline)	96.1%
$Perk^{SMC+/+}$ +HFD	95.2%
$Perk^{SMC-/-}$ +HFD	95.3%

Cell type	Median of genes detected	Cell number
cSMC	3062	27568
minor_SMC	3013	2844
mSMC1	3530	1905
mSMC2	1238	1974
fibroblast1	2095	3440
fibroblast2	3018	663
fibroblast3	2506	2256
macrophage	2365	1493
endothelial1	2517	527
endothelial2	3833	122
epithelial	4150	123
lymphocyte	1288	149
dividing_cell	3939	175

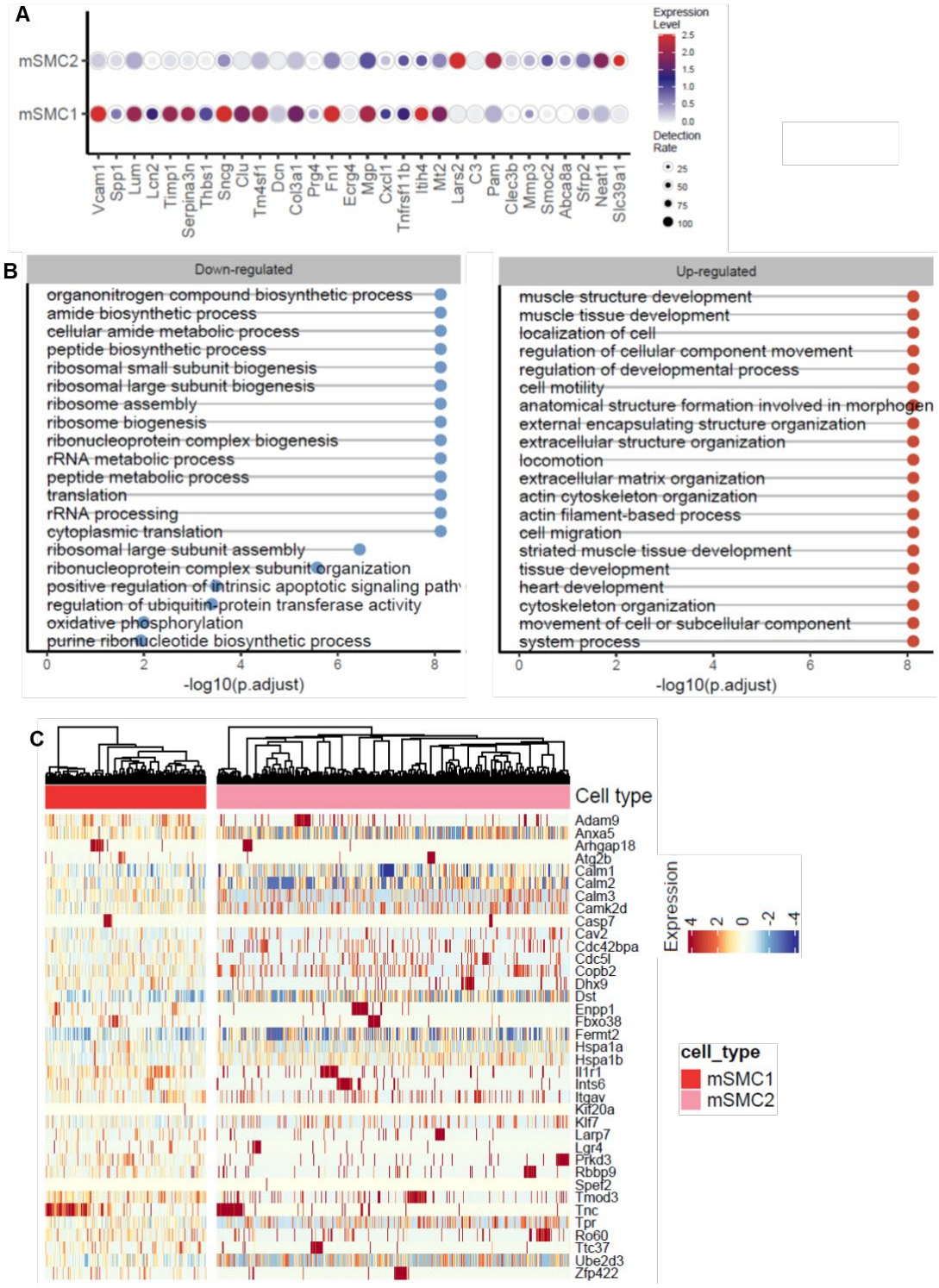
Supplemental Figure S2. Single cell RNA Sequencing (scRNA-Seq) analysis of aortic tissue from *Perk*$^{SMC+/+}$ and *Perk*$^{SMC-/-}$ mice at baseline and following hyperlipidemia and a high fat diet. **A**. Uniform Manifold Approximation and Projection (UMAP) analysis of various clusters obtained after scRNA-Seq. **B**. Representation of the number of cells obtained in each cluster. **C**. Identification of each cell cluster based on their top 5 most highly expressed genes. **D**. Fraction reads in cells for each of the four samples that were subjected to scRNA-seq. **E**. Cell numbers and median of genes detected by scRNA-seq in every cell type. **F**, **G**. Comparison of the two macrophage clusters with the mSMC2 cluster based on UMAP and the top 10 most highly expressed genes. **H**. Comparison of the genomic signatures of contractile markers, endothelial-to-mesenchymal transition (EMT) markers, endothelial markers, phenotypic modulation markers and pericyte markers in cSMC, mSMC1, mSMC2, macrophage, endothelial1 and endothelial2 cell clusters.



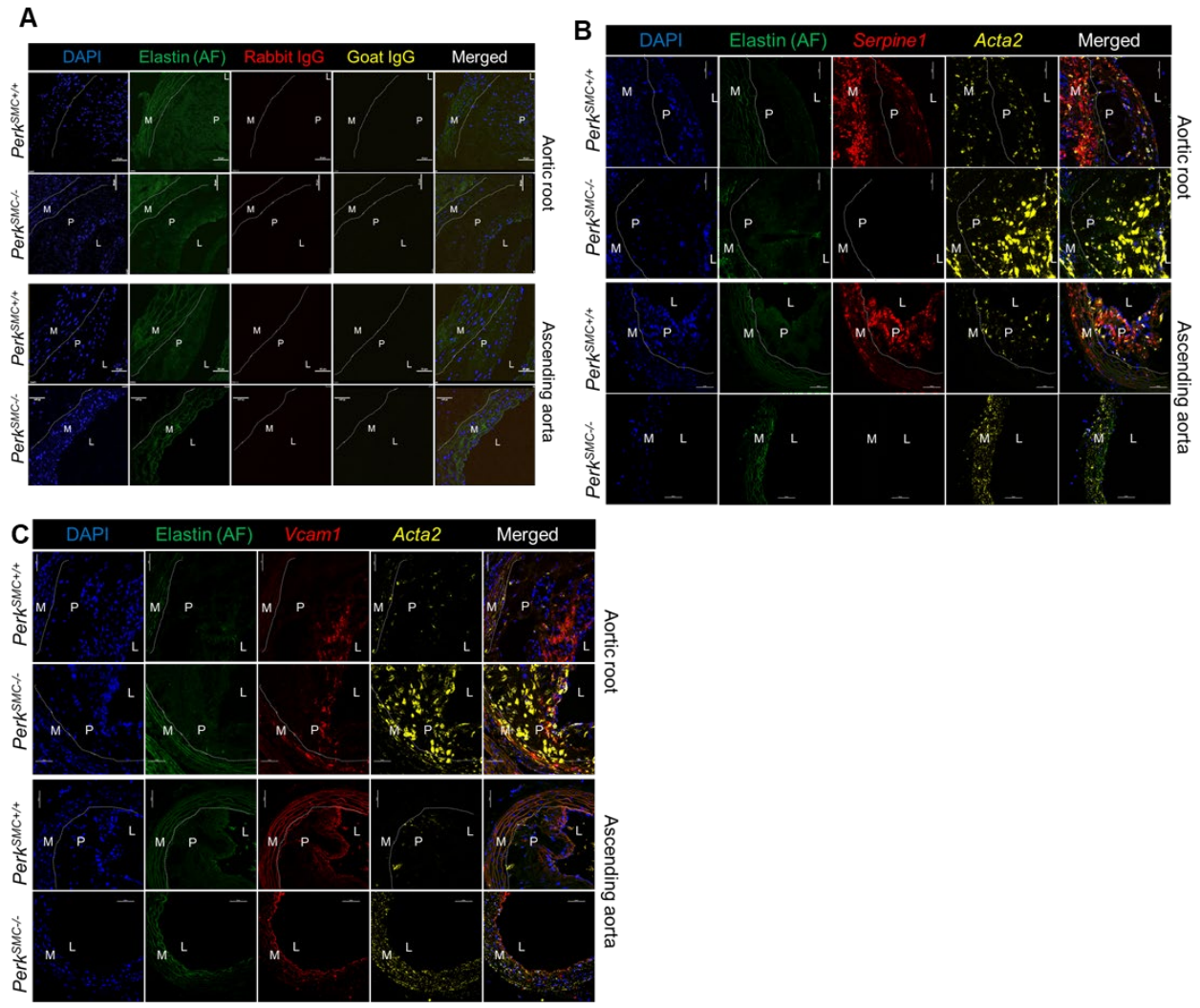
Supplemental Figure S3. Perk deficiency abrogates cholesterol-induced phenotypic modulation of SMCs. A. SMCs explanted from *Perk*$^{SMC+/+}$ aortas reduce expression of contractile markers *Acta2, Tagln* and *Cnn1* when exposed to free cholesterol, but those explanted from *Perk*$^{SMC-/-}$ aortas do not de-differentiate. **B, C.** Deletion of Perk block cholesterol-induced upregulation of SMC modulation markers like *Lgals3* (**B**) and *Spp1*, *Prg4*, *Bgn* and *Dcn* (**C**). **D**. Perk deletion does not affect the expression of a subset of genes in the complement activation pathway. **E**. Perk deletion significantly reduces migration of SMCs. Each qPCR experiment is representative of three independent experiments. Statistical significance for gene expression by qPCR was calculated using two way ANOVA, followed by Tukey's multiple comparisons test for *Lgals3* expression and by Kruskal-Wallis test followed by Dunn's multiple comparisons test for all other genes. Data are represented as mean ± SD.



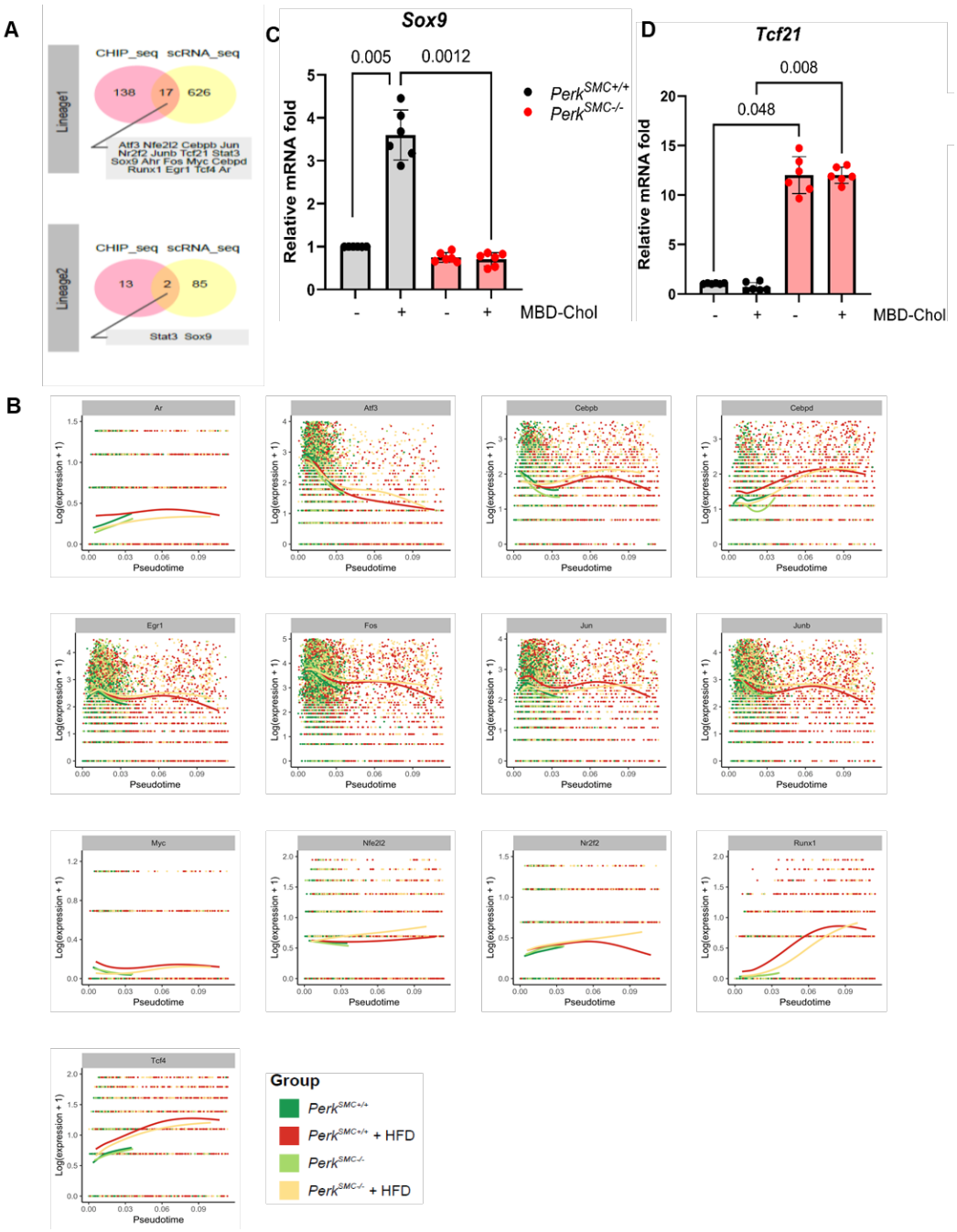
Supplemental Figure S4. Perk deficiency inhibits cholesterol-induced upregulation of genes in the Perk signaling pathway. A. Genes downstream of Perk in the Perk arm of the UPR that are upregulated by cholesterol in *Perk*$^{SMC+/+}$ SMCs do not undergo similar upregulation in *Perk*$^{SMC-/-}$ SMCs. Cellular proliferation (**B**) and apoptosis (**C**), in response to MBD-Chol showed highly variable results. Each qPCR experiment is representative of three independent experiments. **D**. Co-treatment with the Ire1a inhibitor Kira6 inhibits the cholesterol-induced upregulation of RNP genes in both *Perk*$^{SMC+/+}$ and *Perk*$^{SMC-/-}$ SMCs. Each qPCR experiment is representative of three independent experiments. Statistical significance was calculated using Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data are represented as mean ± SD.



Supplemental Figure S5: Comparison of mSMC1 and mSMC2. A. Top differentially expressed genes in mSMC1 and mSMC2. **B**. Top up- and downregulated pathways in mSMC2 cluster compared to mSMC1, following Gene ontology (GO) analysis. **C**. Heat map showing expression of RIDD target transcripts in mSMC1 and mSMC1.



Supplemental Figure S6. Localization of mSMC1 and mSMC2 cell clusters in aortic tissues of *Perk*$^{SMC+/+}$ and *Perk*$^{SMC-/-}$ mice by RNAscope. **A**. Negative control immunofluorescent staining of *Perk*$^{SMC+/+}$ and *Perk*$^{SMC-/-}$ aortic roots and ascending aortas using rabbit IgG (as isotype control for rabbit anti-Pai1 and rabbit anti-Vcam1) and goat IgG (as isotype control for goat anti-α-SMA). **B**. RNAscope demonstrated strong positive staining for *Serpine1* mRNA in the lesions and the media of *Perk*$^{SMC+/+}$ aortic roots and ascending aortas, while the corresponding *Perk*$^{SMC-/-}$ tissues demonstrated very weak to no signal. **C**. Staining for *Vcam1* mRNA was observed in the medial cells as well as in the fibrous cap region in the aortic roots of both *Perk*$^{SMC+/+}$ and *Perk*$^{SMC-/-}$ mice. *Vcam1* transcripts were present in both the media and the fibrous cap of the lesions in the ascending aortas of *Perk*$^{SMC+/+}$ mice and in the while the medial layer in the *Perk*$^{SMC-/-}$ ascending aortas. The positive staining for Vcam1 observed in the elastic laminae in the *Perk*$^{SMC+/+}$ ascending aortas is most likely an experimental artifact. M – medial layer, P – plaque, L – lumen; AF - autofluorescence.



Supplemental Figure SV7: Expression of lineage-specific transcription factors. A. Analyses of ChIP-seq database identified 17 transcriptional factors linked with lineage 1, and 2 transcriptional factors linked with lineage 2. **B**. Transcription factors affected by Perk enriched in lineage 1, in addition to Sox9, Stat3, Tcf21 and Ahr. **C**. Cholesterol-induced upregulation of *Sox9* is blocked by Perk deletion. **D**. Perk deletion causes a significant upregulation of *Tcf21* in $Perk^{SMC-/-}$ cells compared to $Perk^{SMC+/+}$ cells, both at baseline and with cholesterol exposure. Each qPCR experiment is representative of three independent experiments. Statistical significance for gene expression by qPCR was calculated Kruskal-Wallis test followed by Dunn's multiple comparisons test t. Error bars represent ±SD. HFD – hypercholesterolemia, followed by a high fat diet.

Major Resources Table

Animals (*in vivo* studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Mus musculus STOCK Eif2ak3$^{tm1.2Drc}$/J	The Jackson Laboratory	C57BL/6J	M/F	https://www.jax.org/strain/023066
Mus musculus STOCK B6.Cg-Tg(Tagln-cre)1Her/J	The Jackson Laboratory	C57BL/6J	M/F	https://www.jax.org/strain/017491
Perk$^{SMC-/-}$	This paper	C57BL/6J	M/F	N/A

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration or dilution	Persistent ID / URL
Rabbit monoclonal anti-Gapdh	Cell Signaling Technology	Cat# 2118	0.1μg/mL	https://www.cellsignal.com/products/primary-antibodies/gapdh-14c10-rabbit-mab/2118
Rabbit monoclonal anti-Perk [Clone C33E10]	Cell Signaling Technology	Cat# 3192	1μg/mL	https://www.cellsignal.com/products/primary-antibodies/perk-c33e10-rabbit-mab/3192
Goat polyclonal anti-α-Smooth muscle actin	Abcam	Cat# ab21027	5μg/mL	https://www.abcam.com/alpha-smooth-muscle-actin-antibody-ab21027.html
Rabbit polyclonal anti-Pai1	Abcam	Cat# ab66705	10μg/mL	https://www.abcam.com/pai1-antibody-ab66705.html
Rabbit monoclonal anti-Vcam1 [Clone EPR5047]	Abcam	Cat# ab134047	4.73μg/mL	https://www.abcam.com/vcam1-antibody-epr5047-ab134047.html
Rat monoclonal anti-F4/80	ThermoFisher Scientific	Cat#MF48000	1μg/mL	https://www.thermofisher.com/antibody/product/F4-80-Antibody-clone-BM8-Monoclonal/MF48000
Peroxidase-AffiniPure Goat Anti-Rabbit IgG, F(ab')2 Fragment Specific antibody	Jackson ImmunoResearch Labs	Cat# 111-035-006	1:4000	https://www.jacksonimmuno.com/catalog/products/111-035-006

Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	ThermoFisher Scientific	Cat#A-11012	1:100	https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11012
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	ThermoFisher Scientific	Cat#A-11007	1:100	https://www.thermofisher.com/antibody/product/Goat-anti-Rat-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11007
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647	ThermoFisher Scientific	Cat#A-21447	1:250	https://www.thermofisher.com/antibody/product/Donkey-anti-Goat-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21447

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
Perk$^{SMC+/+}$	This paper	F, M	N/A
Perk$^{SMC-/-}$	This paper	F, M	N/A

Table S1: List of Mouse SYBR Green primers used for quantitative real time PCR (qPCR)

Gene	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
18s rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Ago2	CAAGTCGGACAGGAGCAGAAAC	GACCTAGCAGTCGCTCTGATCA
Atf4	GGGTTCTGTCTTCCACTCCA	AAGCAGCAGAGTCAGGCTTTC
Bgn	ATTGCCCTACCCAGAACTTGAC	GCAGAGTATGAACCCTTTCCTG
C1qa	TCTCAGCCATTCGGCAGAC	TGGTTGGTGAGGACCTTGTCA
C1qb	TCACCAACGCGAACGAGAA	AAGTAGTAGAGGCCAGGCACCTT

C1qc	ACACATCGCATACGGCCAA	AACATGTGGTCGCAGAAGCTG
C2	CTCATCCGCGTTTACTCCAT	TGTTCTGTTCGATGCTCAGG
C3	GACGCCACTATGTCCATCCT	CCAGCAGTTCCAGGTCCTTTG
C4b	GGAGAGTGGAACCTGTAGACAG	CACTCGAACACGAGTTGGCTTG
C5	GGATTCAAGCGCATAATAGCA	ACCCGGATGTTGACTCCTC
Celf1	AAATGAACGGCACCCTGGACCA	CTAATGCAGCTTTACGGGTGT
Dcn	TTCCTACTCGGCTGTGAGTC	AAGTTGAATGGCAGAACGC
Ddit3	CCACCACACCTGAAAGCAGAA	AGGTGAAAGGCAGGGACTCA
Edem	CTACCTGCGAAGAGGCCG	GTTCATGAGCTGCCCACTGA
eIF2s2	GTACTTCAAGGACAGGCACAAGCTG	TGGCAATTGCTTTGTTCCTGC
Fn1	CGAGGTGACAGAGACCACAA	CTGGAGTCAAGCCAGACACA
Hsp90b1	AAGAATGAAGGAAAAACAGGACAAAA	CAAATGGAGAAGATTCCGCC
Hspa5	TTCAGCCAATTATCAGCAAACTCT	TTTTCTGATGTATCCTCTTCACCAGT
Klf4	CTGAACAGCAGGGACTGTCA	GTGTGGGTGGCTGTTCTTTT
Lgals3	AGGAGAGGGAATGATGTTGCC	GGTTTGCCACTCTCAAAGGG
Prg4	GAAAATACTTCCCGTCTGCTTGT	ACTCCATGTAGTGCTGACAGTTA
Rps27	AAGAAACGCCTGGTGCAGAGCC	TGTAGGCTGGCAGAGGACAGTG
Rps28	ATCAAGCTGGCTAGGGTAACC	GGCCTTTGACATTTCGGATGA
Sox9	GGCAAGCTCTGGAGGCTG	CCTCCACGAAGGGTCTCTTCT
Spp1	TCACCATTCGGATGAGTCTG	ACTTGTGGCTCTGATGTTCC
Vcam1	CCGGCATATACGAGTGTGAA	GATGCGCAGTAGAGTGCAAG
Xbp1 (spliced)	CTGAGTCCGAATCAGGTGCAG	GTCCATGGGAAGATGTTCTGG
Xbp1 (unspliced)	CAGCACTCAGACTATGTGCA	GTCCATGGGAAGATGTTCTGG

Sample size estimation: Sample sizes for atherosclerosis studies were based on recommendations of the American Heart Association.[13]

Inclusion/exclusion criteria: We did not exclude any data from the analyses, except on some rare occasions when the histology slides had folded or damaged tissue sections.

Randomization: Mice were used for experiments based on their genotypes. Genotypes were identical except for the presence of a Cre-recombinase allele in the knockout mice. Both male and female mice were used for the experiments.

Blinding: Since tissues from 4 mice were pooled for each group for the single cell RNA-seq, it was not possible to blind researchers to the genotypes of the mice, in order to preserve genotype information of each sample. For the same reason, blinding was also not possible for histopathology and immunofluorescence. During quantification of immunofluorescence data, researchers who performed the analyses, were blinded to the identities of the mouse genotypes.