Materials and Methods

Generation of SPRR3$^{-/-}$ Mice. Generation of SPRR3$^{-/-}$ Mice. A targeting vector was designed containing a Neo cassette flanked by both LoxP and FRT sites and carrying a 5' long arm of homology to the SPRR3 allele (7.89 kb), a short homology arm of 1.6 kb, along with a combined total of 17.8 kb of flanking genomic sequence on either side. A forward primer (LAN1) targets sequence inside the Neo cassette (5'-CCAGAGGCCACTTGTGTAGC-3') and two reverse primers target sequences 3' of the cassette to aid in genotyping (5'-CGCACACTGGGCATCTGTACATAG -3'; 5'- CAGCAAGGACTCTTCAGAAAGATCC -3').

High fidelity Red/ET recombineering was used to introduce the targeting vector into a hybrid 129SvEv and C57Bl/6J (129/C57) ES cell line. Cells underwent selection by neomycin and appropriate clones were microinjected into 128-cell stage blastocysts and implanted into foster mothers; the resulting chimaeras were bred to homozygosity. Development of SPRR3$^{-/-}$ mice was carried out by inGenious Targeting Laboratories. The Neo cassette was removed by crossing SPRR3$^{-/-}$ mice with ACTB-Flp mice (Jackson Laboratories, stock #005703). Finally, Neo deleted mice were then backcrossed onto the C57Bl/6 background 15 generations. All mice used in these studies were 6 months old unless otherwise specified. Mice were anesthetized using Isofluorane (HenrySchein).

Mice were maintained in microisolator cages on a rodent chow diet containing 4.5% fat by weight with 13% of calories from fat (diet 5001, St. Louis, MO) or a high fat diet containing 9% fat by weight with 21% of calories from fat, (diet 5021; PMI, St Louis, Missouri, USA). Animal care and experimental procedures were performed according to the regulations of Vanderbilt University's Institutional Animal Care and Usage Committee.

Cells. Immorto-VSMCs were isolated from the aortas of transgenic H-2K^b-tsA58 mouse expressing heat-labile T-antigen and maintained as described[1]. SPRR3 cDNA subcloned into LZRS-MS-IRES-GFP retroviral expression vector (a kind gift of Alyssa Weaver, Vanderbilt University) or the empty vector were used for retroviral transduction of T-antigen expressing

VSMCs. Transduced GFP- or SPRR3-VSMCs were routinely sorted for GFP expression by flow cytometry to enrich for GFP-positive cells.

Primary VSMCs were isolated from the aortas of $SPRR3^{-/-}ApoE^{+/+}$ mice as described and pooled[1]. An immortalized line of SPRR3-KO VSMCs was generated via retroviral transduction with pZipSVtsA58 expressing heat-labile T-antigen (a generous gift from Parmjit Jat). Primary endothelial cells were isolated from the lungs of $SPRR3^{-/-}ApoE^{+/+}$ or WT C57/Bl6 mice as described[2].

Cholesterol analysis. **Cholesterol analysis.** Mice (n=5 WT mice; n=6 $SPRR3^{+/+}ApoE^{-/-}$, $SPRR3^{-/-}ApoE^{+/+}$, and $SPRR3^{-/-}ApoE^{-/-}$ mice) were fasted for 4 hours before blood was collected for total plasma cholesterol and triglyceride measurements, which were determined as described previously[3].

Aortic lesion analysis*.* En face of aortas was carried out as previously described[3]. In brief, aortas were dissected from the ascending aorta to the iliac bifurcation, pinned out, and stained with Sudan IV. Images were captured with a frame grabber and a color video camera, and then analyzed using the KS 300 system. Surface area of aortic lesions was calculated as a percent of total aortic area in 13 mice/group normal chow and 8 mice/group high fat diet. For aortic root analysis, aortic valves were dissected as described[4]. Briefly, the heart and upper aorta were removed and fixed in OCT (Optimum Cutting Temperature) mounting medium. Hearts were sliced in 10 µm sections and stained with MOMA to quantify plaque macrophage content or CD3 to quantify T-cell content (Santa Cruz). Bright field images were captured using an Olympus DP71 camera, while fluorescent images were collected using a CoolSNAP Hq CCD camera (Photometrics).

Immunoblotting. Ly294002 (25 µM; Sigma) PI3K/Akt inhibitor was used to determine SPRR3 involvement in PI3K/Akt signaling. To identify potential upstream regulators of SPRR3-mediated phospho-Akt signaling, recombinant IGF-1 (20 ng/mL; R & D Systems) was added to serum-starved cells for five minutes, then cells were washed and incubated one hour in full

serum before protein collection. . For protein collection, cultured cells were washed twice with PBS and lysed using RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors (Roche). Whole cell lysates were rocked 30 minutes at 4 °C then spun 15 minutes at 13,000xg and the supernatant collected. Protein concentrations were determined using a bicinchoninic acid (BCA) kit (Thermo Scientific). Proteins were denatured 10 minutes at 95 °C in SDS sample buffer before being resolved by SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane (PerkinElmer). Blots were blocked in 5% nonfat milk and probed with antibodies to phospho-Akt (Cell Signaling, #4058S), Akt (Cell Signaling, #9272), β-actin (Sigma, #A5441), active caspase-3 (Cell Signaling, #9665), or SPRR3 (Alexis, #ALX-210-902-R100) overnight at 4 °C. After washing with 0.1% Tween-20-supplemented TBS, blots were incubated with species specific secondary antibodies for 1 hour at room temperature and chemiluminescence (PerkinElmer, NEL104) was detected by film or visualized using a Syngene GBox (Syngene, Cambridge, United Kingdom). ImageJ version 1.38x (National Institutes of Health) software was used for desitometry analysis of the appropriate lanes; values are normalized to β-actin loading control.

RNA isolation, cDNA synthesis, and real-time PCR. For RNA isolation from tissue, 3 mice per group were used. Total RNA was isolated using Trizol (Invitrogen) following the manufacturer's instructions. cDNA was generated using iScript cDNA Synthesis kit (Bio-Rad) from 1µg RNA. The cDNA was then used for quantitative real-time PCR (real time qRTPCR) as described[5]. Primers used for real time qRT-PCR were mSPRR3 forward (5'-CCCTTTGTCCCACCTCCT-3') and mSPRR3 reverse (5'-TTGGTGTTTCCTGGTTGTG-3'). A T_m of 59°C was used and a 134bp product was produced.

Immunofluorescent labeling. For caspase-3 immunofluorescence (IF), GFP-VSMCs and SPRR3-VSMCs grown on coverslips were fixed 10 minutes in cold acetone and permeabilized 5 minutes in 0.2% Triton/PBS. Cells were then stained as described[6]. Briefly, VSMCs were blocked with 10% goat serum before they were incubated with anti-active

caspase-3 antibody (Promega). In the dark, secondary antibody conjugated to Cy3 was incubated with the cells. Vectashield Hard Set mounting medium with Dapi (Vector H-1500) was used to attach coverslips to slides.

For TUNEL IF (Roche), cells grown on coverslips or frozen tissue cryosections were fixed for 1 hour in 1% paraformaldehyde in PBS, pH 7.4. Coverslips were rinsed twice with PBS, then permeabilized 2 minutes at 4 °C with 0.1% Triton X-100 in 0.1% sodium citrate. Following PBS rinse, coverslips were labeled with TdT per manufacturer instructions using the *In Situ* Cell Death Detection Kit, TMR red (Roche). To assess apoptosis within the lesion, paraffin-embedded tissue was deparaffinized and labeled with 1:2 TdT:TUNEL dilution buffer per manufacturer instructions (Roche). Smooth muscle α actin (α-SMA; Sigma)-TUNEL colocalization was assessed in n=5 mice/group 6 months HFD. MOMA-2-TUNEL colocalization was assessed in n=3 mice/group 6 months normal diet. vWF (DAKO #A0082, 1:200) and TUNEL colocalization was assessed in n=3 mice/group.

For assessment of VSMC content, frozen sections in OCT were collected as described above from 6 mo. normal diet fed mice. Sections were fixed in acetone for 10 minutes at 4°C, blocked with 10% goat serum for one hour, and then treated overnight at 4°C with primary α-SMA (1:2000) or α-smooth muscle myosin heavy chain (SM-MHC, 1:300; Alfa Aesar #J64817) in 3% goat serum. Coverslips were affixed to slides using Vectashield Hard Set mounting medium with Dapi (Vector H-1500). Staining was assessed by confocal analysis using a LSM510 (Zeiss) microscope to capture 1 µm optical slices (z stack); the images were analyzed with Metamorph v5.0 (Universal Imaging Corp.). VSMC plaque content was calculated as the number of VSMCs divided by plaque cellularity per mm^2 in 8-10 fields of view per mouse. For the α-SMA experiment, N ($SPRR3^{+/+}ApoE^{-/-}$) = 8 mice; N ($SPRR3^{-/-}ApoE^{-/-}$) = 5 mice. N=3 mice/group for the α-SM-MHC experiment.

Bone marrow transplantation. For BMT, unfractionated BM from donor mice was obtained as described[7]. Recipients were preconditioned with 10Gy of γ-radiation from a $_{137}Cs$

source before injection through the lateral tail vein with 5×10^6 nucleated donor cells. PCR confirmed full hematopoietic engraftment. Recipient mice were allowed 2 months for recovery and engraftment post-transplant before high fat diet was initiated.

Proliferation assays. Proliferation was measured *in vivo* using paraffin-embedded aortic root sections from 6 month high fat diet-fed SPRR3$^{+/+}$ApoE$^{-/-}$ and SPRR3$^{-/-}$ApoE$^{-/-}$ mice (n=3/group) stained by immunohistochemistry with Ki67 (Novocastra Labs, NCLKi67p). *In vitro* Proliferation of GFP-VSMCs and SPRR3-VSMCs was assessed via BrdU incorporation. 96-well plates were seeded at 500 and 1,000 cells/well and cultured in 15% FBS/DMEM. BrdU incorporation assay was carried out according to manufacturer's protocol (BD Biosciences).

Viability assays. Viability was assessed using MTT. Cells were seeded at 25,000 cells/well in a 96-well plate and allowed 4 hours to adhere. Cells were then serum-starved 24 hours with 0.1% FBS/DMEM and 50ng/mL PDGF. After 24 hours starvation, MTT was added to the wells to a final concentration of 1.2mM and cells were incubated 4 hours at 37 °C. The supernatant was then removed from the cells and they were lysed in DMSO for 10 minutes at 37 °C before measuring OD at 540nm. Results represent three independent experiments performed in triplicate.

Cyclic strain was applied in pulse assays as described previously[1]. Cells pretreated for 48 hours with 200pM TGFβ were seeded at 1×10^5 cells/well in a 6 well plate coated with type I collagen and allowed to adhere overnight prior to application of strain.

Statistical analyses. Statistical significance was determined by Student's *t*-test or ANOVA followed by Tukey's multiple comparison test for parametric data and Mann-Whitney or Kruskal-Wallis for nonparametric data using GraphPad Prism (San Diego, CA). $p \leq 0.05$ were considered statistically significant.

Supplemental References

1. AL Pyle, JB Atkinson, A Pozzi, J Reese, B Eckes, JM Davidson, DL Crimmins, PP Young. Regulation of the atheroma-enriched protein, SPRR3, in vascular smooth muscle cells through cyclic strain is dependent on integrin α1β1/collagen interaction. *Am J Pathol.* 2008;173:1577-1588.
2. A Pozzi, PE Moberg, LA Miles, S Wagner, P Soloway, HA Gardner. Elevated matrix metalloprotease and angiostatin levels in integrin knockout mice cause reduced tumor vascularization. *Proc Natl Acad Sci USA.* 2000;97:2202-2207.
3. VR Babaev, PG Yancey, SV Ryzhov, V Kon, MD Breyer, MA Magnuson, S Fazio, MF Linton. Conditional knockout of macrophage PPAR(1) increases atherosclerosis in CC57Bl/6 and low-density lipoprotein receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 2005;25:1647-1653.
4. B Paigen, A Morrow, PA Holmes, D Mitchell, RA Williams. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis*. 1987;68:231-240.
5. PP Young, V Modur, AA Teleron, J Ladenson. Enrichment of genes in the aortic intima which are associated with stratified epithelium: implications of underlyling biomechanical and barrier properties of the arterial intima. *Circulation*. 2005;111:2382-2390.
6. MP Alfaro, A Vincent, S Saraswati, CA Thorne, CC Hong, E Lee, PP Young. sFRP2 suppression of bone morphogenic protein (BMP) and Wnt signaling mediates mesenchymal stem cell (MSC) self-renewal promoting engraftment and moyocardial repair. *J Biol Chem*. 2010;285:35645-35653
7. Babaev VR, PG Yancey, SV Ryzhov, V Kon, MD Breyer, MA Magnuson, S Fazio, MF Linton. Conditional knockout of macrophage PPARgamma increases atherosclerosis in C57Bl/6 and low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol.* 2005;25:1647-1653.