Supplemental Material

The *FES* gene at the 15q26 coronary-artery-disease locus inhibits atherosclerosis

(*FES* at 15q26.1 CAD locus inhibits atherosclerosis)

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Expanded Methods

Bioinformatics analysis

Information of DNase I hypersensitivity footprints and histone modification marks was obtained from https://genome.ucsc.edu/ and https://epgg-test.wustl.edu/browser/. Data on LD between rs17514846 and other SNPs were taken from LDlink (https://ldlink.nci.nih.gov/?tab=ldproxy). RegulomeDB ranks were obtained from http://www.regulomedb.org/. eQTL data were from the cited references, HaploReg, and the GTEx Portal (https://www.gtexportal.org/home/).

Cell culture

Under University of Leicester Medicine and Biological Sciences Research Ethics Committee approval, monocytes from peripheral blood samples of healthy individuals were isolated using the density gradient centrifugation and CD14 positive selection method. In brief, the blood samples were mixed with the density gradient medium Lymphoprep (STEMCELL Technologies, 07851) and centrifugated at 800g for 30 minutes to isolate mononuclear cell populations. Next, CD14$^+$ cells were positively selected using CD14 MicroBeads (Miltenyi Biotec, 130-050-201) following the manufacturer's protocol: CD14$^+$ cells were labeled with CD14 MicroBeads and the sample loaded onto a MACS Column (Miltenyi Biotec, 130-042-201) placed in the magnetic field of a MACS Separator; CD14$^+$ labeled cells retained within the column were washed and then eluted after removing the column from the magnetic field. Trypan blue staining carried out thereafter indicated that >95% of the freshly isolated cells were viable. Flow cytometric analyses showed that approximately 85% of the cells obtained were CD14$^+$ (Figure S12A), approximately 75% of the cells obtained were within the monocyte gate according to cell size and granularity (Figure S12A), an about 43%-49% reduction in the numbers of cells in the monocyte gate with a proportional increase in the numbers of cells outside the monocyte gate (likely due to cell differentiation) at 48 hours post-transfection with either control siRNA or *FES* siRNA (Figure S12A), and approximately 90% of cells were viable at 48 hours post-transfection with either control siRNA or *FES* siRNA (Figure S12B).

Human THP-1 monocytic cells were from the American Type Culture Collection (ATCC, TIB-202). Primary human coronary artery SMCs were from Cell Applications (350K-05a). RAW264.7 cells were also from ATCC (TIB-71).

CD14$^+$ primary monocytes (passage 0) and THP-1 monocytic cells (up to passage 8) were cultured in RPMI-1640 media supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Primary human coronary artery SMCs (up to passage 8) and RAW264.7 cells (up to passage 8) were cultured in Dulbecco's Modified Eagle Medium with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were cultured in a humidified incubator with 5% CO_2 at 37°C.

CRISPR-mediated genome editing to generate isogenic cell lines

With the use of the CRISPR (clustered regularly interspaced short palindromic repeats) technique, genome editing of THP-1 monocytes with the C/C genotype of rs17514846 was conducted to generate isogenic cell lines with either the C/C or A/A genotype for this SNP. In brief, guide RNA (5'-TCTGTGGGGGTCTCATTTTC-3' and 5'-CCCACATCCTCTGTTAAATG-3) were separately cloned into the gRNA/Cas9 expression vector containing a green fluorescent protein reporter (p-U6-sgRNA-EFS-Cas9-T2A-EGFP-WPRE). In addition, a homology-directed repair donor vector was prepared by inserting 5'

and 3' homology arms either side of the to-be-edited site (the inserts were generated by PCR amplification using DNA from THP-1 cells which were of the C/C genotype for rs17514846 and A/A for rs1894401), followed by site-directed mutagenesis to create a donor vector containing the A allele of rs17514846. A PGK-Neo-PolyA cassette was also inserted in-between the 5' and 3' arms in these vectors to provide a means for selection of edited THP-1 cells after transduction. THP-1 cells were transduced with lentivirus particles containing the 2 guide RNAs and the donor vector in a 1:1:1 ratio. The cells were incubated for 96 hours and then subjected to fluorescence-activated cell sorting by flow cytometry. Sorted cells were cultured in 96-well plates under G418 selection to select for successfully edited cells. Subsequently, single-cell clones were expanded in 6-well plates. Every 24 hours, the cell suspension was removed from the wells and centrifuged to remove dead cells. The cell pellet was then resuspended in fresh media containing G418 and returned to the incubator. Daily G418 replenishment and dead cell removal were performed for 2 weeks. The selected cells were then screened via PCR and Sanger sequencing to confirm successful editing of rs17514846 from the parental C/C genotype to A/A. Parental THP-1 cells (of C/C genotype for rs17514846 and A/A for rs1894401), isogenic THP-1 cells (containing the Neo cassette) with the C/C genotype, and isogenic THP-1 cells (containing the Neo cassette) with the A/A genotype were subsequently subjected to FES immunoblotting analysis and monocyte migration, proliferation, and apoptosis assays.

The same CRISPR-mediated genome editing procedure described above was employed to generate isogenic monocyte cell lines with either the A/A or G/G genotype of rs1894401, using 5'-GGCAAAGTGCTGGGAATGTA-3' as the guide RNA. Selected cells were screened via PCR and Sanger sequencing to confirm successful editing of rs1894401 from the parental A/A genotype to G/G. Parental THP-1 cells (of A/A genotype for rs1894401 and C/C for rs17514846), isogenic THP-1 cells (containing the Neo cassette) with the A/A genotype, and isogenic THP-1 cells (containing the Neo cassette) with the G/G genotype were subsequently subjected to FES immunoblotting analysis and monocyte migration, proliferation and apoptosis assays.

CRISPR-mediated or siRNA-mediated gene knockdown
A THP-1 stable cell line expressing Cas9 (THP-1 2×Cas9) was generated by two-round infection of lentiCas9-Blast (Addgene, 52962) packaged in lentiviruses, followed by selection with 30μg/ml blasticidin (ThermoFisher, A1113903) for 5 days. Three guide RNAs targeting *PRDM1* were constructed into lentiGuide-Puro (Addgene, 52963) and packaged using HEK293FT cells. The packaged lentiviruses were used to infect THP-1 2×Cas9 cells for 24h, followed by selection using 10μg/ml puromycin (ThermoFisher, A1113803) for 5 days. Thereafter, live cells were isolated using gradient centrifugation with Histopaque-1077 (Sigma, H8889) and used for assays. The sequences of the guide RNAs targeting *PRDM1* were: CGTACAACGCTCACTACCCC (targeting the antisense strand), CTTGAACGCGTCCTACGGCA (targeting the sense strand), and CCTACGGCATGAATTGTAAT (targeting the antisense strand). Knockdown of PRDM1 was verified by immunoblot analysis (Figure S1).

Freshly isolated CD14$^+$ monocytes from human peripheral blood were transfected with either *FES* siRNA (ThermoFisher, HSS103636, 1299001) or control siRNA (ThermoFisher, 12935300). In brief, 2.5-3 × 10^6 cells were suspended in 100μl Opti-MEM I Reduced Serum Medium (ThermoFisher, 31985070) containing either the *FES* siRNA (150pmol) or control siRNA (150pmol), and electroporation was conducted using an NEPA21 electroporator (Nepagene). Cells were then transferred to warm RPMI 1640 media (Sigma, R8758)

supplemented with 20% fetal bovine serum, 1% sodium pyruvate, 1% nonessential amino acids and 1% L-glutamine, and incubated for 1 hour in a humidified incubator with 5% CO_2 at 37°C. Thereafter, macrophage-colony stimulating factor (10ng/μl Miltenyl Biotech, 130-093-963) was added and the cells were incubated for a further 16 hours. Subsequently, the media with non-attached cells were collected and centrifugated at 200g for 10 minutes. The cell pellets were re-suspended in RPMI 1640 media (Sigma, R8758) supplemented with 10% fetal bovine serum, 1% sodium pyruvate and 10ng/μl macrophage-colony stimulating factor, and incubated for 48 hours prior to downstream assays. Knockdown of FES was verified by immunoblot analysis (Figure S2A).

THP-1 monocytes and primary human coronary artery smooth muscle cells (SMCs) (Cell Applications, 350K-05a) at passage 8 were transfected with either *FES* siRNA (10nM, ThermoFisher, HSS103636) or control siRNA (10nM, ThermoFisher, 12935300), with the use of Lipofectamine RNAiMAX transfection reagent (Invitrogen, 13778150) and Opti-MEM I Reduced Serum Medium (ThermoFisher, 31985070). FES knockdown was verified by immunoblot analysis (Figures S2B & S2C).

Electrophoretic mobility shift assay and super-shift assay
Fluorescent fluorescein-labeled, 25mer double-stranded oligonucleotide probes corresponding to the sequence containing and surrounding the rs1894401 site, with either A or G at the rs1894401 site, were individually incubated with or without nuclear protein extracts from THP-1 monocytes, in the presence or absence of unlabeled A allele oligonucleotide, unlabeled G allele oligonucleotide, an unlabeled unrelated oligonucleotide, a mouse anti-PRDM1 antibody (ThermoFisher, MA1-16874), and a mouse isotype control antibody (ThermoFisher, MA5-14453), at 25°C for 20 minutes. The mixes were subjected to non-denaturing polyacrylamide gel electrophoresis, followed by fluorescence detection. The same approach as described above, with the exception of the presence of an antibody, was used to assess the rs17514846 site with the use of either C or A allele-containing oligonucleotides.

Chromatin immunoprecipitation (ChIP) assay
In a ChIP assay to investigate PRDM1 binding to the rs1894401 site, THP-1 monocytic cells were crosslinked by incubation in formaldehyde and then incubated with glycine to quench formaldehyde. ChIP was carried out using the Pierce Agarose ChIP kit (ThermoFisher, 26156). DNA, in the chromatin immunoprecipitated by an anti-PRDM1 antibody (ThermoFisher, MA1-16874) or an isotype control antibody (ThermoFisher, MA5-14453), was subjected to quantitative PCR analysis of the DNA sequence containing and surrounding the rs1894401 site (primers: 5'-GTTTTGCACAAGAGGCCCTG-3' and 5'-GGAGGCGAGTACCGATTCTG-3') and the housekeeping gene GAPDH (primers: 5'-AAAGTAGGGCCCGGCTACTA-3' and 5'-TCGAACAGGAGGAGCAGAGA-3').

To investigate histone acetylation (H3K9ac) at the rs1894401 site, ChIP was performed on THP-1 monocytic cells with and without PRDM1 knockdown (described earlier), using the Pierce Agarose ChIP Kit (ThermoFisher, 26156) following the manufacturer's protocol. At the various steps in the protocol that include protease inhibitors, the HDAC inhibitor sodium butyrate was also added to a final concentration of 10mM. A total of 4×10^6 cells were fixed with 1% formaldehyde in complete media for 10 minutes at room temperature. The fixation was quenched with glycine and the cells incubated for 5 minutes at room temperature. The cells were pelleted by centrifugation at 300g for 5 minutes and washed with cold phosphate buffered saline by centrifugation. Following isolation of nuclei using the kit reagents, the

chromatin was digested using 8U of micrococcal nuclease per 4×10^6cells. The samples were incubated at 37°C in a water bath for 15 minutes, after which the reaction was stopped and the kit protocol continued for nuclear lysis. Prior to immunoprecipitation, 10% of the sample volume was retained as an input control. Of the remaining sample, 50% was incubated at 4°C with 5µg of rabbit IgG isotype control antibody (Abcam, ab37415) and 50% was incubated with 5µg rabbit anti-H3K9ac antibody (Sigma-Aldrich, 06-942). After overnight incubation, the ChIP kit protocol was continued and recovered DNA was subjected to quantitative PCR to quantify the abundance of H3K9ac at the rs1894401 region.

Luciferase reporter gene assay
Two plasmid constructs were generated by inserting a DNA fragment corresponding to a 152bp sequence containing and surrounding the SNP rs1894401 site, with either A or G at the rs1894401 site, into the pGL3-Control Vector (Promega, E1741). These constructs were individually mixed with a pRL-TK plasmid (Promega, E2241) containing a *Renilla* luciferase gene and used to transfect monocytes/macrophages (RAW264.7). Thereafter, cells were lysed and subjected to luciferase activity assay using a Dual-Glo Luciferase Assay System (Promega, E2920). Results are expressed as the ratio of firefly luciferase activity over *Renilla* luciferase activity.

Reverse transcription polymerase chain reaction (RT-PCR)
Total RNA was extracted from THP-1 cells with CRISPR-mediated knockout of PRDM1 (described above), with the use of Trizol (ThermoFisher, 15596018). RNA was then reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, 4368813). Subsequently, PCR of *FES* and the reference housekeeping gene *RPLP1* was performed using SYBR-green qPCR master-mix (Qiagen, 208057) and primers with the following sequences: *FES* forward primer: AGGCCAAGTTTCTACAGGAAGCGA; *FES* reverse primer: TGCACAAGCTCCATGACGATGTAG; *RPLP0* forward primer: CGACCTGGAAGTCCAACTACTTCCTTAAG; *RPLP0* reverse primer: CATGCGGATCTGCTGCATCTGCTTGGAG. The PCR thermocycling conditions were: 95°C for 5 minutes, followed by 40 cycles of 95°C (denaturation) for 10 seconds and 60°C (annealing and extension) for 30 seconds. The relative expression level of *FES* was determined using the ΔCt method with the *RPLP1* Ct value as a reference.

DNA and RNA samples from a previously described[14] collection of primary human umbilical vein endothelial cells were subjected to rs17514846 genotyping and *FES* RT-PCR analysis, respectively. rs17514846 genotypes were determined using a TaqMan SNP genotyping assay. RNA samples were reverse transcribed with the use of random primers (Promega, C1181) and M-MLV reverse transcriptase (Promega, M1701), followed by real-time PCR of *FES* and the reference housekeeping gene *18S rRNA* with the use of TaqMan gene expression assays (Applied Biosystems, Hs00171375_m1 for *FES*, and Hs99999901_s1 for *18S rRNA*) and a real-time quantitative PCR instrument (Applied Biosystems, ABI 7900HT real-time PCR System). The thermocycling condition was: 95°C for 10 minutes, followed by 40 cycles of 95°C (denaturation) for 15 seconds and 60°C (annealing and extension) for 1 minute. The ΔCt method was used to determine the relative expression level of *FES* in each sample, standardized against the Ct value of *18S rRNA*.

Immunoblot analysis
With the use of a lysis buffer (150mM NaCl, 50mM Tris, 5mM EDTA, and 1% Triton X100) supplemented with a protease inhibitor cocktail (Medchemexpress, HY-K0010), lysates were

prepared from parental THP-1 cells, THP-1 isogenic cells generated by CRISPR-mediated genome editing, THP-1 cells transfected with either *FES* siRNA or control siRNA, and human coronary artery SMCs transfected with either *FES* siRNA or control siRNA. Protein concentrations in the cell lysates were determined using BCA Protein Quantification Kit (ThermoFisher, 23227). Equal amounts of protein from each sample were subjected to electrophoresis with 10% (29:1) Bis-Tris polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking with a TBST buffer (Tris-buffered saline with 0.1% Tween 20) containing 5% bovine serum albumin, the membrane was incubated with a rabbit IgG anti-FES antibody (Abcam, ab108418) and a rabbit IgG anti-β-actin antibody (Sangon Biotech, D110001), diluted in TBST buffer containing 5% bovine serum albumin. After washing the membrane with TBST buffer three times, the membrane was incubated with either a goat-anti-mouse IgG dyelight680-linked secondary antibody (Immunoway Biotechnology, RS23710) or a goat-anti-rabbit IgG IRDye800CW-linked secondary antibody (Li-Cor Bioscience, 926-32211), diluted in TBST buffer containing 5% bovine serum albumin. After washing the membrane with TBST buffer three times, the bands on the membrane were detected using a ChemiDoc MP Imaging system (Bio-Rad).

To validate PRDM1 knockdown efficiency, lysates of THP-1 2×Cas9 cells infected with either *PRDM1* sgRNAs or scramble sgRNA were prepared using a buffer consisting of 50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X100, supplemented with protease inhibitor cocktail (ThermoFisher, 78443). Protein concentrations were determined using the BCA Protein Quantification Kit (ThermoFisher, 23227). Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis and transferred onto a nitrocellulose membrane (BIO-RAD, 1620094). After blocking the membrane with 5% (w/v) milk in TBST, the membrane was incubated with a mouse IgG anti-PRDM1 antibody (ThermoFisher, MA1-16874) or a rabbit IgG anti-FES antibody (Abcam, ab108418) overnight, followed by washing with TBST three times. Subsequently the membrane was incubated with an anti-mouse IgG HRP antibody (Santa Cruz, SC-516102) or a mouse anti-rabbit IgG HRP antibody (Santa Cruz, SC-2357), diluted in TBST containing 5% milk (w/v). After washing with TBST three times, the bands on the membrane were detected using an iBright FL1500 Imaging System (ThermoFisher).

Lysates of human primary peripheral blood monocytes transfected with either *FES* siRNA or control siRNA were prepared using a lysis buffer (150mM NaCI, 50mM Tris, 5mM EDTA, and 1% Triton X100) supplemented with cOmplete Mini Protease Inhibitor Cocktail (Sigma-Aldrich, 4693124001). Protein concentrations in the cell lysates were determined using BCA Protein Quantification Kit (ThermoFisher, 23227). Equal amounts of protein from each sample were subjected to electrophoresis with 4-12% Bis-Tris polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. After adding TBST buffer containing 5% milk, the membrane was incubated with a rabbit IgG anti-FES antibody (Abcam, ab108418) and a mouse anti-GAPDH antibody (Protein tech, 60004-1), diluted in TBST buffer containing 5% milk. After washing with TBST buffer three times, the membrane was incubated with either an anti-rabbit IgG peroxidase-linked secondary antibody (Sigma-Aldrich, A6154) or an anti-mouse IgG peroxidase-linked secondary antibody (Sigma-Aldrich, A4416), diluted in TBST buffer containing 5% milk. After washing the membrane with TBST buffer three times, the bands on the membrane were detected by chemiluminescence using ECL Plus Western Blotting Detection Reagent (VWR, RPN2109) and an ImageQuant LAS 4000 system (GE Healthcare).

To validate Fes knockout in Fes$^{-/-}$/Apoe$^{-/-}$ mice, protein extracts from tissues of Fes$^{-/-}$/Apoe$^{-/-}$ and Fes$^{+/+}$/Apoe$^{-/-}$ mice were prepared using T-PER Tissue Protein Extraction Reagent (ThermoFisher 78510) supplemented with cOmplete Mini Protease Inhibitor Cocktail (Sigma-Aldrich, 4693124001). Protein concentrations in the cell lysates were determined using BCA Protein Quantification Kit (ThermoFisher, 23227). Equal amounts of protein from each sample were subjected to electrophoresis with 4-12% Bis-Tris polyacrylamide gel (ThermoFisher, NP0335BOX) and transferred onto a polyvinylidene fluoride membrane. After adding TBST buffer containing 5% milk, the membrane was incubated with a rabbit IgG anti-Fes antibody (Abcam, ab108418) and a mouse anti-Gapdh antibody (Protein tech, 60004-1), diluted in TBST buffer containing 5% milk. After washing with TBST buffer three times, the membrane was incubated with either an anti-rabbit IgG peroxidase-linked secondary antibody (Sigma-Aldrich, A6154) or an anti-mouse IgG peroxidase-linked secondary antibody (Sigma-Aldrich, A4416), diluted in TBST buffer containing 5% milk. After washing the membrane with TBST buffer three times, the bands on the membrane were detected by chemiluminescence using ECL Plus Western Blotting Detection Reagent (VWR, RPN2109) and an ImageQuant LAS 4000 system (GE Healthcare).

Flow cytometry analysis
Freshly isolated human peripheral blood monocytes, and human peripheral blood monocytes transfected with either *FES* siRNA or control siRNA for 48 hours, were subjected to flow cytometry analysis of CD14. In brief, non-attached cells in culture media were collected and centrifugated at 200g for 10 minutes. Cell pellets were then washed twice with phosphate buffered saline and re-suspended in phosphate buffered saline with 1% bovine serum albumin at a concentration of 10^5 cells/100µl. Subsequently, a 2µl aliquot of anti-human CD14-FITC antibody (Miltenyi Biotec, 130-110-576) or isotype control (IgG1-FITC, Miltenyi Biotec, 130-113-437) was added, and the cells were incubated at 4°C for 15 minutes. Thereafter, cells were washed three times with phosphate buffered saline with 1% bovine serum albumin, and then analyzed using a CytoFLEX Flow Cytometer with CytExpert Acquisition and Analysis software (Beckman Coulter). CD14$^+$ monocytes of the freshly isolated samples were gated using forward and side light scatter, enabling discrimination by cell size and granularity, respectively. This monocyte gate was also used in the analysis of cells transfected with either *FES* siRNA or control siRNA for 48 hours.

Migration assay
Human peripheral blood monocytes transfected with either *FES* siRNA (ThermoFisher, HSS103636, 1299001) or control siRNA (ThermoFisher, 12935300) were seeded onto the apical surface of trans-well Boyden chambers (8µm pore size, Millipore, MCEP12H48) in serum-free complete RPMI-1640 media. RPMI supplemented with 10% (v/v) fetal calf serum was used as the chemoattractant below the trans-well, and cells were incubated for 3 hours. The monocytes in the bottom chamber were collected and pelleted, and cell populations were quantified using 1× PrestoBlue (Thermofisher, A13261) following the recommended manufacturer's protocol. Fluorescence intensity was measured on a BMG labtech NOVOstar plate reader (excitation/emission: 530/590nm). Fluorescence intensity was standardized to cells incubated with serum free complete RPMI in the bottom chamber (to adjust for transient migration). Migration was quantified in 3 replicates of each cell type per independent experiment.

Migration assay of THP-1 monocytic cells and human coronary artery SMCs, respectively, were carried out using 24-well plates with trans-well inserts (8µm pore size, Millipore, MCEP24H48). In brief, cells in serum-free medium were added to the upper chamber, and

culture medium with 10% fetal bovine serum was placed in the lower chamber of each trans-well. In the THP-1 cell migration assay, at 4 hours after placing cells in the trans-well insert, the number of cells in the lower chamber were counted using a hemocytometer. In the SMC migration assay, at 12 hours after placing cells in the trans-well insert, the number of cells on the reverse side of the insert were stained with Giemsa and counted using a microscope.

Apoptosis assay

Human peripheral blood monocytes transfected with either *FES* siRNA (ThermoFisher, HSS103636, 1299001) or control siRNA (ThermoFisher, 12935300) were incubated with 10μM staurosporine (Alfa Aesar, J62837) for 4 hours, and then stained with FITC-Annexin V and propidium iodide using a Dead Cell Apoptosis Kit (ThermoFisher, V13242) following the manufacture's protocol. Cells were analyzed using a CytoFLEX Flow Cytometer with CytExpert Acquisition and Analysis software (Beckman Coulter).

An apoptosis assay of THP-1 monocytic cells transfected with either *FES* siRNA (ThermoFisher, HSS103636) or control siRNA (ThermoFisher, 12935300) was performed using the above procedure and FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, 556547).

Proliferation assay

Proliferation assays of THP-1 monocytic cells transfected with *FES* siRNA (ThermoFisher, HSS103636) or control siRNA (ThermoFisher, 12935300) and human coronary artery SMCs transfected with *FES* siRNA (ThermoFisher, HSS103636) or control siRNA, were performed using Cell Counting Kit-8 (Enzo Life Sciences). Briefly, cells were cultured in 96-well plates ($5×10^{-4}$ cells/well), and a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium solution was added into each well 24- or 48-hours later. At 2 hours after adding the reagent, a colorimetric assay was carried out using a microplate reader which measured the absorbance at 450nm.

Proteomics and phosphoproteomics analyses

Monocytes (THP-1) transfected with either *FES* siRNA (ThermoFisher, HSS103636) or control siRNA (ThermoFisher, 12935300) (n = 5 for each condition) were subjected to quantitative proteomics and phosphoproteomics analysis. In brief, protein extracts from cells were reduced with dithiothreitol, alkylated with iodoacetamide, washed with acetone, and then dissolved in a solution containing 0.1M triethylammonium bicarbonate (pH 8.5) and 6M urea. The concentration of total protein in the samples was quantified by the Bradford method, and protein quality assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie Brilliant Blue R-250 staining. The protein samples were digested by incubation with trypsin and subjected to tandem mass tag (TMT) labeling using a mass tagging kit (ThermoFisher, 90113). Phosphopeptides in the samples were enriched using PHOS-Select Iron Affinity Gel (Sigma-Aldrich, P9740). Subsequently, liquid chromatography-mass spectrometry was performed with the use of an EASY-nLCTM 1200 System (ThermoFisher) and an Q Exactive HF-X mass spectrometer (ThermoFisher).

The liquid chromatography-mass spectrometry data were analyzed using Proteome Discoverer Software 2.4 (ThermoFisher) with the following parameters: a mass tolerance of 10 ppm for precursor ion scans and a mass tolerance of 0.02 Da for the product ion scans. Phosphorylation of serine (S)/threonine (T)/tyrosine (Y), oxidation of methionine (M) and TMT-plex of lysine (K) were specified as dynamic modifications. Carbamidomethyl was set as a fixed modification. TMT-plex, acetylation, and loss of methylation were specified as N-

terminal modifications. Each protein was identified with at least 1 unique peptide at a false discovery rate (FDR) < 1%. Proteins with similar peptides that could not be distinguished based on the mass spectrometry data were combined as protein groups. Quantification based on intensity was performed. Any difference in the quantity of each protein between cells with FES knockdown and cells transfected with the control siRNA was ascertained by Mann-Whitney test.

The motif-x algorithm was used to identify motifs enriched within a set of phosphosites.[15] All enrichments were carried out for 7 residues surrounding the central residue with occurrences > 20 and $p < 10^{-6}$. WebLogo[16] was used to build the motif figures. To predict kinase-substrate relationships, all identified serine, threonine and tyrosine phosphorylation sites (pS/T/Y) were scored with the NetPhorest algorithm[17].

A pathway enrichment analysis was conducted on proteins that showed differences in modification between cells with siRNA-mediated FES knockdown and cells transfected with the control siRNA ($p < 0.05$ by Mann-Whitney analysis, log2 fold change > 0.26 or < -0.32) (Table S1), using the default setting of Gene Ontology (http://geneontology.org). Pathway enrichment bubble plot was generated with the use of SPplot (http://www.bioinformatics.com.cn/srplot).

Single-cell RNA-sequencing of human atherosclerotic plaques
The study was approved by the Medical Ethical Committees of University Medical Center Utrecht and all participants provided informed consent. Human carotid atherosclerotic plaque samples (n = 18) from the Athero-Express Biobank[18] were subjected to single-cell RNA-sequencing as previously described[19]. Single-cell RNA-sequencing data were analyzed with the use of SEURAT (http://www.satijalab.org/seurat), employing the global-scaling normalization (LogNormalize) method to standardize the expression levels of *FES* or *PRDM1* against global gene expression levels in each cell, which are then multiplied by a factor of 10,000 and natural logarithm transformed. The single-cell RNA-sequencing data are available from Dr Sander W. van der Laan (s.w.vanderlaan-2@umcutrecht.nl).

Genotyping and immunohistochemical analysis of human atherosclerotic plaques
The study was approved by the East London & City Research Ethics Committee. The samples examined were from archival tissues collected in the 1980s without requirement of informed consent. The study fully complied with Good Clinical Practice guidelines and the Human Tissue Act regulations.

Formaldehyde-fixed paraffin-embedded blocks of human coronary atherosclerotic plaques were obtained at autopsies performed by R.N.P. in the 1980s, during which the coronary arteries were serially transected. The arterial samples were taken for paraffin embedding from the central regions of morphologically identifiable plaques which showing maximal thickening of the wall by atherosclerosis and were orientated to obtain transverse sections of the arteries upon cutting.

Sections from these arteries were genotyped for rs17514846 using the TaqMan genotyping method and further sections were subjected to immunostaining for CD68 and SMA, double fluorescent immunostaining of FES and Iba1, and double fluorescent immunostaining of FES and SMA, as described below.

For CD68 immunostaining, atherosclerotic plaque sections were deparaffinized with xylene, rehydrated with ethanol, and then incubated with 10mM sodium citrate at 95ºC for 30 minutes to retrieve antigens, followed by incubation with 10% fetal bovine serum and then a peroxidase blocking solution (0.3% H_2O_2). Thereafter, the sections were incubated with a mouse anti-human CD68 antibody (Dako, clone PG-M1), a previously validated specific anti-macrophage monoclonal antibody,[20] and then with a biotin-conjugated goat anti-mouse secondary antibody (Vector Laboratories, BA-2020-.5), followed by incubation with avidin-conjugated horseradish peroxidase and then with 3,3'-diaminobenzidine. For SMA immunostaining, the above procedure was carried out, except a mouse anti-human SMA antibody (Dako, M-0635), instead of the mouse anti-human CD68 antibody, was used. Analysis was performed blind to genotyping results. Images of up to 14 randomly selected areas across each stained section were acquired using a digital camera-equipped microscope. Areas of atherosclerotic plaques were determined, and the plaques classified, by one investigator (K.C.) in accordance with standard American Heart Association definition and classification[21]. The results were independently verified by an expert pathologist (R.N.P.) who was blinded to the findings, with complete agreement.

To analyze the atherosclerotic lesion content of macrophages and SMCs, Image-Pro Software (Media Cybernetics) was employed. On a digital arterial image, an area of interest was defined by lines drawn manually along the arterial inner aspect, and the outer boundary of the medial SMCs, together with straight radial lines drawn perpendicular to the inner surface defining the lateral extent of the plaque at the extremities of the thickened area. This area was further divided into intima and media at the inner boundary of medial SMCs, and in large lesions into segments by further radial lines in multiple digital images. The intimal area was analyzed for macrophages by the determination of the fraction occupied by CD68 staining, which was assessed by hue-saturation-intensity thresholding. The intima was analyzed similarly for SMC content using SMA. A macrophage index was devised that was proportional to the fraction of the total wall area occupied by intimal CD68 staining. The use of the fraction of the intimal area stained and of the total wall area in the macrophage index was to compensate for variation between specimens of the wall thickness and in intima to media ratio. In addition, the ratio was calculated between the fractions of the intimal area that were occupied by intimal macrophages and by intimal SMCs. In specimens requiring segments in multiple images to be analyzed, the mean values of the parameters for the lesion were calculated. By these means, the whole of an atherosclerotic plaque in a transverse arterial section near the longitudinal center of the lesion was analyzed. Only Types IV or V plaques (according to the American Heart Association Histological Classification of Atherosclerotic Lesions[21]) were used in the statistical analyses in relation to genotype.

For double fluorescent immunostaining of FES and the macrophage marker Iba1, sections were deparaffined with xylene, rehydrated with ethanol, and then incubated with a solution containing 0.05% trypsin and 0.1% calcium chloride at 37˚C for 15 minutes to retrieve antigens, followed by incubation with a blocking solution containing 5% goat serum at room temperature for 30 minutes. Thereafter, the sections were incubated with primary antibodies against FES (rabbit IgG, Abcam, ab153841) or rabbit IgG isotype control (ThermoFisher, 10500C), and with Iba1 (mouse IgG, Abcam, ab15690) or mouse IgG isotype control (ThermoFisher, 10400C), respectively, at 4˚C overnight. The sections were then incubated with goat anti-rabbit IgG (Alexa Fluor Plus 488, ThermoFisher, A32731) and goat anti-mouse IgG (Alexa Fluor Plus 647, ThermoFisher, A32728) at 37˚C for 60 minutes, followed by an incubation with DAPI (4',6-diamidino-2-phenylindole) for 5 minutes. After mounting, the sections were examined using an EVOS FL Auto Imaging System (ThermoFisher).

Images were processed with Photoshop software (Adobe). Macrophages (Iba1 positive cells) in each of 10 atherosclerotic lesion images were randomly highlighted, and mean fluorescence intensity (MFI) values of FES staining as well as DAPI for each randomly selected macrophage were measured using Image-Pro Plus 6.0 software (Media Cybernetics), blinded to SNP genotype information. Relative MFI values of FES against DAPI staining in each Iba1 positive cell were calculated, averaged, and presented.

For double immunofluorescent staining of FES and the SMC marker SMA, the immunohistochemical procedure described above for FES and Iba1 was used, but with primary antibodies against FES (rabbit IgG, Abcam, ab153841) or rabbit IgG isotype control (Invitrogen, 10312573), and primary antibodies against SMA (Mouse IgG, Sigma-Aldrich, A5228) or mouse IgG isotype control (ThermoFisher, 10400C), and then with goat anti-Rabbit IgG (Alexa Fluor Plus 488, ThermoFisher, A11008) and goat anti-Mouse IgG (Alexa Fluor Plus 555, ThermoFisher, A21422).

Animal study

The study was conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, under a Home Office Project License (60/4332) and following the guidelines of ARRIVE (Animal Research: Reporting of *In Vivo* Experiments). *Fes*^{-/-} mice[22] were backcrossed onto a C57BL6/J background for seven generations before being intercrossed with *Apoe*^{-/-} mice (B6.129P2-*Apoe*[tm1Unc]/J purchased from Jackson Laboratory) to generate *Fes*^{-/-}/*Apoe*^{-/-} mice and *Fes*^{+/+}/*Apoe*^{-/-} control littermates for the study of atherosclerosis. Knockout of *Fes* in *Fes*^{-/-}/*Apoe*^{-/-} mice were verified by PCR and immunoblotting analyses (Figure S13). Fes[+/-]Apoe^{-/-} were excluded from this study. The study was performed only with males, as it has been reported previously that atherosclerotic plaques develop more reproducibly and with less biological variability in male Apoe^{-/-} mice than in female Apoe^{-/-} mice.[23,24] Male *Fes*^{-/-}/*Apoe*^{-/-} mice and male *Fes*^{+/+}/*Apoe*^{-/-} control littermates were fed a high fat diet (fat 40%, carbohydrate 44%, protein 16%, cholesterol 0.15%) (TestDiet, T-5TJN) for 12 weeks from 6 weeks of age. All mice were housed in a specific pathogen-free facility in an individually ventilated caging system using group housing wherever possible, with routine checks of each animals' health status. Other than weight gain associated with high-fat diet, no mice demonstrated any adverse effects. Because of genotype requirements, mice could not be randomized into groups. Experiments were powered for aortic root analysis of atherosclerosis as the primary objective. A power calculation indicated that group sizes of 8 mice would be sufficient to provide >80% power ($\alpha=0.05$) to detect a difference of 40% in plaque area by aortic root analysis. Our study had 12 *Fes*^{-/-}/*Apoe*^{-/-} mice and 9 *Fes*^{+/+}/*Apoe*^{-/-} mice, and no animals subjected to the high fat diet were excluded from this study.

The aortic root from each mouse was harvested and ninety consecutive sections (10μm in thickness) per aortic root from the appearance of the aortic sinus (identified by the appearance of aortic cusps) were cut. Sections were subjected to MOMA2 immunostaining, Masson's trichrome staining, SMA immunostaining (sections from one *Fes*^{-/-}/*Apoe*^{-/-} mouse were unavailable), and hematoxylin/eosin staining (sections from four *Fes*^{-/-}/*Apoe*^{-/-} mice and one *Fes*^{+/+}/*Apoe*^{-/-} mouse were unavailable), respectively. For MOMA2 and SMA staining, sections were fixed in acetone and endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol. Non-specific binding was reduced by incubation in 2.5% goat serum (Vector Laboratories) before incubation with a rat anti-MOMA2 antibody (MCA519G, BIO-RAD) or a rabbit anti-SMA antibody (Abcam, ab5694). Thereafter, sections were incubated with peroxidase-conjugated anti-rat IgG (MP-7444, Vector Laboratories) or peroxidase-conjugated anti-rabbit IgG (MP-7451, Vector Laboratories), followed by incubation with

3,3'-diaminobenzidine and counterstained with hematoxylin. For Hematoxylin/eosin staining and Masson's trichrome staining, we used commercial kits (H&E Staining Kit, Abcam, ab245880; and Trichrome Stain Kit, Sigma-Aldrich, HT15, respectively) and followed the manufacturer's instruction. Images were acquired using a microscope equipped with a digital camera and analyzed using Fiji software to quantify atherosclerotic lesion sizes and the areas (in atherosclerotic lesions) positively stained for MOMA2, SMA, and collagen, respectively, blinded to genotype. For each mouse, the average value of each measurement in 3 to 9 sections is presented.

PCR analysis of ear snips was performed to determine the genotype. The PCR primers were 5'-CCGCTTCCTCGTGCTTTACGG-3' and 5'-TAACCCACCATGTGTAGACAGG-3', designed to generate a 700bp PCR product from the $Fes^{-/-}/Apoe^{-/-}$ mice but not from the $Fes^{+/+}/Apoe^{-/-}$ mice (Figure S13A). Immunoblotting analysis of lung samples were performed with the use of an anti-Fes antibody (Abcam, ab108418) and an anti-Gapdh antibody (Protein tech, 60004-1), which confirmed the presence of FES in $Fes^{+/+}/Apoe^{-/-}$ mice and the absence of FES in $Fes^{-/-}/Apoe^{-/-}$ mice (Figure S13B).

Levels of total cholesterol, very low-density-lipoprotein (VLDL)-cholesterol, low-density-lipoprotein (LDL)-cholesterol, high-density-lipoprotein (HDL)-cholesterol, and triglycerides, in fasted blood samples were measured with the use of assay kits following the manufacturers' protocols (Abcam, ab65390 and ab65336).

Statistical analyses
Statistical analyses were performed with the use of SPSS version 27 and GraphPad Prism version 9.4.1. The Mann-Whitney test or Kruskal-Wallis test (with multiple comparisons adjusting for the number of comparison) was used to ascertain differences between experimental groups in band intensity (after being standardized against β-actin band intensity) in immunoblot analyses, results of migration and proliferation assays of THP-1 and human coronary artery SMCs, and measurements of atherosclerotic lesions and lipid levels in the animal study. The Wilcoxon matched-pairs signed-rank test was performed to ascertain differences in migration and apoptosis between primary human blood monocytes from different individuals transfected with either FES siRNA or control siRNA. The T-test was used to ascertain a between-genotype difference in FES level in monocytes/macrophages in human coronary atherosclerotic plaques. Linear regression analyses were performed with the independent variable being the rs17514846 C/C, C/A, and A/A genotypes coded as 0, 1, and 2, respectively, and the dependent variable being monocyte/macrophage abundance index, SMC content, or macrophage/SMC ratio, in human coronary atherosclerotic plaques, adjusting for lesion types defined according to the American Heart Association Histological Classification of Atherosclerotic Lesions[21]. The human coronary atherosclerotic plaque collection had n ≥ 10 for each of the rs17514846 genotypes (C/C, C/A, and A/A, respectively), with 80% power to detect a genotypic effect size of 0.2 at α=0.05.
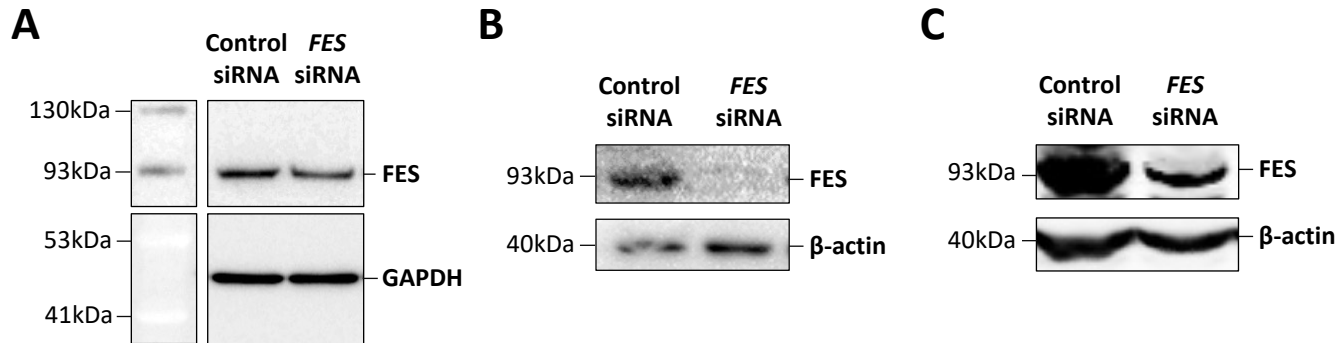
References
14. Ng FL, Boedtkjer E, Witkowska K, et al. Increased NBCn1 expression, Na+/HCO3- co-transport and intracellular pH in human vascular smooth muscle cells with a risk allele for hypertension. *Hum Mol Genet*. 2017;26:989-1002.
15. Wagih O, Sugiyama N, Ishihama Y, et al. Uncovering Phosphorylation-Based Specificities through Functional Interaction Networks. *Mol Cell Proteomics*. 2016;15:236-245.

16. Crooks GE, Hon G, Chandonia JM, et al. WebLogo: a sequence logo generator. *Genome Res*. 2004;14:1188-1190.

17. Miller ML, Jensen LJ, Diella F, et al. Linear motif atlas for phosphorylation-dependent signaling. *Sci Signal*. 2008;1:ra2.

18. Verhoeven BA, Velema E, Schoneveld AH, et al. Athero-express: differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. *Eur J Epidemiol*. 2004;19:1127-1133.

19. Depuydt MAC, Prange KHM, Slenders L, et al. Microanatomy of the Human Atherosclerotic Plaque by Single-Cell Transcriptomics. *Circ Res*. 2020;127:1437-1455.

20. Falini B, Flenghi L, Pileri S, et al. PG-M1: a new monoclonal antibody directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. *Am J Pathol*. 1993;142:1359-1372.

21. Stary HC, Chandler AB, Dinsmore RE, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation*. 1995;92:1355-1374.

22. Zirngibl RA, Senis Y, Greer PA. Enhanced endotoxin sensitivity in fps/fes-null mice with minimal defects in hematopoietic homeostasis. *Mol Cell Biol*. 2002;22:2472-2486.

23. Liu M, Zhang W, Li X, et al. Impact of age and sex on the development of atherosclerosis and expression of the related genes in apoE deficient mice. *Biochem Biophys Res Commun*. 2016;469:456-462.

24. Zhang G, Li C, Zhu N, et al. Sex differences in the formation of atherosclerosis lesion in apoE(-/-)mice and the effect of 17beta-estrodiol on protein S-nitrosylation. *Biomed Pharmacother*. 2018;99:1014-1021.
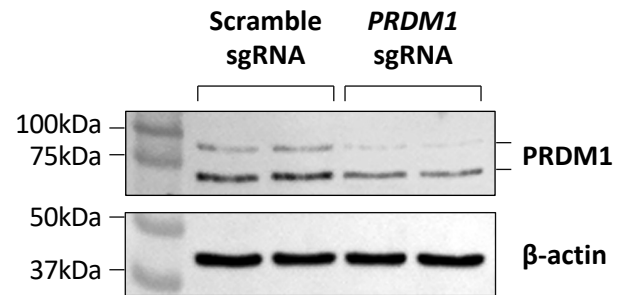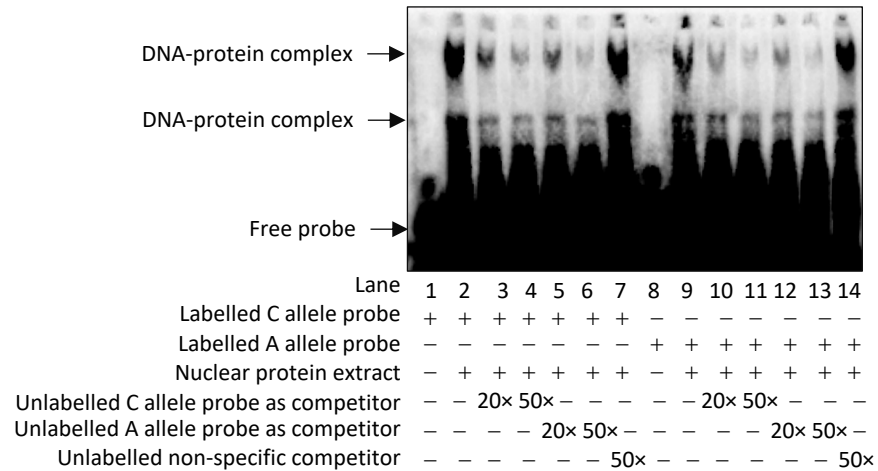
Figure S1. Verification of PRDM1 knockdown
Human monocytic cells (THP1 cells) were transduced with either scramble sgRNA
or sgRNA for CRISPR-mediated knockdown of *PRDM1* as described in the
Supplemental Material (Detailed Methods), and subsequently subjected to
immunoblot analysis with antibodies for PRDM1 and β-actin, respectively.

A

130kDa —
93kDa — Control *FES*
53kDa — siRNA siRNA
41kDa —

— FES

— GAPDH

B

Control *FES*
siRNA siRNA

93kDa — — FES

40kDa — — β-actin

C

Control *FES*
siRNA siRNA

93kDa — — FES

40kDa — — β-actin

Figure S2. Verification of FES knockdown

Human peripheral blood monocytes (**A**), THP-1 monocytic cells (**B**), and human coronary artery smooth muscle cells (**C**), were transfected with either negative control siRNA or *FES* siRNA and subjected to immunoblot analysis at 48 hours post transfection.

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Labelled C allele probe	+	+	+	+	+	+	+	−	−	−	−	−	−	−
Labelled A allele probe	−	−	−	−	−	−	−	+	+	+	+	+	+	+
Nuclear protein extract	−	+	+	+	+	+	+	−	+	+	+	+	+	+
Unlabelled C allele probe as competitor	−	−	20×	50×	−	−	−	−	−	20×	50×	−	−	−
Unlabelled A allele probe as competitor	−	−	−	−	20×	50×	−	−	−	−	−	20×	50×	−
Unlabelled non-specific competitor	−	−	−	−	−	−	50×	−	−	−	−	−	−	50×

Figure S3. Results of electrophoretic mobility shift assay of the rs17514846 site

FAM-labeled, double-stranded DNA probes corresponding to the A or C allele of rs17514846 were incubated with nuclear extracts of THP-1 cells and competitors (in 20- or 50-fold molar excess), followed by polyacrylamide gel electrophoresis. The image is an average representative of 3 experiments.
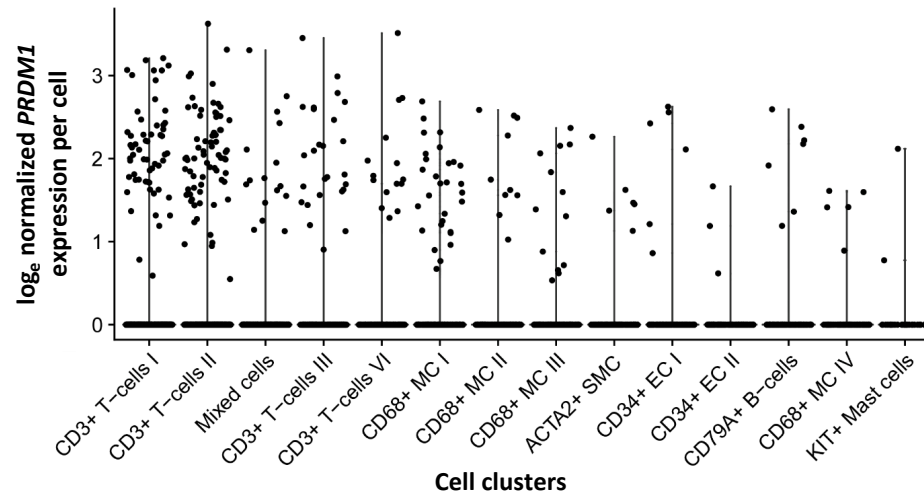
Figure S4. Single-cell transcriptomic analysis of *PRDM1* in human atherosclerotic plaques
Graph illustrates that in atherosclerotic plaques, cells (represented by black dots) which
express *PRDM1* are predominantly macrophages (MC) and T-cells. X-axis: different cell clusters;
y-axis: normalized *PRDM1* expression levels with natural logarithm transformation.
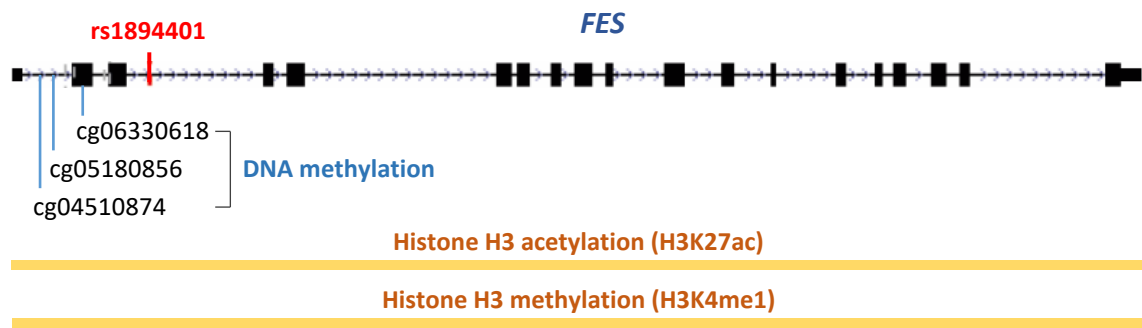
Histone H3 modification (data from QTLbase)

SNP	SNP position	Effect allele	Alternative allele	Histone modification	Histone modification Position	beta	P value	References (PMID)
rs1894401	Chr15:91429042 (hg19)	G	A	H3K27ac	Chr15:91410860-91451973	-0.0196	0.0967	31598699, 27863251
rs1894401	Chr15:91429042 (hg19)	G	A	H3K4me1	chr15:91337464-91511069	0.0083	0.0852	31598699, 27863251

Chromatin-DNA methylation (data from mQTLdb)

SNP	SNP position	Effect allele	Alternative allele	CpG	CpG Position	beta	P value	Reference (PMID)
rs1894401	Chr15:91429042 (hg19)	G	A	cg04510874	Chr15:91427884	0.3393	8.20×10^{-19}	27036880
rs1894401	Chr15:91429042 (hg19)	G	A	cg05180856	Chr15:91427885	0.1882	1.47×10^{-11}	27036880
rs1894401	Chr15:91429042 (hg19)	G	A	cg06330618	Chr15:91427886	0.2395	4.50×10^{-18}	27036880

Figure S5. Reported effect of rs1894401 on histone modification and DNA methylation

A bioinformatics analysis using QTLbase (http://www.mulinlab.org/qtlbase/index.html) and mQTLdb (http://www.mqtldb.org/) showed that compared with the rs1894401 A allele, the rs1894401 G allele has lower histone H3 acetylation level (beta = -0.0196), higher histone H3 methylation level (beta = 0.0083) and higher chromatin-DNA methylation level (e.g. beta = 0.3393 for cg04510874), in blood cells, as described in the tables above.
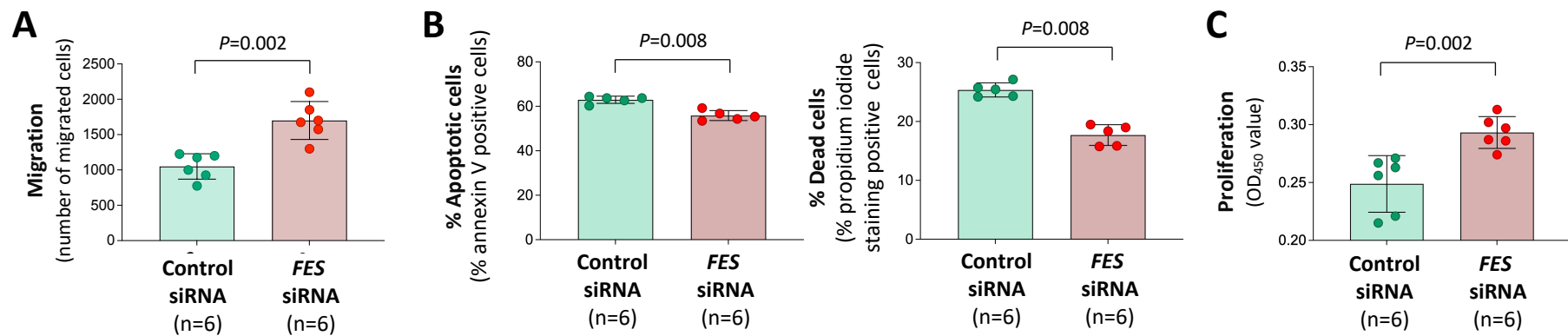
Figure S6. Effects of *FES* knockdown on behavior of THP-1 monocytic cells

Shown in the figure are results of trans-well migration assay (**A**), apoptosis assay (**B**) and proliferation assay (**C**) of THP1 monocytic cells transfected with either negative control siRNA or *FES* siRNA (knockdown of FES is shown in Figure S2B). Columns and error bars represent mean ± standard deviation values; *P* values are from two-tailed Mann-Whitney test.
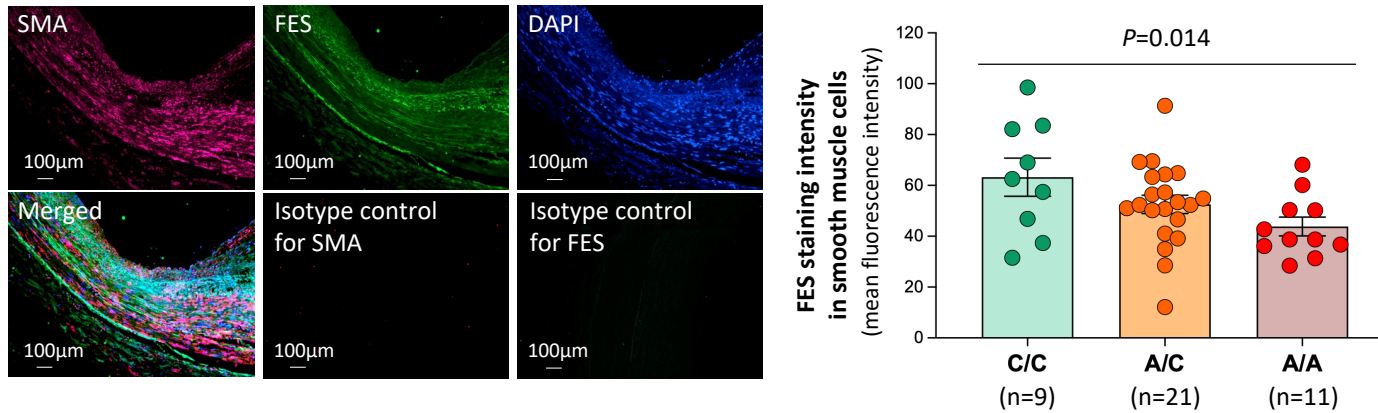
Figure S7. FES expression in smooth muscle cells in atherosclerotic plaques by rs17514846 genotype
Human coronary atherosclerotic plaques from different individuals were subjected to double fluorescent immunostaining for FES and the smooth muscle cell marker SMA. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Fluorescent immunostaining images were analyzed using Image-Pro Plus image analysis software. Left: Average representative images. Right: FES mean fluorescence intensity in smooth muscle cells (SMA positive) in atherosclerotic plaques from individuals of the C/C, C/A, and A/A genotypes, respectively. Columns and error bars represent mean ± standard error of the mean. Numbers of individuals per group are indicated in the Figure. *P* values are from linear regression analysis with the dependent variable being FES staining intensity in smooth muscle cells and the independent variable being the rs17514846 C/C, C/A, and A/A genotypes coded as 0, 1, and 2, respectively.
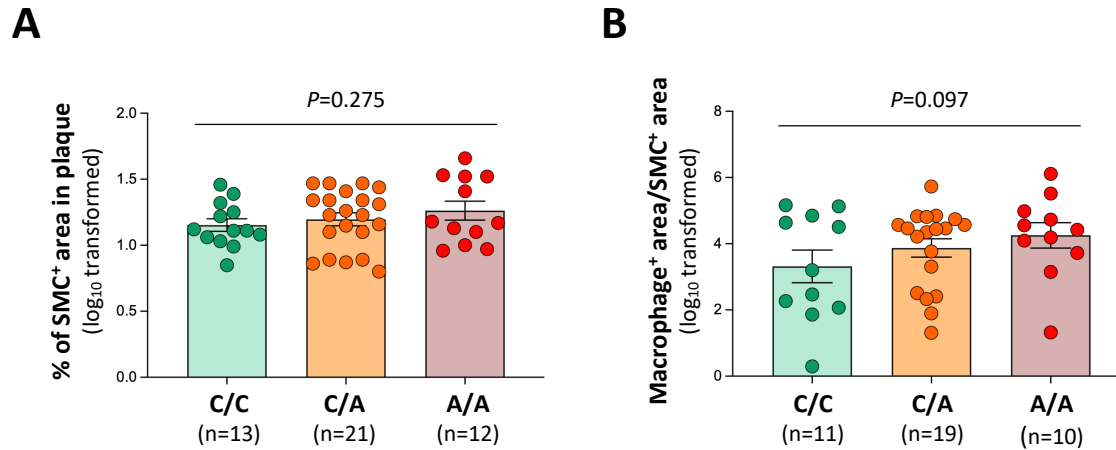
**Figure S8. Smooth muscle cell (SMC) content and macrophage/SMC ratio
in human atherosclerotic plaques by rs17514846 genotype**

A. SMC content. **B**. Macrophage/SMC ratio. Human coronary atherosclerotic plaques from different individuals were subjected to genotyping for rs17514846 and immunostaining for the smooth muscle cell marker SMA and the monocyte/macrophage marker CD68. Immunohistochemical images were analyzed with the use of Image-Pro Plus image analysis software. Columns and error bars represent mean ± standard error of the mean; numbers of individuals per group are indicated in the Figure. *P* values are from linear regression analysis with the dependent variable being % of SMC+ area in plaque (normalized by $\log_{10}$ transformation) or macrophage+ area/SMC+ area ratio (normalized by $\log_{10}$ transformation) and the independent variable being the rs17514846 C/C, C/A, and A/A genotypes coded as 0, 1, and 2, respectively, and with adjustment for lesion types defined according to the American Heart Association Histological Classification of Atherosclerotic Lesions.
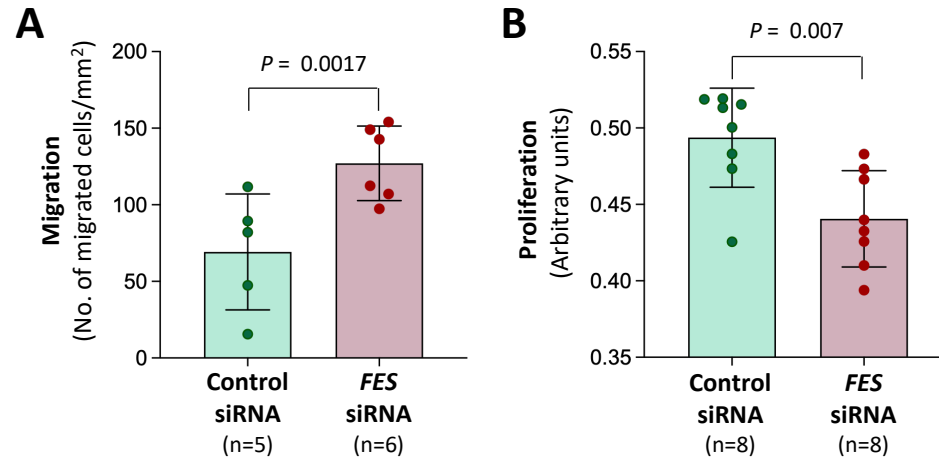
Figure S9. Effect of FES knockdown on vascular smooth muscle cell behavior
Primary human coronary artery smooth muscle cells were transfected with either negative control siRNA or *FES* siRNA (knockdown of FES is shown in Figure S2C), and subsequently subjected to trans-well migration assay (**A**) and proliferation assay (**B**), respectively. Columns and error bars represent mean ± standard deviation; *P* values are from two-tailed Mann-Whitney test.
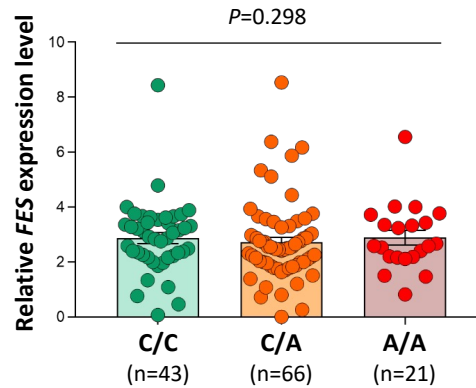
**Figure S10.** *FES* **expression level in vascular endothelial cells by rs17514846 genotype**
DNA and RNA samples were prepared from cultured human umbilical vein endothelial cells from different individuals.
DNA genotypes of rs17514846 were determined by TaqMan SNP genotyping assay. RNA reverse transcription was
carried out with the use of random primers and M-MLV reverse transcriptase. The resulting cDNA was subjected to
real-time polymerase chain reaction of *FES* and the reference housekeeping gene *18S rRNA* with the use of TaqMan
gene expression assays. The ΔCt method was used to determine the relative expression level of *FES* in each sample,
standardized against the Ct value of *18S rRNA.* Columns and error bars represent mean ± standard error of the mean;
the numbers of subjects in each group are indicated in the figure; the *P* value is from a linear regression analysis with
the dependent variable being *FES* expression level and the independent variable being rs17514846 C/C, C/A, and A/A
genotypes coded as 0, 1, and 2, respectively.
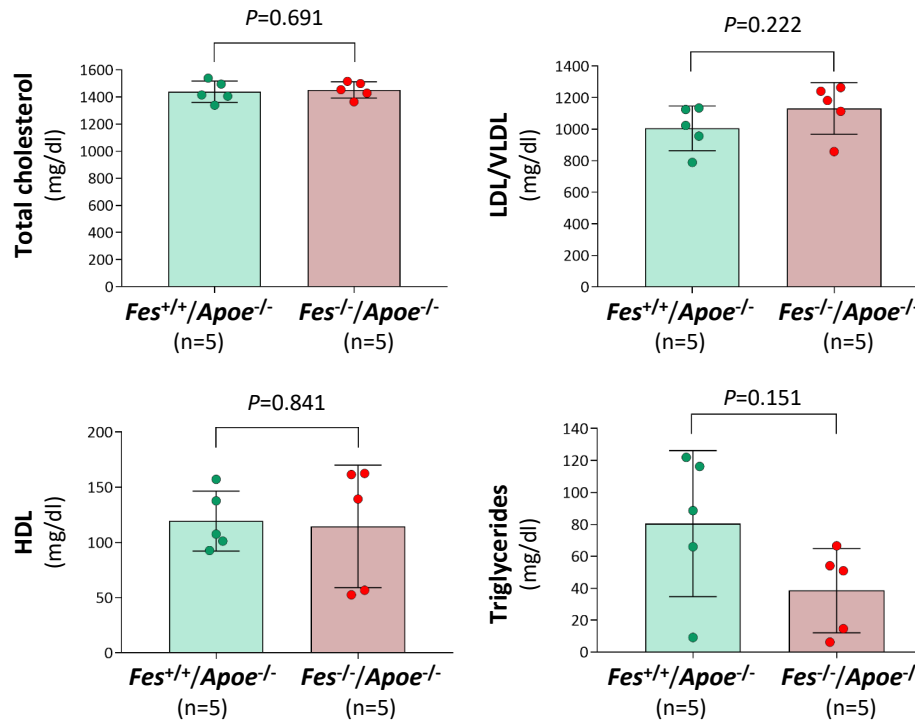
Figure S11. Plasma lipid levels in *Fes^{+/+}/Apoe^{-/-}* and *Fes^{-/-}/Apoe^{-/-}* mice

Mean ± standard deviation values in Fes^{+/+}/Apoe^{-/-} and Fes^{-/-}/Apoe^{-/-} groups, respectively, were 1439 (78.85) mg/dl and 1452 (59.93) mg/dl for total cholesterol, 1006 (141.8) mg/dl and 1132 (163.4) mg/dl for LDL/VLDL (low density lipoprotein/very low density lipoprotein), 119.3 (27.16) mg/dl and 114.6 (55.55) mg/dl for HDL (high density lipoprotein), and 80.41 (45.72) mg/dl and 38.52 (26.43) mg/dl for triglycerides. P values are from two-tailed Mann-Whitney test.
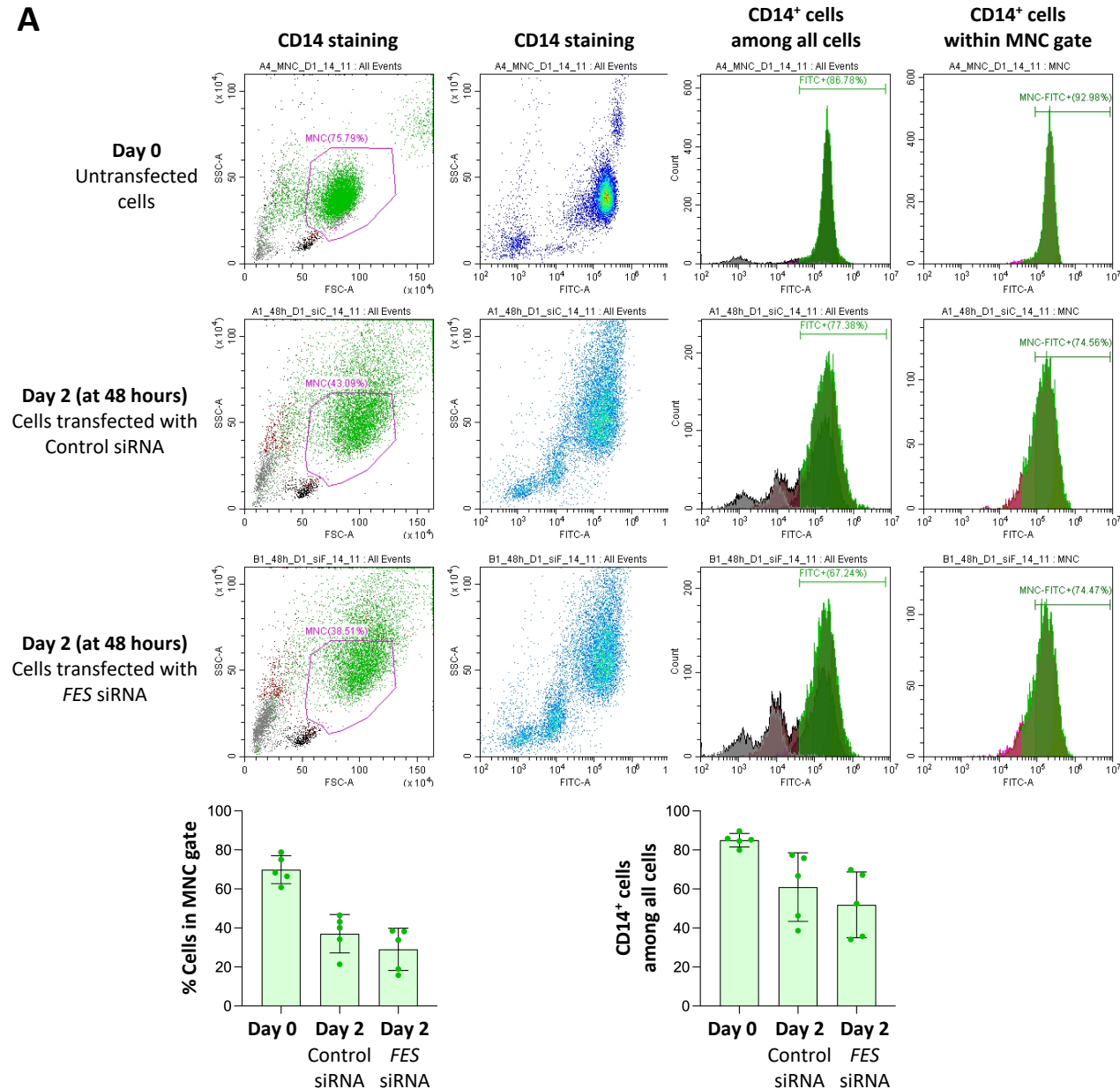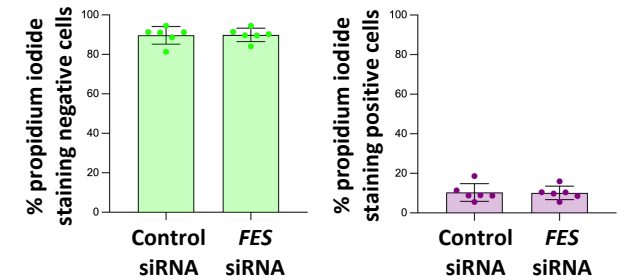
Figure S12. Blood monocyte purity and viability
Human monocytes were isolated from peripheral blood samples from healthy donors with the use of the density gradient centrifugation and CD14 positive selection method as described in the Supplemental Material (Detailed Methods), and subsequently subjected to flow cytometric analyses which showed that 85% ± 1% (mean ± standard deviation) of all freshly isolated cells were CD14 positive (**A**) and 90% of cells were viable (propidium iodide staining negative) at 48 hours post transfection with either control siRNA (90% ± 4%) or *FES* siRNA (90% ± 3%) (**B**).
A. Results of flow cytometric analysis of CD14 of freshly isolated monocytes (day 0) and at 48 hours post transfection with either control siRNA or *FES* siRNA. Average representative flow cytometric analysis diagrams are shown in the figure. Columns and error bars in the column charts represent mean ± standard deviation values from n = 5 donors per group.
B. Results of flow cytometric analysis of monocytes stained with propidium iodide at 48 hours post transfection with either control siRNA or *FES* siRNA. Columns and error bars represent mean ± standard deviation values from n = 6 donors per group.
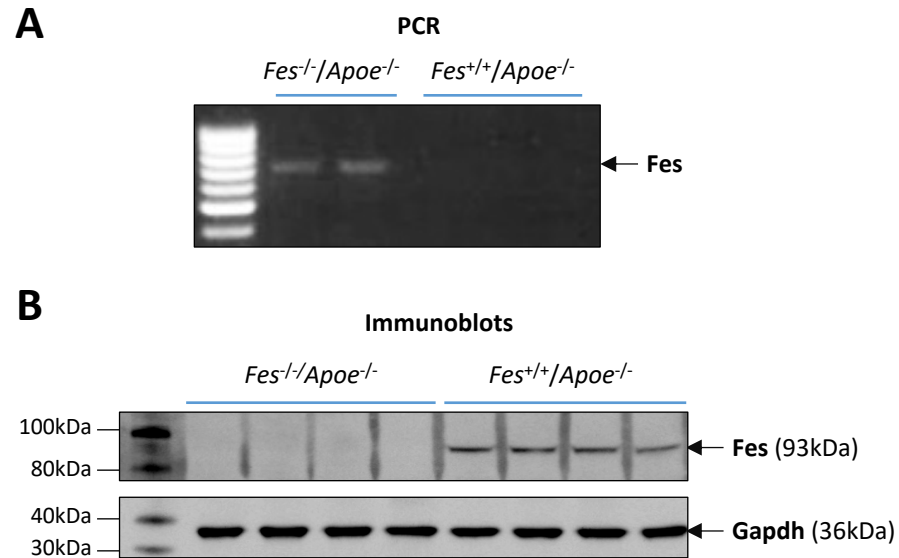
Figure S13. Verification of Fes knockout

A. Results of PCR analysis [described in the Supplemental Material (Detailed Methods)] of ear snips from *Fes⁺/⁺/Apoe⁻/⁻* and *Fes⁻/⁻/Apoe⁻/⁻* mice, respectively. As expected, a PCR band of 700bp was detected in *Fes* knockout mice but not in wildtype mice.

B. Results of immunobloting analyses of lung tissues from *Fes⁺/⁺/Apoe⁻/⁻* and *Fes⁻/⁻/Apoe⁻/⁻* mice, with antibodies for Fes and Gapdh, respectively. A Fes band (93kDa) was detected in *Fes⁺/⁺/Apoe⁻/⁻* mice but not in *Fes⁻/⁻/Apoe⁻/⁻* mice.