SUPPLEMENTAL MATERIAL

Reagents: Antibody directed against Wnt5a (1/200) (Cat No. AF645) was purchased from R&D Systems, Minneapolis, MN. Antibodies directed against p-mTOR (1/1000) (Cat No. 2971S), mTOR (1/1000) (Cat No. 2938S), p-AKT (1/1000) (Cat No. 9271S), AKT (1/1000) (Cat No. 9272S), p-P70S6k kinase (1/1000) (Cat No. 9234S), P70S6k kinase (1/1000) (Cat No. 9202S), p-4EBP1 (1/1000) (Cat No. 9456S), 4EBP1 (1/1000) (Cat No. 9644S), P-NFKB p65 (1/1000) (Cat No. 3033S), NFKB p65 (1/1000) (Cat No. 8242S) were purchased from Cell Signaling (Danevrs, MA). Anti p-Tyrosine clone 4G10 (1/1000) (Cat No. 05-321), anti-GAPDH (1/5000) (Cat No. MAB374), anti-EEA1 (1/200) (Cat No. 07-1820), and anti-eGFP (1/5000) (Cat No. MAB3580) antibodies were purchased from Merck, Darmstadt, Germany. Anti-TOM20 (Cat No. sc11415) and anti-Caveolin (Cat No. sc70516) and anti β Tubulin (1/5000) (Cat No. – sc-5274) antibodies were obtained from Santa-Cruz, Dallas, TX. Anti-CD45 (1/50) antibody (Cat No. PA5-96061), Alexa-Fluor 488 (1/500) (Cat No. A11008), Alexa-Fluor 546 (1/500) (Cat No. A-11003), NBD Cholesterol (Cat No. N1148) and oxLDL (Cat No. L34357) were purchased from Thermo Fischer, Waltham, MA. Anti-PI3kinase p85 alpha (1/500) (Cat No. ab71925), anti-PI3kinase p-p85 (1/1000) (Cat No ab278545.), anti-Ga-lectin-3 (1/500) (Cat No. ab53082), anti-ACTA-2 (1/1000) (Cat No. ab5694),), ERp72 (1/1000) (Cat No, ab155800) antibodies and rosiglitazone maleate (Cat No. ab142461) were acquired from Abcam, Cambridge, UK. Insulin (Cat No. I1882), 3-isobutyl-1-methylxanthine (IBMX) (Cat No. I5879), dexamethasone (Cat No. D49020), FK-506 (Cat No. F4679), 4-methylumbel-liferyl oleate (4-MUO) (Cat No. 75164), Everolimus (Cat No. SML2282), Wortmannin (Cat. No 12-338), DOPC (Cat No. P6354), N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) (Cat No.394866), anti-ACAT1 (1/1000) (Cat No. HPA004428) and anti-Flag-M2 (1/1000) (Cat No. F3165)antibodies were purchased from Sigma-Aldrich, St Louis, M.O. TMP153 (Cat No. BML-EI317-0020) was achieve from Enzo Life Science, Farmingdale, NY. Fura-8TM (Cat No. 21055) was obtained from AAT Bioquest, Sunnyvale, CA. Recombinant human Wnt5a (Cat No. MBS949468) was purchased from MyBioSource, San Diego, CA. Cholesterol (Cat No. 110195000) and β-sitosterol (Cat No. 132720050) were achieve from Thermo-Fisher Scientific (Waltham, MA). Anti-p-GSK3 (1/1000) and anti-His (1/1000) (gift from Mustapha Oulad-Ab-delghani, IGBMC, Strasbourg), anti-HMG-CoA red (1/1000) (gift from Russell Debose-Boyd, UT Southwestern Medical Center at Dallas). Anti-CD31 antibody (1/20) (Cat No. AF3628, R&D SystemsMinneapolis, MN (gift from Nathalie Niederhoffer, Univeristy of Strasbourg).

Anti-Lamp1 (1/200) (Cat No. H4A3) and anti-CD63 (1/100) (Cat No. H5C6) antibodies, from DSHB (gift from Fabien Alpy, IGBMC, Strasbourg). Anti-ApoB100 (1/1000) (Cat No. 20A-G1b) antibody from Academy Bio-Medical Company, Houston, TX (gift from Toshihide Kobayashi, University of Strasbourg). Anti-LIPA (1/1000) (Cat No. TA30973 was purchased from Origene, Rockville, MD). Normal rabbit serum and mouse IgG control antibody (dilutions used same as the corresponding primary antibody) were purchased from Vector Laboratories (Burlingame, CA).

Animals: Wnt5a^{flox} mice were generated using the plasmid EUCOMM-Wnt5a Vectors (target-final) as described[11]. We achieved smooth muscle-specific Wnt5a inactivation by crossing SM22Cre transgenic mice (a gift from J. Herz laboratory[34]) with Wnt5a^{flox} mice. Mice carrying a Wnt5a allele into which loxP sites had been integrated were generated by gene targeting in embryonic stem cells as described[34]. Schematic representation of *Wnt5a1*^{LacZ,flox,cre} alleles is shown in Online Figure SIA. Cre-mediated recombination is expected to result in deletion of the third exon of Wnt5a, thus rendering the allele nonfunctional. Animals were also crossed to LDL receptor knockout (LDLR-/-) mice (Jackson laboratory) to generate SM22Cre, Wnt5a$^{flox/flox}$, LDLr-/- mice. Mutant mice were then referred to as SM22Cre+ (SM22Cre+, Wnt5a$^{flox/flox}$, LDLr-/-) & their littermate controls are referred as SM22Cre- (SM22Cre-, Wnt5a$^{flox/flox}$, LDLr-/-). Three months old animals were fed a Paigen diet for 5 months and kept on a 12h light/dark cycle as described[10]. Experiments were conducted according to standard procedures approved by the Institutional Animal Care and Use Committee at University of Strasbourg, France. All male and female mice used in the experiments were age and sex-matched and littermates, whenever possible. No animals were excluded from analysis. We calculated sample size using G-Power 3.1 software (Franz Faul Universität Kiel, Germany). For a 5% significance level and power =0.80, a grouper of 6 mice was required. The whole study was performed using a double-blind experimental design.

Quantification of Atherosclerotic Lesions: At the end of high cholesterol diet feeding, mice were euthanized with an overdose of anesthetic solution (ketamin/xylazin), and then perfused via left ventricle using Dulbecco's Phosphate-Buffered Saline (DPBS). The whole aortas of mice were dissected. Adventitial and adipose tissues were carefully removed, opened, and stained with Sudan IV to reveal lipid-filled atherosclerotic lesions[10]. The stained vessels were imaged using a camera and analyzed using Image-Pro Plus software 6.0 (Media Cybernetics).

The percentage of the Sudan IV-positive plaque area was determined as a measurement of the plaque burden.

Cholesterol accumulation protocols:

Cholesterol accumulation cocktail: Cells were seeded in 100-mm dishes and grown to 60% confluence in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) new born calf serum (NCS). For cholesterol loading, medium was changed for modified Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and supplemented with methylisobutylxanthine (0.5 mM), dexamethasone (0.25 μM), rosiglitazone (1μM) and insulin (10 μg/ml). Every 2-3 days this medium was withdrawn and replaced with medium supplemented only with insulin (10 μg/ml) and rosiglitazone (1μM) until day 10 as described[35].

Ox LDL protocol: Wnt5a+/+ and Wnt5a-/- VSMC were treated with E Coli lipopolysaccharide (2μg/ml-Sigma-Aldrich, St Louis, MO) for 8 hours in DMEM supplemented with 20% fœtal Bovin serum (FBS). Then, medium was replaced with fresh 20% FBS-DMEM containing 100μg/ml of oxLDL (Thermofischer, Waltham, MA) for 2 days. Medium was replaced with fresh oxLDL – 20%FBS-DMEM each 2 days for 12 days.

Immunostaining analysis & electronic microscopy: For histological studies, mice were transcardially perfused with a 4% paraformaldehyde solution in phosphate-buffered saline, aorta of mice were embedded in paraffin, and cut in 5 μm slices. For hematoxylin/Eosin staining paraffin fixed tissue sections were deparaffinized with washes of histosol, decreasing concentration of ethanol (100%, 95%) and water. Fixed tissue sections were colored with hematoxylin solution followed by differentiation with 0.1% HCl in ethanol 70% solution and coloration with eosine solution. Mounting slides with Eukitt, tissue sections were dehydrated with ethanol 100% and histosol washes. Non-immune controls corresponding to normal rabbit serum and mouse IgG control antibody (Vector Laboratories (Burlingame, CA). were used under the same experimental conditions. Electron microscopy was performed as described[36].

Cell culture: We generated Human Vascular Smooth Muscle cells (CMLVs) Wnt5a-/- using the Dharmacon™ (Lafayette, CO) Edit-R™ CRISPR-Cas9 Genome Engineering system. crRNA was directed against exon 3 of the Wnt5a gene. Human Vascular Smooth Muscle cells (CMLVs) and Human Embryonic Kidney cells (HEK) cells were seeded in 100 mm dishes and grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM)

(Invitrogen/Thermofischer, Waltham, MA) supplemented with 10% (v/v) new-born calf serum, penicillin/streptomycin and ultra-glutamine. All cells were maintained in an incubator at 37°C, 5% CO2. For CMLVs and MEFs, cells were utilized at passage range 5-10 in experiments. Cholesterol accumulation was induced using an adipogenic cocktail as described [11]. Cells were fixed in 4% paraformaldehyde and neutrals lipids were stained with Oil-Red-O (Sigma-Aldrich, St Louis, MO). Wnt5a Knockout human CMLVs were treated with varying doses of reagents as described in results section. Monolayers of human embryonic kidney (HEK) 293 cells were transfected with plasmids using Fugene 6 Reagent (Promega, Madison, WI) according the manufacturer's protocol.

Oil Red O staining: Cells were fixed with 10% PFA for 15 min. After 3 washes with PBS, cells were stained with 0.05% Oil- Red-O filtered solution for 30 min at room temperature. 3 additional washes are performed to remove unincorporated Oil-Red-O.

Quantitative real time PCR analysis: RNA was isolated using TRIzol reagent (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. A total of 50 ng of RNA was converted to cDNA using the high-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). PCR amplification was performed using SYBRGreen PCR master mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Thermocycle condition were as follows: Taq enzyme activation at 95°C for 10 min followed by denaturation at 95°C for 15 sec, annealing/extension at 60°C for 60 sec, repeated for 40 cycles and followed by a melt curve analysis for 1min30sec. Gene expression was then analyzed by delta-delta Ct method.

Immunoblotting: Cells were washed with PBS and lysed at 4°C using RIPA buffer (Radio ImmunoPrecipitation Assay Buffer, Tris-HCl 50mM, pH 7,8, NaCl 150mM, Na deoxycholate 0.25%, Na Orthovanadate 1mM, Na Fluoride 1mM, Nonidet 13P-40 1%, EDTA 1mM supplemented with PIC (Protease Inhibitor Cocktail) without EDTA 1/100 tablet/ml and PMSF (1/1000)) followed by homogeneisation with 22G seryngue and centrifugation at 12 000g, 4°C, for 30min. Proteins quantification were determined by standard colorimetric assay at 595 nm according to manufacturer's procedures (Bio-Rad Detergent-Compatible protein assay). Proteins were denatured by adding Laemil 4X buffer (0,5% SDS) containing β-mercaptophenol (1/20) and heated for 10min at 95°C. 40-50µg of proteins were separated on a polyacrylamide gel in the presence of 0.2% SDS (SDS-PAGE) with a Tris-Glycine buffer containing 20% SDS.

Proteins were then transferred to a nitrocellulose membrane with a Tris-Glycine containing 100% ethanol. Non-specific binding was saturated by incubating the membrane with 5% non-fat milk or BSA in 1X Tris Buffer Saline, 0.1% Tween 20 during 1h at room temperature. The membrane was incubated with primary antibody, overnight at 4°C. After several washes (5x5min) with TBST, the membrane was incubated with the secondary antibody coupled with HRP (Horseradish peroxidase) for 1h at room temperature. After several washes (5x5min), chemiluminescence signal was detected using Clarity Western ECL substrate kit (BioRad) with Image Quant TM LAS 4000 Imaging System (Amersham) or ChemiDoc XRS+. Densitometry analysis was performed using ImageJ software.

Immunoprecipitation experiments: Immunoprecipitation experiments were performed as described previously[10]. Briefly, cells were washed with cold PBS and lysed in phosphate lysis buffer supplemented with protease inhibitor cocktail on ice for 20min. Lysates were precleared, incubated with indicated antibodies and protein A/G-plus agarose beads at 4 °C overnight. Immunoprecipitates were washed twice with lysis buffer. Proteins were eluted from beads with SDS sample buffer, separated by SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and blotted with the indicated antibody. For mass spectrometry analysis, all immunopurified samples were prepared as three biological replicates. Immunoprecipitated proteins were digested in-gel and peptides were analysed by nanoLC-MS/MS.

Immunofluorescence: Immunofluorescence was performed using CMLVs knockout for Wnt5a and the controls as per the protocol described [37]. Cells were labelled with GFP-D4. Antibody used was mouse anti-Lamp1 H4A3 (1:50; DSHB) and Alexa Fluor 546 secondary antibody. Slides were mounted in fluorescence mounting medium (Dako/Agilent, Glostrup, Denmark). Immunofluorescence-labelled cells were analyzed using a Leica TSC SPE confocal microscope with the 63x oil immersion objective. GFPD4 positive lamp1 vesicles in cells were counted with Image-J. For immunostaining experiments, non-immune antibodies corresponding to the host species of the primary antibody were used under the same experimental conditions to distinguish genuine target staining from unspecific background and validate antibody specificity. Secondary antibody and primary antibody were also used alone as controls under the same experimental conditions to determine background staining.

Lipofuscin measurement: VSMCs WT & KO for wnt5a were seeded on coverslips in 24-well glass bottom plates. Cells were labelled with mouse anti-Lamp1 H4A3 (1:50; DSHB) antibody and Alexa Fluor 546 secondary antibody. Slides were mounted in fluorescence mounting medium (Dako/Agilent, Glostrup, Denmark). Immunofluorescence-labelled cells were analyzed using a Leica TSC SPE confocal microscope with the 63x oil immersion objective. Lipofuscin was detected by its auto-fluorescence.

Lysotracker red staining: VSMCs WT & KO for Wnt5a were seeded on coverslips in 24-well glass bottom plates. Cells were incubated with 100nM lysotracker red (Thermofischer, Waltham, MA) for few minutes at 37°C. After the cells were fixed with paraformaldehyde 4% for 10 minutes. Washed three times with cold PBS and stained with DAPI for 10 minutes to obtain nuclear staining. Slides were mounted in fluorescence mounting medium (Dako/Agilent, Glostrup, Denmark). Lysotracker-labelled cells were analyzed using a Leica TSC SPE confocal microscope with the 63x oil immersion objective.

Liposome's preparation: Stock solutions of DOPC and Cholesterol were prepared in Chloroform. Solutions of DOPC with or without cholesterol were dried under liquid nitrogen stream. Lipid film is further dried under vacuum for an hour. HEPES-Potassium acetate buffer at pH 6.8-8 is used to dissolve the dried lipid film, vortexed to prepare the liposomes.

Neutral lipids and sterol analysis: Lipids corresponding to 1 million of cells were extracted according to Bligh and Dyer[38] in dichloromethane/methanol/water (2.5 :2.5 :2.1, v/v/v), in the presence of the internal standards : 4 µg of stigmasterol, 4 µg of cholesteryl heptadecanoate, 8 µg of glyceryl trinonadecanoate. Dichloromethane phase were evaporated to dryness and dissolved in 30µl of ethyl acetate. 1µl of the lipid extract was analyzed by gas-liquid chromatography on a FOCUS Thermo Electron system using a Zebron-1 (Phenomenex, Torrance, CA) fused silica capillary columns (5m X 0,32mm i.d, 0.50 µm film thickness). Oven temperature was programmed from 200°C to 350°C at a rate of 5°C per min and the carrier gas was hydrogen (0.5 bar). The injector and the detector were at 315°C and 345°C respectively. After evaporation, the extract was then submitted to derivatisation in *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide/acetonitrile (50:50 ; v/v) for 1h at 80°C and solubilized in 20ml heptane for analysis by gas chromatography-mass spectrometry. It was performed on a ThermoScientific Trace GC coupled to a Trace TSQ Mass selective detector (Thermofischer, Waltham, MA). The sillylated

sterols were separated on an Agilent J&W HP-5MS capillary column (30 m, 0.25 mm, 0.25 µm phase thickness). The oven temperature program was as follows: 180°C for 1 min, 20°C/min to 250°C, 5°C/min to 300°C where the temperature was kept for 8 min, and then 35°C/min to 325°C. High purity helium was used as carrier gas at a flow rate of 0.8 mL/min in constant flow mode. The samples were injected in a splitless mode with an injection volume of 1 µL. The injector, transfer line and source temperature was 270°C, 280°C and 250°C respectively. The mass spectrometer was operated in the selected ion-monitoring (SIM) mode. Peak detection, integration and quantitative analysis were executed using Xcalibur Quantitative browser (Thermofischer, Waltham, MA) based on calibration lines built with commercially available sterol standards (Sigma-Aldrich, St Louis, MO).

Analysis of free cholesterol in the ER and LELs fractions was performed as described previously [39]. Briefly, prior to extraction 10 µL of β-sitosterol solution (0.5 mg/mL in ethyl acetate (analytical grade, Acros, Geel, Belgium) was added to the cellular fraction as an internal standard. Lipids were then extracted 3 times by ethyl acetate. The combined organic extracts were evaporated and dried under nitrogen flow. Then the sterol residue was converted to trimethylsilyl ethers with 15 µL of pyridine and 15 µL of MSTFA (N-Methyl-N-(trimethylsilyl)trifluoroacetamide, Sigma-Aldrich, St Louis, MO) at room temperature in the dark overnight and then, diluted with 20 µL of isooctane (analytical grade, Merck, Darmstadt, Germany). One microliter of each sample was injected into the GC–MS system. GC–MS analyses were performed on a Varian STAR 3400 GC instrument equipped with an on-column SPI injector coupled to a Varian SATURN 2000 mass sensitive detector (Varian, France) with an Electronic Impact as ionization source (EI, ionization energy of 70 eV) working with a range of mass from 40 to 600 m/z. Data acquisition and processing were done on Varian Saturn Work Station 5.11 software. Analytes were separated in a VF-5ms capillary column (phase stationary: 5 % phenyl–95 % dimethylpolysiloxane, thickness of 0.1 m, 60 m × 0.25 mm, Varian, France). The column temperature gradient was programmed from 55°C (hold for 1 min) to 320°C at 7°C/min and hold for 6 min. The injector operating conditions were as follows: injection volume 1µL; initial injector temperature of 55°C was increased to 300°C at 100°C/min (hold for 37 min). Helium (purity 99.9995%) was used as a carrier gas with a flow rate of 1 mL/min. Cholesterol was quantified compared to β-sitosterol as internal standard as described[39].

Quantification of total cholesterol (free + esterified) in the ER and LELs fractions was also performed as described [39]. Briefly, a previous step of saponification (before extraction) was necessary. It consisted of adding to the cellular fraction, ethanol (Carlo Erba, France) and saturated potassium hydroxide (KOH, from Merck Darmstadt, Germany) aqueous solution. The

sample was purged with nitrogen, and was put into a rotary shaker (Edmund Buhler, Johanna Otta GmbH, Hechingen, Germany) at ambient temperature in the dark overnight (15 h). Then the extraction was done as previously described [39].

Fluorescent ACAT assay: ACAT activity was measured as previously described [40]. Briefly, human wild type and Wnt5a-/- VSMC were cultured in 96 wells plates to a confluence of 80%. Cells were incubated with NBD Cholesterol (Invitrogen/Thermofischer, Waltham MA) at 2µg/ml for 1- 8h or NBD cholesterol and TMP153 (Enzo life Science, Farmingdale, NY) an ACAT inhibitor, at a final concentration of 450nM for 1-8h. Fluorescence was read at 485nm excitation and 535nm emission wavelength (SAFAS Xenius XM plate reader, Monaco). After reading, cells were lysed in 0.5M NaOH for 1h and cellular protein content was determined by Bradford method. ACAT activity was calculated by subtracting the background fluorescence of the NBD cholesterol - TMP153 treated cells from total fluorescence of NBD cholesterol treated cells.

Lipase activity: Acid lipase activity was measured with homogenate from VSMCs WT & KO for Wnt5a using the fluorescent substrate 4-methylumbelliferyl oleate (4-MUO) (Sigma-Aldrich, St Louis, M.O) as described [41]. Cells homogenate were prepared by sonication and centrifugation in 0.2M citrate buffer (pH4.6). Briefly, 50µg of proteins were incubated in sodium acetate buffer (pH=5) containing 4-MUO (100nM) during 30min at 37°. The fluorescence corresponding to the catalytic cleavage of oleate by lipase was measured with a fluoromax 4 spectrofluorometer using an excitation wavelength of 327nm and an emission wavelength of 449nm.

***In vitro* interaction experiments**. For these experiments, we used Wnt5a recombinant human protein from My Bio Source, and the ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich, St Louis, M.O). pCMV-NPC2-LVPRGS-His-10-FLAG encodes human NPC2 protein, a thrombin cleavage site, 10 histidines and a FLAG tag under the control of the CMV promoter as described[7]. This plasmid was constructed from pCMV-NPC2-His-6-FLAG using the forward oligonucleotide; 5'-CATATGCGTTTCCTGGCAGCTAC-3' and the reverse oligonucleotide 5'-CTACTTATCGTCATCGTCTTTATAGCGTGATGGTGATGGT GATG GTGATGGCTGCTCGGGGGACTAAGAGATGAGAAACGATC-3'. pCMV-NPC2-LVPR GS-His-10-FLAG was expressed and purified from BL21 E. coli bacteria. Bacteria was grown

in LB media containing ampicillin (100µg/mL) and expression of NPC2 was induced using IPTG (1mM). After induction, pellet of bacteria was collected and stored at -20°C. Proteins extractions were realized with bacterial protein extraction reagent (B- PER® ThermoFisher, Waltham MA) in the presence of imidazole (10mM) and DNAse (5µg/mL). After incubation at room temperature and centrifugation, a supernatant containing the soluble fraction and a pellet containing the insoluble fraction (inclusion bodies) were obtained. Pellet were resuspended with a lysis buffer (buffer A; 6M Gu-HCl, 0.1M NaH_2PO_4, 0.01M Tris-Cl, pH 8) and incubated during 30min at room temperature. After centrifugation, supernatant was incubated with Ni-NTA Agarose beads during 30min to1h at room temperature. AKTA column was preequilibrated with 10 volumes of buffer B (8M urea, 0.1M NaH_2PO_4, 0.01M Tris-Cl, pH 8) and the sample was injected. The column was then washed with 10 volumes of buffer C (8M urea, 0.1M NaH_2PO_4, 0.01M Tris-Cl, pH 6.3). After a gradient of buffer C and buffer N (50mM NaH_2PO_4, 300mM NaCl, pH 8) and an equilibration of the column with buffer N, bound proteins were eluted in the presence of buffer N with imidazole (250mM). Eluted fractions containing NPC2 proteins were then pooled and dialyzed against PBS at 4°C. Purification of NPC2 was analyzed by SDS/PAGE followed by a Coomassie staining. 20µg of purified NPC2-LVPRGS-His10-FLAG were incubated with ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich, St Louis, M.O) during 1h at room temperature, allowing NPC2 to bind to the beads. After 5 washes Tris lysis buffer, 10 µg of human recombinant Wnt5a (MyBioSource, San Diego, CA) were added, in the presence or the absence of cholesterol (1µM) or a mix of cholesterol (1µM) and 25-hydroxy cholesterol (0.1µM) and incubated during 1h at room temperature. After 5 washes, complex of proteins was eluted with a glycine buffer (pH 2.8) and with Tris buffer (1M HCl pH 9). Eluted sample were then analyzed by western blotting.

LDL binding of Wnt5a: Purified human LDL were incubated during 4h at 37°C with 100ng of recombinant Wnt5a (MyBioSource, San Diego, CA) and 0.6% Chaps as described [23]. Samples were filtered through 100kDa cut-off protein concentrator (Merck Milipore, Molsheim, France). High molecular fraction (>100kDa) and low molecular fraction (<100kDa, flow-through) were then analyzed by western blotting.

Nuclear localization of TFEB: Wild type and Wnt5a-/- VSMCs were seeded on coverslips prior to transfection. pEGFP-N1-TFEB (Addgene, MA) was transfected using FuGENE® HD or Lipofectamine 3000 transfection reagent following manufacturer's instructions. Transfected cells were treated for cholesterol accumulation in presence or absence of 13µM FK506 (Sigma-

Aldrich, St Louis, MO). After 48 hours of treatment, cells were fixed with 4 % PFA and stained with DAPI for immunofluorescence confocal microscopy. Nuclear localization of TFEB was assessed with a ratio cytoplasm/nucleus with ImageJ software.

Ca2+ measurements: Cells were incubated in Ringer solution containing 2 mM Ca2+ and 5 µM Fura-8TM (AAT Bioquest, Inc., Sunnyvale, CA, USA) for 30 min, washed, and incubated in 2 mM Ca2+ Ringer solution for 30 minutes. The Fura-8 fluorescence ratio was recorded for 10 s on a SP8 UV confocal microscope (Leica, Wetzlar, Germany) to determine the resting cytosolic Ca2+ (n=4 independent experiments with a total of 200 cells per condition).

Mass spectrometry analysis:

Sample preparation: Immunoprecipitated proteins were concentrated in a stacking gel. Protein bands were cut in pieces and washed by four successive additions of 50µL ammonium bicarbonate (25mM)/acetonitrile (50/50 v/v). After dehydration with 50µL of acetonitrile, proteins were reduced with 50µL of dithiothreitol (DTT) 10mM during 30min at 60°C and 20min at room temperature. Proteins were then alkylated with 50µL of iodoacetamide (IAA) 55mM during 20min in the dark at room temperature. Bands were washed three times with 50µL of ammonium bicarbonate 25mM and 50µL of acetonitrile (ACN). After dehydration with 50µL ACN, proteins were digested with 30µL of sequencing grade trypsin (Promega) at 6.7ng/µL in ammonium bicarbonate 25mM overnight at 37°C. Peptides were extracted twice during one hour under agitation at room temperature: First with ACN/Formic acid (FA) (60/0.1 v/v) and second with pure ACN. Peptides were vacuum drying and solubilised in 80µL of H_2O/ACN/FA (98/2/0.1 v/v/v).

NanoLC-MS/MS analysis: Samples were analysed on an ultra-high pressure nanoflow chromatography system coupled to a trapped ion mobility-quadrupole time-of-flight mass spectrometer (nanoElute coupled to TimsTOF Pro, Bruker Daltonics, Bremen, Germany) equipped with a CaptiveSpray source operating in positive mode. Peptides were loaded on a trapping column (Acclaim PepMap 100, C18, 2cm x 100µm, 5µm, 100Å, Thermo Scientific) and separated on a reversed-phase C18 column (Aurora 2, 25 cm x 75 µm i.d., 1.6 µm, IonOpticks, Australia). The column was heated at 50°C and used with a constant flow of 300nL/min. Composition of A and B was as followed: A contains H_2O/ACN/FA (98/2/0.1 v/v/v) and B contains ACN/FA (100/0.1 v/v/v). The 60 min gradient started after 2 min at 2% B by ramping B from 2 to 15% within 18 min, then to 25% within 25 min and then to 40% within 17 min. MS data were collected over a m/z range of 100 to 1700 and the Ion mobility coefficient (1/K0) was set

from 0.7 to 1.25 V.s.cm^{-2}. LC-MS/MS data were acquired using the PASEF method with a total cycle time of 1.88s and a duty cycle locked at 100%. One PASEF cycle includes 1 TIMS MS scan and 10 PASEF MS/MS scans. The 10 PASEF scans (166ms each) contained at maximum 12 MS/MS scans per PASEF scan. TIMS, MS operation and PASEF were controlled and synchronized using the control instrument software Otof Control (v.6.0, Bruker Daltonik).

Data processing: Raw data were converted into mascot generic files (.mgf) using Data Analysis software (v. 5.3, Bruker Daltonik) before being subjected to a search against a concatenated target-decoy database including both forward (target) and reversed (decoy) SwissProt Human sequences (2019/11 release, 20 506 entries) using Mascot search algorithm (v.2.6.2). Searches were performed with a mass measurement tolerance of 15 ppm for precursor and 0.05 Da for fragment ions. Oxidation of methionine residues, and N-terminal acetylation were set as variable modifications, and carbamidomethylation as well as of cysteine residues as fixed modification. A maximum of one missed cleavage was allowed. ProlineStudio software (v. 2.0) was used to validate the identification results. Peptide Spectrum Matches (PSM) were filtered out according to following criteria: pretty rank ≤ 1, a minimal length of 7 amino acids and a maximum false discovery rate (FDR) of 1 % on the adjusted e-value. Then, proteins were filtered out in order to obtain a final list with a maximum FDR of 1 % based on the modified protein MudPit score.

Blinding Procedure: While data collection could not be completely blinded, authors ensure that the outcomes measured are as objective as possible. All experiments were done multiple times by several independent investigators for rigor and reproducibility. The RT-PCR analysis and western blotting were done by operator 1 while in the same animals, operator 2 performed aorta physiological measurements without being informed of the status of the animal. Mass spectrometry analysis was performed by the core facility at the University of Strasbourg, France which was verified by operator 1. Representative images were selected based on their quality and accurate representation of similarity with the average value of each experimental group.

Statistical analysis: The statistical analyses were performed using GraphPad Prism version 9.0. Outliers were determined according to the ROUT method with Q to 1% with Graph Pad software. Shapiro-Wilk test was used to determine normality for all the tests relying on the central limit theorem. Normally distributed data were expressed as mean ± SEM. Statistical significance for individual experiment is described in the figure legends with their significance

values. Online Table SII includes detailed information about statistical tests performed for each figure. A value of $p<0.05$ was considered statistically significant.

Table S1: Primers List

Genes	Human
Wnt5a short	Forward – 5'-ATTAATTCTGGCTCCACTTGTTG-3' Reverse – 5'-GACATTGCACTTCCAGCCATC-3'
Wnt5a long	Forward – 5'-CTTTTGCACAATCACGCCCA-3' Reverse – 5' –TTTCCAACGTCCATCAGCGA-3'
Myo CD	Forward – 5'-TCAACATGACACTCCTGGGG-3' Reverse – 5'-CTGGACGTTTCAGTGGTGGT-3'
KLF4	Forward – 5'-CCCACATGAAGCGACTTCCC-3' Reverse – 5'-CAGGTCCAGGAGATCGTTGAA-3'
SREBP2	Forward – 5'-CCCTATTCCATTGACTCTGAGC-3' Reverse – 5'-GAGTCCGGTTCATCCTTGAC-3'
HMGCOA RED	Forward – 5'-CCTTGGTGATGGGAGCTTGT-3' Reverse – 5'-TGCTCTGCAGCCTCTATTGG-3'
NPC1	Forward – 5'-CTTACTGGGAGCCACTCACG-3' Reverse – 5'-CGACCGACCCTTAGACACAG-3'
DGAT2	Forward – 5'-GCCTGTGTTGAGGGAGTAC-3' Reverse – 5'-CAGGGCCAGTTTCACAAAGC-3'
Hoxa9	Forward – 5'- TGGCATTAAACCTGAACCGC-3' Reverse – 5'- ACCGCTTTTTCCGAGTGGAG-3'
CTSF	Forward – 5'-AATGAGGATCCCCTGTCCCA-3' Reverse – 5'-TTGCCAGGCTCTTTCCTCAG-3'
SGSH	Forward – 5'-AGACCGTGTACCCGTTTGAC-3' Reverse – 5'-AAGTAAGGCACCAGCACGTC-3'
ATP6V	Forward – 5'-TCAACGTCTCCTTGGTGCG-3' Reverse – 5'-GCAGCCTTGGCAGCAATAAT-3'
TPP1	Forward – 5'-TGTTCCCACGGCCTTCATAC-3' Reverse – 5'-ATGCTGCTGGTAGAGCCTTG-3'
18S (internal control)	Forward – 5'-TGTGGTGTTGAGGAAAGCAG-3' Reverse – 5'-TCCAGACCATTGGCTAGGAC-3'

Table S2: Statistical assessment

Figure	Statistical test	Post hoc correction	p-values
1C	Mann-Whitney	N/A	raw
1D	Mann-Whitney	N/A	raw

1F	Mann-Whitney	N/A	raw
2A	Shapiro - Wilk and Two tailed Unpaired t-Test	N/A	raw
2B	Shapiro - Wilk and Two tailed Unpaired t-Test	N/A	raw
2C	Mann-Whitney	N/A	raw
3B	Mann-Whitney	N/A	raw
3C	Shapiro - Wilk and Two tailed Unpaired t-Test	N/A	raw
3F	Mann-Whitney	N/A	raw
4B	Mann-Whitney	N/A	raw
4C	Mann-Whitney	N/A	raw
4G	Mann-Whitney	N/A	raw
5G	Mann-Whitney	N/A	raw
6B	Mann-Whitney	N/A	raw
6C	Mann-Whitney	N/A	raw
7C	one Way ANOVA, Tukey post hoc analysis	N/A	raw
S1C	Mann-Whitney	N/A	raw
S2A	Mann-Whitney	N/A	raw
S3D	Mann-Whitney	N/A	raw
S3F	Mann-Whitney	N/A	raw
S3H	Mann-Whitney	N/A	raw
S4D	Mann-Whitney	N/A	raw
S4E	Mann-Whitney	N/A	raw
S4F	Mann-Whitney	N/A	raw
S5E	Mann-Whitney	N/A	raw

Table S3: Inhibitors/chemicals solubility

S.No	Inhibitors/Chemicals	Cat. No.	Solubility
1	Insulin from bovine pancreas	I1882 (Sigma-Aldrich)	Water
2	3-isobutyl-1-methylxanthine (IBMX)	I5879 (Sigma-Aldrich)	DMSO (Cat No. D2650, Merk)
3	Rosiglitazone maleate	ab142461 (Abcam)	DMSO
4	Dexamethasone	D49020 (Sigma-Aldrich)	Ethanol
5	FK-506	F4679 (Sigma-Aldrich)	DMSO
6	NBD Cholesterol	N1148 (Thermofisher)	Ethanol
7	TMP153	BML-EI317-0020 (Enzo life Science)	DMSO
8	4-methylumbelliferyl oleate (4-MUO)	75164 (Sigma-Aldrich)	DMSO
	Fura-8TM	21055 (AAT Bioquest)	DMSO
9	Everolimus	SML2282 (Sigma-Aldrich)	DMSO
10	Wortmannin	12-338 (Sigma-Aldrich)	DMSO

Legends

Figure S1-S5

Figure S1. Generation of mice inactivated for Wnt5a in vascular smooth muscle cells. A, schematic representation of *Wnt5a1*^{LacZ,flox,cre} alleles. Exons are shown as grey boxes and marked by a number. SA is a splicing acceptor; IRES is an internal ribosome entry site; lacZ is the lac operon (lactose operon); pA is a poly(A) signal; Neo is neomycine-resistant gene driven by lacZ promoter; FRT is *flp* recombinase; CRE is *cre* recombinase. There are two LoxP sites for Cre recombinase on the 5′ and 3′ ends of the third exon of Wnt5a. *Rxfp1*^{fl} allele is produced by *flp*-induced recombination, and the deleted allele without exon 3 is produced by *cre*-induced recombination as shown. **B,** smWnt5a+, smWnt5a-, and heterozygote SM22Cre+/Wnt5a$^{flox/wt}$/LDLR-/-, SM22Cre+/Wnt5a$^{flox/wt}$/LDLR-/- mice alive at birth, compared to the expected percentage of surviving animals (Cre- expected, Cre+ expected) (n=236). **C,** plasma cholesterol levels in mice. The plot shows individual values with mean ± SEM (n=7 sm22Wnt5a+, n=6 sm22Wnt5a-). Data were analyzed using a Mann-Whitney test. **D**, A representative immunoblot shows the protein expression of Galectin 3 in human Wnt5a-/- VSMCs and controls untreated (D0) and treated for cholesterol accumulation during the indicated time (n=3). **E**, A representative immunoblot shows the protein expression of the indicated genes in the livers from mutant and control mice, and in control cells (Hela) (n=3).

Figure S2. Accelerated atherosclerotic lesions formation in smWnt5a- mice. A, Oil Red O, Hematoxylin and eosin (H&E) and immuno-histochemical staining of mouse thoracic aortas. Scale bars, are 120µm for H&E and Galectin-3, and 30□m for Oil-Red-O and CD31. The subpanels are higher magnification images (2.5X) of the areas outlined in black. Figure shows representative images (n=6 mice/group). **B**, representative electron micrographs of hearts and aortas from smWnt5a+ and smWnt5a- mice fed a Paigen diet (n=6 mice/group). White arrows show large lipofuscin pigment granules (Lipo), and accumulation of lipid droplets (LD) in mutants vs controls. Mitochondria are indicated (M). Scale bars, are 2µm.

Figure S3. Cholesterol accumulation in human Wnt5a-/- VSMCs. A, Relative transcript levels of Wnt5a long and short isoforms in human VSMCs taking GAPDH as internal control. The plot shows individual values with mean ± SEM (n=4 Wnt5a+/+, n=4 Wnt5a-/-). **B,** Representative western blot analysis for Wnt5a in LMTK cells stably transfected with an expression vector coding for Wnt5a (Ctrl+/+/) or mock (Ctrl-/-), and human VSMCs transfected with a CRISPR/Cas9 Wnt5a targeted vector (-/-) or a non-targeted vector (+/+) (n=4). **C**,

Representative western blot analysis of Wnt5a protein levels in human VSMCs over the course of a 10 days cholesterol accumulation protocol (n=4). **D**, Quantification of triglycerides in human Wnt5a-/- VSMCs and controls (Wnt5a+/+) untreated (D0) (n=6 Wnt5a+/+, n=7 Wnt5a-/-) or treated (D10) (n=6 Wnt5a+/+, n=7 Wnt5a-/-) for cholesterol accumulation during 10 days. **E,** Representative plates and micrographs of human Wnt5a-/- VSMCs and controls treated for cholesterol accumulation using a differentiation protocol and stained with Oil Red O. Scale bars are 50µm (n=20). **F,** A representative immunoblot shows the protein expression of the HMGCoA reductase in human Wnt5a-/- VSMCs and controls untreated (D0) and treated (D10) for cholesterol accumulation using a differentiation protocol (n=3). **G**, mRNA levels of DGAT2 in human Wnt5a-/- VSMCs and controls (Wnt5a+/+) untreated (D0) (n=4 Wnt5a+/+, n=4 Wnt5a-/-) or treated (D10) (n=4 Wnt5a+/+, n=4 Wnt5a-/-) for cholesterol accumulation during 10 days. The plot shows individual values with mean ± SEM. Statistical significance was assessed using a Mann-Whitney test for **A**, **D**, and **G**. **A**, P=0.028 indicate significance relative to Wnt5a+/+. ns indicates not significant.

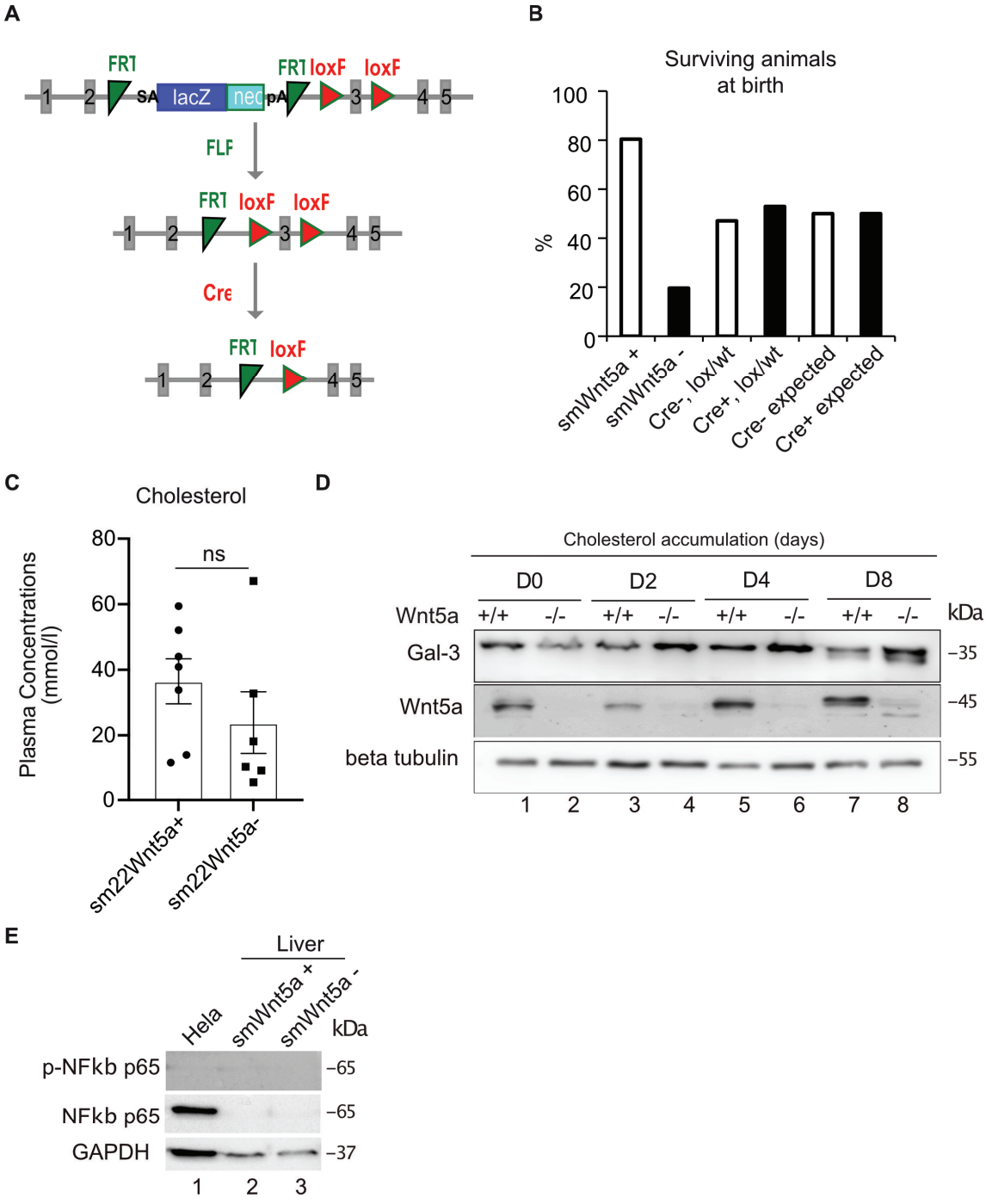
Figure S4. Cholesterol accumulated in Lamp1 positive cytosolic vesicles in human Wnt5a-/- VSMCs. A, Representative plates and micrographs of human Wnt5a-/- VSMCs and controls treated for cholesterol accumulation using a LDLox protocol during 12 days and stained with Oil Red O (n=4). Scale bars are 50µm. **B**, Electronic microscopy analysis of human Wnt5a+/+ and Wnt5a-/- VSMCs upon cholesterol accumulation. White arrows show large lipofuscin pigment granules (Lipo), and accumulation of lipid droplets (LD) in mutants (n=3). Scale bars are 2µm. **C,** To visualize lipofuscin granule formation upon cholesterol accumulation, human Wnt5a+/+ and Wnt5a-/- VSMCs were labeled with anti-Lamp1 antibodies (red) a LELs marker, and analyzed by confocal imaging using green fluorescence. Nuclei were stained in blue (Dapi). Merge images of lipofuscin green fluorescence and Lamp1 signals are shown. Arrows indicated lipofuscin pigment granules. Scale bars are 5µm (n=3). **D**, Quantification of relative protein levels of Lamp1 in human VSMC Wnt5a-/- (n=5) and controls (n=4) treated for cholesterol accumulation for 10 days (D10) and taking GAPDH as internal control. **E**, and **F,** Relative mRNA levels of Hoxa9 (D0, ctrl, n=4; D0, Wnt5a-/- n=3; D10, ctrl, n=4; D10, Wnt5a-/- n=3), CTSF (D0, ctrl, n=4; D0, Wnt5a-/- n=4; D10, ctrl, n=3; D10, Wnt5a-/- n=4), SGSH (D0, ctrl, n=4; D0, Wnt5a-/- n=4; D10, ctrl, n=4; D10, Wnt5a-/- n=4), ATP6V (D0, ctrl, n=4; D0, Wnt5a-/- n=3; D10, ctrl, n=7; D10, Wnt5a-/- n=7), and TPP1 (D0, ctrl, n=4; D0, Wnt5a-/- n=4; D10, ctrl, n=3; D10, Wnt5a-/- n=3) in human VSMC Wnt5a-/- and controls untreated (D0) and treated for cholesterol accumulation (D10), taking GAPDH as internal control. The plot shows

individual values with mean ± SEM. Statistical analysis was done using a Mann-Whitney test for **D**, **E**, and **F**. **D**, P=0.016; **E**, P=0.028; **F**, P=0.028 and P=0.006 indicate significance relative to Wnt5a+/+. ns indicates not significant.

Figure S5. Wnt5a decreases mTORC1 activity. A, A representative immunoblot shows the protein expression of p-mTORC1 and total mTORC1 during the course of cholesterol accumulation in Wnt5a-/- VSMCs and controls (n=3). **B**, Representative western blot analysis of Wnt5a in Wnt5a-enriched conditioned medium (CM Wnt5a+) and mock medium (CM Wnt5a-) (n=5). **C**, Representative western blot analysis showing p-mTORC1, total mTORC1, p-Akt, total Akt, and Wnt5a in Wnt5a-/- VSMCs and controls treated with Everolimus® (30nM) (n=5). **D**, Oil red O staining of Wnt5a-/- VSMCs and controls upon 10 days of cholesterol accumulation cocktail plus treatment with the p-mTORC1 inhibitor, Everolimus® (30nM) (+ Everolimus) or vehicle (UT) (n=3). **E**, Quantification of total cholesterol (UT, n=7; + Everolimus n=5) and cholesteryl esters (UT, n=6; + Everolimus n=4) in Wnt5a-/- VSMCs treated with Everolimus® (30nM) (+ Everolimus) or vehicle (UT). **F**, A representative immunoblot shows expression of the indicated proteins in Wnt5a-/- VSMCs and controls treated with Wortmanin (30nM) (n=3). The plot shows individual values with mean ± SEM. Data were analyzed using a Shapiro-wilk to test normality followed by Mann Withney test. ns indicates not significant.

Table S4. Mass Spectrometry analysis of the Wnt5a/NPC1 and Wnt5a/NPC2 interactions. Data reported the molecular weight and the total number of identified peptide sequences (peptide spectrum matches, PSMs) averaged between three biological replicates for each prey protein in IP NPC1 and NPC2 (demonstrating that they were detected consistently in both co-IP) (n=3).

Figure S1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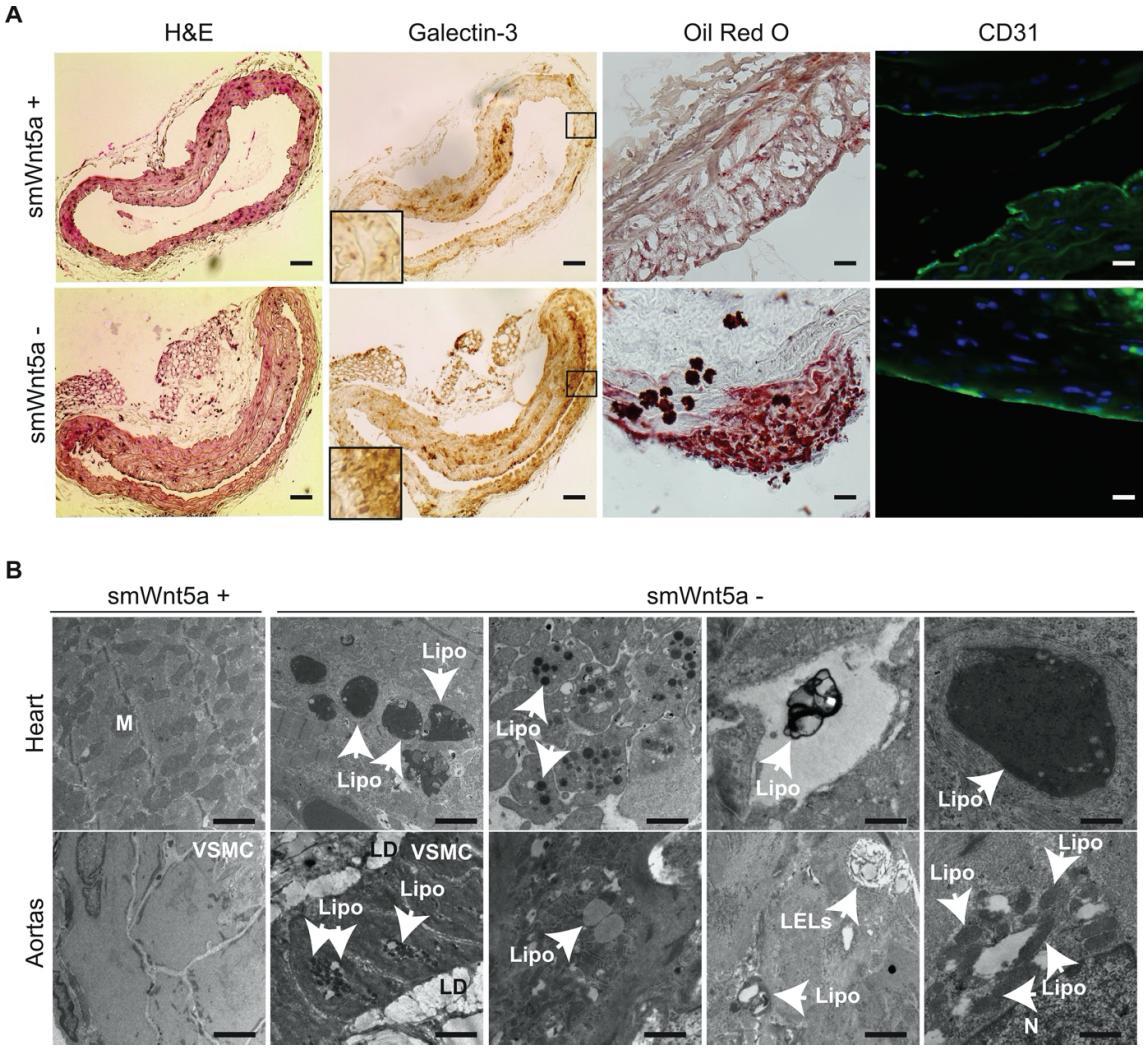
1 2 SA lacZ neo pA 3 4 5
FLP
FRT loxF loxF
1 2 3 4 5
Cre
FRT loxF
1 2 4 5
B
Surviving animals at birth
100
80
60
40
20
0
%
smWnt5a +
smWnt5a -
Cre-, lox/wt
Cre+, lox/wt
Cre- expected
Cre+ expected
C
Cholesterol
Plasma Concentrations (mmol/l)
80
60
40
20
0
ns
sm22Wnt5a+
sm22Wnt5a-
D
Cholesterol accumulation (days)
D0
D2
D4
D8
Wnt5a
+/+ -/- +/+ -/- +/+ -/- +/+ -/-
kDa
Gal-3
Wnt5a
beta tubulin
-35
-45
-55
1 2 3 4 5 6 7 8
E
Liver
Hela
smWnt5a +
smWnt5a -
kDa
p-NFkb p65
NFkb p65
GAPDH
-65
-65
-37
1 2 3

Figure S2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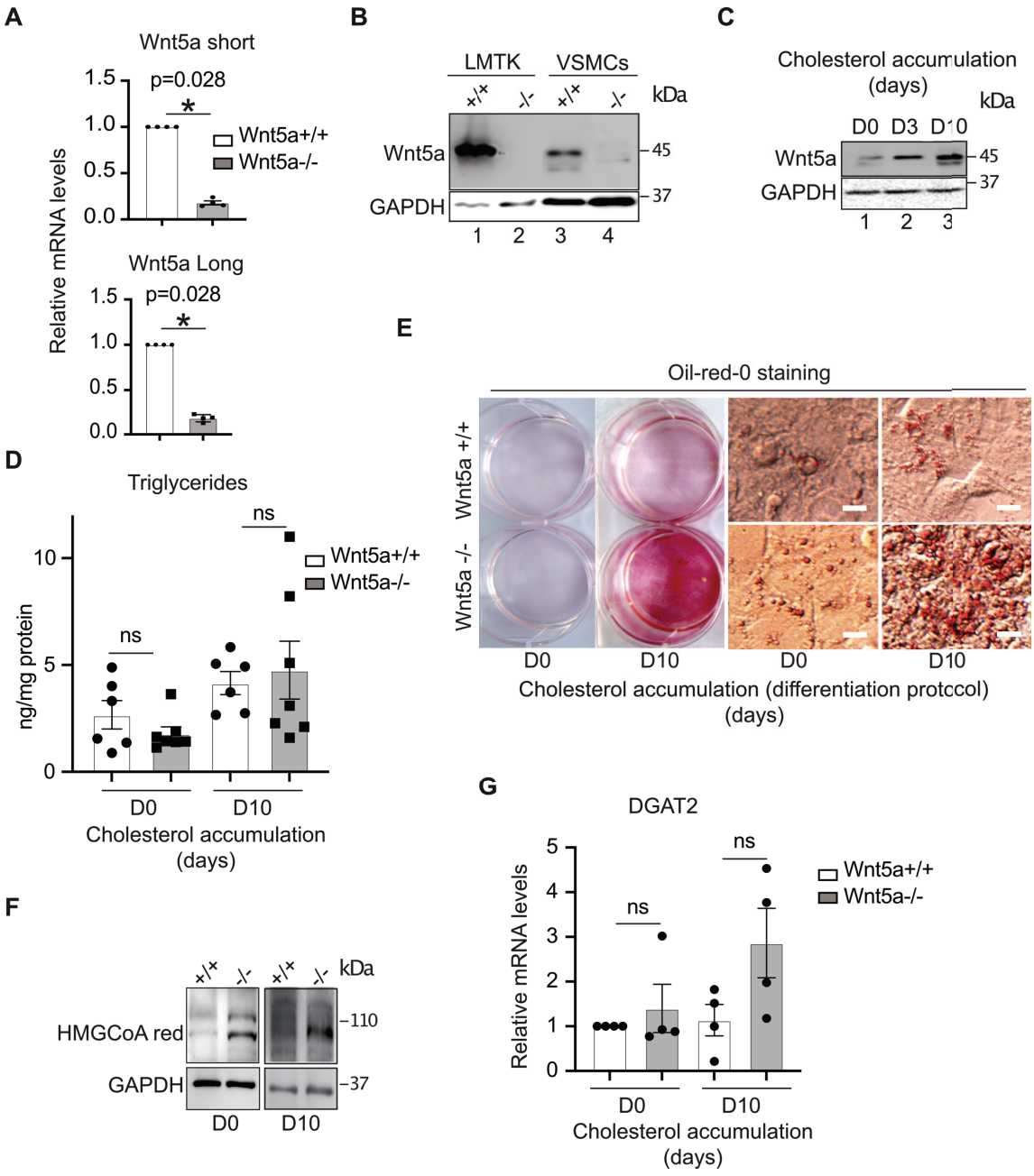
H&E
Galectin-3
Oil Red O
CD31
smWnt5a +
smWnt5a -
B
smWnt5a +
smWnt5a -
Heart
Aortas
M
Lipo
VSMC
LD
LELs
N

Figure S3


A
Wnt5a short
p=0.028
*
Wnt5a+/+
Wnt5a-/-
Relative mRNA levels
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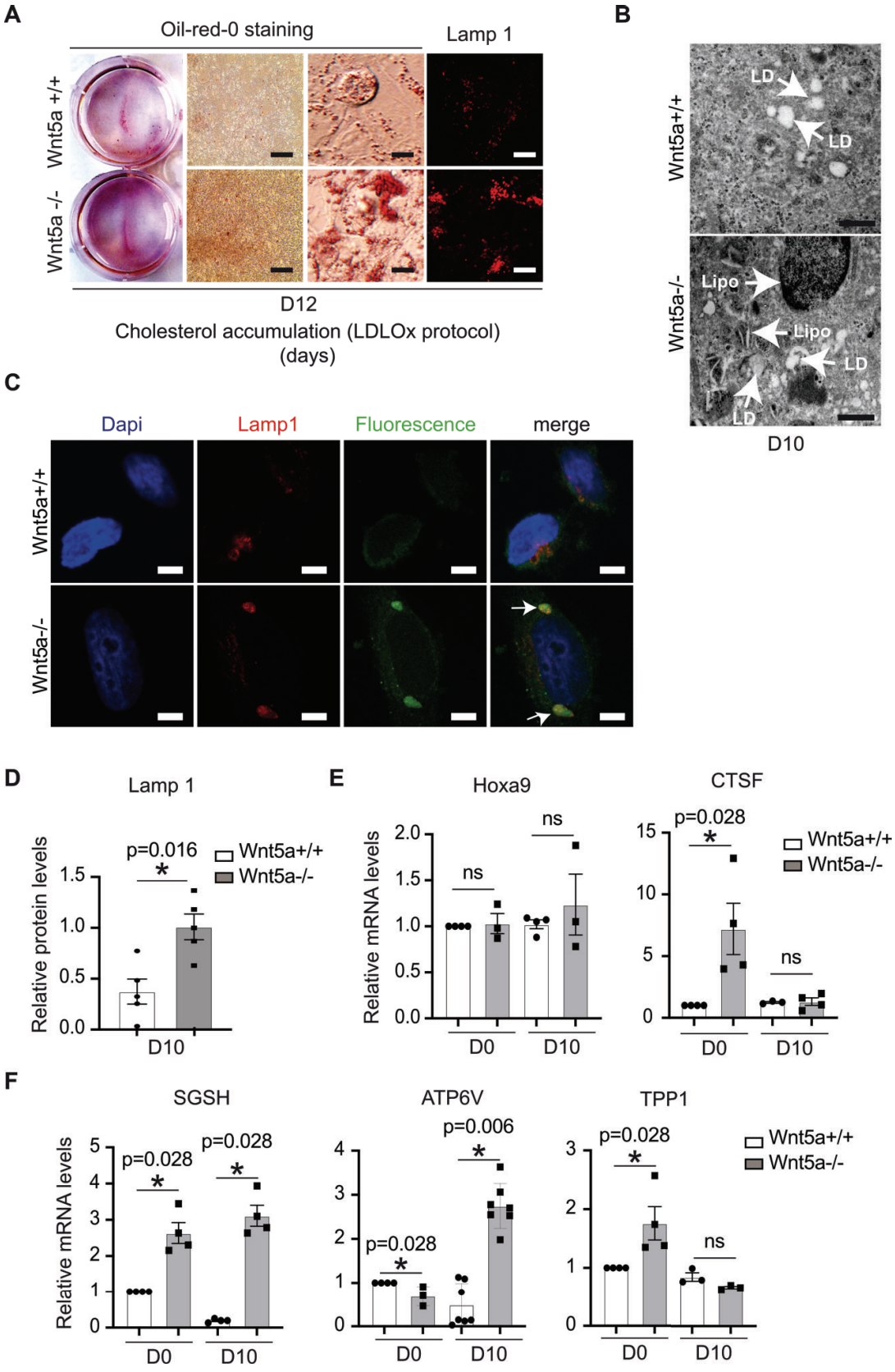
1.0
0.5
0.0
Wnt5a Long
p=0.028
*
B
LMTK
VSMCs
+/+
-/-
+/+
-/-
kDa
Wnt5a
GAPDH
45
37
1 2 3 4
C
Cholesterol accumulation
(days)
kDa
D0 D3 D10
Wnt5a
GAPDH
45
37
1 2 3
D
Triglycerides
ns
ns
Wnt5a+/+
Wnt5a-/-
ng/mg protein
10
5
0
D0
D10
Cholesterol accumulation
(days)
E
Oil-red-0 staining
Wnt5a +/+
Wnt5a -/-
D0
D10
D0
D10
Cholesterol accumulation (differentiation protccol)
(days)
F
+/+
-/-
+/+
-/-
kDa
HMGCoA red
GAPDH
110
37
D0
D10
G
DGAT2
ns
ns
Wnt5a+/+
Wnt5a-/-
Relative mRNA levels
5
4
3
2
1
0
D0
D10
Cholesterol accumulation
(days)

Figure S4


A
Oil-red-0 staining
Lamp 1
Wnt5a +/+
Wnt5a -/-
D12
Cholesterol accumulation (LDLOx protocol)
(days)
B
Wnt5a+/+
Wnt5a-/-
LD
Lipo
D10
C
Dapi
Lamp1
Fluorescence
merge
Wnt5a+/+
Wnt5a-/-
D
Lamp 1
Relative protein levels
p=0.016
Wnt5a+/+
Wnt5a-/-
D10
E
Hoxa9
CTSF
Relative mRNA levels
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D0
D10
F
SGSH
ATP6V
TPP1
p=0.028
p=0.006

Figure S5

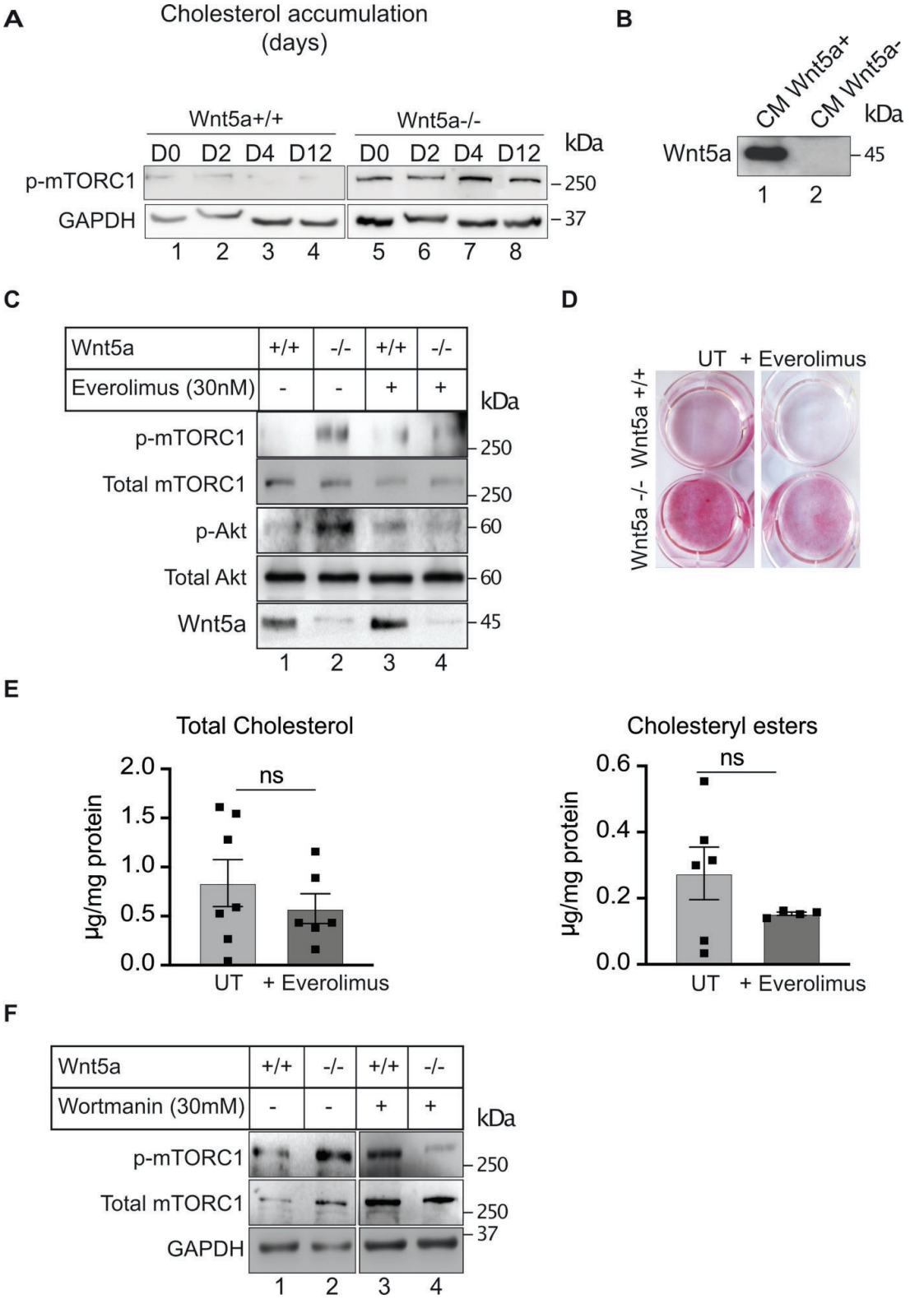
A
Cholesterol accumulation
(days)
Wnt5a+/+
Wnt5a-/-
D0 D2 D4 D12 D0 D2 D4 D12
kDa
p-mTORC1
GAPDH
250
37
1 2 3 4 5 6 7 8
B
CM Wnt5a+
CM Wnt5a-
kDa
Wnt5a
45
1 2
C
Wnt5a +/+ -/- +/+ -/-
Everolimus (30nM) - - + +
kDa
p-mTORC1
Total mTORC1
p-Akt
Total Akt
Wnt5a
250
250
60
60
45
1 2 3 4
D
UT + Everolimus
Wnt5a +/+
Wnt5a -/-
E
Total Cholesterol
µg/mg protein
2.0
1.5
1.0
0.5
0.0
ns
UT + Everolimus
Cholesteryl esters
µg/mg protein
0.6
0.4
0.2
0.0
ns
UT + Everolimus
F
Wnt5a +/+ -/- +/+ -/-
Wortmanin (30mM) - - + +
kDa
p-mTORC1
Total mTORC1
GAPDH
250
250
37
1 2 3 4

Table S4. Mass Spectrometry analysis of the Wnt5a/NPC1 and Wnt5a/NPC2 interactions:

Prey proteins	MW (kDa)	IP Bait NPC1	IP Bait NPC2
NPC1	142.2	165	12
NPC2	16.6	4	11
Wnt5a	42.3	110	48