Supplemental Materials

METHODS

Mice

All CARMN$^{+/+}$ and CARMN$^{-/-}$ animal experiments were performed in accordance with the Animals (Scientific Procedures) Act (UK) 1986 and under the auspices of UK Home Office Project and Personal Licenses (number I34D4F056) held within The University of Edinburgh facilities. CARMN NtacCARMN knock-out mice and C57BL/6Ntac littermate wild-type controls were created by TACONIC Bioscience, lnc. (NY, USA). In order to obtain constitutive knock-out for CARMN, the first exon of the CARMN transcripts and a portion of 4.8kb of the gene promoter were deleted, leaving exons 2/3 and the miRNA stem loops intact. We aimed to prevent bias by keeping environmental cues, housing conditions and handling practices comparable between groups by randomization. All mice used in in vivo experiments underwent a process of randomisation prior to be subjected to experimental procedures and processing, where each animal was randomly assigned to random numbers in an excel sheet. Moreover, cages were placed in a random order and shelf on the rack, and number of mice per cage varied depending on nest size. Meta-analysis studies registered a predominance in carotid atherosclerosis in men than women[2,3]. In addition, studies in animals confirmed that sex is a biological variable, and generally male animals show more inflamed plaque phenotype[4]. Only one gender was used to minimize variation within groups and keep standard deviation low and power high. For the purpose of this study, only male animals were included to remain comparable with a dose-response study with AAV-PCSK9, and this therefore represents a limitation to the understanding of underlying mechanisms of sex-related differences. Male, 8-week-old CARMN$^{-/-}$ (n=16) and CARMN$^{+/+}$ (n=19) animals were

injected intraperitoneally (IP) with $1X10^{12}$ vg/mouse of Adeno-Associated Vector serotype 8 (AAV-8) expressing Protein Convertase Subtilisin/kexin type 9 (PCSK9). At 1-week post injection, all mice were fed with high cholesterol diet (DBM Food Hygiene Supply, Scotland) for 18 weeks. During the time of the study, plasma was collected from the tail for cholesterol measurements immediately previous injection and at 6-,10-weeks post-injection and at sacrifice. Twenty-four hours prior sacrifice, mice were injected IP with 200 mg/kg of sterile 5-ethynyl-2'-deoxyuridine (Invitrogen™, A10044) in sterile PBS. All mice were then sacrificed at 27 weeks post injection for collection and processing of plasma and tissues. The processing of animal tissues used for downstream analysis was performed in a blinded way in which a blinded number was assigned to each sample. The key list of number and genotype was not accessible to the person acquiring raw measurements until data analysis. Non-responder mice (n=3), in which no change in plasma cholesterol and no formation of atherosclerotic plaque in their aortic valves and brachiocephalic arteries were observed, were removed from the analysis of the plaque volume, size and composition. LDLR knock-out studies were carried out in accordance with institutional guidelines and regulations of the Animal Welfare Committee of the Royal Netherlands Academy of Arts and Sciences under project license number PV2018-011. Low density lipoprotein receptor knockout ($LDLR^{-/-}$) mice originated from Jaxx (B6.129S7-Ldlrtm1Her/J Stock No: 002207). Experimental mice were obtained from an in-house breeding colony in Maastricht University, which is refreshed every 10 generations from JACC source to avoid genetic drift. Male low-density lipoprotein receptor deficient mice ($LDLR^{-/-}$) of 8-12 weeks old were fed chow (controls) or high-cholesterol diet (HCD, 0.25%, 824171, Tecnilab-BMI) for 16 weeks. All mice were euthanized with an overdose pentobarbital (100mg/kg) injected intraperitoneally. Brachiocephalic arteries, aortic roots, spleen, lymph nodes and blood were collected for further analysis.

1

Blood count and flow cytometry

2

3 Immune cell subsets were quantified in a blinded way in blood, spleen and lymph nodes using
4 flow cytometry (see Online Figure 5-7 for gating strategy) and absolute cell counts were
5 obtained using TruCount (BD, Cat No 340334). Blood was subjected to erythrocyte lysis prior
6 to antibody labeling (8.4g NH_4CL + 0.84g $NaHCO_3$ in 1 litre H_2O, pH 7.2-7.4). Combinations of
7 different antibodies were used to identify leukocytes (CD45 Biolegend clone 30-F11), B-cells
8 (B220 BD clone RA3-6B2), T-helper cells (CD3 eBioScience clone 17A2/CD4 BD clone GK1.5),
9 cytotoxic T-cells (CD3/CD8 eBioScience clone 53-6.7), NK cells (CD8/NK1.1 BD clone PK136)
10 NKT cells (CD3/NK1.1), monocyte subsets (Ly6C Milteni clone 1G7.G10/CD11b BD clone
11 M1/70) and eosinophils (SiglecF BD clone E50-2440/CD11b clone M1/70). Spleens and lymph
12 nodes were dissociated into single-cell suspensions and enzymatically digested for dendritic
13 cell separation using liberase and DNAse (both 0.2 mg/ml, Roche) for 30 min in RPMI medium.
14 Spleen and lymph nodes were then subjected to erythrocyte lysis as stated above. Antibody
15 staining was performed using the following antibodies to detect granulocytes ($CD11b^{high}$,
16 $Ly6G^{high}$ BD, Cat. 561114 and BD, Cat. 560602 respectively), T cells ($CD3\varepsilon^{+}$, BD Cat. 45003180),
17 T helper cells ($CD4^{+}$; BD, Cat. 560246), cytotoxic T cells ($CD8a^{+}$; BD Cat. 100711),
18 effector/memory T cells ($CD44^{high}$, $CD62^{low}$; BD, eBioscience, Cat. 560181 and Cat. 110081
19 respectively), naïve T cells ($CD44^{low}$, $CD62^{high}$), regulatory T cells ($CD4^{+}$, $CD25^{+}$, $FoxP3^{+}$;
20 eBioscience cat. 560246), B cells ($B220^{+}$; BD Cat. 561227), NK cells ($NK1.1^{+}$; BD, Cat. 561046)
21 and monocytes ($CD11b^{high}$, Ly6G-, $Ly6C^{high/int/low}$; BD, eBioscience and Miltenyi, Cat. 561114,
22 Cat. 560602, Cat. 130102899 respectively). The gating strategy is depicted in Online Figure 6.
23 Tibia and fibula were flushed with PBS using a 23G needle and gently pressed through a 70
24 µm strainer. Bone marrow cells were subjected to erythrocyte lysis as stated before. All

1 lineage negative cells (CD3$^-$, B220$^-$, CD11b$^-$, Ly6G$^-$, NK1.1$^-$ and Ter-119$^-$) were analyzed further
2 for bone marrow stem cells (lin/Sca-1/c-kit; eBioscience, Bd, Cat. 455981, Cat. 47117182
3 respectively), common myeloid progenitors (CD16/32^{int}, CD34^{int}; eBioscience and BD Cat.
4 14016182), granulocyte-macrophage progenitors (CD16/32^{high}, CD34^{high}), erythrocyte-
5 megakaryocyte progenitors (CD16/32$^-$, CD34$^-$). Gating strategy for bone marrow progenitors
6 is shown in Online Figure 7. All data was acquired and analyzed using a FACSCanto II and
7 FACSdiva software (BD Bioscience).
8
9 **Processing of Aortic roots**
10 Aortic roots of LDLR$^{-/-}$ mice were embedded in OCT and frozen at 80°C till further use for laser
11 capture microdissection. Aortic roots of CARMN$^{+/+}$ and CARMN$^{-/-}$ were fixed in
12 paraformaldehyde (1%, 24h), paraffin embedding, and serially sectioned (4µm per section),
13 and stained with haematoxylin and eosin (H&E) and imaged. Specifically, following steps of
14 deparaffination in xylene and ethanol (100%, 96%, 70%, 50%) solutions at room temperature,
15 slides were washed in RNase-Free water and Rnase-Free PBS. Slides were then incubated with
16 Haematoxylin solution for 3 minutes followed by wash in RNase-Free water. Following bluing
17 step for 3 minutes, slides were then washed in running tap water for 5 minutes and
18 counterstained with Eosin for 3 seconds. Sections were then washed in tap water and
19 dehydrated (95%, 100% EtOH, Xylene) and mounted with xylene-based mounting medium to
20 be imaged. Sections were used for immunohistochemical staining within a 100 µm interval
21 here a fully developed media within the aortic valves was present. The sum of plaque area in
22 all three valves was analysed in a blinded way in five consecutive H&E sections at 20 µm
23 intervals using computerized morphometry (QuPath v0.1.2, Open-source software for digital
24 pathology image analysis) and averaged per mouse. Two sections per mouse were stained

1 with alpha-SMA, Lgals3, EdU and Sirius Red. The signal was quantified and averaged per
2 mouse.
3
4 **Laser Capture Microdissection (LCM)**
5 Plaques from frozen sections of aortic roots (9 slides, 18 sections/mouse) were captured from
6 Low Density Lipoprotein Receptor (LDLR) knock-out mice in a random order. Blinding was not
7 possible at this stage due to clear differences between morphology. However, for subsequent
8 analysis of RNA and qRT-PCR analysis each sample was assigned to a random number and the
9 group to which they belonged to was revealed only at the end of the analysis. To perform
10 microdissection PALM Robo laser capture microdissection (LCM) machine (New York, NY),
11 was used as previously described [4]. Atherosclerotic plaques were collected in Qiazol reagent
12 (Qiagen) and RNA was extracted using miRNeasy Micro Kit (Qiagen). Samples were quantified
13 using QuBit technology (Thermo Fisher Scientific) according to manufacturer's instructions.
14
15 **Optical Projection Tomography (OPT)**
16 Formalin (Sigma)-fixed aortic arches and major branches (left carotid artery, left subclavian
17 artery and brachiocephalic trunk) from $CARMN^{-/-}$ and $CARMN^{+/+}$ were embedded in 1.5% low
18 melting point agarose (Invitrogen,16520-050) and dehydrated with 24 hours serial incubation
19 washes with 100% MetOH, BABB solution made up with Benzyl alcohol (Sigma,402834) and
20 benzyl benzoate (Sigma,B6630) and 100% MetOH. Samples were then placed in a calibrated
21 tomography machine (Edinburgh, UK) for further processing of the vessel performed by an
22 independent operator blinded for genotype. Tomographic reconstruction of the pictures was
23 obtained using CTAn software[2].
24

Processing of brachiocephalic arteries

Following OPT analysis, brachiocephalic arteries were serially sectioned (5µm per section). The processing of the samples was performed in a blinded way. Five equally dispersed sections were stained with haematoxylin and eosin (H&E) and imaged. Sections within the plaque interval were used for immunohistochemical staining. Plaque areas were analysed five consecutive H&E sections using ImageJ software and averaged per mouse. Two sections per mouse were stained with alpha-SMA, Lgals3, EdU and Sirius Red and the signal was averaged per mouse. Sirius Red staining was performed to quantify plaque collagen and detailed procedure can be found in the Methods in “Collagen detection in tissue” section below. The signal was quantified only in the plaque area and the medial layer was not included in the quantification of the immunostainings.

Immunohistochemistry (IHC)

Paraformaldehyde-fixed tissues were deparaffinised in xylene and ethanol (100%, 96%, 70%, 50%) solutions at room temperature and washed in RNase-Free water and Rnase-Free PBS. Tissues were then incubated (microwave, 90W) with antigen retrieval buffer HIER (made up with 1X citrate antigen retrieval buffer, pH 6.0 diluted from 10X stock (S2031, Dako) in mQ water for 10 minutes. Slides were then washed in TBS buffer slides and incubated with goat serum (MP-7404, Impress kit) accordingly with manufacturer’s instructions for 30 minutes at room temperature. Primary antibody anti-alpha-SMA at a concentration of 1:3000 diluted in TBT (F3777, Sigma) or Lgals3 (Cedarlane, CL8942AP) in a concentration of 1:4000 diluted in 1% goat serum in PBS or IgG (Dako, X0910) control for 30 minutes at room temperature. Tissues were then washed (3X) in TBT and incubated with secondary antibodies, poly-anty-FITC^{HRP} diluted 1:600 in TBT for alpha-SMA staining and ready to use goat anti-rat IgG

1 secondary antibody (Vector Impress kit MP-7404) for Lgals3 staining, for 30 minutes at room
2 temperature. Following TBT washes (3X) signal was developed with DAB diluent
3 (ImPACT^{TM}DAB) accordingly with manufacturer's instructions for 2 minutes at room
4 temperature. Reaction was then quenched with tap water and sections were counterstained
5 with haematoxylin. Slides were dehydrated (70%, 96%, 100% EtOH washes followed by
6 xylene) and mounted in coverslip with xylene-based mounting medium.
7
8 **Plasma cholesterol measurements**
9 Detection of cholesterol was performed using Cholesterol FS* kit (DiaSys Diagnostic System
10 GmbH, Germany) accordingly to manufacturer's instructions. The absorbances of samples
11 and standards were measured using plate reader at 490 nm.
12
13 **EdU detection in tissue**
14 Paraformaldehyde-fixed tissues were deparaffinised in xylene and ethanol (100%, 96%, 70%,
15 50%) solutions at room temperature and washed in RNase-Free water and RNase-Free PBS.
16 Tissues were then incubated (microwave, 90W) with antigen retrieval buffer (made up with
17 1X citrate antigen retrieval buffer, pH 6.0 diluted from 10X stock (S2031, Dako) in mQ water
18 for 10 minutes. After rinsing in PBS/BSA, slides were then treated with PBS-TritonX-0.5% for
19 20 minutes. EdU cocktail was prepared accordingly with manufacturer's instructions (Click-IT
20 EdU Proliferation kit for Imaging kit). Tissues were then incubated with the cocktail for 30 min
21 at room temperature and protected from light. After washes with BSA/PBS and PBS/Twin-20
22 for 3 min, slides were stained with DAPI at a dilution of 1:1000 in PBS for 3 minutes protected
23 from light and mounted with water-based mounting medium.
24

Collagen detection in tissue

Paraformaldehyde-fixed tissues were deparaffinised in xylene and ethanol (100%, 96%, 70%, 50%) solutions at room temperature and washed in RNase-Free water and RNase-Free PBS. After wash with tap water and rinsed in demineralised water, slides were incubated with 0.2% Phosphomolybdic Acid (PMA) in distilled water solution for 5 minutes and incubated with 0.1% Sirius-Red solution in saturated picric acid for 90 minutes. Tissues were then washed with 0.01M HCl (made up with 1 volume of 1M HCl and 99 volumes of distilled water) for 2 minutes and rinsed in water. Slides were then dehydrated with serial washes (70%, 96%, 100% EtOH and xylol and mounted in xylene-based mounting media.

Human atherosclerotic samples

As previously described[3], patients with symptomatic carotid artery stenosis scheduled undergo carotid endarterectomy were recruited from neurovascular clinics at the Royal Infirmary of Edinburgh. At the time of surgery, plaques were collected immediately following excision and biopsy specimens for RNA analysis were immediately frozen and stored at -80°C. Carotid artery tissue collection for in situ hybridization was part of the Maastricht Pathology Tissue Collection and further storage and use of the tissue was in line with the Dutch Code for Proper Secondary use of Human Tissue and the local Medical Ethical Committee (protocol number 16-4-181). Carotid arteries were collected from patients undergoing carotid endarterectomy. Formalin-fixed, paraffin embedded 5mm-segments were used for histology.

Human cell culture

Human Coronary Arterial Smooth Muscle Cells (hCASMS), purchased from Lonza (Basel, Switzerland), were cultured in Smooth Muscle Cells Growth Medium 2 (PromoCell)

supplemented with 10% foetal bovine serum (Life Technologies, Paisley, UK), Supplement (PromoCell), 50µg/mL penicillin and 50µg/mL streptomycin (Gibco, Paisley, UK) and L-glutamin (Gibco, Paisley, UK). Cells were maintained in culture in humidified atmosphere 37°C (5% CO2) and used between passages 2-6.

5' and 3' Rapid Amplification of cDNA Ends (RACE)

5' and 3' RACE was performed using the SMARTer® RACE 5'/3' Kit (Takara) according to the manufacturer's instructions. Briefly, nuclear RNA was isolated from CASMCs using PARIS kit (Invitrogen) and RACE-ready cDNA was prepared separately for 5' and 3' RACE as described in kit protocol. Following cDNA synthesis, 5'- and 3'-RACE PCR products were amplified using Universal primer (supplied with kit) and gene-specific primers (see Table 2). RACE PCR products were analysed using agarose gel and further purified and cloned into the linearized pRACE vector with In-Fusion® HD Cloning supplied with RACE kit. At least two clones were sequenced using M13 forward primer for each band visualized on agarose gel. Sequencing data was analysed with BLAST and mapped on human genome assembly GRCh38.p13 using Ensemble gene browser.

Subcellular Fractionation

RNA fractionation was performed using the PARIS™ Kit (Thermo Fisher) according to the manufacturer's instructions.

Long-read Nanopore sequencing

1 Long-read sequencing was performed following enrichment of the nuclear fraction obtained
2 through subcellular fractionation (PARIS™ Kit,Thermo Fisher) using cDNA-PCR Sequencing kit
3 (SQK-PCS109) following manufacturer's instructions. Briefly, ribosomal RNA depletion was
4 performed with Ribominus Eukaryote System V2 (Thermo Fisher) and 296ng of RNA was used
5 for polyadenylation step using Lucigen Poly(A) Polymerase Tailing Kit (Lucigen). Following
6 RNA purification with Agencourt RNA cleanup XP Kit (Beckman Coulter), 5ng of
7 polyadenylated RNA was used for Nanopore library preparation using cDNA-PCR Sequencing
8 kit (SQK-PCS109) and sequenced using Oxford Nanopore Technologies' (ONT) MinION
9 sequencer using FLO-MIN106 flow cell. Sequencing was performed using MinKNOWN
10 127.0.0.1 software. Base-calling was performed using Guppy (https://nanoporetech.com/).
11 We obtained 6.8M reads for replicate 1 and 7.1M reads for replicate 2. Fastq reads were
12 mapped to the human genome (GENCODE GRCh38 primary assembly fasta file) using
13 minimap2 (Li Bioinformatics 2018). To focus on CARMN locus, we kept all mapped reads
14 overlapping any CARMN exons and/or miR143/miR145 loci using Samtools
15 (http://www.htslib.org/).
16
17 **RNA-seq analysis of CASMC RNA-seq from ENCODE data**
18 Reads for caSMCs (n=2) were obtained from gene expression omnibus (GSE78534) and
19 mapped to the human genome using STAR[5] (indexed with GENCODEv33, parameters –
20 sjdbOverhang 100). StringTie[6] was then used to assemble any non-GENCODE transcripts
21 merge these with GENCODE v33 (using -m 300). RSEM[7] was used to obtain transcript FPKMs
22 against this new reference (generated using –bowtie2).
23
24 **Transfection of hCASMCs with GapmeR and Mimics**

Antisense oligonucleotides (GapmeR) targeting CARMN transcripts (Exiqon, Denmark) were transiently transfected in hCASMCs using RNAimax Lipofectamine reagent (Invitrogen, Cat.13778-150) in Opti-MEM reduced serum medium (Gibco) for 5 hours and then added complete smooth muscle cells medium overnight. GapCARMN (A*T*A*G*G*T*G*T*C*A*G*G*T*G*T*C), GapCARMN2 (T*T*G*A*G*G*T*A*G*C*T*A*A*G*A*G) and GapCARMN3 (T*C*T*G*T*G*A*A*A*G*G*T*G*A*T*G) target a common region to the CARMN transcripts while GapCTR (A*A*C*A*C*G*T*C*T*A*T*A*C*G*C) was used as standard negative control. Mimics reagents were provided by miRagen Therapeutics as double stranded oligonucleotides in their mature sequence (hsa-miR-143-3p and has-miR-145-5p). In the case of transfection, mimics were added in combination with GapmeR reagents in a concentration of 5nM.

RNA sequencing (RNA-seq) analysis

RNA sequencing was performed in stimulated hCASMCs (PDGF-BB treatment, scratch stimulus and cholesterol loading) or corresponding basal conditions (described 3 basal) following transfection with GapCARMN and GapCTR. Total RNA was obtained using the miRNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. The quality of samples was assessed with Agilent RNA 6000 Nano kit (Agilent technology lnc., California US) accordingly with manufacturer's instructions and samples with RIN > 9.8 to 10 were used. PolyA-enriched strand-specific RNA libraries were prepared by GENEWIZ, lnc. (South Plainfield, New Jersey, USA). Libraries were sequenced with Illumina HiSeq at an average of 30 million reads per samples (paired end 2 x 150bp). Gene quantification (read count and normalised expression value as FPKM) was obtained using RSEM (options: -bowtie2 -forward-prob 0 -paired-end), based on GENCODE annotation version 26 (primary assembly). The

differential expression was assessed using DESeq2 by comparing treated conditions with the untreated control cells. We considered a threshold of absolute Fold Change >=2 and adjusted pvalue <0.01 to identify significant changes between two conditions. We also applied an expression value threshold of 2 FPKM (average of the three replicates) in the considered groups. Sample clustering was evaluated using the Principal component analysis (PCA) tool available in DESeq2 on the regularized log transformed data. Heatmaps were generated using the CRAN package heatmap. The gene ontology analysis was done using topGO (Alexa A and Rahnenfuhrer J (2016)) on enriched genes over a background of expressed genes (FPKM>2 in at least one condition). Fisher's exact test was used to calculate the p-values.

Cell proliferation assay

hCASMCs were plated at a density of $1X10^5$ cells/well in 6-well plate and starved in 0.2% Foetal Bovine Serum (FBS) medium for 48 hours following transfection using RNAiMax reagent (Invitrogen, Cat.13778-150) and Opti-MEM medium (Gibco) for 5 hours and then smooth muscle complete medium (15% FBS) was added overnight. Following 48 hours of starvation with 0.2%FBS medium, cells were then treated with Platelet Derived Growth Factor (PDGF)-BB (R&D System) 20ng/mL, or untreated with 0.2% FBS fresh medium for 48 hours. In both conditions (treated and untreated), cells were simultaneously supplemented with EdU (10µM) accordingly with manufacturer's instructions. 48 hours following the stimulation, cells were harvested and fixed with 70% of ethanol. EdU incorporation was quantified using Click-it EdU Proliferation kit assay (Life Technologies) with Alexa Fluor 594 antibody according to manufacturer's protocol and analysed by Fortessa Analytic Flow Cytometry (FACS).

In the case of GapmeR transfection, hCASMCs were plated to a confluence of $1X10^5$ in 6-well plates. 24 hours after plating, cells were transiently transfected with GapCARMN (or

GapCARMN2), GapCTR and un-transfected cells (Mock) for 5 hours and then added complete smooth muscle cells medium. Cell proliferation assay was carried out 24 hours following transfection.

Cell migration assay

hCASMCs were plated at a density of 1.8X10^5 cells/well in 6-well plate and starved in 0.2% Foetal Bovine Serum (FBS) medium for 48 hours following transfection using RNAiMax reagent (Invitrogen, Cat.13778-150) and Opti-MEM medium (Gibco) for 5 hours and then smooth muscle complete medium (15% FBS) was added overnight. Following starvation with 0.2%FBS, cells in monolayer were then scratched with a sterile pipet (~500μm-wide wounds) and replaced with fresh medium. At 10 hours following the scratch cells were harvested for quantification of the relative migration. Pictures were captured at 0- and 10-hours post-scratch and the migration distances were analysed by using ImageJ software.

In the case of GapmeR transfection, hCASMCs were plated to a confluence 1.8X10^5 in 6-well plates. 24 hours after plating, cells were transiently transfected using RNAiMax with GapCARMN (or GapCARMN2 GapCTR and un-transfected cells (Mock) for 5 hours and then added complete smooth muscle cells medium.

Cholesterol loading assay

hCASMCs were plated at a density of 2X10^5 cells/well in 6-well plate and, following transfection (as above explained), cells were treated with water soluble cholesterol-methyl-β-cyclodextrin (Sigma) loading 10μg/mL in 0.2% Bovine Serum Albumin (BSA) medium for 72 hours or in 0.2% BSA medium for the untreated cells. Cells were then harvested for downstream RNA analysis.

In the case of GapmeR transfection, hCASMCs were plated to a confluence $2X10^5$ in 6-well plates. 24 hours after plating, cells were transiently transfected using RNAiMax with GapCARMN (or GapCARMN2), GapCTR and un-transfected cells (Mock) for 5 hours and then added complete smooth muscle cells medium. Cholesterol loading assay was carried out 24 hours following transfection.

hCASMCs loading with ox-LDL particles

hCASMCs were seeded at $2x10^5$ confluence in 6-well plate. After 24 hours from the plating, cells were treated with Ox-LDL particles (Invitrogen L34358) and harvested at 24 hours post treatment to perform RNA extraction and MTT assay. Pictures were acquired with fluorescence microscopy at 24 hours post treatment.

MTT cell viability assay

MTT cell viability assay was performed using MTT assay kit (Abcam, ab211091) following manufacturer's instructions.

RNA-Fluorescent in-situ hybridization

Custom RNA-FISH tiled probe sets were generated to all exons of CARMN as well as UBC and SNORD3 as positive controls (Thermo Fisher Scientific). RNA-FISH was performed according to manufacturer's instructions (ViewRNA™ cell FISH) with minor changes as previously published[8]. Briefly, CASMCs were grown on 16-mm coverslips to 80% confluency, washed in PBS and fixed in 4% paraformaldehyde with 1% glacial acetic acid for 1h. Following detergent QS permeabilization and 1:4000 protease digest, coverslips were incubated with a combination of CARMN, UBC, and SNORD3

probe sets. Probe set buffer was used as a negative control and specificity of. Following probe hybridisation, cover slips were incubated with branched tree technology pre-amplifier for 1h and then with the amplifier for 30 min. Coverslips were then mounted onto glass slides using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories) and imaged using Andor Revolution XDi spinning disk confocal microscope. To quantify the data produced by RNA-FISH, Z stack images of each condition were taken, and quantification performed using semi-automated procedure using Image J Software where an intensity threshold above which a spot is considered an RNA particle was selected under default settings. Once threshold was computationally estimated (and then manually confirmed or adjusted), the images were converted to binary and the number of particles were quantified using the "Analyse Particles" feature on ImageJ. This allowed us to count the number of nuclear transcripts per cell, in a non-biased manner. Cells were only counted if the whole of the nuclei was present in view, and not overlapping other cells.

Gene expression analysis by qRT-PCR

Total RNA from hCASMCs, from plaques isolated from patients with symptomatic carotid artery stenosis and from mouse aortas, was obtained using the miRNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. In the case of tissues, frozen samples were fragmented using liquid N_2 and tissue homogenizer to further ensure the tissue disruption. cDNA for mRNA analysis of gene expression was synthesized from total RNA using the Multiscribe Reverse Transcriptase (Life technologies, Paisley, UK). cDNA for miRNA analysis was obtained from total RNA using specific reverse transcription primers according to the TaqMan MiRNA Assay protocol (Applied Biosystem, Foster City, CA, USA). Quantitative

qRT-PCR was performed using Power SYBR green (Life technologies) with custom PCR primers (Eurofins Scientific, Ebersberg, Germany). In the case of Sybr Green qRT-PCR, samples were subjected to 2 minutes at 50 °C, 10 minutes at 95°C, 40 cycles of denaturation for 15 sec at 95°C, 1 min at 60°C. In the case of TaqMan reaction, qRT-PCR plate underwent to a first step of 2 min at 50°C followed by 10 min at 95°C and 40 cycles at 95°C for 15 sec to finish with 1 min at 60°C. The sequences of the primers used is specified in the Online Supplement- Table I). Ubiquitin C for human and Cyclophilin and 18s for mouse, were selected as housekeeping genes because of their stability across all studied groups. In the case of microRNAs, RNU48 for human and U6 for mouse samples were selected as stable endogenous controls. Fold changes were calculated by using the $2^{-\Delta\Delta ct}$ method.

In Situ Hybridization (ISH)

CARMN was detected in human carotid atherosclerotic plaques fixed in formalin and embedded in paraffin. Slides were deparaffinised in xylene and ethanol (100%, 96%, 70%) solutions at room temperature and washed in RNase-Free water and Rnase-Free PBS. The tissue was then incubated with 1:1000 Proteinase K (miRCURY LNA miRNA ISH Buffer Set, Qiagen Cat.339450) diluted into RNase-Free PBS and incubated in hot humidified plate at 37°C. After serial RNase-Free PBS washes the tissue was incubated with LNA double-DIG labelled probes (Exiqon) detecting CARMN (/5DigN/TCTGGTCCAGGTGTGGCTCCTT/3Dig_N/) and control probe (/5DigN/GTGTAACACGTCTATACGCCCA /3Dig_N/) at 100nM diluted in 1x Formamide-free miRNA ISH buffer (miRCURY LNA miRNA ISH Buffer Set, Qiagen Cat.339450) at 55°C O/N. The tissue slides were then washed (X3) in 5X SSC Buffer (prepared with RNase-free water from 20X SSC, Thermo Fisher Scientific) at 55°C followed by room temperature wash. The tissue was then blocked with 1X Roche DIG blocking buffer (made up in maleic acid

from 10X blocking reagent, Roche Cat. 11585762001) for 1 hour followed by the incubation with Anti-Digoxigenin-AP diluted 1:500 in 1X blocking buffer (Fab fragments, Roche Cat.11093274910) for 1 and ½ hours. The tissue was then washed 3 times with RNase-free PBST and incubated with detection solution prepared by NBT/BCIP tablet (Roche Cat.11697471001) accordingly with manufacturer's instructions. Reaction was stopped after 40 minutes by washing the tissue with RNse-Free PBS. The tissue was then treated with 0.3% H2O2 in MetOH to block endogenous peroxidases for 15 minutes at room temperature. After a wash in demineralised water, the tissue was washed with 0.1% BSA in TBST and incubated with 5% Goat serum diluted in TBST-T buffer for 30 minutes at room temperature. The tissue was then incubated with human alpha-SMA antibody (Dako, Cat. M0851, clone 1A4), diluted 1:2500 in TBST or human CD68 antibody (Dako, Cat. M0814, clone KP1) diluted 1:250 in TBST or human CD45 (Cat. GA75161 Dako) diluted 1:400 or IgG control (abcam, Cat. 37355), (diluted to reach the same concentration of alpha-SMA, CD68 or CD45) for 30 minutes at room temperature. After washing in TBS, the tissue was incubated with ready to use Brightvision anti-mouse HRP (Immunologic, VWR Cat. VWRKDPVM55HRP) for 30 minutes at room temperature. Following 2X washes in TBS, Poly-Detector HRP Green Kit (BioSB, Cat. BSB0130) was applied in the tissue accordingly to manufacturer's instructions for 3 minutes at room temperature. The stain was stopped by washing in tap water and tissue was counterstained with Fast Red solution (Sigma, N3020) for 5 minutes at room temperature, dehydrated with serial washes (96%, 100% EtOH and xylene) and glasses were mounted in coverslip with xylene-based mounting medium.

Pseudo Fluorescent Image Analysis

1 Bright field images for MIR143HG (Purple in situ staining) and CD68/alpha-SMA (green
2 immunohistochemistry staining) in human plaque were converted into pseudo fluorescent
3 images using Image J Software. Images were opened in Image J, and the colour deconvolution
4 tool used, selecting regions of interest for each stain (Purple in situ staining, green
5 immunohistochemistry staining, and pink nuclear red counterstain), generating RGB values
6 for each colour, and splitting the images into these components. The RGB values generated
7 for each colour were kept consistent for further analysis across all samples. Once the images
8 were split into the 3 colours, the nuclear stain was discounted, so that the *in situ* and
9 immunohistochemistry staining could be seen clearly. These were then inverted, brightness
10 adjusted consistently across all samples, and given pseudo colours of red and green
11 respectively, and the resulting images merged to create a dual fluorescent image.
12
13 **Statistical analysis of experimental data**
14 Graphs are presented as bar charts of mean ± standard error of the mean (SEM) with
15 individual datapoints superimposed to show full data distribution. QRT-PCR data in graphs is
16 shown as relative expression to housekeeping control as described by Livak and Schmittgen[3].
17 Statistical tests used to assess statistical significance is indicated in each figure legend with
18 the precise p-value provided in the graphs where statistical significance was observed. For *in*
19 *vitro* experiments, all biological replicates using primary cells correspond to independent
20 experiments from distinct expansions and passage numbers, with technical replicates (precise
21 replicate number indicated in the figure legends). As each experimental data set is an average
22 of a large number of cultured cells, we assumed the data was normally distributed based on
23 the central limit theorem. Statistical analysis of biological replicates was performed using
24 unpaired t-test (2 groups comparison) or one-way ANOVA with Bonferroni correction for

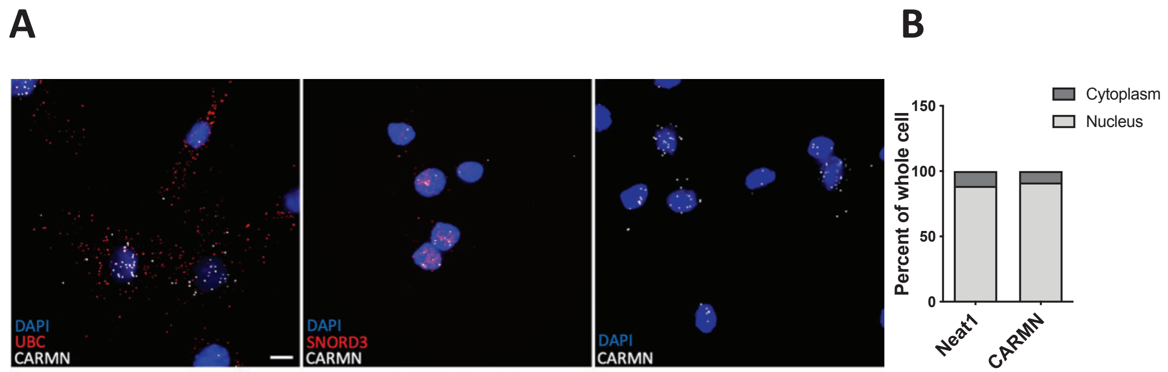
multiple comparisons (>2 groups comparison). Statistical analysis was performed using GraphPad Prism 8.0.0. All the data obtained from *in vivo* experiments were tested for normal distribution using the Kolmogorov-Smirnov test. Data following a normal distribution was analysed using unpaired t-test. In the case of data not-normally distributed or n too small (n<6) to test for normality, statistical significance was analysed using Mann-Witney. Multiple testing correction was used for comparison of groups within ANOVA using the Bonferroni correction. No multiple testing correction was done beyond this and therefore, it might represent a limitation of this study.

Online Table II. Human and mouse SYBR Green primers sequences and TaqMan probes.

HUMAN PRIMERS	SEQUENCE
CARMN_001_Fw	TGGGAATGGAAACCCTTGGT
CARMN_001_Rev	AAGCTGCATGTTCAAGGTCG
CARMN_003_Fw	CTGTAACAAGTGCACAGGCAA
CARMN_003_Rev	TTCTGTTGGGAGGCTTGGAGA
CARMN_005_FW	CCAGGAAAGGCAAGGGCGCTA
CARMN_005_Rev	TGGGAGCGGATGTGGGTGCCG
CARMN_006_Fw	GCTGTAACAAGTGCACAGGCA
CARMN_006_Rev	GTGGGCTCACAGTTCTGTCT
CARMN_007_Fw	GATCCAGAGTAGGAGGGAGCC
CARMN_007_Rev	GGCCCTTGAATCTGCTTGCC
CARMN_008_Fw	AGCCTGGAAGTGGCTGGATGT
CARMN_008_Rev	AAACGCATGCCTGATGGTGT
CARMN_009_Fw	GCTCCCAAAGCAGGAAGACC
CARMN_009_Rev	GCCCAACCTCACAAATCCTCT
CARMN_010_Fw	AATGCAGGAGGCATGGGCCA
CARMN_010_Rev	GCCACTTGAGTCAGTGATGGTG
CARMN _011_Fw	AAAAGTCAGAGGCTGTGGGAC
CARMN _011_Rew	TTGCCCACACAATGCCCTA
CARMN _012_Fw	TGCCTCTTCAGCTCATATAAG
CARMN _012_Rev	GCTAGCGCCCTTGCCTTTCCT
Common primer_Fw	CGCCATGCTGATGTCAGAGA

Common primer_Rev	GTTCTGTCTCCGGGCTGC
Ubc_Fw	TTGCCTTGACATTCTCGATG
Ubc_Rev	ATCGCTGTGATCGTCACTTG
MOUSE PRIMERS	**SEQUENCE**
CARMN _01_Fw	CATTTGAGGGAGCCAGGGGT
CARMN _01_Rev	GTGGGAAGGAACAGTAGGACA
CARMN _02_Fw	AGTGCCAGCCCTGAGGAAAG
CARMN _02_Rev	TCCCCAGATAACCTTTGCTTCGT
Common primer_Fw	GGTTCCAGTGCCAGTTGCTTA
Common primer_Rev	GTGGTTGTGGGTGTTATTGCT
Ppia_Fw	ATTTCTTTTGACTTGCGGGC
Ppia_Rev	AGACTTGAAGGGGAATG
18S_Fw	GTAACCCGTTGAACCCCATT
18S_Rev	CCATCCAATCGGTAGTAGCG
TAQMAN PROBES	**ASSAY ID**
CD68	Hs02836816_g1
LGALS3	Hs00173587_m1
ACTA2	Hs00426835_g1
MHY11	Hs00975796_m1
CCN	**Hs00959434_m1**
TAGLN	Hs06633192_s1
KLF4	**Hs00358836_m1**
UBC	Hs01867132_s1

Hsa-miR-143-3p	477912_mir 1
Hsa-miR-145-5p	477916_mir 2
RNU48	Hs04931161_g1
U6-snRNA	Mm00505971_m1
RACE PRIMERS	**SEQUENCE**
5'RACE	GATTACGCCAAGCTTCCCAGGAGGCTGCTTCTC
3'RACE_1	GATTACGCCAAGCTTAGGCTGGGTCTAATTAGTTGAGA
3'RACE_2	GATTACGCCAAGCTTCAGCCCGGAGACAGAACT



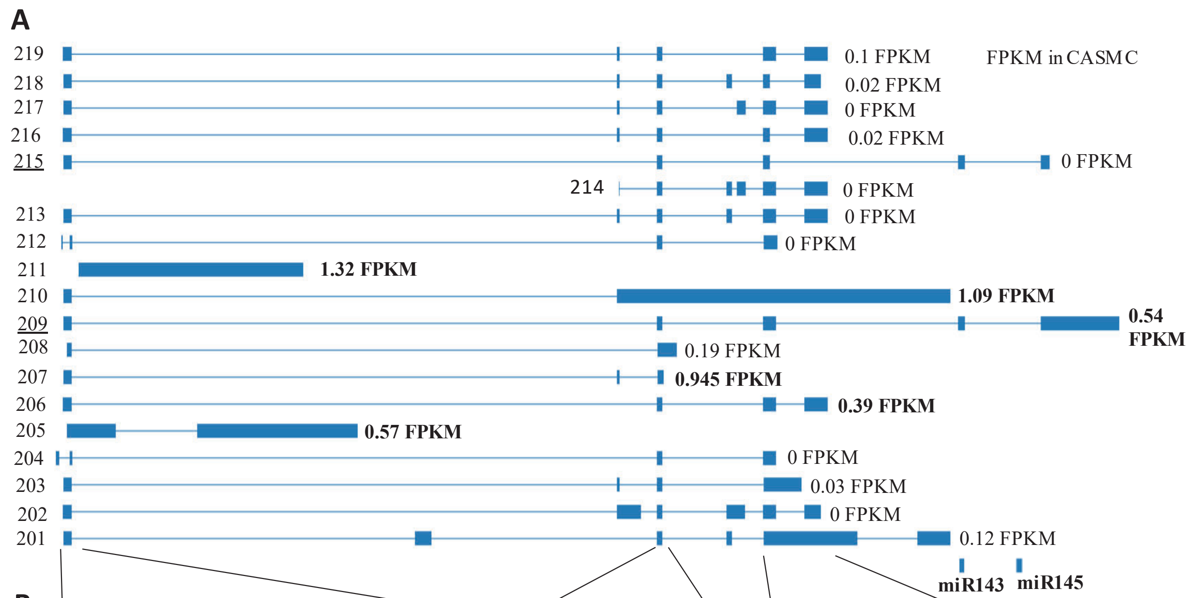
1 **Online Figure I. CARMN localises in the nucleus of hCASMCs at basal conditions.**

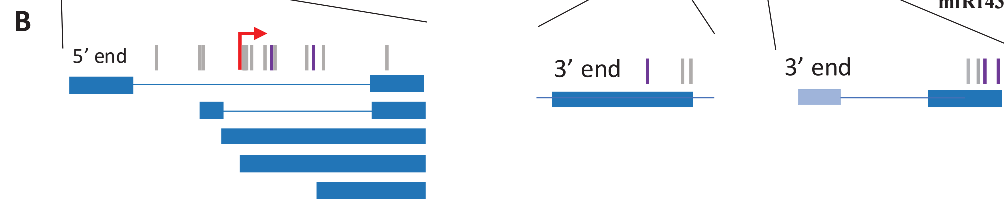
2 A) RNA FISH analysis of CARMN, cytoplasmic UBC mRNA, and nuclear SNORD3 mRNA in

3 quiesced CASMCs. Scale bar = 100um. B) Subcellular fractionation of hCASMCs (n=1) at basal

4 condition. QRT-PCR results indicate CARMN mostly localizes in the nucleic compartment of

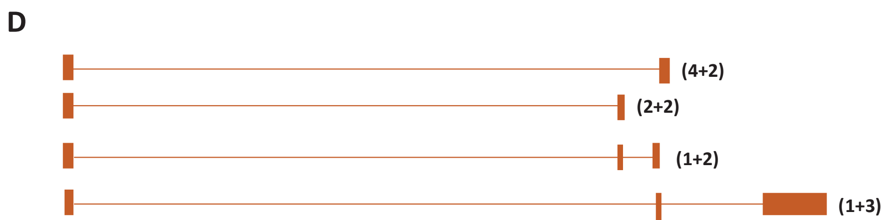
5 cells. Neat1 was used as nuclear control.

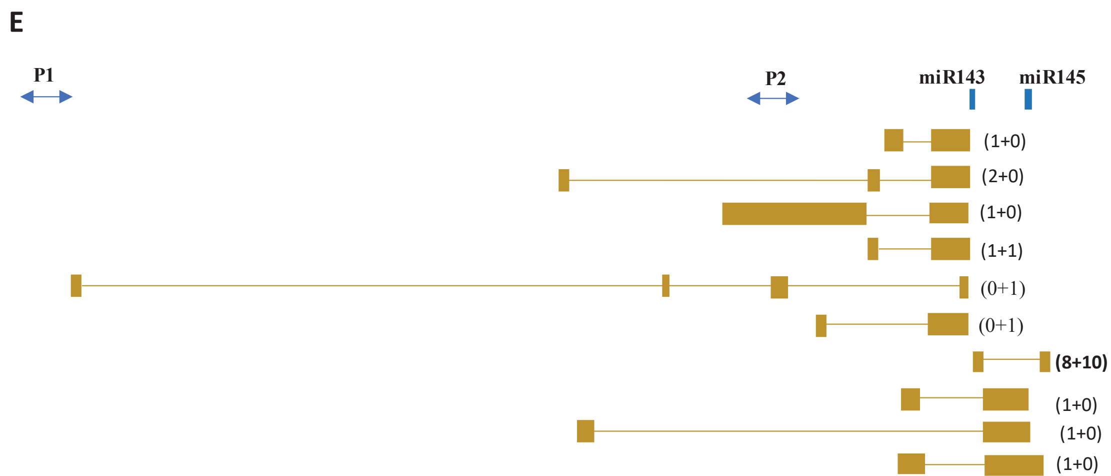




C

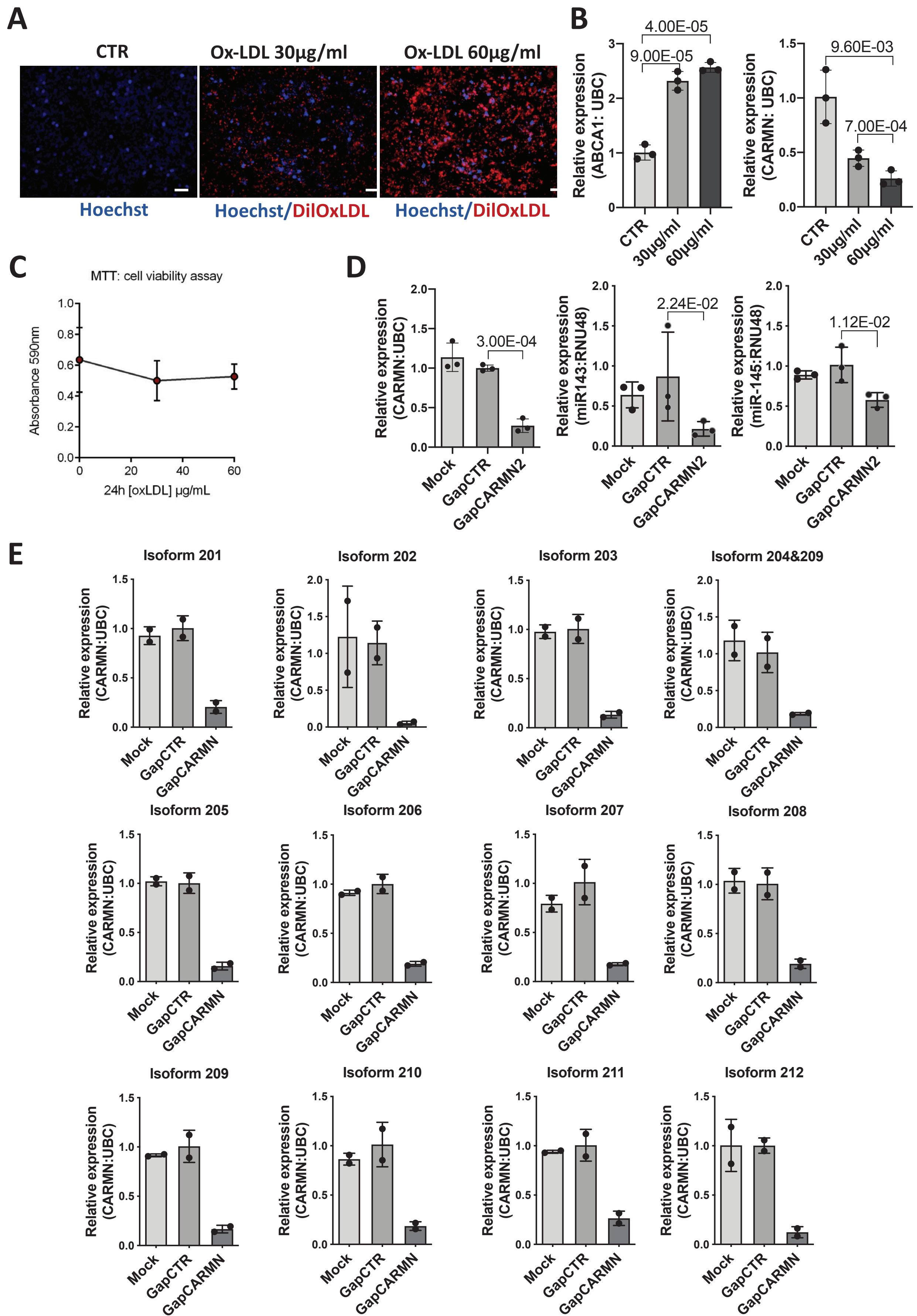
Nanopore spliced reads not overlapping miR143/miR145	total number of reads	number of reads with identical exonic structure in both replicates
5' end near main TSS	30 (17 in Rep1 and 13 in Rep2)	17
Other 5' end	7 (3 in Rep3 and 4 in Rep2)	0





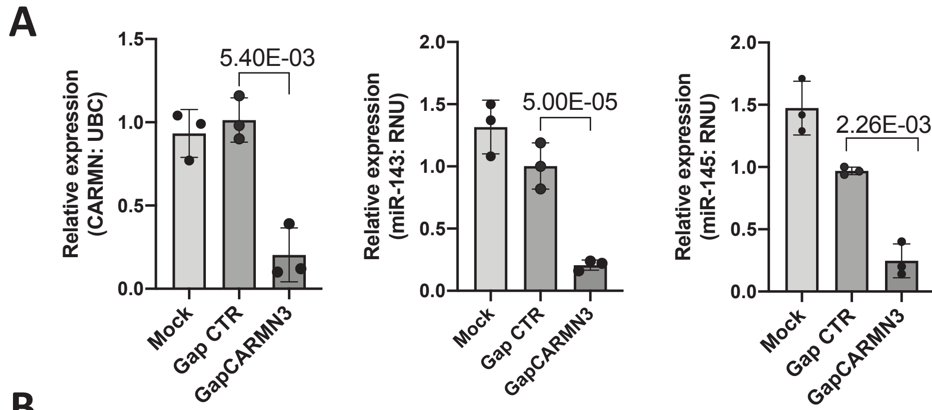
Online Figure II. Characterisation of CARMN/miR-143/145 locus structure.

A) Schematic of CARMN locus and isoforms based on ENSEMBL version p13/GENCODE v33. Expression level of each isoform in CASMC is indicated (average FPKM based on 2 RNA-seq replicates). B) Summary of 5’ and 3’ end. Only region with RACE products is indicated. Nanopore ends are in grey while RACE ends are in purple. The main TSS identified based on RACE and nanopore sequencing is indicated as a red arrow. C) Table of spliced CARMN reads non-overlapping miR143/miR145 loci. D) Schematic of exonic structure detected in both nanopore sequencing replicates. In bracket, number of reads in replicate 1 + number of reads in replicate 2. E) Schematic of potential pri-miRNA read fragments. The two previously described miRNA promoters are indicated (P1 and P2). In bracket, number of reads in replicate 1 + number of reads in replicate 2.

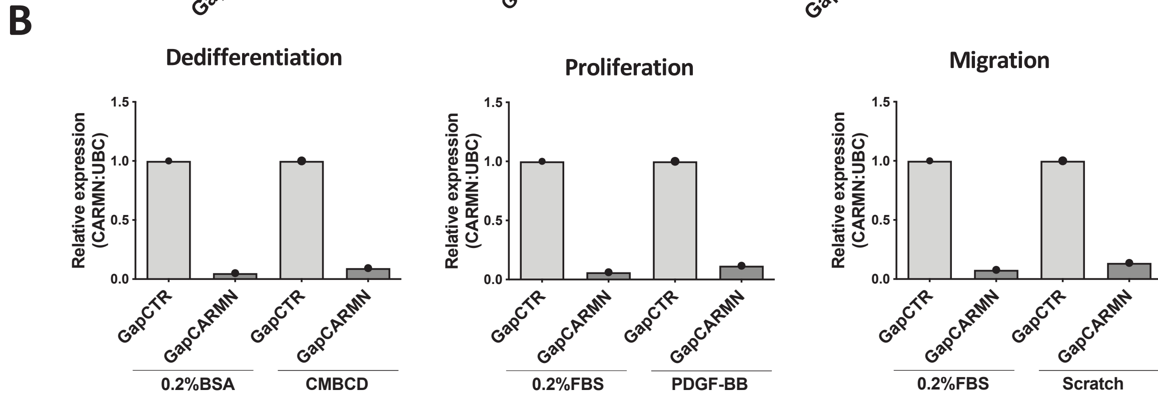

A
CTR
Ox-LDL 30μg/ml
Ox-LDL 60μg/ml
Hoechst
Hoechst/DilOxLDL
Hoechst/DilOxLDL
B
Relative expression (ABCA1: UBC)
4.00E-05
9.00E-05
CTR
30μg/ml
60μg/ml
Relative expression (CARMN: UBC)
9.60E-03
7.00E-04
C
MTT: cell viability assay
Absorbance 590nm
24h [oxLDL] μg/mL
D
Relative expression (CARMN:UBC)
3.00E-04
Relative expression (miR143:RNU48)
2.24E-02
Relative expression (miR-145:RNU48)
1.12E-02
Mock
GapCTR
GapCARMN2
E
Isoform 201
Isoform 202
Isoform 203
Isoform 204&209
Isoform 205
Isoform 206
Isoform 207
Isoform 208
Isoform 209
Isoform 210
Isoform 211
Isoform 212
Relative expression (CARMN:UBC)
Mock
GapCTR
GapCARMN

1 **Online Figure III. CARMN expression is affected by ox-LDL and GapmeR approach**
2 **significantly decreases encoded transcripts.**
3 A) Representative images of hCASMCs treated with Dil-labelled ox-LDL for 24 at a
4 concentration of 30 or 60ug/ml or control. Ox-LDL particles are stained in red, nuclei in blue.
5 B) qRT-PCR relative to ABCA1 and CARMN respectively in hCASMCs treated with ox-LDL
6 particles or control. UBC was used as housekeeper gene. One-way ANOVA with Bonferroni
7 multiple comparison test was used to assess statistical significance indicated with p values. C)
8 MTT cell availability assay performed in hCASMC treated with ox-LDL 30 or 60ug/ml or
9 control. D) qRT-PCR data of CARMN and miR-143/145 in hCASMCs (n=3) following
10 transfection with GapCARMN2 in basal condition versus control. One-way ANOVA with
11 Bonferroni multiple comparison test was used to assess statistical significance indicated with
12 p values. E) Expression of CARMN transcript variants in hCASMCs (n=2) following transfection
13 with GapmeR targeting CARMN (GapCARMN), GapmeR control (GapCTR) and un-transfected
14 cells (Mock). QRT-PCR results were obtained using transcript-specific couple of primers. One-
15 way ANOVA with Bonferroni multiple comparison test was used to assess statistical
16 significance indicated with p values.

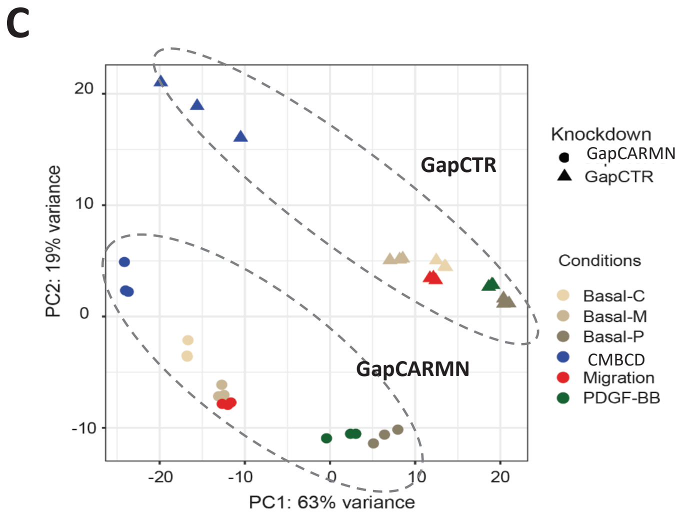
A



B



C

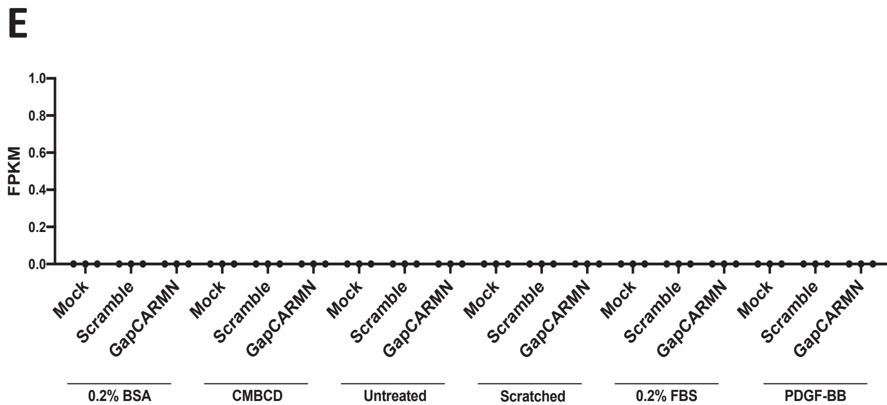


D

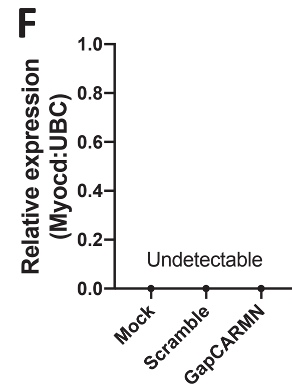
Significant changes
GapCARMN vs GapCTR
(abs(LogFC)>=1 & padj<0.01 & FPKM>=2)

	Total	up	down
Basal-P	469	216	253
PDGF-BB	649	338	311
Basal-M	1103	480	623
Migration	1180	481	699
Basal-C	1322	615	707
CMBCD	734	336	398

E

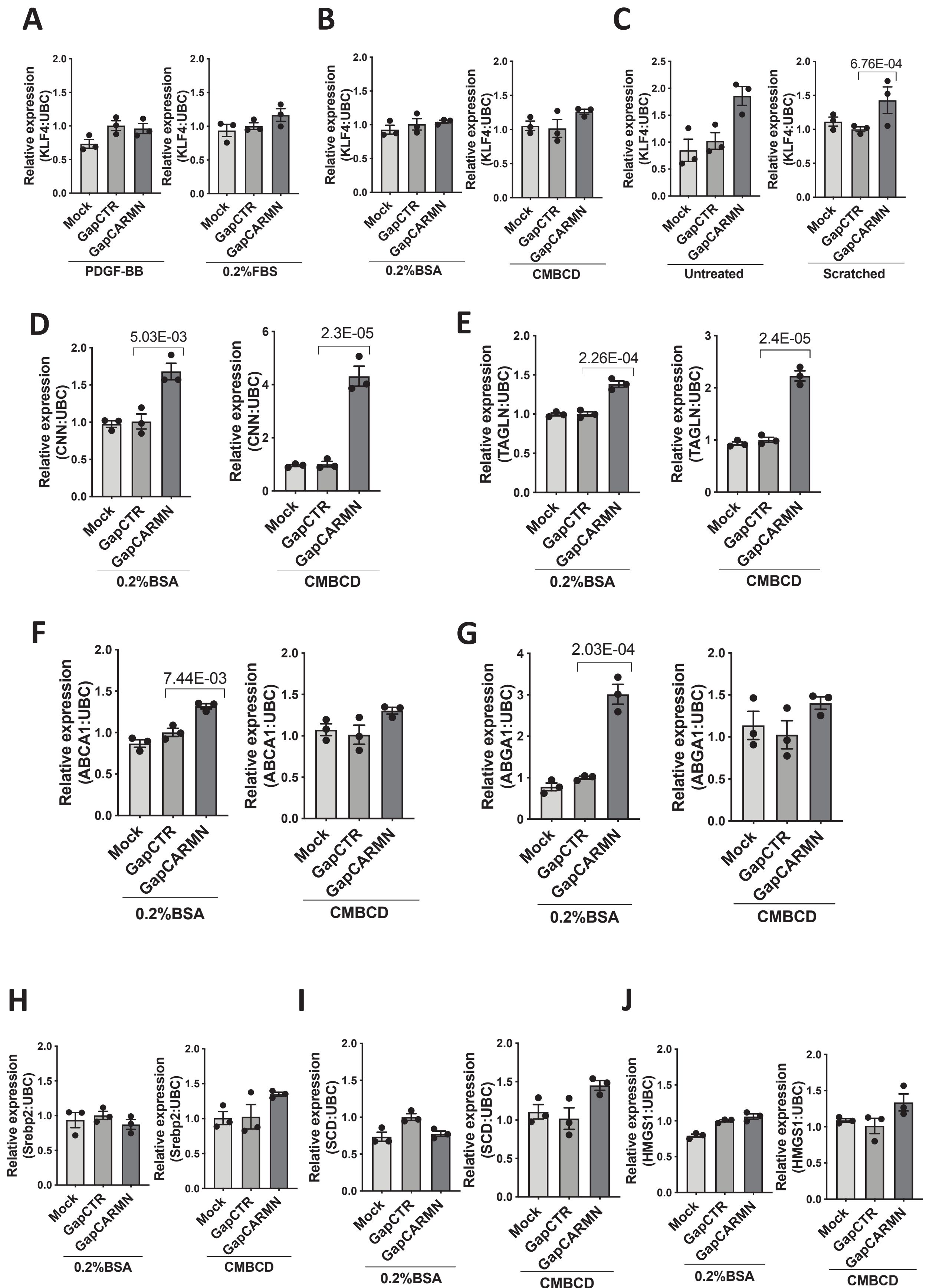


F

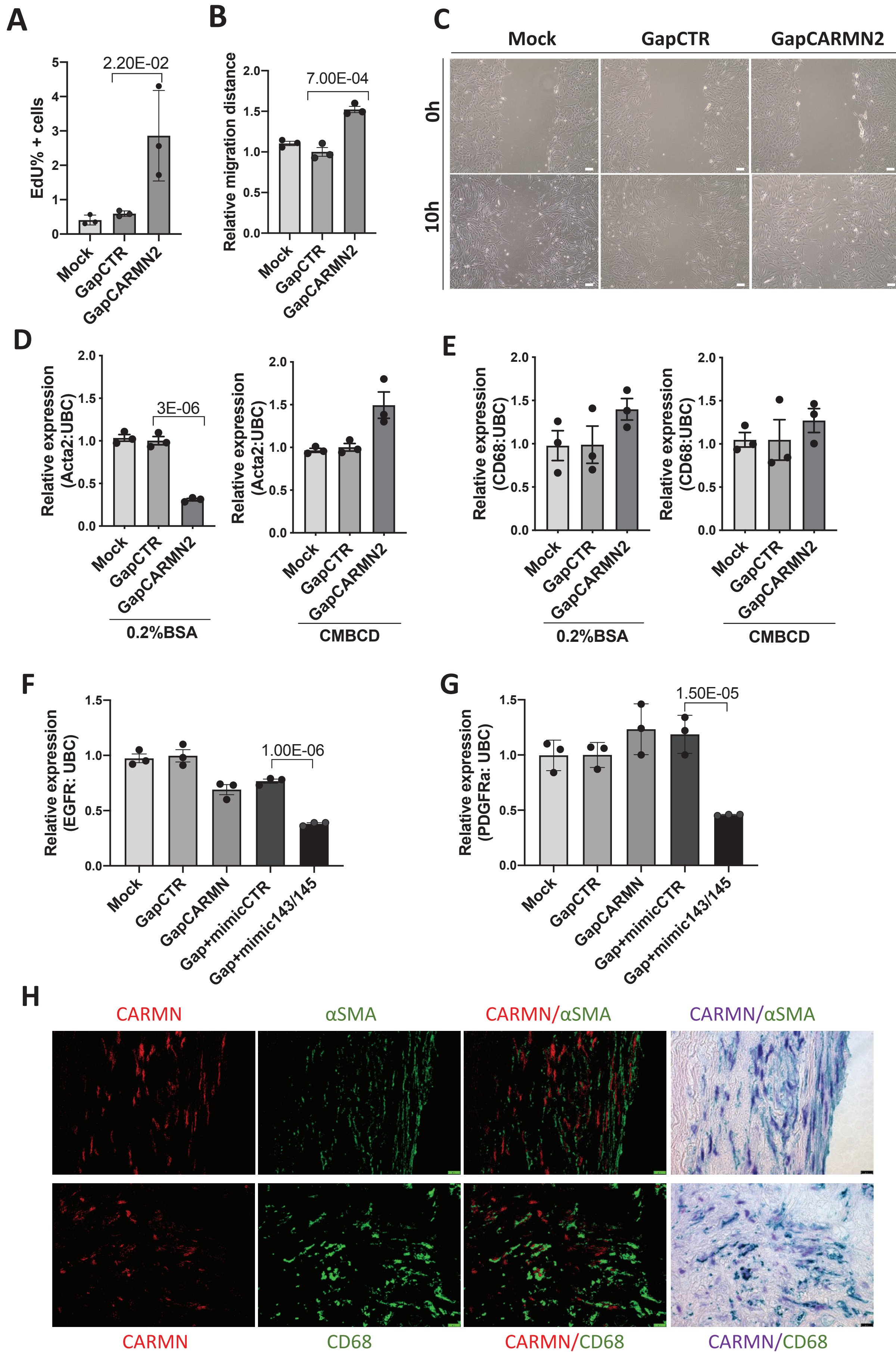


Online Figure IV. Transcriptomic analysis of CARMN depleted hCASMC in biological (basal) and pathological (PDGF-BB, Cholesterol-treated and migration-induced) conditions.

A) QRT-PCR data relative to CARMN and miR-143/145 following 48 hours of transfection with GapmeR targeting common exon to CARMN transcripts, GapCARMN3, versus control. One-way ANOVA with Bonferroni multiple comparison test was used to assess statistical significance indicated with p values. B) QRT-PCR validation of CARMN GapmeR-mediated knock-down in samples used for RNA sequencing (RNA-Seq) experiment. GapCTR indicates the transfection of hCASMCs with GapmeR control and Gap CARMN refers to the transfection with GapmeR targeting CARMN. C) Principal component analysis of all RNA-seq samples obtained using DESeq2. Control and CARMN knockdown samples are highlighted. D) Table showing the number of significant changes (abs LogFC)>=1 & padj<0.01 & FPKM>=2) upon CARMN knockdown samples in the 6 independent conditions. E), F) Expression of Myocardin (Myocd) gene as FPKM and qRT-PCR respectively, in hCASMCs (n=1) under basal or stimulated conditions following CARMN depletion or control. UBC and RNU were used as housekeeper control genes. One-way ANOVA with Bonferroni multiple comparison test was used to assess statistical significance indicated with p values.


A
B
C
Relative expression (KLF4:UBC)
Mock
GapCTR
GapCARMN
PDGF-BB
0.2%FBS
0.2%BSA
CMBCD
Untreated
Scratched
6.76E-04
D
Relative expression (CNN:UBC)
5.03E-03
2.3E-05
E
Relative expression (TAGLN:UBC)
2.26E-04
2.4E-05
F
Relative expression (ABCA1:UBC)
7.44E-03
G
Relative expression (ABGA1:UBC)
2.03E-04
H
I
J
Relative expression (Srebp2:UBC)
Relative expression (SCD:UBC)
Relative expression (HMGS1:UBC)

1 **Online Figure V. Expression of vSMC regulator genes, vSMC identity markers and genes**
2 **involved in lipid homeostasis in CARMN-depleted hCASMCs.**
3 A), B), C) QRT-PCR results of KLF4 gene following transfection with GapCARMN in treated
4 (PDGF-BB, CMBCD, scratch) and untreated hCASMCs (n=3) versus GapCTR. One-way ANOVA
5 with Bonferroni multiple comparison test was used to assess statistical significance indicated
6 with p values. D), E) Expression of CNN and TAGLN genes in hCASMCs (n=3) treated with
7 CMBCD and control (0.2%BSA) following transfection with GapCARMN or GapCTR. One-way
8 ANOVA with Bonferroni multiple comparison test was used to assess statistical significance
9 indicated with p values. F), G), H), I), J) QRT-PCR data of ABCA1, ABGA1, Srebp2, SCD, HMGS1
10 genes respectively in hCASMCs (n=3) following treatment with CMBCD or 0.2% BSA control in
11 GapCARMN transfected cells or control (GapCTR). UBC was used as housekeeper control
12 gene. One-way ANOVA with Bonferroni multiple comparison test was used to assess
13 statistical significance indicated with p values.

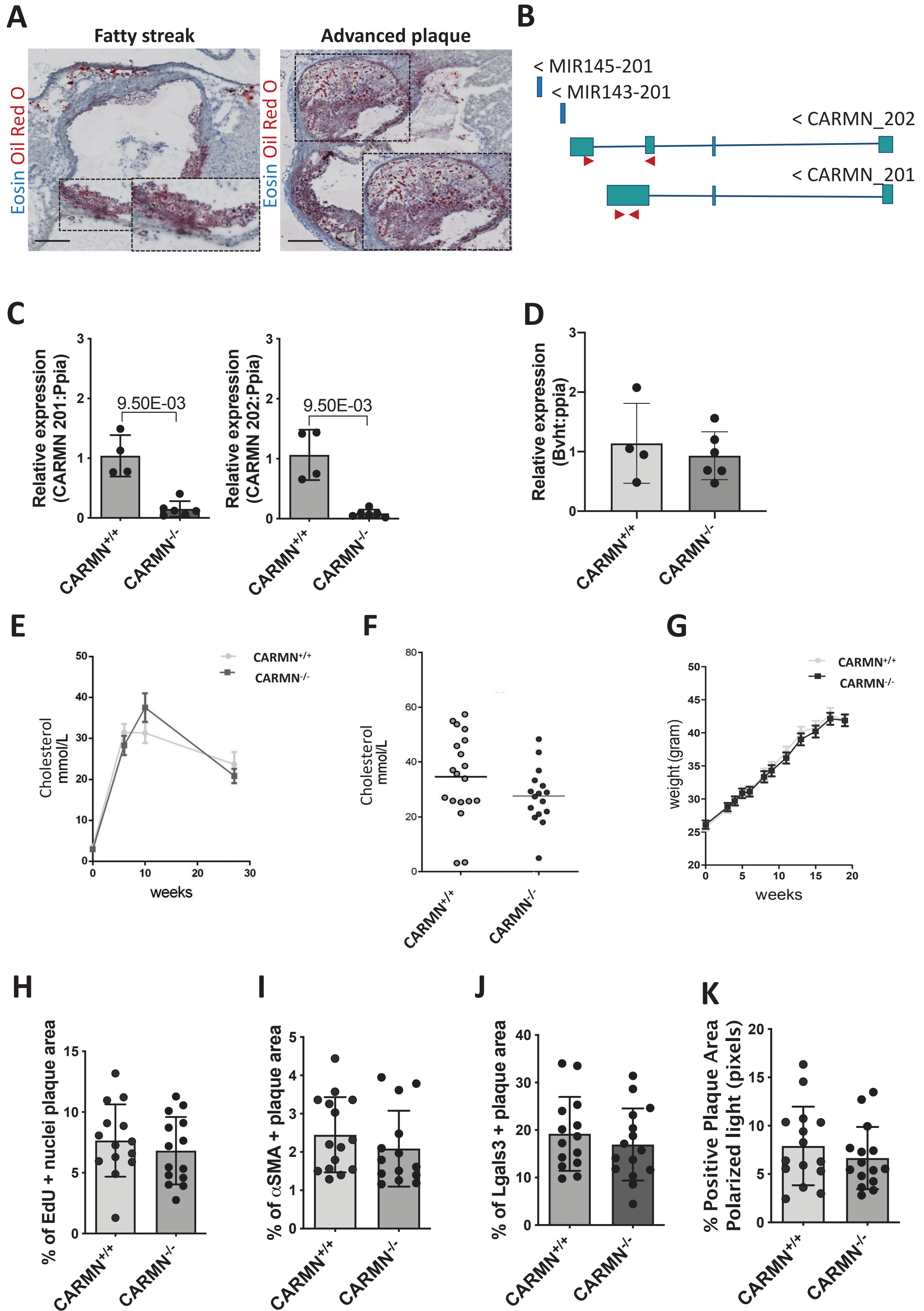

A
EdU% + cells
2.20E-02
Mock
GapCTR
GapCARMN2
B
Relative migration distance
7.00E-04
C
Mock
GapCTR
GapCARMN2
0h
10h
D
Relative expression (Acta2:UBC)
3E-06
0.2%BSA
CMBCD
E
Relative expression (CD68:UBC)
F
Relative expression (EGFR: UBC)
1.00E-06
GapCARMN
Gap+mimicCTR
Gap+mimic143/145
G
Relative expression (PDGFRa: UBC)
1.50E-05
H
CARMN
αSMA
CARMN/αSMA
CD68
CARMN/CD68

1 **Online Figure VI. Assessment of observed phenotypes GapCARMN2 and expression of**
2 **microRNAs target genes following co-transfection of GapCARMN with mimics.**
3 A) Graph showing the percentage of EdU positive hCASMCs (n=3) obtained by FACS analysis
4 following transfection with GapmeR targeting CARMN (GapCARMN2), Scramble GapmeR
5 (GapCTR) and un-transfected cells (Mock) in unstimulated cells (0.2% FBS). Data were
6 analysed with FlowJo software One-way ANOVA with Bonferroni multiple comparison test
7 was used to assess statistical significance indicated with p values. B), C) Quantification of the
8 relative migration distances and representative micrographs of hCASMCs (n=3) acquired at
9 10X (scale bar 100µm) at 0 and 10 hours post scratch assay following transfection with the
10 second GapmeR targeting CARMN transcripts (GapCARMN2), GapmeR control (GapCTR) and
11 un-transfected cells (Mock). The relative migration distance was obtained using ImageJ tool.
12 One-way ANOVA with Bonferroni multiple comparison test was used to assess statistical
13 significance indicated with p values. D), E) Expression levels of dedifferentiation markers
14 Acta2 and CD68 in hCASMCs (n=3) stimulated with CMBCD or in 0.2% BSA for 72 hours
15 following transfection with CARMN GapmeR (GapCARMN2), GapmeR control (GapCTR) or un-
16 transfected (Mock) cells. One-way ANOVA with Bonferroni multiple comparison test was used
17 to assess statistical significance indicated with p values. F), G) QRT-PCR data of EGFR and
18 PDGFRa in hCASMCs (n=3) at basal conditions following transfection with GapCARMN,
19 GapCTR and co-transfection with a combination of GapCARMN and mimic control
20 (GapCARMN+mimicCTR) or in combination with miR-143/145 (GapCARMN + mimic143-145).
21 UBC was used as housekeeping gene. One-way ANOVA with Bonferroni multiple comparison
22 test was used to assess statistical significance indicated with p values. H) Representative
23 pseudo-fluorescent and bright field images of in-situ detection of CARMN co-localizing with

1 CD68 or α-SMA signal in plaques obtained from carotid artery derived from symptomatic

2 patients at carotid endarterectomy. Pictures were acquired at 63X magnification and were

3 converted to pseudo-fluorescent using ImageJ software. Scale bar 10μm.


A
Fatty streak
Advanced plaque
Eosin Oil Red O
B
< MIR145-201
< MIR143-201
< CARMN_202
< CARMN_201
C
Relative expression (CARMN 201:Ppia)
9.50E-03
Relative expression (CARMN 202:Ppia)
D
Relative expression (Bvht:ppia)
CARMN+/+
CARMN-/-
E
Cholesterol mmol/L
weeks
F
G
weight (gram)
H
% of EdU + nuclei plaque area
I
% of αSMA + plaque area
J
% of Lgals3 + plaque area
K
% Positive Plaque Area Polarized light (pixels)

1 **Online Figure VII. The knock-down of CARMN does not affect the expression of Bvht at basal**
2 **conditions or circulating cholesterol levels and plaque composition in the aortic root of**
3 **atherosclerotic CARMN$^{-/-}$ versus CARMN$^{+/+}$animals.**

4 A) Oil Red O staining in aortic roots cross-sections isolated from LDLR$^{-/-}$ mice developing early
5 fatty streak and advanced plaque. Lipids are stained in red, nuclei in blue (eosin). Pictures
6 were acquired at 4X and 10X magnification, scale bar 100μm. B) Graphic representation of
7 mouse CARMN splice variants and pre-microRNAs miR-143 and miR-145 located in mouse
8 chromosome 18 based on the latest release of Ensembl 98. The scheme includes the position
9 of the primers used to specifically detect the two transcripts (red arrows). Arrows next to
10 transcripts name indicate the sense of transcription. C) QRT-PCR showing the expression of
11 mouse CARMN transcript variants in the aortic arches of CARMN$^{-/-}$ (n=6) and CARMN$^{+/+}$ (n=4)
12 animals at baseline. Values are normalized with Ppia housekeeping control. Mann-Whitney
13 was used to assess statistical significance indicated with p values. D) QRT-PCR data relative to
14 Bvht expression in the aortic arches of CARMN$^{-/-}$ (n=6) and CARMN$^{+/+}$ (n=4) animals at
15 baseline. Values are normalized with Ppia housekeeping control. E) Regression line showing
16 the levels of circulating cholesterol in the plasma of CARMN$^{+/+}$ (n=19) and CARMN$^{-/-}$ (n=16)
17 animals during the weeks of the experiment. Values are expressed as mmol/L. F) Circulating
18 levels of cholesterol at 10-weeks post AAV-PCSK9 injection measured in CARMN$^{+/+}$ (n=19) and
19 CARMN$^{-/-}$ (n=16) animals. Values are expressed as mmol/L. G) Regression line showing the
20 weight of CARMN$^{+/+}$ (n=19) and CARMN$^{-/-}$ (n=16) animals during the weeks of the experiment.
21 Weight is expressed in grams. H) Quantification of proliferating cells in the plaques developed
22 in the aortic roots of CARMN$^{+/+}$ (n=14) and CARMN$^{-/-}$ (n=15) animals. Values are expressed as
23 % of positive nuclei over the total cells counted in the plaque. I) and J) Quantification of the

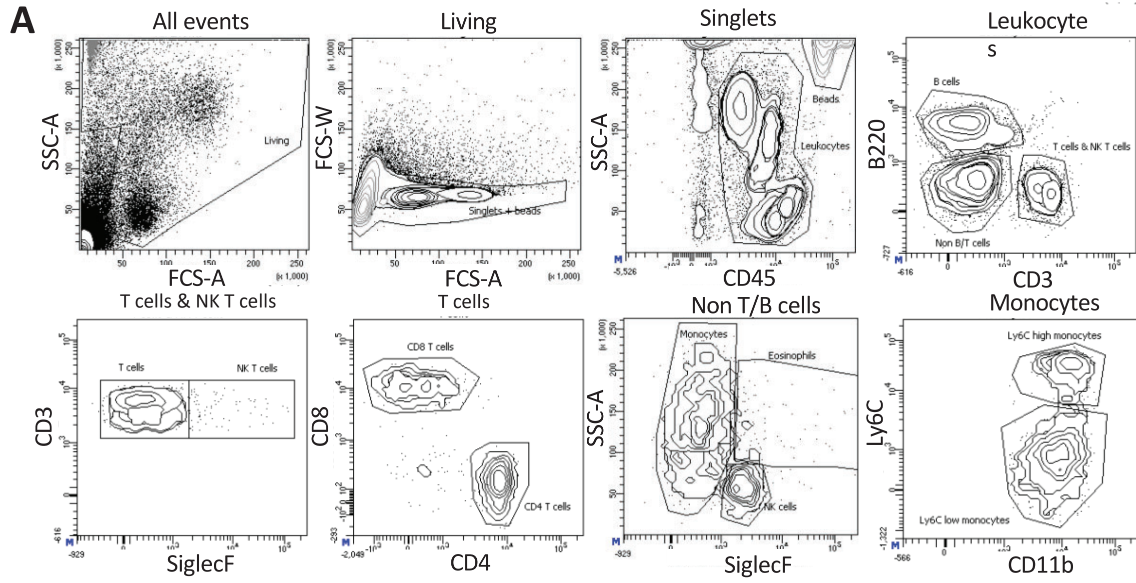
1 content of αSMA and Lgals3 staining respectively in the aortic root plaques of CARMN$^{+/+}$

2 (n=14) and CARMN$^{-/-}$ (n=15) animals. K) Quantification of collagen content in the aortic root

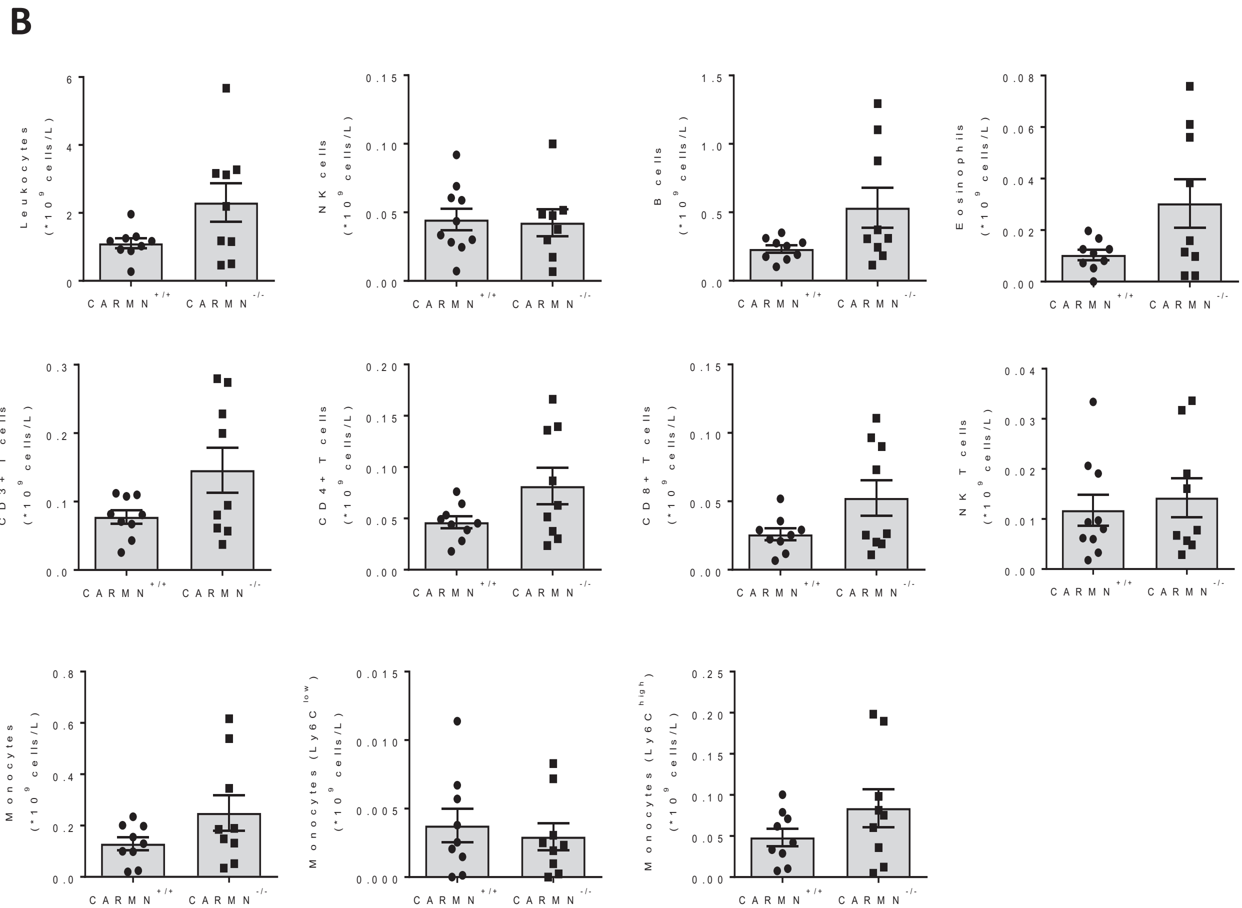
3 plaques of CARMN$^{+/+}$ (n=14) and CARMN$^{-/-}$ (n=15) animals. Mann-Whitney was used to assess

4 statistical significance indicated with p values.

A



B



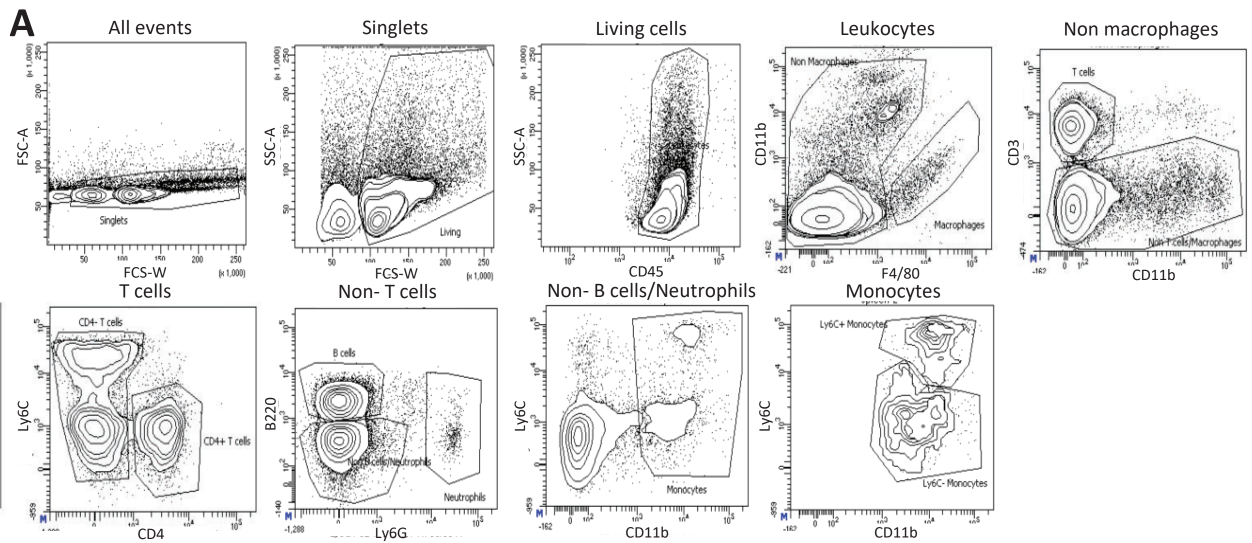
1 **Online Figure VIII. Quantification of blood lymphocytes subsets in CARMN$^{+/+}$ and CARMN$^{-/-}$**

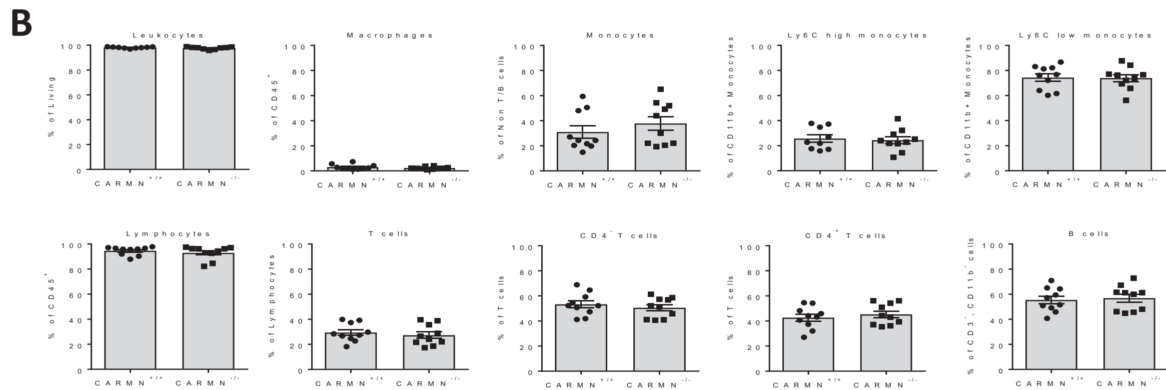
2 **animals.**

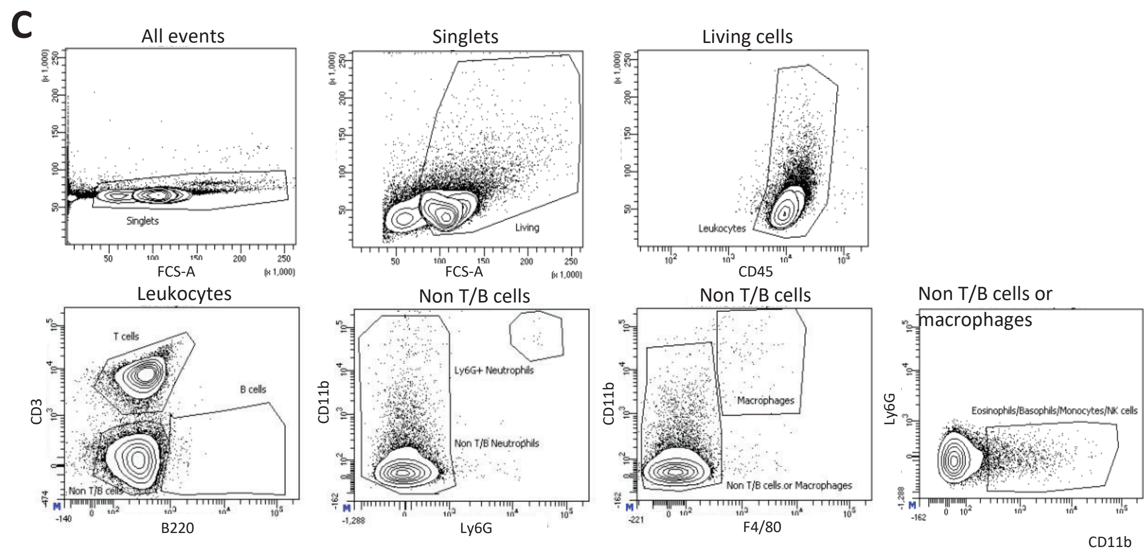
3 A) Gating strategy of flow cytometry analysis for blood TruCount. B) Quantification of blood

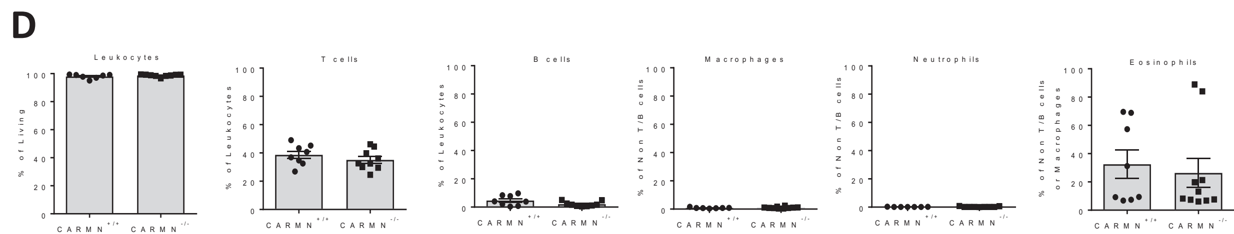
4 lymphocytes subsets.

5


A
All events
Singlets
Living cells
Leukocytes
Non macrophages
FSC-A
FCS-W
SSC-A
CD45
CD11b
F4/80
CD3
Singlets
Living
Non Macrophages
Macrophages
T cells
Non T cells/Macrophages
T cells
Non- T cells
Non- B cells/Neutrophils
Monocytes
Ly6C
CD4
B220
Ly6G
CD11b
CD4- T cells
CD4+ T cells
B cells
Neutrophils
Monocytes
Ly6C+ Monocytes
Ly6C- Monocytes


B
Leukocytes
Macrophages
Monocytes
Ly6C high monocytes
Ly6C low monocytes
Lymphocytes
T cells
B cells
% of Living
% of CD45+
% of Non T/B cells
% of T cells
% of Lymphocytes
CARMN +/+
CARMN -/-


C
All events
Singlets
Living cells
Singlets
Living
Leukocytes
FCS-A
CD45
Leukocytes
Non T/B cells
Non T/B cells
Non T/B cells or macrophages
CD3
CD11b
Ly6G
T cells
B cells
Non T/B cells
Ly6G+ Neutrophils
Non T/B Neutrophils
Macrophages
Non T/B cells or Macrophages
Eosinophils/Basophils/Monocytes/NK cells
B220
Ly6G
F4/80
CD11b


D
Leukocytes
T cells
B cells
Macrophages
Neutrophils
Eosinophils
% of Living
% of Leukocytes
% of Non T/B cells
% of Non T/B cells or Macrophages
CARMN +/+
CARMN -/-

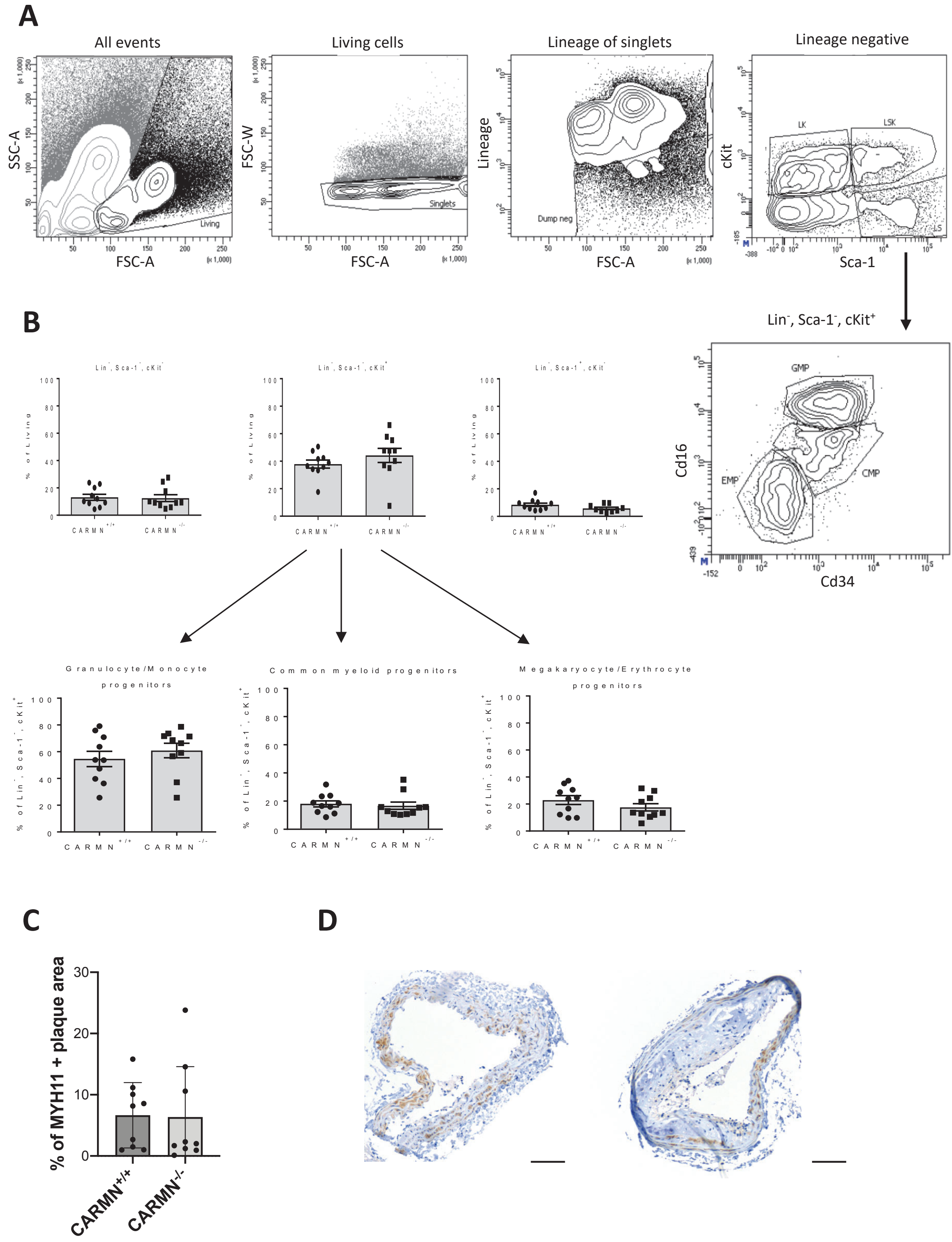
1 **Online Figure IX. Quantification of immune subsets in spleen and lymph nodes of CARMN$^{+/+}$**

2 **and CARMN$^{-/-}$ animals.**

3 A) Gating strategy of flow cytometry analysis for spleen. B) Quantification of immune subsets

4 in murine spleen. C) Gating strategy for lymph node FACS. D) Quantification of immune

5 subsets in murine lymph nodes.


A
All events
Living cells
Lineage of singlets
Lineage negative
SSC-A
FSC-A
FSC-W
Lineage
cKit
Sca-1
Living
Singlets
Dump neg
LK
LSK
LS
B
Lin⁻, Sca-1⁻, cKit⁺
GMP
CMP
EMP
Cd16
Cd34
Granulocyte/Monocyte progenitors
Common myeloid progenitors
Megakaryocyte/Erythrocyte progenitors
C
% of MYH11 + plaque area
CARMN+/+
CARMN-/-
D

1 **Online Figure X. Quantification of lineage negative cells of bone marrow progenitors and**
2 **Myh11 plaque content in CARMN$^{+/+}$ and CARMN$^{-/-}$ animals.**
3 A) Gating strategy of flow cytometry analysis for bone marrow progenitors. B) Quantification
4 of lineage negative cells consisting of: Lineage-, Stem cell antigen-1 (Sca-1)-, cKit- cells (Lin^{-},
5 Sca-1^{-}, cKit^{-}), Lineage-, Sca-1+, cKit- (Lin^{-}, Sca-1^{+}, cKit^{-}) and Lineage-, Sca-1-, cKit+ (Lin^{-}, Sca-1^{-},
6 cKit^{+}). Lower panel displays the different progenitor populations originating from Lin^{-}, Sca-1^{+},
7 cKit^{-} cells. C), D) Quantification of the positive area for Myh11 staining and representative
8 immunostaining pictures in the plaque of CARMN$^{-/-}$ (n=9) and CARMN$^{+/+}$ (n=9) animals.
9 Pictures were acquired at 10X magnification. Scale bar 100µm.