SUPPLEMENTAL MATERIAL

Long non-coding RNA *MIAT* controls advanced atherosclerotic lesion formation and plaque destabilization

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Supplemental Methods

Laser Capture Micro-dissection of Advanced Atherosclerotic Carotid Artery Plaques

Carotid atherosclerotic lesions from the Munich Vascular Biobank[18] were paraffin-embedded, sectioned, and stained with H&E on RNase free glass slides as published before[19,20]. Briefly, the selected sections were pre-treated with UV light (254nm) for 30 min to stimulate adhesion of the paraffin-embedded sections as well as to overcome the hydrophobic nature of the membranes. Five to ten consecutive slides per individual patient were micro-dissected and pooled to assess changes in mRNA expression. In preparation for the laser pressure catapulting procedure, sections were initially de-paraffinized with xylene (2 x 2 min) as well as decreasing ethanol concentrations (100%, 96%, and 70% each for 1 min). Afterwards, sections were washed in RNase free water and stained for 10 min with Mayer's hematoxylin (Sigma-Aldrich, Saint Louis, MO, USA), again washed for 3 min in RNase-free water, and then serially stained for 3 min with eosin (Sigma-Aldrich, Saint Louis, MO, USA). Finally, samples were dehydrated with increasing ethanol concentrations, before briefly being air-dried at room temperature. Upon microdissection and catapulting, the fibrous cap sample (see Figure 1c) was collected into AdhesiveCaps (Zeiss, Oberkochen, Germany), and 350 mL of RLT buffer (Qiagen, Hilden, Germany) was added and mixed by inversion after closure. The lysate was centrifuged for 5 min at 13,000 rpm and stored at -80°C. RNA extraction from catapulted, micro-dissected samples was performed using the RNeasy Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA was quantified by Nanodrop (Agilent Technologies, Santa Clara, CA, USA) and RNA quality verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples required 260/280 ratios > 1.8 and sample RNA integrity numbers > 5 for inclusion. The Taqman High-Capacity cDNA Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for cDNA synthesis, and primer assays for mRNAs and RPLPO (endogenous control; both from Thermo Fisher Scientific, Waltham, MA, USA) were used to detect changes in expression levels.

RNAscope

Co-localization analysis of MIAT and αSMA/CD68 (Abcam, Cambridge, UK) in human and murine tissue was performed using RNAscope Multiplex Fluorescent v2 Assay combined with immunofluorescence staining (ACD, Bio-Techne, technical note 323100). Control slides, positive and negative control probes were used to establish the protocol. All reagents/buffers were prepared with RNAse free (DEPC treated) water. Mouse tissue pre-treatment: Fresh frozen, OCT embedded murine carotid arteries were cut 10 µm and fixed with 10% NFB for 15 min, dehydrated, blocked with Hydrogen Peroxide for 10 min and then digested with Protease IV for 30 min. Human tissue pre-treatment: FFPE human carotid artery lesions were cut into 3 µm, deparaffinized, dehydrated and blocked with Hydrogen Peroxide. Afterwards, standard Antigen Retrieval (15 min) and standard Protease Plus (30 min) treatment was performed. 2-3 drops of the appropriate MIAT and negative control probe were added to the slides and incubated at 40°C for 2h in an oven (Boekel Scientific, Feasterville-Trevose, US). Next, signal amplification and development of HRP by using fluorophore Opal 690 (Akoya Bioscience, Marlborough, US) were performed according to the manufacturer's instructions. Then, the protein staining protocol was initiated. Unspecific binding was prevented by a 1h incubation step in blocking buffer (TBS buffer, 1% BSA, 10% normal goat serum). Primary antibodies αSMA/CD68 were incubated overnight. On the next day, a HRP-secondary antibody was added for 1h, following an incubation with Opal 520 dye (Akoya Bioscience, Marlborough, US) for 10 min. DAPI staining was performed and the slides were mounted with ProLong Gold antifade (Thermo Fisher Scientific, Waltham, MA, USA), sealed with clear nail polish, and stored at 4°C until imaging. Images were acquired with an Olympus FLUOVIEW FV3000 (Olympus, Tokyo, Japan) confocal microscope.

Cell culture and transfection of human carotid smooth muscle cell

Primary human carotid smooth muscle cells (hCASMCs) were obtained from PeloBiotech (#PB-3514-05a) and cultured in Smooth Muscle Cell Growth Medium (PeloBiotech, Munich, Germany), following manufacturer's instructions. THP1 cells were a gift from the Vascular Surgery Unit at Karolinska Institutet (Stockholm, Sweden) and cultured in RPMI Medium + 10% FBS + 1% PenStrep at a density of

400.000 cells/ml. Cells were seeded into the respective flasks/dishes at a density of 100.000 cells/cm2 with 100 ng/ml PMA for 24h to differentiate them into M0 Macrophages. After 24h, medium was changed and the cells were harvested, fixed, or treated.

Transfection of antisense oligonucleotides (ASOs) was performed by using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) mixed with anti-*MIAT* GapmeR (Qiagen, Hilden, Germany) or scrambled controls (Qiagen, Hilden, Germany) for a final concentration of 20 nM. Three different custom anti-*MIAT* GapmeRs were tested for knockdown efficiency and the most potent one (*MIAT* KD2) was chosen for experimental studies. The sequence of anti-*MIAT* GapmeR is: 5'-ACGGGTTAGTAATCGA-3'. For plasmid transfection, the Lipofectamine 3000 (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) transfection protocol was adapted as follows: 250ng of DNA + 0.5 µl of Lipofectamine 3000, for a 12-well multi-well format. Human *MIAT ELK1 BS 1-2* plasmid was obtained from gene synthesis (Eurofins, Ebersberg, Germany) and built on a pcDNA3.1 HisC backbone (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA). The design was based on *in silico* prediction of RNA-protein binding sites by *RegRNA 2.0*[27]. A detailed list of all *MIAT* constructs´ sequences is reported at the end of the Supplementary Materials and Methods section.

RNA immunoprecipitation (RIP)

RNA-Immunoprecipitation was performed using the Magna RIP 17-700 Kit (Millipore, Burlington, MA, USA), following manufacturer's instructions. All steps, including centrifugation, were performed at 4°C. Briefly, THP1 cells were treated with 50 µg/ml oxLDL (Thermo Fisher Scientific, Waltham, MA, USA) for 48h, subsequently washed twice with ice cold PBS and lysed on ice in RIP Lysis Buffer, implemented with RNAse and Protease-Inhibitors. hCASMCs were transfected with *MIAT ELK1 BS1-2* (in 175cm^2 flask with 30ul Lipofectamine 3000 and 7500ng of DNA) and after 48h lysed as described above. Insoluble material was removed by centrifugation at 12.000 rpm and cleared lysates stored at -80°C until RIP was performed. 50 µl of magnetic beads were washed and incubated with 5 µg of specific antibody (for macrophages: anti-NFKB (ab175192, Abcam); for hCASMCs: anti-ELK1 (ab125085, Abcam)) or IgG

control (Millipore, Burlington, MA, USA) for 30 min with mild rotation at room temperature. Antibody/IgG-coated beads were further washed and eventually incubated with equal volumes of cell lysate overnight on a rotary platform at 4°C. About 10-20% of total lysate was kept as input. Beads-antibody-lysate complexes were washed 6 times and finally submitted to protein and RNA analysis.

RNA quantification and gene expression

Total RNA was isolated with a Qiazol-based (Qiagen, Hilden, Germany) RNA isolation protocol, by taking advantage of miRNeasy Micro Kit (Qiagen, Hilden, Germany). RNA was quantified by NanoDrop (Wilmington, DE, US) and RNA and miRNA quality were verified with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples required 260/280 ratios of >1.8 and sample RNA integrity numbers of ≥9 for inclusion. DNase (Qiagen, Hilden, Germany) was applied to avoid artifacts deriving from plasmidic/genomic DNA contamination. First strand cDNA synthesis was performed with the High-Capacity-RNA-to-cDNA Kit (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions, starting from equal amounts (or, for RIP, equal volumes) of purified RNA. Quantitative Real-time PCR (qPCR) reaction was performed on StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) or on QuantStudio3 Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA) by using Sybr-Green PCR Master Mix (Roche, Basel, Switzerland) or TaqMan™ Fast Advanced Master Mix (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA). Oligonucleotide sequences/Taqman assays used in this paper are listed in the Data Supplement Table IV below. Amplified transcripts were quantified by using the comparative Ct method, and relative gene expression calculated by the method of ΔΔCt[69]. and are expressed as mean ± SEM. All experiments included at least 3 replicates per group.

Kinetic assessment of proliferation and apoptosis in human CaSMCs

The IncuCyte Zoom System (Sartorius, Göttingen, Germany) was utilized for real-time assessment of the hCaSMCs status as previously described by our group[20,28]. Cells were transfected with anti-MIAT GapmeRs (20 nM) or *MIAT ELK BS1-2* (250 ng/well

in a 12 multi-well format). A Caspase 3/7 Apoptosis reagent (Sartorius, Göttingen, Germany) was added at a final concentration of 5 µM before the plate was monitored in the IncuCyte with phase/fluorescence and a 2h/imaging pattern. Images were auto-collected and analyzed using the IncuCyte software package.

Protein isolation and Western blotting

Cells were homogenized in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) including protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Saint Louis, MO, USA). Protein concentrations were determined by using the Bicinchoninic Acid assay (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. Protein samples (5-10µg/well) were loaded on 4-15% Tris-Glycine gels. Following electrophoresis and electrotransfer, blots were blocked with 5% milk in Tris-buffered saline + 0,1% Tween-20 and probed with specific antibodies diluted in blocking solution. Signals were revealed after incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam) 1:10000 or with Veriblot HRP (Abcam, for RIP experiment) 1:1000, in combination with ECL (GE Healthcare, Chicago, IL, USA). Antibodies used: #4370 p_ERK, 1:2000 in 5%BSA-TBST, #4695 t_ERK 1:1000 in 5%BSA-TBST, #12173 KLF4 1:1000 in 5%BSA-TBST (Cell Signaling, Danvers, MA, USA). B-actin A1978 (Millipore, Burlington, MA, USA), 1:8000 in 5%BSA-TBST and Vinculin V9131 (Millipore, Burlington, MA, USA), 1:1000 in 5%BSA-TBST were used as loading control. For RIP experiments, immunoprecipitation efficiency was monitored by loading 10% of IP/ IgG fractions on NuPage™ 3-8% Tris Acetate-Gel. Prior to loading, samples were mixed with LDS 4X Sample Buffer and Sample reducing agent (Thermo Fisher Scientific, Waltham, MA, USA), and incubated at 95°C for 5 min for protein denaturation. Image detection was performed with C600 Azure Biosystems Imager (Biozym)/ChemiDoc XRS System (Bio-rad, Hercules, CA, USA). Image quantification was done using ImageJ software.

Cell fractionation

Nucleocytoplasmic fractionation was performed as previously described in Wang *et al*[70]. Fractions were extracted from confluent hCASMCs cultured in T75cm2-flasks (Corning, Corning, NY, USA) and RNA isolated using Qiazol™ reagent, as previously

described. The purity of the nuclear and cytoplasmic fractions was confirmed by real-time quantitative PCR on *GAPDH*/*RPLPO*/*ß Actin* and *NEAT1*, respectively[69].

Murine inducible carotid artery plaque rupture model

All animal protocols were approved by the North Stockholm Animal Ethics Committee and followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). To investigate the role of *Miat* in plaque rupture in atherosclerosis prone mice B6.129P2-Apoetm1Unc/J mice (denoted *ApoE*$^{-/-}$) were purchased from Taconic Biosciences (Silkeborg, Denmark). These mice were subjected to the inducible carotid artery plaque rupture model which has been previously described[19,20]. Briefly, male mice at age of 12-14 weeks received an incomplete ligation (Vicryl 5-0 suture, Ethicon Endo-Surgery Inc, Blue Ash, USA) of the common right carotid artery (proximal to bifurcation) for 4 weeks, triggering intimal hyperplasia and stable carotid atherosclerotic lesion development. Then, to provoke rupture of the developed plaque, a conical polyethylene cuff with diameters of 300 and 150 μm (Promolding BV, The Netherlands) was placed proximal to the ligation site for an additional 4 days, to induce fibrous cap collagen degradation. All animals were anesthetized with 2:1 isoflurane:O_2. Subcutaneous injection of Buprenorphine (0.1mg/kg) was applied before and after surgery for pain relief for both ligation and cuff placement. At day 32 the mice were sacrificed the carotid arteries were embedded in O.C.T and fresh frozen before further immunohistochemistry preparation and morphological analysis.

To investigate the effect of a deficiency of *Miat* on plaque rupture male *Miat*-deficient mice were used. *Miat*-deficient mice (originally denoted Gomafu CDB1347K) on C57BL/6N background were a kind gift from Professor Nakagawa at RIKEN Center for Life Science Technologies, Kobe, Japan[51]. For the inducible plaque rupture model *Miat*-deficient mice with the loxP-PGK-Neo cassette removed were intercrossed with B6.129P2-Apoetm1Unc/N (Purchased from Jackson Laboratories and backcrossed to C57BL/6N for 10 generations) and are denoted *Miat*$^{-/-}$*ApoE*$^{-/-}$. Age mated (10-13 weeks) male mice were then subjected to the inducible plaque rupture model as described above.

Murine carotid ligation model

Miat-deficient mice containing the loxP-PGK-Neo cassette (as above) were maintained on a C57BL/6N background with heterogenous breeding and are denoted *Miat*$^{-/-}$. Male mice at 12 weeks of age were subjected to ligation of the right carotid artery at the bifurcation with 7-0 sutures as described previously[19]. The animals were anesthetized with 2:1 isoflurane: O_2. Subcutaneous injection of Buprenorphine (0.1mg/kg) was applied before and after surgery for pain relief. 28 days after ligation, the mice were sacrificed, and the carotid arteries were embedded in O.C.T and fresh frozen before further immunohistochemistry preparation and morphological analysis.

Tissue histology and immunohistochemistry (human and mouse)

Mouse OCT-embedded frozen tissue was cut into 6 µm thick slides, dried and stored at -80°C for further analysis. Human carotid arterial plaque material was sampled during carotid endarterectomy fixed for 48 hours in 4% paraformaldehyde at room temperature, paraffin-embedded and cut into 5 µm thick slides. Per carotid plaque specimen, 4 slides were stained with hematoxylin and eosin (HE). Plaques were classified as ruptured or stable according to their histomorphologic AHA classification[71] and critical fibrous cap thickness as described by Redgrave *et al*[72]. Ten whole plaque transverse sections from ten different plaques per group (ruptured or stable) were selected for staining. Control arteries (carotid, radial, iliac, or mammary) were obtained from deceased organ donors without any reported history of cardiovascular disease. HE stains were used for basic tissue morphology. Lipids were stained with Oil Red O (Sigma Aldrich, Saint Louis, MO, USA). A subset of sections was stained with primary antibodies and developed by using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA USA), and finally counterstained with Hematoxylin (Sigma-Aldrich, Saint Louis, MO, USA) for immunohistochemical analysis. A second subset was stained with primary antibodies (Biotin, Ki-67, NF-κB, Caspase3, cross-linked Fibrin antibody[19,20] followed by fluorescent secondary antibodies (Alexa Fluor 488/546 goat anti-rabbit/mouse IgG) and counterstained with DAPI (Sigma-Aldrich, Saint-Louis, MO, USA) for immunofluorescent analysis. All histological analyses were at room temperature using Leica Microsystems (Wetzlar, Germany; Fluorescence Light DM4000B and Confocal TCS SP8) microscopes and

analyzed by Leica Imaging Software (Leica, Wetzlar, Germany). αSMA/CD68-positive cell counts (4 high power fields in 10-15 different sections per experimental group) in wild-type or *Miat*-modulated mice were analyzed with the NDP.view2 software (both Meyer Instruments, Hamamatsu, Japan). Image analysis was performed with AZ AnalyserII software (Keyence), as well as Imaris (Oxford Instruments, Zurich, Switzerland) and Fiji ImageJ for high power field (HPF) analysis of cell counts.

Mouse plasma cholesterol and triglyceride measurements

Triglyceride levels were detected using reagents from Randox (Kearneysville, WV, USA) and by following the manufacturer's protocol. To measure cholesterol, a colorimetric HDL and LDL/VLDL Cholesterol Assay Kit was utilized (Abcam, Cambridge, UK).

Mouse primary aortic smooth muscle migration and apoptosis assay

Aortic SMC originating from 6–8-week-old age and sex matched mice were isolated as published previously by others[9]. In brief, 3 aortas from the same genotype (*Miat*$^{-/-}$ or *Miat*$^{+/+}$) were pooled with 3 replicates (total 9 mice per genotype). Cells were propagated in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Thermo Fisher, Waltham, MA, USA), 100 U/ml penicillin/streptomycin (Gibco) and passaged three times before undergoing further studies. For migration studies cells were grown to 90% confluency in IncuCyte ImageLock 96-well Plate (Satorius, Göttingen, Germany), a wound was then created through the SMC monolayer using the IncuCyte WoundMaker, and cells were scanned every 2 hours for total 24 hours. Apoptosis rate of the cells was determined by seeding 2.4 x 10^4 cells per well in 48 well plate and immediately before imaging Annexin V, Alexa Fluor™ 594 conjugate (Thermo Fisher Scientific, Waltham, MA, USA 1 to 100 dilution) was added. Cells were then imaged every two hours for up to three days.

***In situ* hybridization (ISH)**

Qiagen miRCURY locked nucleic acid DIG (digoxigenin)-labeled probes (human MIAT 5'-3' sequence: /5DigN/TCGGAGGCTCAGAGGTGAAGTA/3Dig_N/; mouse MIAT 5'-

3' sequence: /5DigN/TACTGGAGGTGAGGCATGAAAT/3Dig_N/) with the accompanying kit and protocol (Qiagen, Hilden, Germany) were used for ISH. In brief, tissue sections were either de-paraffinized (formalin-fixed paraffin embedded) or thawed (frozen) and rehydrated. Nucleases were permeabilized with proteinase K followed by a 2-hour hybridization at hybridization temperature (54°C for *MIAT*). Slides were washed in saline–sodium citrate buffers with subsequent DIG detection methods as previously described[28]. Nuclear counterstaining was performed with Nuclear Fast Red (Sigma-Aldrich, Saint Louis, MO, USA).

Oxidized LDL stimulation and uptake by macrophages

Lipoproteins were isolated from human plasma and oxidized with copper sulfate $CuSO_4$ (Merck, New Jersey, USA) as described previoulsy[73]. Human monocytes were isolated and differentiated into macrophages[74]. Briefly, human peripheral blood mononuclear cells were isolated from buffy coats by endotoxin-free Ficoll density gradient centrifugation. Monocytes were separated from lymphocytes and platelets by high density hyper-osmotic Percoll density gradient centrifugation and low-density iso-osmotic Percoll density gradient. Then, monocytes were cultured in RPMI-1640 medium supplemented with penicillin-streptomycin, L-glutamine (2mM) and 5% FBS (all purchased from Thermo Fisher Scientific, Waltham, MA, USA). The cells were seeded in 6-well plates at a density of 0.8×10^6 cells/well and differentiated in the presence of recombinant mouse MCSF (100 ng/ml; PeproTech Nordic, Stockholm, Sweden) over seven days. Human macrophages and mice intra-peritoneal derived macrophages were seeded for oxLDL challenging and uptake capability monitoring. To investigate the mechanism, 9 μM of NF-κB nucleus translocation inhibitor SN50 (Merck, New Jersey, USA) was added to cultured macrophages for 15min before oxLDL loading.

Luciferase reporter assay

The luciferase reporter assay was performed as described previously[28]. In brief, transcriptional activity of KLF4 was confirmed by transfecting human or mouse aortic SMCs with pLS-Klf4/KLF4 promoter (or promoter flanking region without predicted *Miat* binding site as control) luciferase vector (Active Motif, Carlsbad, CA, USA), with

or without oxLDL treatment, *MIAT* knockdown (KD) or overexpression (pCAG-*Miat* vector[52]; data presented in Figures 6e,f) using DharmaFECT Duo Transfection Reagent (Active Motif). After a 24-hour transfection period, luciferase activity was quantified using the LightSwitch Luciferase Assay Kit (Active Motif) according to the manufacturer's instructions. *In silico* prediction of lncRNA-DNA binding motifs (presented in Figure 6d) was performed using LongTarget v2.1[39].

Porcine atherosclerotic model

Six Yucatan LDLR$^{-/-}$ mini-pigs were purchased from Exemplar Genetics, IA, USA. The pigs were fed a high-caloric Western style diet once being 6 months of age. Three pigs were sacrificed at 12 months of age (6 months of HFD) and the other three at 18 months of age (12 months HFD). Plaque development in carotid arteries from pigs were assessed using standard histo-morphological analysis with H&E similar to the protocols used in human and mouse, as well as antibodies from Abcam (Cambridge, UK) for IBA-1 and αSMA.

Plasmids sequence list

hMIAT refseq: NR_033320.2

MIAT ELK BS1-2

GTTAAGTGACGGGGATAGAAGCTGTCCTGCACAGGAAGTCACGAGGGGGG
CGTATCCCACGAGGAAGGCAGGAGGGGGCGTGCCCCTCACCGGAAATTAG
CAGAGGGGCGTGTCCCACACCGGAAGTCAGAAAGCGGAGCCTTTCTTACA
CCGGAAGTCAATGAAGCGGGTCTTTCCTACGCTAAAAACCACTGAGTGGA
GTATTTAGTACACAGGAAGTCGGCCAGAGAAACATTTCTCATATTTGAAG
GCCGGAAAGAGGGACATTTCTGACACCGGAAGTCAGTGAGAGGACTCTTT
CCCACACAGGAAGTCAGCTAGAGAGCCGTCTCCCCTCTCTGGAGCCGAGA
GAGGCCGGTTTCCCCCACCGTAAGTAGACGTGGGGCCGTGACCGGAAGTC
CTTGGGAAAGATCCGTCCCATTCCCGGAAGCTAGAGGGCGTTAGTTGTCG
GGTTGAAAAGGGGTGTGGGGAGGGGAAGCAGCTTTACCCCGGGCTCGGAG
TTTGCAGGAGAGAGAAGTGGGGAGCAAGAAGTGAACCTCAGGGGCTCACA
GGGTTCCCGCAGATGCTCAGGCCGGCCAGGAATGCATCTCTGGCTCTCTG
TTCCCACGGACGTCACTGCCTCAGCCAGCCTCCCCCAGAGCCCGCCAGCC
GCTAAGCCGGGGCCACACCTGGGGGTGATTTCATGCCTCACCTCCAGTAG
GCACCTTGGTTTCTTTGGGCTAATCTCTGGCTCCCTTGCGCTAACTCTTG
CTCTCACCCAGCTAATCCCTGCCTCACCCTGACTGCCCCAGGGGCTGACC
ACTAACAACCAACCTGGCCCTGTCTGGGGGTTCCAGGCTCCTGGCCTGGC
CCTGACCGGTTCTTAATTAACCTTTCCTTCACCTTGACTAACTCCTGCCT
TCCTGGTCTGTTCCTTTCAGCAGAAACTAATGGTTTGTGGATTTTTTTCT
GACTAACAACAGGTCTAACATTCCTCGTTACTGTTAACAGCTTGGATGTC

GGCATGGCTGGGAAGGGGCTAACACAGCTTTGAACTTGGCTAACACAGGT
TTGAACTTGGCTAACACAGGTTTGAACTTGACTAACACAGGGAAAAGCAT
AGCTAACAATTTTGGGCGTGGTGGCTGCTCTGAGTCAGAACAATCAGAAG
TCGGTAAAGATGGTAGTTTTCTAAAGGAGGTGCCAGGGCTCTGGTGTGGA
CCAGGCCTGATGGAGCAGTGGTACCCACCAAGGTGGGGTCAGAAGTATAG
CCAGTCTTGCAAGGTTTTGGCCATTGGGCATATCTTCACTCCTCATAGTC
TGCATTTGGTTTCAGTTCTTAAAAAAATATAGCCTTATAGCTACAGTAGT
TTGCACAAGTAGATGCAGCTCTTATAAACCTTAAAATACCTGTCTGGTGT
CTACAGAGCATATGCCATTTTGTATAGAGTCACCCTTCCCCAGGCCAGGG
CCTAGAGTCTTCATTTTGGGGACTTTGTGTTTTGGAAGTTCTAGGACATA
AAGCTTTAGATCAGAGTATTCAGAAGGGTTATAACTCCGTCAGTCATTTA
CATGTTAGTAGTATAAATTATCTGAGCTTCCTGTTCTAACTTTTAGCTCT
TCTAGCATAAACTCTTCTGTGCAATTTAGCTGCACCGAGGAAACGGGAGT
TTTTCTGGAAGGGACTTTTGATCTCTTTAGACTGAGGGAACGTCCTTTGG
GAGTAGAGGGGCAGGGAGCATACGCAAGGGATTCCAGGTGCAGGTAAAAG
GTGGCACTAGTTCAAGGTTTTGCTGACTCAGTCTGGTAGTCAGAGTCTGC
AGGAGAAGACAGTTCAAGGCAGGGCCTGGAGGATTGGATCAGTTTAGGGA
CAGGTCAAAGGCTGGCTTACAGACCTTAGAGGCAGGTTGCTTGGGTCGTT
GAATGCTAGTCTGGTGCTGAGAGCCCTTTTCTCTGGCAACTGTGGACTCA
GAGCTAACCAATTGTAGTTGGCAGTGGGGGTGAAGGGTGATCCAGAGGCC
TGAGCTGCAGAGGGCACAAGAGAGAAAAGATGTCTTAGAAAGAGCTTTGA
GAACATGCCTTGGCTGCTGGCAGGGACCTTGGATGGGGTAGTCTACACCC
GGAAGTGCCTGCCTGCCATCCTCTAGTGGCTGCCTTGCTCCATTTCACTC
AAAGCAGGAAGCTCACACCTCCTATTCCTGAAACTCCTCTTTGTTTAACT

Klf4 promoter_*Miat* (mouse, luciferase reporter assay construct)
GGAAGTCGCTTCATGTGAGAGAGTTCCTCACGCCAACGCTGCAAGAACAGGGAG
GGAGAGAGAAACTGTTAATTGTAGCACCCTGTTGCCACCCCTAATATTGACTTGTT
GGGTGAAGCACACCAACACCCTGCTATAGGAACTGGACAGAACAGGAAGGCAGC
CTCGGGGACTCCAGGGGCCACACTGCAATCTCAGCTAACACCAAGGTAAGAGGT
GGCGCGTTCGTTCTCCTGCAAGCTAGTGCCGTCGCTCCACCAGCCCAGTCCTCT
TCCCCGCCAGATCCGCGGGACGCTCCAGCCGCCCACGGAATTGGGACACTGGG
ACACGGTGGCTTTCTCCCCATGCACCATAGCCACCGGGTCGCCCACGCGCTACG
ATCACTCCGGCCCAAAGGGTCTCGAGACTTGTCCACCTAAAATATAGTGGACTGA
GTGCGCCCACCCCACGGTCCGGACGCCCGAAGACTAGTGGGGAACCGCTGTGC
GCCCCTGGCGGTACCGCAGTGCCGGGACCACACGCACTTAAGGCCGACTCAAG
GGCCGAGGAGCCACCTCGGAAGGCTGTAGAGTTCCAATGGAGGGCAATCCTTGT
CCTAACCAAGGACTCTTGGTGACCCCCGGATTCCGCCCATACACCCCACCCCAT
CTGCAGAAATCTACAGGGCACTAGC

CTRL (control, promoter flanking region without predicted *Miat* binding site, mouse, luciferase reporter assay construct)
TGAACATTTTTCCATGTCATTAGCTTTGCTTTGAATATATTCTTGACCACATAAAATT
CCATCTTCCTGATGCACCTGTTTCCTTTTAAGCAAAGACACATGCTGTGGTTTCAAT
CACACTGGCCTCTTAAAAGTCAAAGAAACTACTTTGCCGATCTCCCATGAGCTCCT
TGTGAATCTACGTTTCTAAATTCCATTTATAAAGCATAATACGGTGTAAATACAACT
CCAGGCACTAGGTACAATGGATGGTTAATAAGCACTCTAAAGAATAAGCCAGTCA

GCTTCTGCTCCTTTCATCTTCTGAGTCACCCACACCACCATTAACATGTCTACAAG
TATCACACAGACTTTTCCATTTTGTGACAGCTAAAAATAGTTTTGCTTCAACCTGGA
GAGGTGGCTACCTCTCCAGGGTTTTGCTCAGTTGCACCCCACCACTGGGCTACA
CAGTCCTGTCCTTCAGGGTTTGAGTAATTTTTAAATTGTTTCCATGCCTTCAGAGAC
ATTCAGGAAGCTTTGAGATACTCTGTAGATAGTATATTTTGGATCTCTTTAGGGATA
ATATATGCAGCATAAATGAAGCCCTTAATCCTCAGGAGGGCACTGTGGAACTACAT
TCTGTTCTTAGGGTTGTTTGCCTGGGGAAGGTTCTTTTGTTACTTAGATTCTGTGGG
GACTACAAGGCCAGGCCTGCACAGAAAATGATTCATAATTTCATTTAAAGACAAAA
TGTAACATGTACATATACACACACGTTTGTGTGTGTATGTGTCTATGTGTATAAATA
TAAATGTATGAAACTAGAGTGTTCTGGCCTACTGTTTAGTGCTCCCTGCTAAAATTG
AGCAGATCAAAGAATAACTGAGCCACCTACCTGGAAATGTGCGTTTGGGTAAGGA
GGTATACTTCTGCATTCGTACTAAACAAACATGAACCAGATGTTTTTTAAGGTCCCA
TCCGTACAGCTCTCAACTCCAACACTACTTCATGTAAAACTGAGTAGTCTAAAAAA
GCAAGTTGCCTACATCCTCTTGCTAAGGTAAGGTTGGCTTCACAGTGTGGTTCTCA
CCAGTGGCCCCCCCCCCCCCCCCGTTAAAAAGTTCTTTTCCAGTTTCGAAAAAAA
AAAACAAAGAGATTGTC

References

See Main Manuscript.

Supplemental Tables

Table I: Patient demographics for tissue specimens included into the carotid plaque microarray analysis from the Biobank of Karolinska Endarterectomies (BiKE). P-value: *<0.01.

GENERAL	asymptomatic	symptomatic	P value
number of patients	40	87	
age (years, mean)	66,4	72,52	0.0002*
gender (male/female)	39/1	61/26	0.0003*
BMI (mean)	27,5	24,67	ns
smoking	19 (47.5%)	42 (48.28%)	ns
SYMPTOM	Not applicable		Not applicable
minor stroke (MS)		32 (36.78%)	
transitory ischemic attack (TIA)		29 (33.33%)	
amaurosis fugax (AF)		26 (29.89%)	
TIME (from symptom to surgery)	Not applicable		Not applicable
days <15		15 (17.24%)	
15 to 30		24 (27.59%)	
>30		48 (55.17%)	
THERAPY			
lipid lowering drugs (ezetimib, HMG-CoA reductase inhibitors)	32 (80.00%)	74 (85.06%)	ns
antidiabetics	10 (25%)	20 (22.98%)	ns
antihypertensives (ACE inhibitors, beta-blockers, diuretics, angiotensin II blockers)	34 (85%)	76 (87.36%)	ns
COMORBIDITIES			
previous myocardial infarction	6 (15%)	20 (22.98%)	ns
inflammatory diseases (rheumatism, psoriasis, multiple sclerosis, inflammatory bowel disease)	1 (2,5%)	3 (3.44%)	ns
hypertension	31 (77.5%)	75 (86.21%)	ns
diabetes	10 (25%)	22 (25.28%)	ns
LAB MEASUREMENTS (mean)			
serum creatinine (mg/dl)	89,54	97,64	ns
serum cholesterol (mmol/l)	4,4	4,55	ns
LDL (mmol/l)	2,75	2,57	ns
HDL (mmol/l)	1	1,11	ns
CRP (mg/l)	9,2	15,86	ns
HbA1c (mmol/mol)	6,33	4,5	ns
Hb (g/dl)	141,62	131,55	0.0077*

Table II: *MIAT* expression in advanced human carotid plaques in relation to clinical parameters of patients with carotid artery disease from the Biobank of Karolinska Endarterectomies (BiKE).

MIAT VS.	PEARSON R	95% CONFIDENCE INTERVAL	P (TWO-TAILED)	P VALUE SUMMARY
CRP (SENS.)	0.082	-0.11 to 0.27	0.3986	ns
HBA1C	0.024	-0.19 to 0.23	0.8223	ns
FIBRINOGEN	-0.024	-0.20 to 0.16	0.7992	ns
S-CREATININE	0.049	-0.14 to 0.24	0.6160	ns
S-CHOLESTEROL	0.046	-0.13 to 0.22	0.6157	ns
TG	0.0026	-0.18 to 0.18	0.9772	ns
LDL	0.046	-0.15 to 0.24	0.6417	ns
HDL	0.051	-0.14 to 0.24	0.6021	ns
HB	0.022	-0.16 to 0.20	0.8119	ns
LPK	-0.021	-0.20 to 0.16	0.8142	ns
PROINSULIN	0.044	-0.14 to 0.23	0.6473	ns
PROINSULIN CV	-0.084	-0.27 to 0.10	0.3810	ns
IL-6	0.047	-0.14 to 0.23	0.6208	ns
L-6 CV	-0.031	-0.22 to 0.16	0.7435	ns
ADIPONECTIN	0.14	-0.048 to 0.32	0.1443	ns
ADIPONECTIN CV	-0.030	-0.21 to 0.16	0.7518	ns
BMI	-0.040	-0.22 to 0.15	0.6773	ns
DIABETES	-0.022	-0.20 to 0.16	0.8113	ns
EXTREM. ISCHEMIA	0.12	-0.059 to 0.29	0.1916	ns
EARLIER INFARCTION	-0.13	-0.30 to 0.043	0.1365	ns
MEDICINES EARLIER TIA/STROKE	0.0060	-0.17 to 0.18	0.9474	ns
MEDICINES INFLAMMATORY DIS.	-0.23	-0.39 to -0.053	0.0111	*
MEDICINES ANGINA PECTORIS	-0.18	-0.35 to -0.0056	0.0434	*
HYPERTONIA TREATMENT	-0.045	-0.22 to 0.13	0.6201	ns

Table III: Expression correlation of *MIAT* with different cell markers from the carotid plaque microarray data of the Biobank of Karolinska Endarterectomies (BiKE).

MIAT VS.	PEARSON R	95% CONFIDENCE INTERVAL	P (TWO-TAILED)	P VALUE SUMMARY
SMC MARKERS				
ACTA2	-0.3039	-0.4547 to -0.1363	0.0005	***
SMTN	-0.3221	-0.4705 to -0.1560	0.0002	***
MYH11	-0.4472	-0.5770 to -0.2954	< 0.0001	****
MACROPHAGE MARKERS				
CD80	0.3469	0.1838 to 0.4914	< 0.0001	****
CD36	0.3667	0.2056 to 0.5084	< 0.0001	****
IGF1	0.4744	0.3266 to 0.5996	< 0.0001	****
CD163	0.5093	0.3671 to 0.6282	< 0.0001	****
CD40	0.2372	0.06496 to 0.3957	0.0075	**
CELL PROLIFERATION				
PCNA	0.2371	0.06557 to 0.3950	0.0073	**
NFKB1	0.3371	0.1724 to 0.4835	0.0001	***
CYTOKINES, GROWTH FACTORS				
IL6	0.2001	0.02682 to 0.3617	0.0241	*
IL10	0.2713	0.1012 to 0.4260	0.0021	**
TGFB1	0.2894	0.1206 to 0.4420	0.0010	**
TNFA	0.2908	0.1221 to 0.4432	0.0010	***
IL1B	0.3065	0.1391 to 0.4569	0.0005	***
PDGFB	0.3545	0.1914 to 0.4985	< 0.0001	****

Table IV: Primers and Taqman Assays

PRIMER ID	SEQUENCE	Provider
RPLPO_human_Fwd	ATGGCAGCATCTACAACCCT	Thermo Fisher
RPLPO_human_Rev	TTGGGTAGCCAATCTGCAGA	Thermo Fisher
MIAT_ELKBS_A_F	GGATTCCAGGTGCAGGTAAA	Thermo Fisher
MIAT_ELKBS_A_R	TGAGTCCACAGTTGCCAGAG	Thermo Fisher
MIAT_ELKBS_B_F	GGATTCCAGGTGCAGGTAAA	Thermo Fisher
MIAT_ELKBS_B_R	CTGAGTCCACAGTTGCCAGA	Thermo Fisher
MIAT_ELKBS_B_F	TTCCAGGTGCAGGTAAAAGG	Thermo Fisher
MIAT_ELKBS_B_R	CCAAGCAACCTGCCTCTAAG	Thermo Fisher
Taqman Assay ID	**Catalogue number**	**Provider**
hRPLPO	Hs00420895_gH	Thermo Fisher
hGAPDH	Hs03929097_g1	Thermo Fisher
hMIAT	Hs00402814_m1	Thermo Fisher
hNKILA	Hs04937740_s1	Thermo Fisher
hNEAT1	HS03453535_S1	Thermo Fisher
hEGR1	Hs00152928_m1	Thermo Fisher
hELK1	Hs00901847_m1	Thermo Fisher
hKLF4	Hs00358836_m1	Thermo Fisher
hACTA2	Hs00426835_g1	Thermo Fisher
hTAGLN	Hs00162558_m1	Thermo Fisher
hMYH11	Hs00975796_m1	Thermo Fisher
hCD68	Hs00154355_m1	Thermo Fisher
hLGALS3	Hs00173587_m1	Thermo Fisher
hHDAC2	Hs00231032_m1	Thermo Fisher
hCD36	Hs00354519_m1	Thermo Fisher
hSRA1	Hs00398296_g1	Thermo Fisher
hHMGCR	Hs00168352_m1	Thermo Fisher
hABCA1	Hs01059101_m1	Thermo Fisher
hLDLR	Hs01092524_m1	Thermo Fisher
hTFCP2	Hs00232185_m1	Thermo Fisher
hVDR	Hs00172113_m1	Thermo Fisher
hEP300	Hs00914223_m1	Thermo Fisher
hNR3C	Hs00353740_m1	Thermo Fisher
hETS1	Hs00428293_m1	Thermo Fisher
hMZF1	Hs01051952_g1	Thermo Fisher
hPARP	Hs00242302_m1	Thermo Fisher
mMiat	Mm01196418_g1	Thermo Fisher
mKLF4	Mm00516104_m1	Thermo Fisher
mEgr1	Mm00656724_m1	Thermo Fisher
mElk1	Mm00468233_g1	Thermo Fisher

Supplemental Figures

Figure I

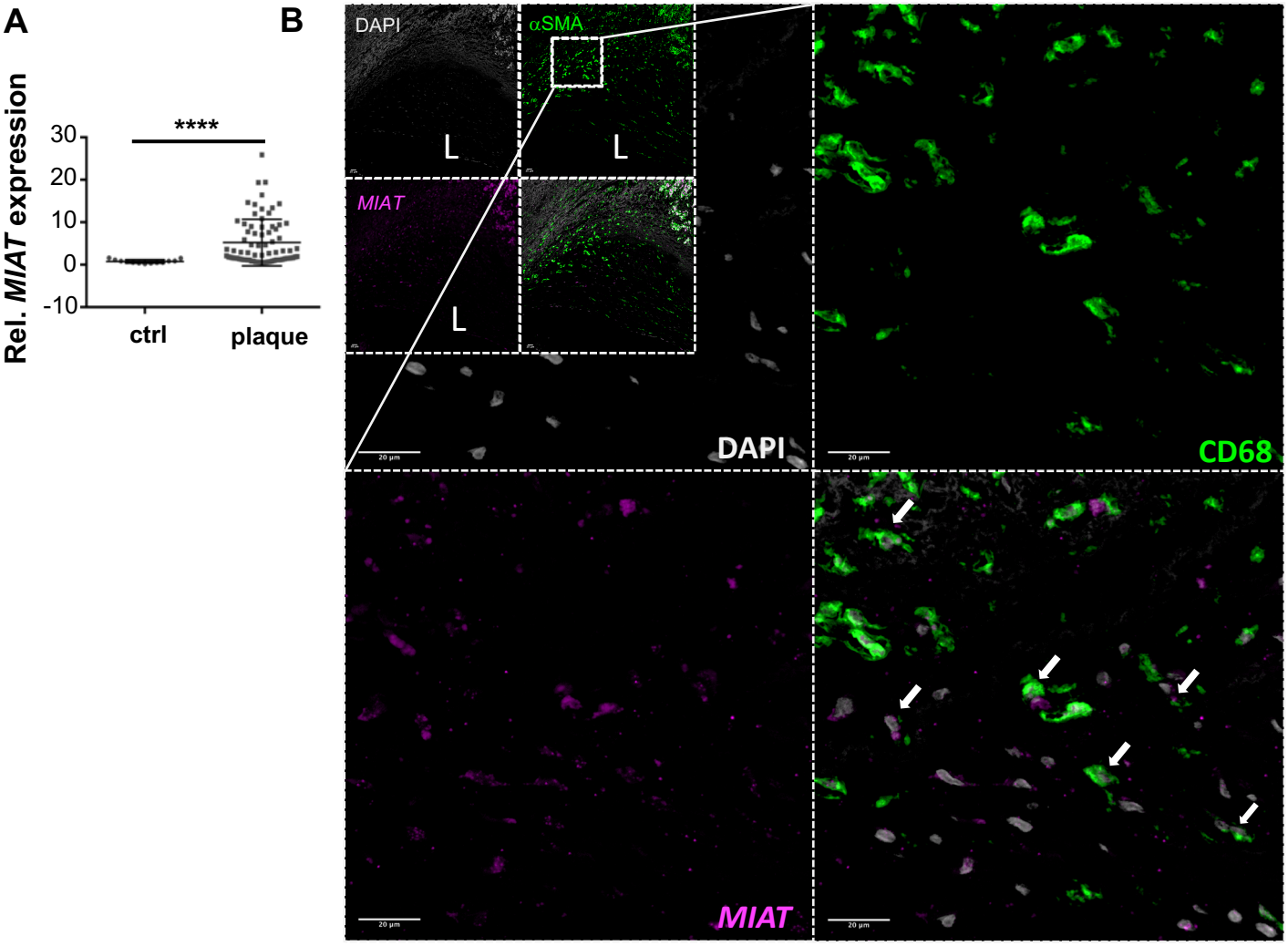

Figure I: *MIAT* in human plaques and co-localizing with CD68. (A) Increased expression of *MIAT* was confirmed with qRT-PCR in human carotid lesions (B) Co-localization analysis of *MIAT* and CD68 protein was preformed by RNAscope protocol. *MIAT* and CD68 protein were detected *via* Opal 690 (purple) and Opal 520 (green), DAPI in grey. The upper left boxes represent overview images of the carotid plaques and the zoomed-in images are magnifications acquired within the plaque shoulder region. White arrows highlight the nuclear detection of *MIAT* in cells expressing the macrophage cell marker (CD68). Scale bar=20 μm. Data were analyzed by Student's t-test. p-value: ****<0.001.

Figure II

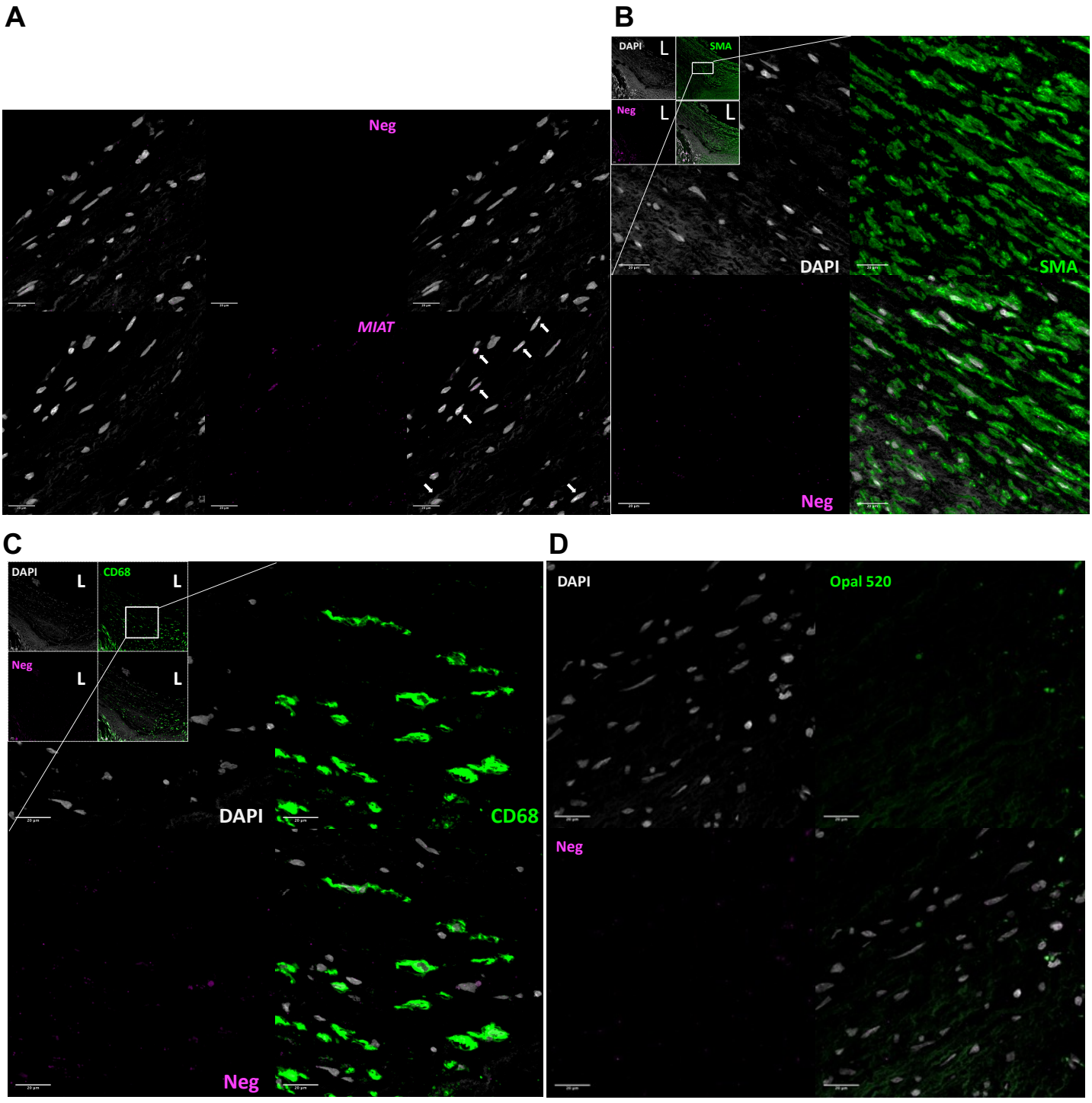

Figure II: RNAscope control stains. Control slides were designed for *MIAT* and αSMA/CD68 separately. In (A), *MIAT* probe was used to localize *MIAT* in absence of primary antibodies and in (B,C) a negative control probe was used in presence of αSMA/CD68 positive staining. Double negative staining for *MIAT* and primary antibodies is shown in D. Scale bar=20 μm.

Figure III

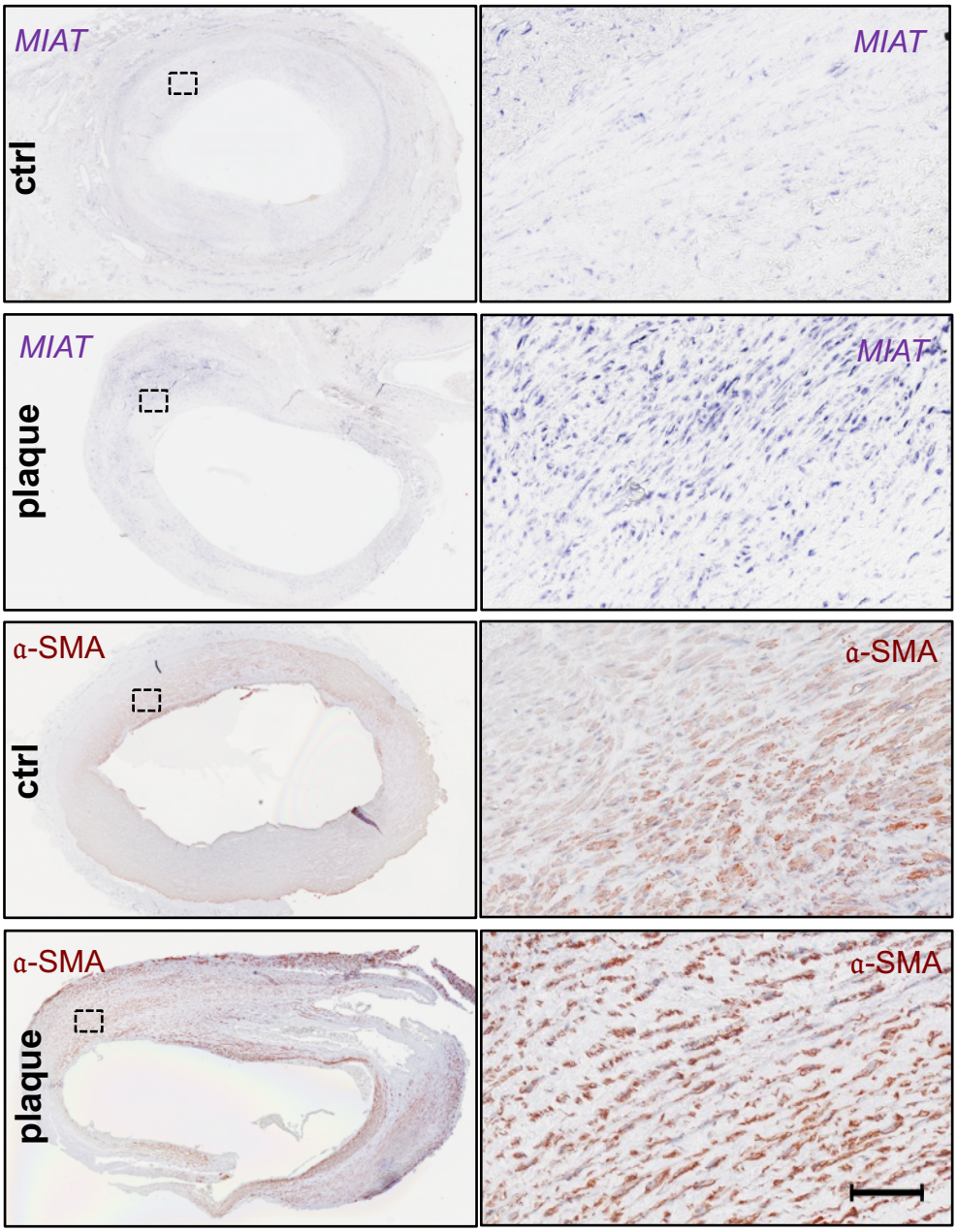

Figure III: *MIAT* expression in human carotid artery plaques. *In situ* hybridization of *MIAT* (upper two panels, purple), immunostaining of smooth muscle cell alpha-actin (αSMA; lower two panels, brown) in non-atherosclerotic control arteries (ctrl) and human stable carotid plaques (plaque). Bar=100 μm.

Figure IV

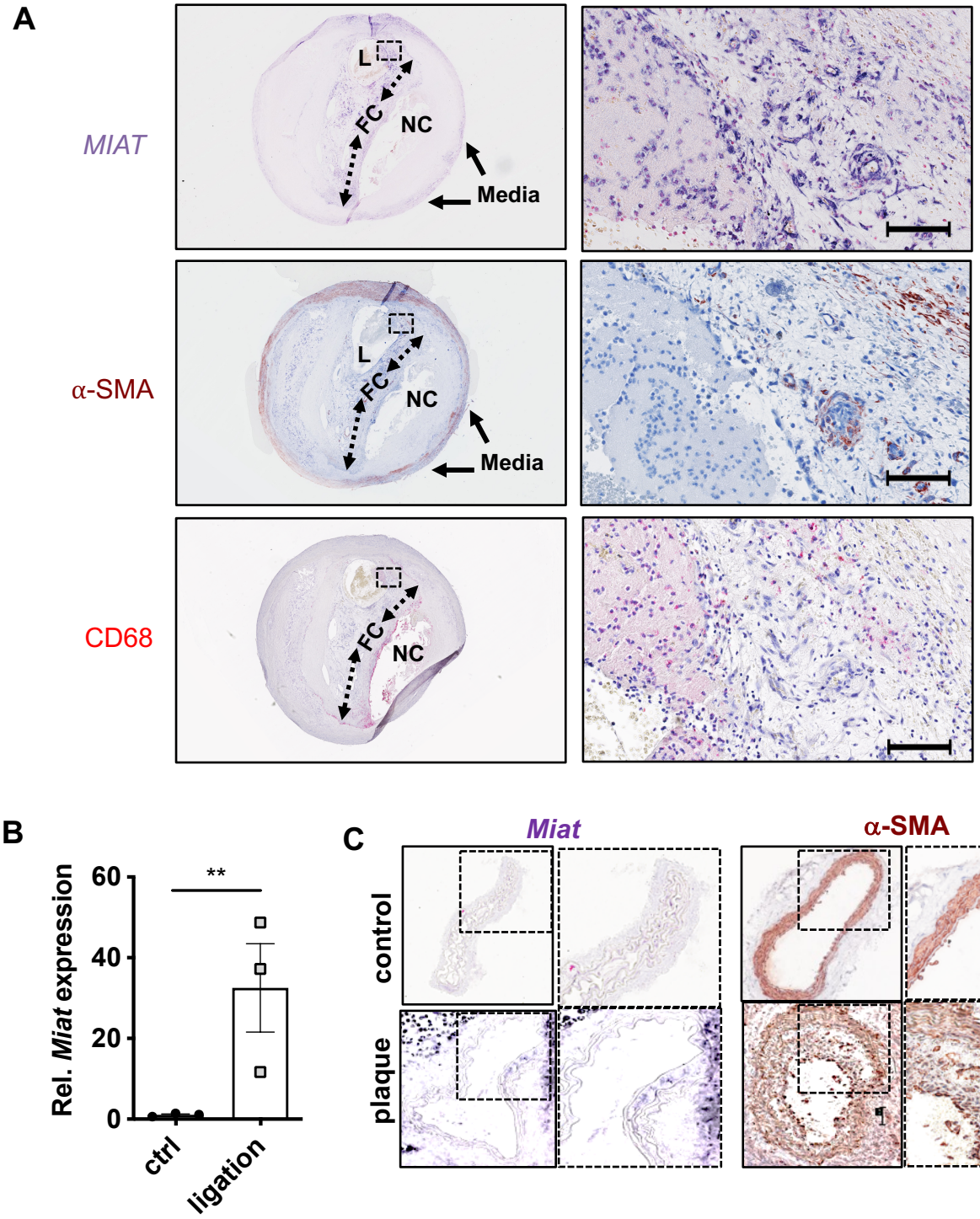

Figure IV: *MIAT* in human and murine lesions. (A) *In situ* hybridization of *MIAT*, immunostaining of smooth muscle cell alpha-actin (αSMA) and human CD68 in consecutive slides of human unstable carotid plaques. Bar=100μm. (B) Increased expression of *Miat* in mouse carotid lesions (in *ApoE*$^{-/-}$ mice upon ligation) using qRT-PCR. (C) *In situ* hybridization of *Miat,* immunostaining of smooth muscle cell a-actin (αSMA) in control and carotid plaques from mouse lesions. Data were analyzed by Student's t-test. p-value: **<0.01. Abbreviations: FC=fibrous cap, NC=necrotic core, L=lumen.

Figure V

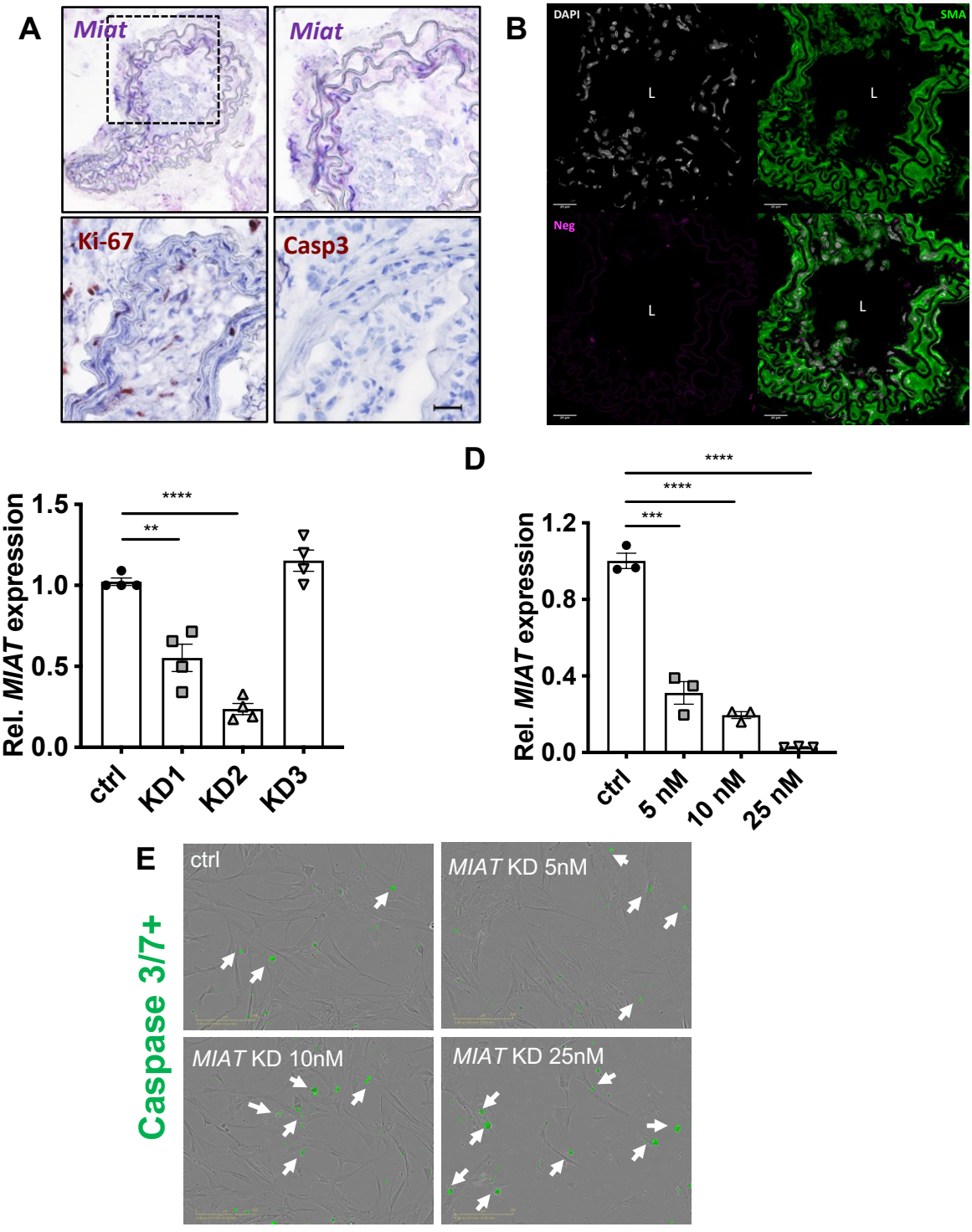

Figure V: (A) Murine carotid lesions indicate increased proliferation (Ki-67 positive cells) and limited apoptosis in relation to elevated *Miat* levels. Scale bar=100 µm. (B) Control murine section for *Miat* RNAscope protocol. A negative control probe was used in presence of αSMA positive staining. Scale bar=20 µm. (C) Assessment of knockdown efficiency by determining the expression of *MIAT* by qRT-PCR in hCASMCs with three different anti-*MIAT* GapmeRs. (D) Expression of *MIAT* with the most potent GapmeR (KD2) in a dose dependent manner. (E) Live-cell imaging detected apoptosis in hCASMCs upon *MIAT* knockdown (KD) in a dose dependent manner. Data were analyzed by One-way ANOVA. p-values: **<0.01, ***<0.001, ****<0.0001.

Figure VI

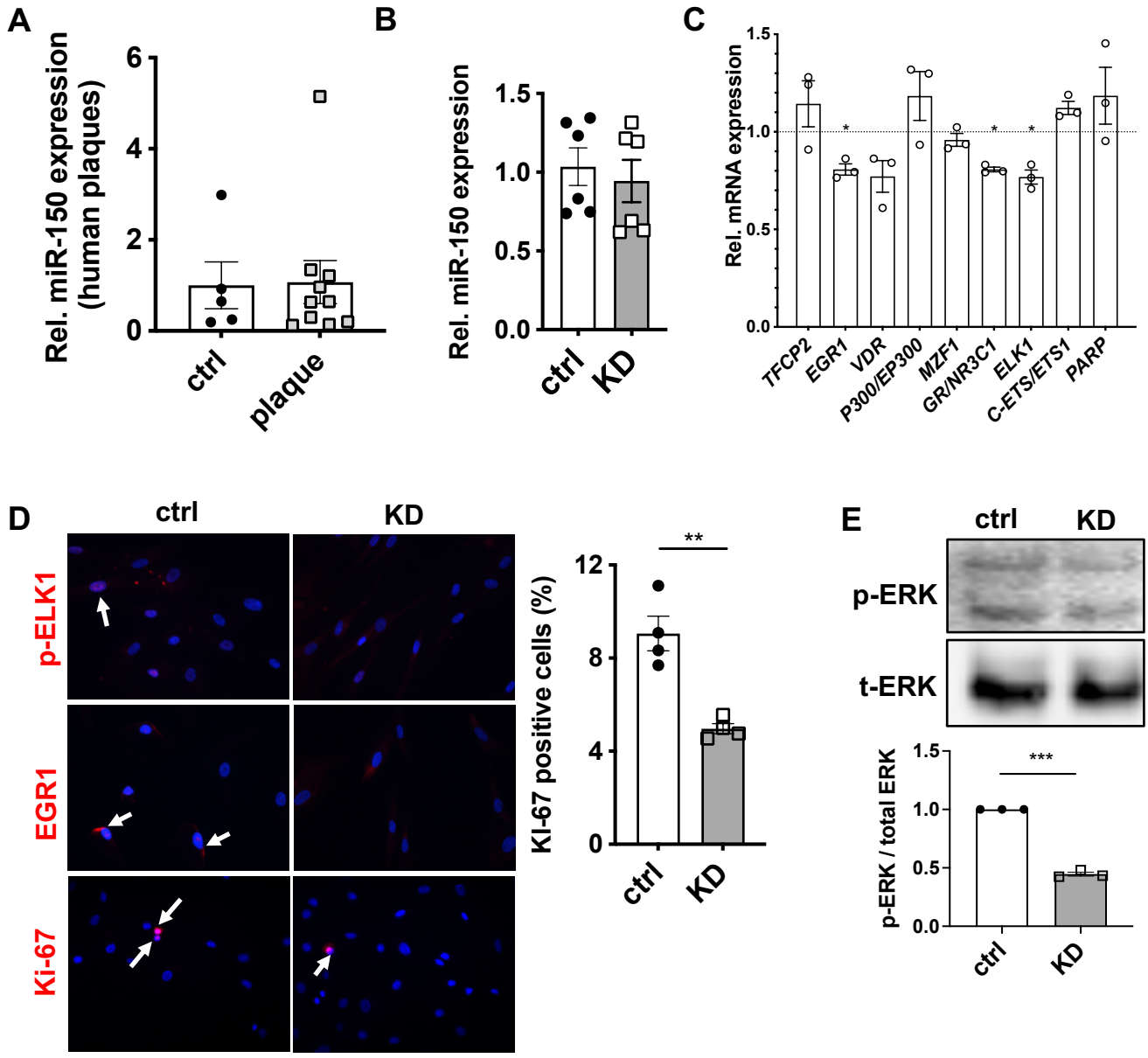

Figure VI: (A) Expression of miR-150 in human carotid lesions. (B) Expression of miR-150 in hCASMCs upon GapmeR-mediated *MIAT* knockdown (KD). (C) *RegRNA2*-based expression analysis of transcription factors upon inhibition of *MIAT*. (D) Immunostaining of p-ELK1/EGR1/Ki-67 upon *MIAT* inhibition in hCASMCs compared to scrambled-mismatch controls (ctrl). Quantification of Ki-67 positive cells is shown on the right. (E) Protein levels measured by Western blot of p-ERK/ERK from the experiments presented in SFig6D. Data were analyzed by Student's t-test. p-values: *<0.05, **<0.01, ***<0.001

Figure VII

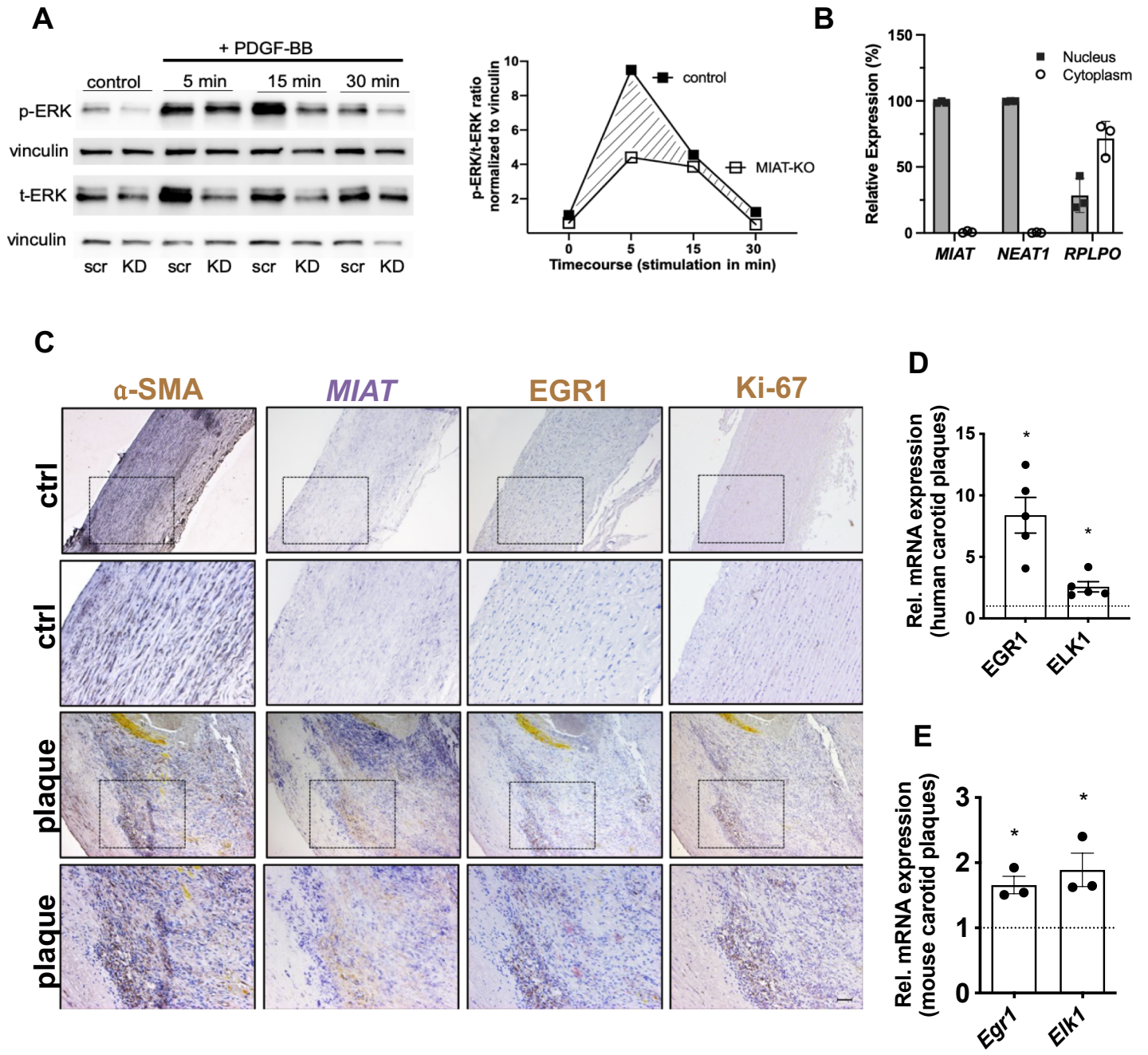

Figure VII: (A) PDGF-BB pre-stimulated hCASMCs show limited phosphorylation of ERK over time when *MIAT* is knocked-down. (B) Isolation of nuclear/cytoplasmic fractions followed by qRT-PCR reveals nuclear *MIAT* expression (distribution controls *NEAT* and *RPLPO*; n=3). (C) *In situ* hybridization of *MIAT*, immunostaining of αSMA, EGR1 and Ki-67 in consecutive slides of non-atherosclerotic control arteries (ctrl) and human stable carotid plaques (plaque). (D,E) Expression of *EGR1* and *ELK1* in human (D) or mouse (E) carotid plaques determined by qRT-PCR. Bar=100 µm. Data were analyzed by Student's t-test. p-value: *<0.05.

Figure VIII

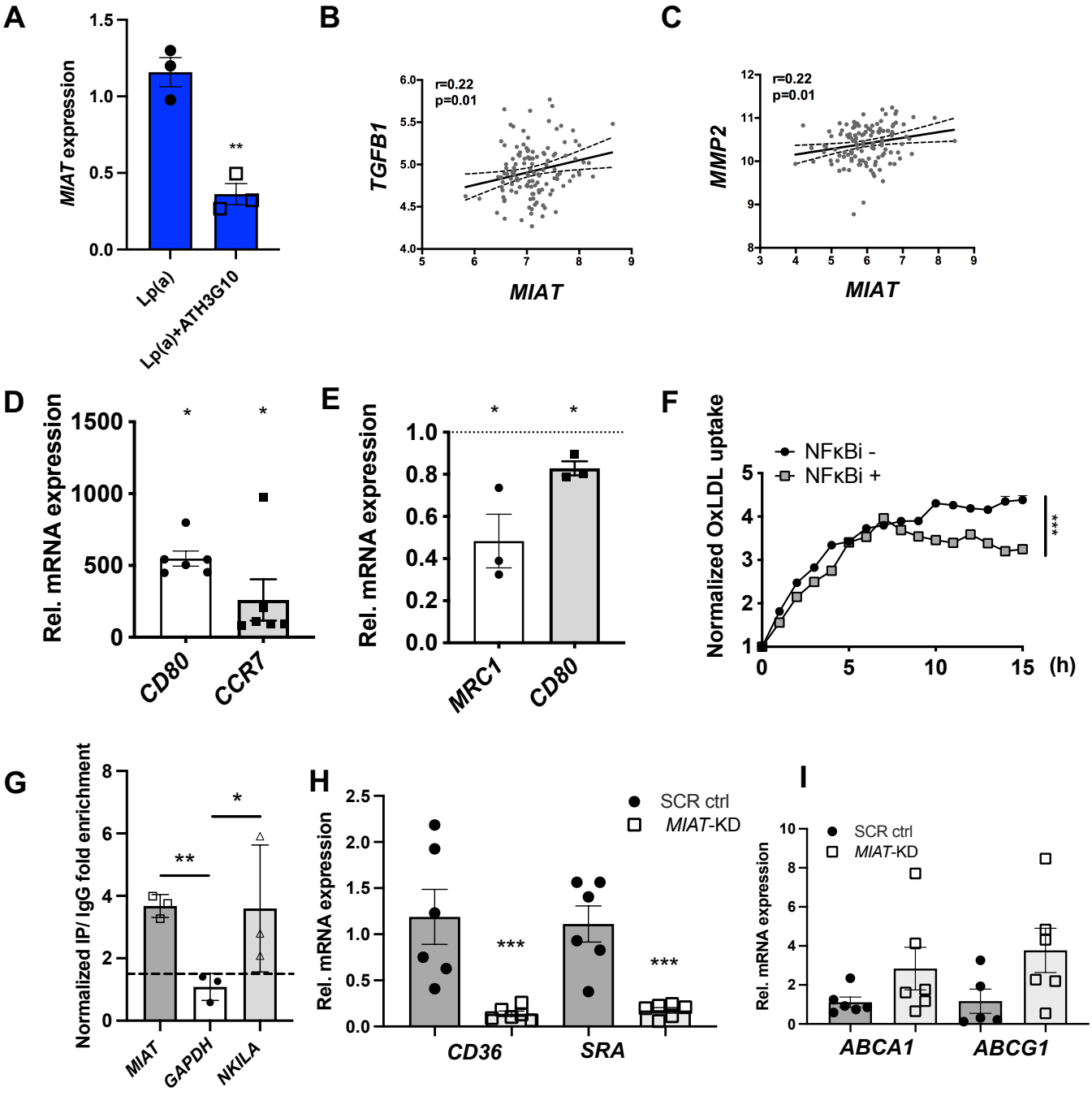

Figure VIII: (A) *MIAT* expression in Lp(a) stimulated hCASMCs with and without ATH3G10 treatment. (B,C) Expression correlation (Pearson, r) of *MIAT* with *TGFB1* (B) and *MMP2* (C) in human carotid plaques of the Biobank of Karolinska Endarterectomies (BiKE). (D,E) Gene expression in THP1-derived macrophages stimulated with LPS (D) or IL4 (E). (F) Uptake of florescence-labeled oxLDL in human monocyte-derived macrophages with or without NFκB inhibitor treatment. (G) Binding of endogenous *MIAT* and lncRNA *NKILA* to NFKB2/p52/p100 in oxLDL-stimulated THP1 cells. (H) *CD36*, *SRA* (I) and *ABCA1*, *ABCG1* mRNA expression in human BMDMs upon knockdown of *MIAT* (vs. scrambled mismatch control). Data were analyzed by Student's t-test or AUC (oxLDL uptake experiment in panel F) p-values: *<0.05; **<0.01, *** <0.001.

Figure IX

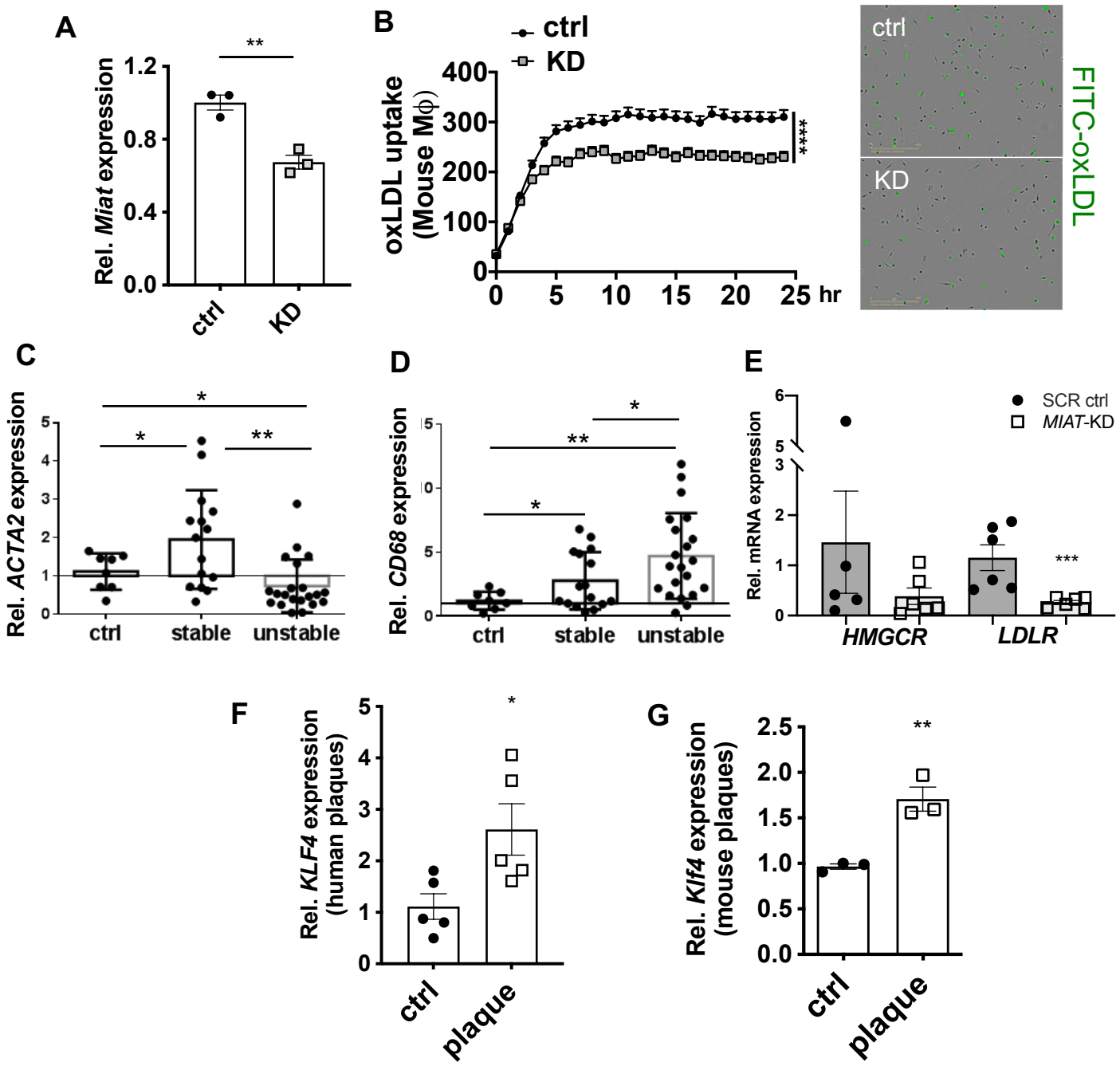

Figure IX: (A) Expression of *Miat* in mouse monocyte-derived macrophages upon *Miat* knockdown (KD) *vs.* scrambled mismatch control (ctrl). (B) Uptake of fluorescence labeled oxLDL in mouse monocyte-derived macrophages with or without *Miat* knockdown (KD). Data were analyzed by Two-way Repeated Measures ANOVA. (C,D) Expression of *ACTA2* (SMC marker) and *CD68* (phagocytosis/macrophage marker) in human carotid plaques (stable and unstable/ruptured) *vs.* non-diseased controls from Munich Vascular Biobank. (E) *HMGCR* and *LDLR* expression in hCASMCs upon oxLDL stimulation and *MIAT* knockdown (*vs*. scrambled mismatch control; scr). (F, G) Expression of *KLF4* in human (F) and *ApoE-/-* mouse (upon ligation/cuff injury) carotid plaques (G). Data were analyzed by Student's t-test (A,E,F,G), One-way ANOVA (C,D) or AUC (oxLDL uptake experiment in panel B). p-values: *<0.05; **<0.01; ***<0.001.

Figure X

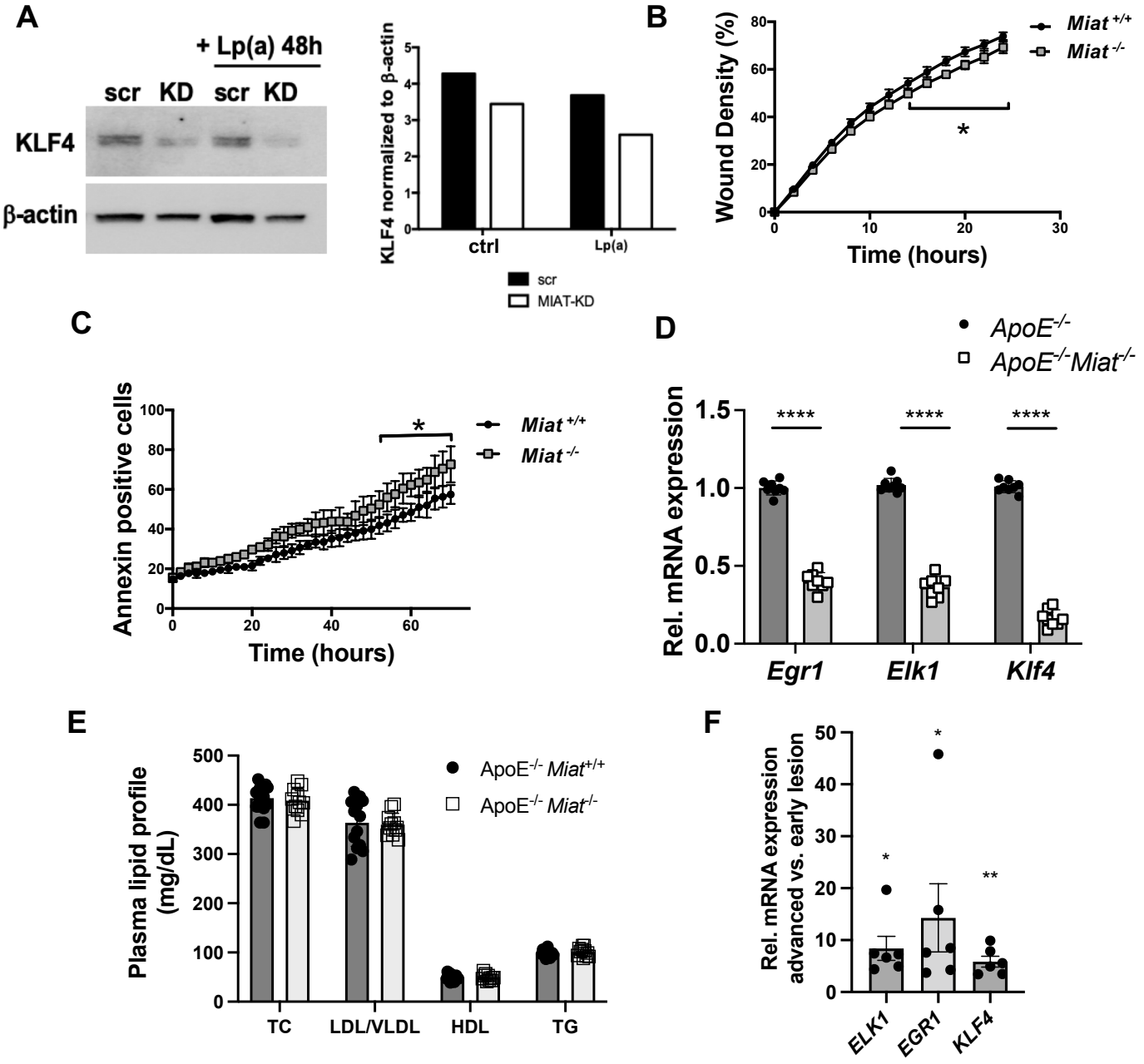

Figure X: (A) KLF4 protein levels with and without Lp(a) stimulation (for 48h) and treated with either scrambled mismatch control or *MIAT* knockdown. (B) Smooth muscle cells (SMCs) derived from *Miat*$^{-/-}$ mice show a lower migratory capacity than SMCs isolated from *Miat* wildtype mice over time (24h). (C) Isolated SMCs of *Miat*$^{-/-}$ mice present with increased apoptosis (Annexin positivity) compared to SMCs from *Miat* wildtype animals (*Miat*$^{+/+}$) in live cell imaging experiments (up to 72h). (D) Expression of downstream *Miat* targets in *Miat*$^{-/-}$*ApoE*$^{-/-}$ mice (*vs.* littermate *ApoE*$^{-/-}$ *Miat* wildtype controls). (E) Plasma lipid profile of *Miat*$^{-/-}$*ApoE*$^{-/-}$ mice *vs.* littermate *ApoE*$^{-/-}$ *Miat*$^{+/+}$ (wildtype controls) indicate equal levels in between both strains. (F) Relative *MIAT* target expression in advanced *vs.* early stage atherosclerotic lesions from carotid arteries of the Yucatan LDLR$^{-/-}$ mini-pig model. Data were analyzed by Student's t-test (D,E,F), multiple t-test (B,C). p-values: *<0.05; **<0.01, ****<0.0001.