SUPPLEMENTAL MATERIAL

Palmdelphin regulates nuclear resilience to mechanical stress in the endothelium

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EXPANDED METHODS

Isolation of human valvular endothelial and interstitial cells

Human regurgitant aortic valves were obtained after written consent, according to the Declaration of Helsinki and approval by the local ethics committee for the use of human material (approval #2012/1633-31/4) from patients that underwent surgical aortic valve replacement. Valves were collected in cold sterile PSS buffer (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.17 mM MgSO4, 25 mM NaHCO3, 1.18 mM KH2PO4, 0.026 mM EDTA, and 5.5 mM glucose). Tissue from aortic regurgitation patients was selected because regurgitant valves yield more endothelial cells as compared to stenotic valves. The tissue was transported to a sterile bench, treated with collagenase I (Sigma) dissolved in Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher Scientific) for 5-10 min at 37°C, 5% CO2. The endothelial cell layer was subsequently removed by rotating a sterile cotton swab onto the surface of the leaflets. VEC were recovered in endothelial cell growth medium (ECGM; Medium 199 (Sigma), 20% FBS (Gibco), 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Gibco), 2 mM L-glutamine (Gibco), 2.5 µg/ml Amphotericin B (Sigma), 50 units/ml penicillin - 50 µg/ml streptomycin (Gibco), 50 µg/ml Endothelial Cell Growth Supplement (Corning), 100 µg/ml heparin (Sigma) and centrifuged for 5 min at 400 x g. Cells were washed once with PBS (Gibco), and seeded onto gelatin-coated polystyrene tissue culture flasks containing ECGM and grown at 37°C, 5% CO2 until the density of the VEC reached near confluency. Culture medium was changed every 48 h. The cells were detached, labeled with anti-human CD31 antibody coupled to magnetic beads and purified by positive selection using a LS-column on a MidiMACS™ Separator (Miltenyi Biotec). The purified VEC were seeded onto gelatin-coated tissue culture flasks, grown until near confluency and used for experiments between passages 2 and 6. The remaining valvular tissue was further digested using collagenase I and dispase II (Sigma) in DMEM (Gibco) for 16-18 h at 37°C, 5% CO2. The digested tissue was passed through a 40 µM cell strainer, and the isolated VIC were centrifuged for 5 min at 400 x g. Cells were washed once with PBS and seeded in a polystyrene tissue culture flask in DMEM containing: 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 2.5 µg/ml Amphotericin B, 100 units/ml penicillin, 100 µg/ml streptomycin. Culture medium was changed every 48 h. The isolated VIC were used for experiments between passages 1 and 6.

Pre-embedding immunogold electron microscopy

Mice were anesthetized with pentobarbital and perfused with 4% paraformaldehyde (PFA; Sigma-Aldrich), 0.05% glutaraldehyde, 100 mM sodium phosphate, pH 7.5, for fixation. After 10 min of perfusion, cerebella were removed and post-fixed overnight at 4°C with pentobarbital and perfused with 4% paraformaldehyde, 0.05% glutaraldehyde, 100 mM sodium phosphate, pH 7.5; washed 3x with cold PBS, and stored at 4°C in PBS with 0.05% sodium azide. Cerebella were embedded in 4% agarose, 100 mM sodium phosphate, pH 7.4, and frontal sections of 65 µm thickness cut on a Leica VT 1005 vibratome. Sections were subjected to heat-induced antigen retrieval in 10 mM sodium citrate pH 6.0 (microwave, 400 W, total heating time 4 min), permeabilized for 30 min in 1% $NaBH_4$ in PBS, incubated with the

primary antibody at a dilution of 1:20 in PBS, 10% normal goat serum, 0.02% Triton X-100, 0.05% NaN_3, first for 2.5 h at room temperature and then for 67 h at 4°C, and finally incubated with the secondary antibody diluted 1:400 in PBS, 0.5% BSA, 0.4% fish gelatin, 1% normal goat serum, for 2.5 h at room temperature and for 18 h at 4°C. Bound antibodies were finally fixed in 2.5% glutaraldehyde, 100 mM sodium phosphate, pH 7.5 for 45 min. Before and after incubations, sections were thoroughly washed and blocked with appropriate buffers. The primary antibodies were affinity-purified rabbit anti-mouse Palmd sera #1 or #17.[2] The secondary antibody was Nanogold-coupled goat anti-rabbit Fab' (Nanoprobes). Silver-enhancement of nanogold particles, osmium tetroxide postfixation, uranyl acetate contrasting, embedding in Araldite 502, ultrathin sectioning, and final counterstaining with uranyl acetate and lead citrate was according to standard procedures. The endothelial labeling pattern shown in Figure 1a was typical, being consistently observed in numerous capillary cross-sections of mouse and rat cerebella, with both sera. Immunoreaction was not seen in capillaries of *Palmd*$^{-/-}$ mouse specimens processed in parallel.

PALMD siRNA silencing

Where indicated, cells were transfected using a commercial human *PALMDELPHIN* siRNA (Santa Cruz Biotechnology, sc-88247), a commercial human *RANGAP1* siRNA (Sigma-Aldrich, SASI_H02_00334671), or a negative siRNA control (Lo GC Duplex#2, Invitrogen, 12935110) at a final concentration of 10 pM. Transfections were carried out using Lipofectamine RNAiMax (Invitrogen). RNA was isolated using High Pure RNA Isolation kit (Roche Diagnostics), cDNA was synthesized with iScript (Bio-Rad), and quantitative PCR (qPCR) was performed using Sybr Green Fast mix (Applied Biosystems) on a ViiA7 (Applied Biosystems). The 60S ribosomal protein L19 (RPL19) was used for normalization. The comparative Ct method was used to calculate fold differences in gene expression. Primers are listed below.

Gene	Amplification fragment	Sequence
h*PALMD*	FWD	AGACTCCAGGCCATCACAGATA
	REV	TTTCCGCTGCTGATTCCATCT
-KKVI	FWD	GAGAAACCGTCCTACCACCC
	REV	CCCAGCTTTGCCATTCTCATC
-CaaX	FWD	GAGAAACCGTCCTACCACCC
	REV	CAGTGGCATGCCTTGTCATTT
h*RPL19*	FWD	GAACATCTCCCCCTTCTCCTT
	REV	ATTGCGGACACCCTCTAGGAA

In vitro flow experiments

HUVECs transfected with a *Control* or a *PALMD* siRNA were seeded at confluence on fibronectin-coated (1.5µg/cm^2; Sigma) slides (µ-Slide I0.6 Luer; Ibidi), cultured for 24 h and subjected to laminar flow (10 dynes/cm^2) in a parallel plate flow chamber system (Ibidi Pump System), or kept under static conditions for 48 h. Thereafter, cells were fixed, stained and mounted using Ibidi Mounting Medium (Ibidi). Images were acquired using a Leica TCS SP8

Confocal Microscope with PMT and HyD detectors and LAS X Navigator software version 3.5.2.18963 (Leica). Quantifications were done using ImageJ software.

***In vitro* wound assay**

HUVECs were seeded on fibronectin-coated (1.5µg/cm^2) ImageLock 96-well plates (Essen Bioscience). Confluent cell monolayers were scratched using a 96-pin mechanical device (WoundMaker, Essen Bioscience), producing a 700-800 µm wound. Cell migration was recorded for 24 h using a 10× objective with IncuCyte ZOOM (Essen Bioscience). The integrated CellPlayer Cell Migration Software Module (Essen Bioscience) enabled automated analysis of IncuCyte cell migration, and relative wound density analysis was performed in multiple scratches from *siControl* and *siPALMD* HUVECs.

***In vitro* cell proliferation**

Proliferation of *siControl* and *siPALMD* HUVECs was examined by seeding 30,000 cells/well on 1% gelatin-coated (Sigma, G1890) plates, left to grow for 12h, 24h and 36h. At the defined endpoints, cells were collected and quantified by normalizing to the total number of cells collected at 12h for each condition.

Cell adhesion assay

siControl and *siPALMD* HUVECs were seeded on fibronectin-coated (1.5 µg/cm^2) 96 well plates for 30 and 60 minutes. At the defined time points media were removed and unattached (floating) cells were counted. Quantification is shown as the fold change of cell attachment with the number of seeded cells (30,000 cells/well) set to 100%.

Atomic force microscopy (AMF) indentation

AFM indentation was carried out using JPK NanoWizard3 mounted on an Olympus inverted microscope. The protocol was adapted from previous study.[47] A modified AFM tip (NovaScan) attached with 10 µm diameter bead was used to indent the cytoplasmic area. The spring constant of the AFM tip cantilever was approx. 0.03 N/m and the AFM indentation loading rate 0.5 Hz with a ramp size of 3 µm. AFM indention force was set at a threshold of 2 nN. The datapoints below 0.3 µm indentation depth were used to calculate Young's modulus to ensure small deformation and minimize substrate contributions.[47] The Hertz model is shown below:

$$F = \frac{4}{3}\frac{E}{(1-\nu^2)}\sqrt{R\delta^3}$$

where F is the indentation force, E is the Young's modulus to be determined, v is the Poisson's ratio, R is the radius of the spherical bead, and δ is the indentation depth. The cell was assumed incompressible and a Poisson's ratio of 0.5 was used.

Lentiviral transduction

PALMD cDNA was transduced into HUVECs by lentivirus-mediated transfer. The plasmid for lentiviral package was purchased from OriGene (clone for human PALMD, Myc-DDK-tagged, RC203079L1). Lentivirus stocks were generated using the packaging cell line HEK293FT.

Supernatants containing viral particles were collected at 48 and 72 h after transfection, diluted 1:4 and supplemented with polybrene (8 µg/mL) for infection of HUVECs. The virus-containing medium was replaced with fresh medium 24 hours after infection.

Antibodies

Antibodies used in cell and tissue immunostainings, and for immunoblotting.

Antibody	Host	Producer	Reference number	RRID
anti-mouse Palmdelphin	rabbit	Kilimann lab	Hu et al. 2005 [2]	
anti-human Palmdelphin	rabbit	Sigma	HPA017959	RRID:AB_1854949
anti-VE-cadherin	goat	R&D	AF1002	RRID:AB_2077789
anti-GAPDH	mouse	Millipore	MAB374	RRID:AB_2107445
anti-phospho-FAK (Tyr397)	rabbit	Abcam	ab39967	RRID:AB_955850
anti-integrin β1 12G10 (h-active integrin)	mouse	Abcam	ab30394	RRID:AB_775726
anti-integrin β1 9EG7 (m-active integrin)	rat	BD	553715	RRID:AB_395001
anti-Erg	rabbit	Abcam	Ab92513	RRID: AB_2630401
anti-RANGAP1	rabbit	Sigma	HPA050110	RRID:AB_2681018
Anti-RANGAP1	mouse	Origene	TA501265	RID:AB_11125787
anti-XPO1/CRM1	rabbit	Sigma	HPA042933	RRID:AB_2678229
anti-XPO1/CRM1	mouse	Santa Cruz Technologies	sc-74454	RRID:AB_1122704
anti-CD31	mouse	DAKO	M0823	RRID:AB_2114471
anti-p21	human	ThermoFisher	PA5-14456	RRID:AB_2077674
anti-TP53	human	Atlas Antibodies	AMAb90956	RRID:AB_2665731
Phalloidin – F-actin Alexa 488, 555, 647		Molecular probes	A12379 - A34055 - A22287	
Hoechst		Molecular probes	H3570	

Immunofluorescent staining

Confluent cells were fixed for 20 minutes in 4% PFA in PBS at room temperature. Samples were permeabilized using 0.2% Triton X-100 in PBS for 20 min and blocked using Protein Block Serum-Free ready-to-use solution (DAKO,) for 1 h at RT. Primary antibodies were incubated in blocking solution at 4°C overnight while secondary antibodies were incubated at RT for 2 h.

Human aortic valves sections (8 µm) were collected for immunostaining. The sections were fixed in 4% PFA for 10 min. Samples were washed with PBS, permeabilized and blocked with 3% BSA/0.2% Triton X-100 in PBS, followed by incubation with primary antibody at 4°C overnight and with second antibody at room temperature for 1 h. Samples were mounted in Fluoromount-G (Southern Biotech) mounting medium. Images were acquired using a Leica TCS SP8 Confocal Microscope with PMT and HyD detectors and LAS X Navigator software version 3.5.2.18963 (Leica). Quantifications were done using ImageJ software.

Immunoblotting

Cells and tissues were lysed in 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP40, supplemented with protease inhibitors (Roche,), phosphatase inhibitors (Thermo Scientific), 1 mM NaF, and 1 mM Na_3VO_4. Protein concentration was determined using the BCA assay system (Pierce). Lysate aliquots (20 µg) were resolved on NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen) and processed for immunoblotting. Immunodetection of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Blots were incubated at RT for 1 hour with a horseradish peroxidase–conjugated secondary antibody and the peroxidase activity was detected by Amersham ECL Prime western blotting detection reagent (GE Healthcare).

Immunoprecipitation

Cells were lysed in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 2 % Igepal, supplemented with protease and phosphatase inhibitors (Roche). For preclearing, 300 µg lysate was incubated with protein G Sepharose beads (GE Healthcare) at 4°C for 30 min. Beads were spun down and lysates incubated with 0.8 µg/ml of antibody, overnight at 4°C on a rotor. 50 µl protein G Sepharose beads were added and rotated for 3 h at 4°C. Samples were washed with lysis buffer 5 times and processed for SDS-PAGE as described above.

GTPase activation assay

400 µg freshly prepared total cell lysate (prepared as for immunoblotting) were used/sample, following the manufacture's protocol (Cytoskeleton). For each GTPase, the Cytoskeleton rhotekin-RBD (RHOA) or PAK-PBD (CDC42-RAC1) beads were used.

Scanning electron microscopy (SEM)

Mice were anaesthetized with ketamine:xylazine (100 mg/kg:20 mg/kg bodyweight) and perfused via cardiac puncture with PBS and a fixative buffer (2.5 % v/v glutaraldehyde, 2 % v/v PFA, 2 % w/v sucrose and 2 mmol $CaCl_2$) for 5 min. After perfusion, aortas were dissected and stored overnight in fixative buffer at 4°C. After brief washings with the fixative buffer 3 times, aortas were post-fixed in 1 % w/v unbuffered osmium tetroxide for 1 h, followed by washing 3 times, and dehydration through a series of graded ethanol. Samples were dried in an Agar E3000 critical point dryer (Quorum Technologies Ltd.) using liquid CO_2 as the drying agent, mounted on stubs, and coated with gold using an Emitech K550X sputter device (Emitech Ltd.). Observations were made using a Philips XL 30 ESEM (Philips, Eindhoven, Netherlands) operated at 10 kV accelerating voltage with images recorded digitally.

For SEM imaging of aortic valve tissue, human stenotic aortic valves were obtained after written consent according to the Declaration of Helsinki and approval by the local ethics committee for the use of human material (approval #2012/1633-31/4) from patients that underwent surgical aortic valve replacement. The tissue was fixed in cold 2.5% glutaraldehyde, and the fixed tissue was macroscopically dissected into calcified and non-calcified sections as described,[44] before serial dehydration in 70%, 95%, and 100% ethanol for 10 minutes as well as in acetone twice for 10 minutes. The dehydrated tissue was critical point dried using carbon dioxide (Leica EM CPD 030; Leica Camera AG), and mounted on aluminum

stubs using silver paint to be sputter-coated with platinum (Quorum Q150T ES; Quorum Technologies Ltd). SEM images were acquired at 100,000 x magnification using a Zeiss Gemini Ultra 55 field emission scanning electron microscope (Carl Zeiss AG) equipped with an Everhart-Thornley SE detector (SE2) at 3 kV.

Mass spectrometry (MS)

Sample preparation: HUVECs were transduced using viral supernatants containing the Myc-DDK-tag alone or PALMD-Myc-DKK-tag (described above). The virus-containing medium was replaced with fresh medium 24 h after infection. After transduction, cells were lysed as described for immunoprecipitation (IP) and IP was performed using Myc-Trap magnetic beads (Chromotek) following the manufacture´s protocol. After IP, samples were washed with 25 mM ammonium bicarbonate.

LC-ESI-MS/MS Q-Exactive-HF: Online liquid chromatography mass spectrometry (LC-MS) was performed using a Dionex UltiMate™ 3000 RSLCnano System coupled to a Q-Exactive-HF mass spectrometer (Thermo Scientific). 1 µg was injected/sample. Samples were trapped on a C18 guard-desalting column (Acclaim PepMap 100, 75 µm x 2 cm, nanoViper, C18, 5 µm, 100 Å), and separated on a C18 column (Easy spray PepMap RSLC, C18, 2 µm, 100 Å, 75 µm x 50 cm). The nanocapillary solvent A was 95% water, 5% DMSO, 0.1% formic acid; and solvent B was 5% water, 5% DMSO, 95% acetonitrile, 0.1% formic acid. At a constant flow of 0.25 µl min^{-1}, the curved gradient went from 2% B up to 40% B in 180 min, followed by a steep increase to 100% B in 5 min. Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTMS) master scans with 70,000 resolution (and mass range 300-1700 m/z) were followed by data-dependent MS/MS (35 000 resolution) on the top 5 ions using higher energy collision dissociation (HCD) at 30-40% normalized collision energy. Precursors were isolated with a 2 m/z window. Automatic gain control (AGC) targets were $1e^6$ for MS1 and $1e^5$ for MS2. Maximum injection times were 100 ms for MS1 and 150-200 ms for MS2. The entire duty cycle lasted ~2.5 s. Dynamic exclusion was used with 60 s duration. Precursors with unassigned charge state or charge state 1 were excluded. An underfill ratio of 1% was used.

Peptide and protein identification. The MS raw files were searched using Sequest-Percolator or Target Decoy PSM Validator (build date 20180628) under the software platform Proteome Discoverer 1.4 (Thermo Scientific) against human Uniprot database and filtered to a 1% FDR cut off. A precursor ion mass tolerance of 10 ppm, and product ion mass tolerances of 0.02 Da for HCD-FTMS were used. The algorithm considered tryptic peptides with maximum 2-missed cleavage; carbamidomethylation (C) as fixed modifications; oxidation (M) as variable modifications. MS analyses were carried out at the Chemical Proteomics and Proteogenomics SciLifeLab facility (www.scilifelab.se/facilities/proteogenomics).

Protein partner identification: Proteins that were uniquely identified in Myc-DDK-tagged PALMD expressing HUVECs samples and never observed in the samples from control HUVECs. These uniquely identified proteins were considered as potential PALMD interacting proteins. The list was further filtered to remove common contaminants, such as keratins. The latter list was then analysed by processing the data in Proteomaps Software[48] to highlight functionally relevant proteins (Supplemental Data set 3).

Genotyping of human samples

Illumina Human610-Quad BeadChip and Infinium Global Screening Arrays were used to obtain genotype data on patients undergoing surgical aortic valve replacement due to CAVS. Genotyping of human endothelial cells isolated from valves retrieved from patients suffering from aortic valve regurgitation was performed using single base primer extension (SBE) with detection of the incorporated allele by mass spectrometry using a MassARRAY analyzer from Agena Bioscience. Raw data from the mass reader was converted to genotype data using the Typer software (Agena Bioscience). Analyses were performed by the SciLifeLab SNP&SEQ Technology facility.

Transcriptomic analysis

Bulk RNAseq: HUVECs transfected with *siControl* or *siPALMD* were grown under static or laminar flow conditions. RNA samples collected from 4 (static) or 5 (flow) independent experiments were assessed using the FragmentAnalyzer or TapeStation to verify that the quantity and quality was acceptable for library preparation. From total RNA, mRNA purification was performed and one sequencing library prepared from each sample using the TruSeq stranded mRNA library preparation kit with polyA selection (Illumina Inc.). Cluster generation and 50 cycles paired-end sequencing of the 18 libraries were performed in 1 NovaSeq SP lane using the NovaSeq system and v1 sequencing chemistry (Illumina Inc.) and at least 320 million read pairs per lane, with >75% of the bases having a base quality score of 30 or higher for samples fulfilling the QC requirements set. Sequencing was performed by the SciLifeLab SNP&SEQ Technology facility. Data pre-processing was done to filter out genes with no counts or with only a single count across all samples. Variance stabilizing transformation (VST) was used and data was normalized to account for different sequence depth in each sample using Bioconductor DESeq2 package (version 1.22.2) in R program (version 3.5.1). All comparisons were performed by adding batch as a covariate to account for the variance of batch effect. Differentially expressed genes were filtered to give lists of genes with an adjusted p-value <0.05 and with a log2 fold change of >1.0 or < -1.0.

Gene expression analyses of CAVS samples: Gene Chip Affymetrix Human Transcriptome 2.0 (HTA 2.0 arrays, Affymetric) was used for CAVS transcriptome analyses at the core facility for Bioinformatics and Expression Analysis at the Karolinska Institute, Sweden. Raw data was normalized with Signal Space Transformation-Robust Multi-Chip Analysis (SST-RMA). Standard quality control and batch control was carried out using Transcriptome Analysis Console (TAC) Software (ThermoFisher Scientific). A total of 58 patients were included in the transcriptomic analysis. Data pre-processing was done to filter out genes with no counts or with only a single count across all samples. Differential gene expression was assessed using linear model with coefficient evaluation by moderated t-test. P-values were adjusted using the false discovery rate (FDR). Limma package (version 3.38.3) and R software (version 3.5.1) were used for all the analysis. Differentially expressed genes were filtered to give lists of genes with an adjusted p value <0.05 and with a log2 fold change of >1.0 or < -1.0.

Gene Set Enrichment Analysis

HUVEC gene expression data was analyzed by gene set enrichment analysis (GSEA software, UC San Diego and Broad Institute[49]), using the Gene Ontology - Biological Process gene set collection (GO_c5.bp.v7.0.symbols.gmt), filtered to gene sets with 15-200 members: The analysis was run using gene set permutation with 1000 permutations and results were filtered to gene sets with a $p<0.05$ and $q<0.1$. For patient samples, gene set enrichment analysis was performed using Qlucore Omics Explorer 3.4 (Qlucore AB) using phenotype permutation with 1000 permutations on the same gene set collection (GO_c5.bp.v7.0.symbols.gmt). The resulting lists of gene sets were compared to identify gene sets that were enriched in HUVEC's and patient samples.

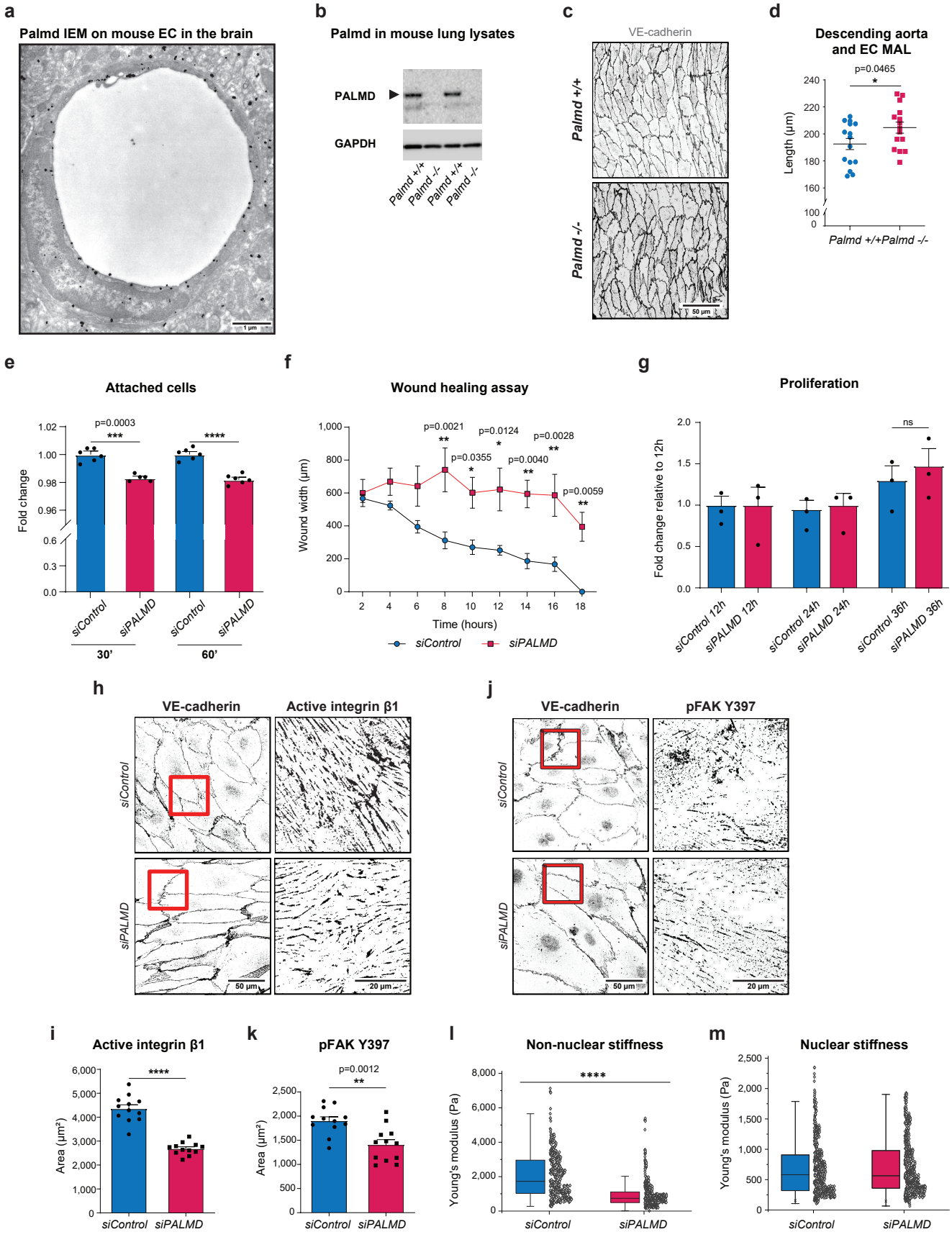
Image analyses

MAL detection: fields of view were acquired and the perimeter of cells was detected based on VE-cadherin immunostaining. Measurements were acquired either automatically using Cell Profiler or manually using ImageJ. The two methods showed similar results on the EC MAL in all models and conditions.

Cortical PALMD mean intensity: cell-cell junctions were defined using VE-cadherin immunostainings and a mask was created using ImageJ. This mask was then dilated 15 times creating a broader area to detect cell-cell junctions. Images were analyzed only in the basal part of the cells in the PALMD channel using the VE-cadherin mask to define the aggregation of PALMD close to the junctions.

Active integrin β1 and pFAK: fields of view showing immunostaining for matrix-adhered, active integrin β1 and pFAK Y397 were analyzed by thresholding the positive area of maximum intensity projection images.

RANGAP1 and XPO1 relocalization: using fields of view from the different HUVEC cultures, nuclei were defined using Hoechst 33342 and a mask was created with ImageJ. This mask was then dilated 3 times for RANGAP1 and 8 times for XPO1 to determine perinuclear aggregation. Images were analyzed only in the central aspect of the cells in the RANGAP1 or the XPO1 channel using the Hoechst 33342 mask to define the perinuclear aggregation.



Supplemental Figure I. EC behavior in the presence and absence of *PALMD*

a. Palmd immune-EM of mouse cerebellum capillary.

b. Immunoblot for Palmd in lung lysates from *Palmd*$^{+/+}$ and *Palmd*$^{-/-}$ mice. Two mice from each genotype are shown.

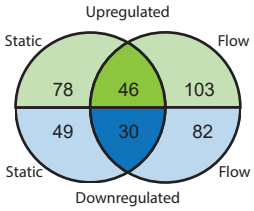
c. Aorta *en face* from *Palmd*$^{+/+}$ and *Palmd*$^{-/-}$ mice showing ECs outlined by VE-cadherin (grey).

d Quantification of EC major axis length. n=3 mice/genotype, 15 FoVs. *p=0.0465; unpaired t-test.

e. Adhesion assay; *siControl, siPALMD* HUVECs on fibronectin at 30 and 60 min. n=3 (duplicate samples in all but siPALMD 30 min). ***p=0.0003; ****p<0.0001; unpaired t-test.
f. Wound healing assay, *siControl, siPALMD* HUVECs. n=3. **p=0.0021; *p=0.0355; *p=0.0124; **p=0.0040; **p=0.0028; **p=0.0059; 2-way ANOVA with Sidak correction.
g. Proliferation assay; fold change in *siControl* and *siPALMD* cell numbers over 36 h, normalized to the 12h value for each condition. n=3. ns; not significant.
h-i. VE-cadherin (left) and active integrin β1 (right) immunostaining in *siControl* and *siPALMD* HUVECs. Active integrin β1 area in μm^2 (i). n= 3; 12 fields of view (FoV). ****p<0.0001; unpaired t-test.
j-k. VE-cadherin and pFAK Y397 immunostaining in *siControl* and *siPALMD* HUVECs. pFAK area in μm^2 (k). n= 3, 12 FoV. **p=0.0012; unpaired t-test.
l. Non-nuclear stiffness by AFM in *siControl, siPALMD* HUVECs. Young´s modulus analysis. n=3, 80 cells, 5 measurements/cell. ****p<0.0001; unpaired t-test.
m. Nuclear stiffness by AFM in *siControl, siPALMD* HUVECs. Young´s modulus analysis. n=3, 120 cells, 5 measurements/cell.

a

DEGs after *PALMD* silencing



b

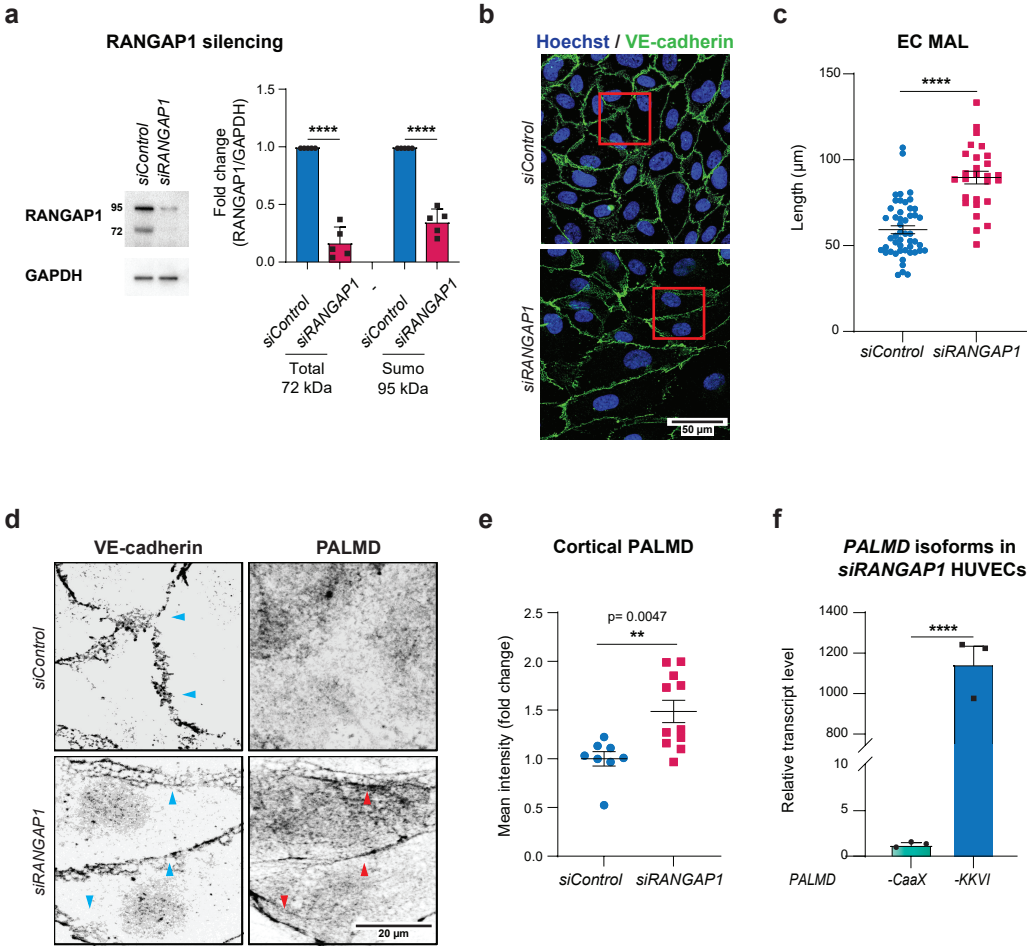
List of DEGs after *PALMD* silencing

	Static				Shared		Flow				
Upregulated	ABCA4	CMPK2	LCP2	TPPP3	BCL2	MATN3	AC025857.2	FAM136A	LPAR5	RRAGD	TXLNB
	AC000078.1	CNN1	LRRC8E	TSPAN2	CA13	MYOZ2	AC093010.3	FERMT1	LRCH2	SAMD8	YOD1
	AC004918.1	COL12A1	LZTS3	UNC5B	CDKN1C.1	NHLRC3	AC108463.1	FHOD3	LRFN1	SCG3	ZNF341-AS1
	AC004918.3	CPM	MAGI2		CHIC1	NHS	AC140479.3	FOXN2	LRRC36	SIX4	
	AC022075.1	CSF2	NCKAP5		CHRNA1	NLRC3	ACADM	GAL	LRRC8B	SLAIN1	
	AC083862.2	CSF3	NFIA-AS2		COL25A1	PDE1C	ADAMTSL2	GALNT13	N4BP3	SLC16A14	
	AC107959.3	CSPG4	NKX3-1		CXCR4	PGM2L1	APOD	GALNT15	NCALD	SNTA1	
	AC112198.2	CXCL12	NTNG1		DAPK2	PIKFYVE	ARHGAP45	GALNT4	NEB	SNTB1	
	ACSM3	DLL4	NUDT4B		DENND1B	PLEK2	ARID5B	HDAC9	NOG	SOWAHC	
	ADA	EFCAB14	OCIAD2		ESM1	RASD1	BCL2L11	HIST2H2BE	NOTCH3	SPOCK2	
	AFAP1L2	EFNB2	PDZD2		EXOC3L1	RIPK3.1	CAND2	HS6ST2	NR4A3	SPTBN5	
	AKAP12	F2RL2	PLVAP		F2RL1	SEMA7A	CDH6	HSD17B2	NRK	STK26	
	AL009178.2	FBLN1	PPM1H		FAXC	SGPP1.1	CDHR3	HVCN1	NUAK2	SULT1C4	
	ANKRD44	FLT1	PRDM1		FBXO32	SHISAL1	CHGB	IL11	OGFRL1	SYT1	
	APOE	GBP1	RASGRF2		GIPC3	SHTN1	COL21A1	IL16	PCSK1	SYT17	
	ARHGEF16	GJA4	RGS9		GPNMB	SLC26A2	COL6A2	KCNMB1	PDGFB	SYT7	
	ATXN1	HEY1	RNASE10		GPRIN3	SPTA1	CPXM1	KIF1A	PGF	TENT5C	
	BANK1	HTRA3	RND1		GRIK4	TGFBR1	CRMP1	KIT	PNPLA3	TGFBI	
	C2CD4A	INSR	SEMA3G		IGF2.1	UHMK1	DDI2	KLHL28	POU2F2	TGFBR3	
	CCDC113	IRF6	SLC45A4		IGIP	VAT1L	DENND6A	KRTAP5-AS1.1	PPIP5K2	THAP5	
	CCIN	KCNE3	SLC46A3		KCNN2	ZNF365	DIAPH2	LAYN	PURA	TMEM229B	
	CD101	KCNF1	SYT9		KLHL24		DUSP10	LDLRAD4	PWAR6	TOM1L1	
	CDC42BPG	KIAA1147.1	TCIM		KYNU		EBF1	LINC00702	RAB11FIP1	TRIM23	
	CHST1	KIF26A	TENT5B		LCP1		ENKUR	LINC01629	RAP1GAP2	TRPS1	
	CITED4	KLK6	TMOD1		LPP		F2RL3	LMBR1	RCAN2	TTTY14	
Downregulated	ACP5	KLF2			ACKR3	SERPINB2	ACER2	DNASE1L3	NRCAM	TFAP2A	
	ADTRP	KRT19			ADAMTS1	SLC40A1	ACR.1	DOC2B.1	NTN1	TGFB3	
	AL122020.1	LNCTAM34A			ADAMTS18	SULF1	ADAMTS15	FMOD	PCDH7	TLL1	
	AL133517.1	MATN2			ADIRF	SULT1E1	ADAMTS5	FOXD2-AS1	PCDHB12	TMEM100	
	AMIGO2	MELTF			AK5	ZNF219	AKR1B10	G0S2	PLCD4	TMEM216	
	ANGPT1	MEST			CBLN2		AL158206.1	GLIS3	PLEKHB1	TPBGL	
	AP002387.1	MGARP			CLDN11		ALDH3A1	HAS3	POSTN	VTN	
	B9D1	NDRG4			CMTM6		ANKRD35	HOXD3	PPFIBP2		
	CLMP	PCDH17			CYBRD1		AP001189.3	HR	PRODH		
	CLU	PKHD1L1			FAM167B		AP001189.4	HSPA2	PRPF40B		
	CPA4	PLAT			GABBR2		AQP5	IGFBP5	PTAFR		
	CSKMT	RPS27AP16			GDF3		ARRDC4	IL10RB	PTX3		
	DIRAS3	RUNDC3B			GNS		BTK	IL17RD	QSOX2		
	DOK5	SERPIND1			HOXA1		C6orf132	IL33	RNASE1		
	E2F2.1	SKP2			IL6R		CA4	IL6	RTN4RL1		
	ECM1	SLC7A8			MAFB		CCDC189	INHBB	SDC1		
	ELMOD1	SLCO4A1			MAN1C1		CD14	INPP5B	SELL		
	EPHB6.1	SULT1B1			MT1E		CDH23	ITGA9	SEMA3D		
	GDF6	SYBU			MYRIP		CEBPD	LAMP3	SFRP5		
	HCFC1R1	TAF7L			NRG1		CLSTN2	LINC00520	SHISA2		
	HFE	TMEM158			PALMD		CNTNAP1	LPXN	SLCO2B1		
	HTR2B	TRBC2.2			PLAC9		CPXM2	MASP1	ST8SIA2		
	INPP4B	VIT			RECK		CROT	MOBP	SYCE1L		
	IQCN	ZNF804A			SELP		CXCL1	MT1X	TACSTD2		
	KHK				SEMA3A		CXCL8	NPPC	TENM4		

Supplemental Figure II. DEGs in *siPALMD* cultures

a. Venn diagram of DEGs in *siPALMD* cells cultures in static or flow conditions. Green shows upregulated genes and blue downregulated genes.

b. List of DEGs in *siPALMD* cells cultures in static or flow conditions. Green shows upregulated genes and blue, downregulated genes.



Supplemental Figure III. RANGAP1 characterization

a. HUVECs ± *siRANGAP1*, blotting for RANGAP1. n=5; ****p<0.0001; unpaired t-test.

b-c. HUVEC ± *siRANGAP1*. VE-cadherin (green) and Hoechst 33342 (blue). Red boxes magnified in (d). Quantification of major axis length (MAL) of the cell body in µm (c). n=3 expt, 49-27 cells. ****p<0.0001; Mann-Whitney.

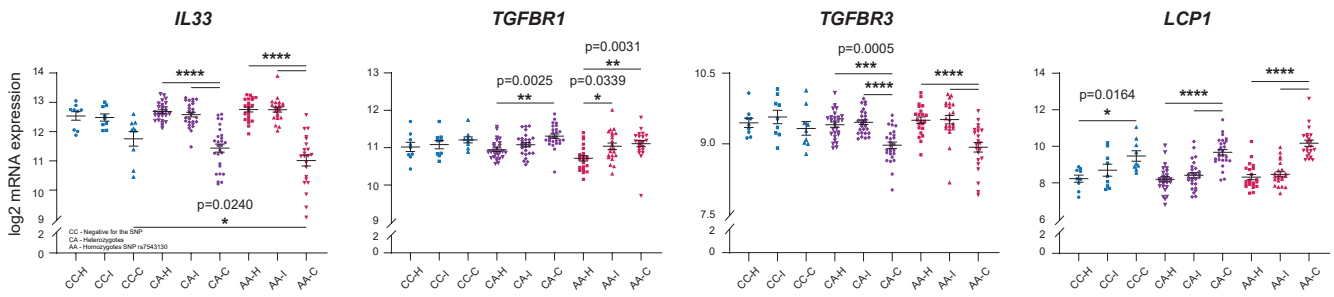
d. Black-on-white insets of panels indicated in (b), *± siRANGAP1*. VE-cadherin (left) and PALMD (right). Blue arrowheads indicate VE-cadherin+ cell-cell borders, red arrows indicate PALMD localized at cell-cell borders in the siRANGAP1 ECs (denoted cortical PALMD).

e. Quantification of cortical PALMD ± *siRANGAP1*. n=3 expt, 8-11 FoV. **p=0.0047; unpaired t-test.

f. PALMD splice variants in HUVECs ± *siRANGAP1.* n=3, ****p<0.001; unpaired t-test.

Mean±S.E.M shown in all graphs.

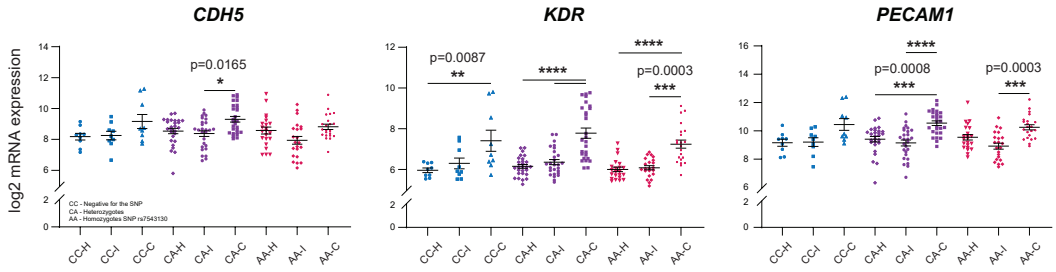
a



b

	HUVECs		Tissue		
DEGs in Actin related GO:BPs	Static	Flow	Healthy	Intermediate	Calcified
# Total Upregulated DEGs	124	149	761	456	1832
# Upregulated DEGs in actin-related GO:BPs	17	18	12	6	51
% Upregulated DEGs in actin-related GO:BPs of total DEGs	13.7	12.1	1.6	1.3	2.8
# Downregulated DEGs	79	112	721	817	2120
# Downregulated DEGs in actin-related GO:BPs	1	3	31	19	61
% Downregulated DEGs in actin-related GO:BPs of total DEGs	1.3	2.7	4.3	2.3	2.9

c

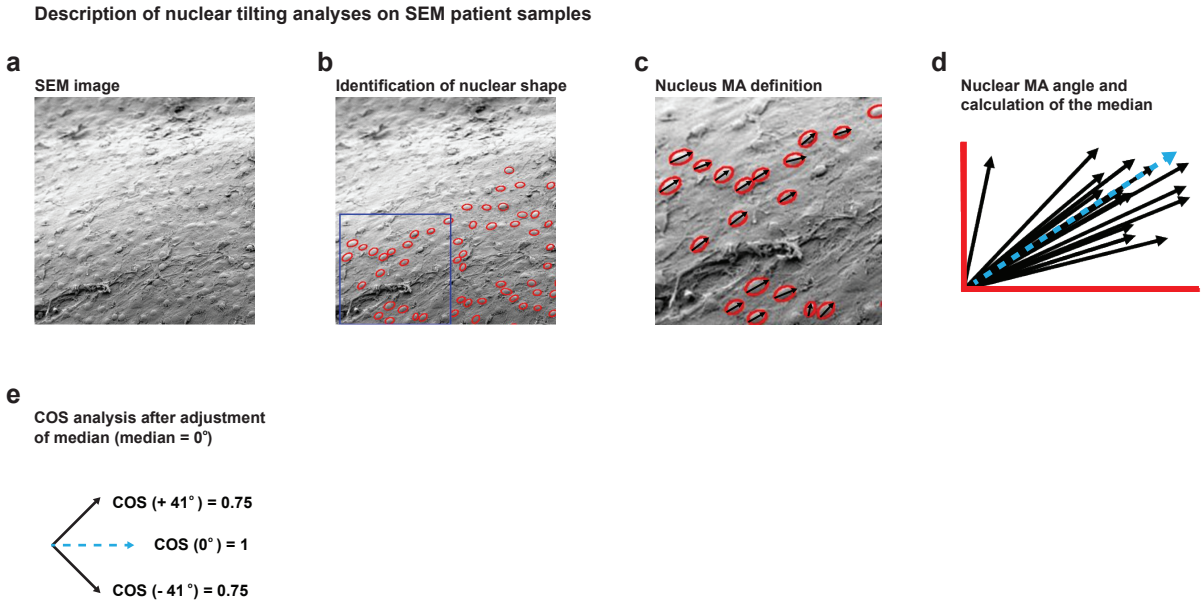


Supplemental Figure IV. Transcript levels and GO processes in relation to tissue culture model, in patient valve samples

a. *IL33*, *TGFBR1*, *TGFBR3* and *LCP1* levels in the 58-patient cohort grouped by genotype (CC, CA and AA) and by valve region (H, I or C); n = 9 (CC), 27 (CA) and 22 (AA). *p=0.0240; *p=0.0339; **p=0.0025; **p=0.0031; ***p=0.0005; *p=0.0164; **** p<0.0001; 2-way ANOVA with Tukey correction.

b. Accumulation of differentially expressed genes (DEGs) and accumulation of actin-related DEGs in Gene Ontology Biological Processes (GO:BP) in *siPALMD* HUVECs in static or flow conditions and in patient valve samples; healthy, intermediate and calcified regions. Number (#) of total up- (green) or down- (blue) regulated DEGs, and number of up- or downregulated DEGs in actin-related GO:BPs are given, as well as the percentage of the up- or downregulated actin-related DEGs within the total regulated DEGs.

c. *CDH5*, *KDR* and *PECAM1* levels in the 58-patient cohort grouped by genotype (CC, CA and AA) and by valve region (H, I or C); n = 9 (CC), 27 (CA) and 22 (AA). *p=0.0165; **p=0.0087; ***p=0.0003; ***p=0.0008; ****p<0.0001.



Supplemental Figure V. Schematic step-by step description of calculation of the nuclear MA angles in patient valve samples

Schematic describing nuclear mis-alignment analyses on SEM patient samples. a) Selection of region of interest (ROI). b) Outline of nuclei in the ROI. c) Outline of major axis (MA) of each nucleus. d). Identification of the nuclear MA angle in relation to a set line indicating flow. Calculation of the mean of all angles and the deviation of individual nuclear MA from the mean. e) Calculation of COS in relation to the median.