Supplemental Material

Locus-conserved circular RNA cZNF292 controls endothelial cell flow responses

Authors: Andreas W. Heumüller[1,2], Alisha N. Jones[3,4], André Mourão[3,4], Marius Klangwart[1], Chenyue Shi[5], Ilka Wittig[6], Ariane Fischer[1], Marion Muhly Reinholz[1], Giulia K. Buchmann[7], Christoph Dieterich[8], Michael Potente [5, ,9,10,11,12], Thomas Braun [5,11,12], Phillip Grote[1], Nicolas Jaé[1], Michael Sattler[3,4], Stefanie Dimmeler (*) [1, ,11,12]

Expanded Materials and Methods

RNA Sequencing

RNA sequencing data was either publicly available[5,11] or generated at the European Molecular Biology Laboratory (EMBL, Heidelberg, Germany) with paired-end 2 × 75 nucleotide reads. The removal of rRNA was performed with the Ribo-Zero Gold (Epicentre Biotechnologies, Madison, Wisconsin, USA; Cat# RS-122-2301) or NEBNext rRNA Depletion kit (New England Biolabs, Frankfurt, Germany; Cat# E6310L). Newly generated RNA-Sequencing data has been stored in the GEO repository under the ID XXXXXXX.

The datasets (HUVEC RNaseR, HCMEC/HAOEC) were analysed as described previously[11]. Briefly, Flexbar (version 3.5.0) was used to perform quality clipping and adapter removal, excluding all bases with a Phred score <28. Potential rRNA reads were removed using Bowtie2 (version2.3.5.1) by comparison against the human 45S precursor sequence (NR_046235.1). Remaining reads were aligned using the STAR read-mapping software (with chimeric alignments support (version 2.6.1d)) to the Ensembl *Homo sapiens* genomic reference sequence. CircRNAs were detected by at least two backsplice-site reads in at least two samples using DCC of the circtools software (version 1.1.0.8).

CircRNAs of the basal HUVEC dataset were identified in the RNA-seq dataset stored under the accession GSE107033 of the GEO repository as previously described[5].

Cell culture

Endothelial cells were purchased from Lonza and cultured as previously described[5]. All experiments were initiated with HUVECs of either the second or the third passage. HeLa (purchased from ATCC), Lenti-X 293T (Takara) and murine H5V cells were cultured in DMEM containing 10% (v/v) FCS and antibiotics (Penicillin 100 U/ml and Streptomycin 100 µg/ml).

RNA Isolation

For RNA isolation, cultured cells were washed once with PBS and afterwards scraped in 700 µl Qiazol. Cell pellets were resuspended in 50 µl of PBS prior to lysis by Qiazol. RNA was subsequently isolated using the miRNeasy-Kit (217004; Qiagen) with additional DNase I (79254; Qiagen) digestion according to the manufacturer's protocol. RNA was eluted in water and quantified using the NanoDrop2000 (ThermoScientific).

RNase R digestion

6 µg RNA were digested by 6 U RNase R (RNR-07250, epicentre) in 1x RNase R buffer at 37°C for 10 min followed by heat inactivation at 95°C for 3 min. Control samples were treated identical except for the exclusion of RNase R.

Fractionation of poly-A$^{+/-}$ RNA

Enrichment of poly-A tail containing RNA was achieved using oligo-d(T)^{25}-Magnetic Beads (S14195, NEB) as previously described[5].

cDNA synthesis

RNA was reverse transcribed using random hexamers and the MuLV Reverse Transcriptase (N808-0018, Invitrogen) in a total volume of 40 µl following the suppliers' instructions. Synthesized cDNA was diluted to equal a final concentration 5 ng RNA per µl.

PCR

Circular and linear RNA were amplified using qRT-PCR or RT-PCR as mentioned in the figure's legends. qRT-PCR were performed using Fast Sybr Green Mastermix (Life Technologies, #4385612)

and the ViiA7 qPCR System (Life Technologies). Primers used in this study are listed in the table below:

Target	Forward Primer	Reverse Primer
cZNF292	GCTCAAGAGACTGGGGTGTG	AGTGTGTGTTCTGGGGCAAG
cZNF292 Ex1A-Ex2	AGTAGCTCCCTTAAAGAATGAGGT	TCTGAAGTTTTCCATTTCTCTGCAT
cZNF292 Ex1-Ex2	CAGGAGAGGTTGAGTTGCGG	TCTGAAGTTTTCCATTTCTCTGCAT
ZNF292	GCAAAGCTGTGTTCTGACCA	CTTGTTGGAGCTGACGTGAC
SDOS	TGCCACGCCATGCTGTAC	CCAGCAGCCCGTCGAAAC
SDOS (mutated)	TTCCCCAACTTCCTGAGCAA	AGGGCCTTCTTCTGCTTCTC
RPLP0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC
SDC4	GAGCCCTACCAGACGATGAG	AGGGATGGACAACTTCAGGG
cZfp292 (murine)	GGAGACTGGGGTGTGGAAAA	GACCACGTAACAATCAGCCC
Zfp292 (murine)	CCTGGAAAGGTGTCGGCA	CCAGCCCCTTCAGTCTCTG
Rplp0 (murine)	TTTGACAACGGCAGCATTTA	CCGATCTGCAGACACACACT
Gapdh (murine)	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA

Step	Purpose	Temperature	Time	Ramp Speed
1	Denaturation	95°C	20 s	1.9°C/s to Step #2
	2-Step Cycle (40x):			
2	Denaturation	95°C	1 s	1.6°C/s to Step#3
3	Annealing	60°C	20 s	1.9°C/s to Step#2, 1.9°C/s to Step#4
	Melt Curve:			
4	Denaturation	95°C	15 s	
5	Annealing	60°C	60 s	0.05°C/s to Step #6
6	Denaturation	95°C	15 s	

Programs KAPA2G Fast PCR Mastermix					
Step	Purpose	°C	Time	Cycle Length by experiment	
1	Denaturation	95°C	20 s		
	3-Step Cycle:			Genotyping	Colony-PCR
2	Denaturation	95°C	10 s	37x	35x
3	Annealing	60°C	20 s		

Step	Purpose	°C	Time	Cycle Length by experiment	
4	Extension	72°C	20 s		
5	Final extension	72°C	2 min		
6	Hold	4°C	---		
KOD Xtreme Hot Start					
Step	Purpose	°C	Time	Cycle Length by experiment	
1	Denaturation	95°C	20 s		
	3-Step Cycle:			Glycerol Gradient	RIP Assay
2	Denaturation	95°C	10 s	37x	33x
3	Annealing	60°C	20 s		
4	Extension	68°C	30 s		
5	Final extension	72°C	2 min		
6	Hold	4°C	---		

Glycerol-gradient centrifugation

For the analysis of protein association by glycerol gradient centrifugation, HUVECs were grown to confluency in T75 cell culture flasks washed with ice-cold PBS and scraped and collected in 1.5 ml ice-cold PBS. Cells were pelleted at 800x g for 10 min at 4°C and cells were carefully resuspended in five times the packed cell volume of Buffer A (10 mM HEPES pH=8, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT). Cells were incubated for 10 min on ice and homogenized by a Dounce homogenizer. Cytosolic extracts (supernatant) were collected after centrifugation at 1900x g for 10 min at 4°C. Extracts were divided three ways: one third was stored on ice, one third was submitted to RNA isolation by Qiazol and one third was supplemented with 0.5% SDS and treated with Proteinase K (40 µg/ml) for 1 h at 37°C. Glycerol gradients were prepared using 1.5 ml of each of the different glycerol solutions (10%, 15%, 20%, 25%, 30%, each containing 0.5 mM DTT, 20 mM HEPES pH=8, 1.5 mM $MgCl_2$, 150 mM KCl). Gradients were allowed to diffuse for 4h at 4°C after which lysates were overlayed. Samples were submitted to ultracentrifugation at 33,000 rpm and 4°C for 16 h (MLA-50 Rotor, Beckman Coulter). Samples were fractionated and analysed by semiquantitative PCR after RNA precipitation and reverse transcription.

RNA affinity purification/ pulldown

For each RNA pulldown experiment, HUVECs were grown to full confluency on three 15 cm cell culture dishes per antisense-oligo. Cell were washed twice with ice-cold PBS, scraped in PBS and pelleted. Cell pellets were resuspended and lysed in 100 µl Buffer R (50 mM Tris HCl pH=8, 50 mM NaCl, 0.5% (v/v) NP-40, 0.1 mM $MgCl_2$ and 1x Protease Inhibitor Cocktail) for 30 min on ice. Cell debris was cleared by 5 min centrifugation at full speed and 4°C and supernatant was transferred to a new tube. Supernatant was diluted with preheated 10x RNase R buffer (RNR-07250, epicentre) and water to a final concentration of 1x RNase R buffer and a total volume of 1100 µl. For partial linear RNA digestion, 20 U RNase R were added and lysates were incubated at 37°C for 10 min. Lysates were immediately placed on ice afterwards and 80 U of RNase Inhibitor (N8080119, Life Technologies) were added. For input samples, 50 µl lysate was taken twice and either resuspended in Qiazol or tagged along for the reminder of the protocol without further treatment. For bead preparation, 100 µl Dynabeads MyOne Streptavidin-Beads C1 were washed thrice with Wash Buffer (50 mM Tris-HCl pH=8, 50 mM NaCl, 1 mM EDTA, 0.05% (v/v) NP-40), blocked with 0.2 mg/ml yeast tRNA and 0.2 mg/ml glycogen diluted in Wash Buffer for 2h at 4°C on a turning wheel and washed again thrice with wash buffer. Lysate was precleared with 50 µl for 2h at 4°C in a turning wheel. Afterwards lysates were cleared from beads and split in two equal samples. Volumes were adjusted to 1.5 ml with Wash Buffer and NaCl was adjusted to 100 mM. For pulldown, 1 µl of 100 µM 2'O-Me biotinylated RNA-oligos (IDT) was added (Scr-Probe: 5-mGmCmAmAmGmGmAmAmCmGmUmGmUmGmAmGmAmCmUmA/iSp9/rArCrGrArUrC/3deS BioTeg-3, cZNF292-probe: 5-mUmUmUmAmAmGmGmGmAmGmCmUmAmCmUmA-mGmAmUmC/iSp9/rArCrGrArUrC/3deSBioTeg-3) and incubated at 4°C over night on a turning wheel with gently rotation. The next day, 25 µl of preblocked beads were added to each sample and incubated for an additional hour at 4°C. Tubes were placed in a magnetic rack and beads were washed thrice with Wash Buffer. For elution, beads were resuspended in 52.5 µl of 50 µM D-Biotin solution and incubated 1 h at room temperature with agitation. Samples were replaced to the magnetic rack and 2/3 of the supernatant were submitted for mass-spectrometry and 1/3 was used for RNA analysis.

Mass spectrometry

Eluate was diluted 2-fold with 8M Urea, 50 mM Tris/HCl, pH 8.5 and thiols were reduced with 10 mM DTT. Samples were incubated at 22°C for 30 min. Reduced thiols were alkylated with 40 mM chloroacetamid and samples were diluted with 25 mM Tris/HCl, pH 8.5, 10% acetonitrile to obtain a final urea concentration of 2 M. Proteins were digested with 1 µg Trypsin/LysC (sequencing grade, Promega) overnight at 22°C under gentle agitation. Digestion was stopped by adding trifluoroacetic acid to a final concentration of 0.1 %. Peptides were loaded on multi-stop-and-go tip (StageTip) containing a stack of six C18-disks. Peptide purification by StageTips was performed as described in [25]. Eluted peptides were dried and resolved in 1% acetonitrile, 0.1 % formic acid.

Liquid chromatography / mass spectrometry (LC/MS) was performed on Thermo Scientific™ Q Exactive Plus equipped with an ultra-high-performance liquid chromatography unit (Thermo Scientific Dionex Ultimate 3000) and a Nanospray Flex Ion-Source (Thermo Scientific). Peptides were loaded on a C18 reversed-phase precolumn (Thermo Scientific) followed by separation on a with 2.4 µm Reprosil C18 resin (Dr. Maisch GmbH) in-house packed picotip emitter tip (diameter 100 µm, 30 cm long from New Objectives) using an gradient from mobile phase A (4% acetonitrile, 0.1% formic acid) to 60 % mobile phase B (80% acetonitrile, 0.1% formic acid) for 60 min with a flow rate 300 nl/min.

MS data were recorded by data dependent acquisition Top10 method selecting the most abundant precursor ions in positive mode for HCD fragmentation. Lock mass option [26] was enabled to ensure high mass accuracy between multiple runs. The Full MS scan range was 300 to 2000 m/z with resolution of 70000, and an automatic gain control (AGC) value of $3*10^6$ total ion counts with a maximal ion injection time of 160 ms. Only higher charged ions (2+) were selected for MS/MS scans with a resolution of 17500, an isolation window of 2 m/z and an automatic gain control value set to 105 ions with a maximal ion injection time of 150 ms. Selected ions were excluded in a time frame of 30s following fragmentation event. Fullscan data were acquired in profile and fragments in centroid mode by Xcalibur software.

For data analysis MaxQuant 1.5.3.30 [27], Perseus 1.5.6.0 [28] and Excel (Microsoft Office 2013) were used. N-terminal acetylation (+42.01) and oxidation of methionine (+15.99) were selected as variable modifications and carbamidomethylation (+57.02) on cysteines as a fixed modification. The human reference proteome set (Uniprot, December 2016, 70947 entries) was used to identify peptides and proteins with a false discovery rate (FDR) less than 1%. Minimal ratio count for label-free quantification (LFQ) was 1. Reverse identifications and common contaminants were removed. To identify significant interacting proteins, LFQ intensities were logarithmized, and histograms were inspected to verify approximate normally distributed data. Data-set was reduced to proteins that were identified in at least 6 of 8 samples in one experimental group. Missing values were replaced by random values imputed from normal distribution according to the default options of Perseus (which shrink the distribution by a factor of "0.3" and shift it down by "1.8"). Data was quality checked by the analysis of the Pearson correlation of the LFQ intensities between samples and exceeded "0.7" in all comparisons and correlations within the Scr or AS-Oligo groups were higher than between the groups. Significant interacting proteins were then determined by an unpaired two-tailed Student's t-test, excluding results with a log2(Fold Change AS-Oligo/Scr) below 0.1, followed by an analysis for permutations-based FDR with 250 randomizations and a threshold of 0.05.

Immunoprecipitation assays

For RNA immunoprecipitations (RIP) experiments, $2*10^6$ HeLa cells were seeded onto a 10 cm cell culture dish and were transfected with overexpression or control plasmids 24 h afterwards. The following day, cells were washed with PBS and harvested by scraping. RIP experiments were performed using the EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (17-701, Merck Milipore) following the manufacturers' instructions. The subsequent antibodies dilutions were used: 1:1000 anti-Myc-Tag (#2276, Cell Signalling). Respective isotype controls (17-701, Merck Milipore) were adjusted to match the respective antibody concentrations. For Complex-Immunoprecipitations (CoIP), $2*10^6$ HeLa cells were seeded onto a 10 cm cell culture dish and were transfected with overexpression or control plasmids on the next day. Membrane & Membrane-associated protein lysates were generated 48h after transfection using the Mem-PER™ Plus Membrane Protein

Extraction Kit for which two cell culture dishes were pooled per sample. For coupling, 4 µg antibody were bound to Dynabeads Protein A/G for 4h at room temperature in binding buffer (50 mM Tris/HCl pH=8, 150 mM NaCl, 1mM EDTA, 0.05% NP-40), after which beads were washed trice with binding buffer. Precipitations were carried out in 100 µl membrane lysate diluted to 1 ml with dilution buffer (50 mM Tris/HCl pH=8, 150 mM NaCl, 1mM EDTA) overnight at 4°C. The next day, beads were washed thrice with binding buffer before samples were boiled in 1x Laemmli loading dye and analysed by Western Blotting.

Protein expression

SDOS genes (6-211) wild type and mutant (5Alanine, H29A, R55A, F66A, E138A, D164A) were ordered as gene optimized DNA gene block from IDT with a C terminal 6-histidine extension and cloned into a pETM13 vector. Positive clones were sequenced verified and transformed in *E coli* Bl21 cells. Expression and purification was performed as previously described[29]. Proteins were flash frozen in liquid nitrogen and stored at -80°C until further usage.

RNA transcription and purification

DNA template containing the T7 promoter region for each RNA (Exon4: 5′-GGAAAAACCCGGTA-CTGTGCACTATTCTTTCCCAGGAACCATTGGATAAGGATAAA-3', Exon4 + Exon1A: GGAA-AAACCCGGTACTGTGCACTATTCTTTCCCAGGAACCATTGGATAAGGATAAAGCTTGCCC CAGAACACACACTATAGAGCAGATGCAGTAGCCACAAAT, Exon4_Mt: GGAAAAACGTGG-TACTGTGCACTATTCTTTCGTAGGAACCACTACATAAGGATAAA, Exon4+1A_Mt: GGAAA-AACGTGGTACTGTGCACTATTCTTTCGTAGGAACCACTACATAAGGATAAAGCTTGCTTC AGAACACACACTATAGAGCAGATGCAGTAGCCACAAAT full-length[5]) was prepared by PCR. RNA was transcribed *in vitro* using in-house prepared T7 polymerase as previously described[29]. Briefly, 600 ng of DNA was incubated at 37ºC for 3 hours with 0.04 M MgCl2, 8 mM of rNTPs, 5% PEG 8000, 1X transcription buffer (40 mM Tris-HCl pH 8, 2.5 mM Spermidine, 0.01% w/v Triton X-100), and 0.03 mg of T7 polymerase. Reactions were quenched with 0.08 M EDTA pH 8. The RNAs

were purified under denaturing conditions using polyacrylamide gel electrophoresis. RNA was extracted from the gel by electroelution. RNA was equilibrated against a high to low NaCl salt gradient, followed by equilibration against RNAse free water.

Recombinant circular RNA was produced similar to the procedure described by Jost et al. [30]. In short, DNA template containing the T7 promoter was generated by PCR and RNA was *in vitro* transcribed using T7 polymerase (NEB, #E2040S) in a modified transcription buffer (1x T7 buffer (NEB, #E2040S), 10 mM rNTPs, 100 mM GMP) for 4 h at 37°C. Template was digested using RQ1 DNase (Promega, #M6101) for 1 h at 37°C and RNA was precipitated by phenol/chloroform/isoamyl precipitation. After reconstitution, single nucleotides were removed by gel filtration using mini Quick Spin RNA Columns (Sigma, #11814427001). For circularization, DNA splints were added in a molar ration of 1:1.2 RNA to DNA splint, and RNA was ligated using T4 RNA ligase (NEB, #M0204S) following the suppliers' instructions. Samples were precipitated by phenol/chloroform/isoamyl precipitation and resuspended in formamide-loading buffer (1xTBE, 90% formamide, Bromophenol blue, Xylene Cyanole) and analysed by denaturing Urea-PAA gels. Circular RNA bands were excised from gels, slices were crushed and incubated in Proteinase K buffer (100 mM Tris/HCl pH 7.5, 150 mM NaCl, 12.5 mM EDTA, 1% SDS) for 1.5 h at 50°C. Samples were cleared by centrifugation using SpinX Columns (Sigma, #CLS8162-96EA) and RNA was precipitated by phenol/chloroform/isoamyl precipitation.

Electrophoretic mobility shift assays (EMSA)

RNAs were dephosphorylated using CIP (NEB), purified using an RNeasy MinElute clean up kit (Qiagen), and subsequently rephosphorylated with γ-^{32}P ATP and T4 PNK (NEB). RNAs were purified again using the RNeasy MinElute clean up kit.

5 nM ^{32}P-labeled RNA was incubated with increasing concentrations of SDOS protein (wildtype or mutant) in buffer containing 10 mM HEPES, 100 mM NaCl and 1 mM beta-mercaptoethanol. Reactions were run on a 0.7% agarose dissolved in 1X TBE. Gels were exposed for two hours on a

GE-phosphor plate prior to scanning on a Typhoon 9000 imager. Band intensity was quantified using ImageJ, followed by fitting in Kaleidograph for determination of the dissociation constant with the Hill-equation. Errors indicated represent the error of fit. At least two biological replicates were run for each EMSA. For EMSAs carried out on unlabeled RNA, the RNA concentration was 100 nM, supplemented with 0.5% glycerol (for loading into the gel). EMSAs were carried out on a 20% native acrylamide gel, followed by fluorescent scanning (390 nm) on a Typhoon 9000 imager.

RNA knockdown using siRNAs

For gene silencing by siRNAs, $4*10^5$ HUVECs were seeded in 60 mm cell culture dish and cultured for 24h. Afterwards, cells were transfected using GeneTrans II (#0203B Mobitec) at a final concentration of 60 nM following the suppliers' instructions. The following siRNAs were used in this study and purchased from Sigma: si_Scr: 5-GUGGGCACCGAUAUCUUGA-3, si_cZNF292 #1: 5-CAGAACACACACUAUAGAG-3, si_cZNF292 #2: 5-AUCUAGUAGCUCCCUUAAA-3, si_SDOS #1: 5-TTTGGGTTGTTGTTGGATT-3, si_SDOS #2: 5-CGAAGCTGTCGAAGGAGAA-3, si_SDC4 #1: 5-GAGAATCTCACCCGTTGAA-3, si_SDC4 #2: 5-GAAACCCATCTACAAGAAA-3.

Laminar flow

For analysis of the endothelial response to laminar flow the Ibidi Perfusion System was used. A total of 2.5 $*10^5$ HUVECs were reseeded 24h after siRNA silencing or 48h after lentiviral overexpression to 0.4 Luer Ibidi µ-Slides and were allowed to attach for 3h. Cells were exposed to unidirectional laminar flow (12 dyn/cm², 40h) using the Ibidi Perfusion System following the suppliers' instructions. For static controls, cells were not exposed to flow and media was changed daily. Following, cells were washed once with PBS containing calcium and magnesium and fixed with 4% formaldehyde/PBS for 10 min at room temperature.

Immunofluorescence staining

For morphological analysis cells were reseeded 24h after siRNA silencing to fibronectin coated 4-well (1*10^5 HUVECs) or 8-well (4*10^4 HUVECs) chamber μ-slides (Ibidi) and cultured for an additional 24 h. Afterwards, cells were washed once with PBS, fixed with 4% formaldehyde/PBS for 10 min at room temperature, permeabilized with 0.1% TritonX-100 for 10 min at RT, blocked in 10% normal donkey serum for 1 h at RT and stained with primary antibodies in the blocking solution overnight at 4°C. Antibodies used for staining were as follows: PXN (abcam, ab32084, 1:250), Cd144 (BD, #555289, 1:200), CD31 (BD, #553370, 1:200), ERG (abcam, ab92513, 1:200), VE-Cadherin (#AF938, 1:50). Cells were washed thrice with PBS containing 0.05% Tween-20 before incubation with fluorescent labelled secondary antibodies (1h, RT in PBS). F-Actin staining were performed with Oregon Green 488 Phalloidin (ThermoFisher Scientific, #O7466) and included during the secondary antibody incubation at a dilution of 1:50. Secondary antibodies coupled to Alexa Fluorophores produced in Donkey were purchased from Invitrogen and used at a dilution of 1:400 (Invitrogen: #A-21206, #A-32794, #A-31573, #A-32766, #A-31570, #A-32787, #A-27040, #A-48272). DAPI staining was included during the secondary antibody incubation at a dilution of 1:200. Samples were compared to secondary antibody only controls to distinguish detected signal from background signal. Cells were mounted in Fluoromount-G and imaged using a NikonTie2 Eclipse microscope or Leica SP8 confocal microscope.

Lentiviral overexpression

Lentivirus stocks were generated using Lenti-X 293T cells (Takara) and using psPAX2 (Addgene, #12260) and pMD2.g (Addgene, #12259) as packaging vectors and GeneJuice (Merck Milipore, 70967) as transfection reagent following the manufacturers' instructions. Constructs for lentiviral overexpression were cloned from SDOS-Myc expressing vector (Origene, RC202638) into pLenti4V5/DEST Gateway Cloning Vector using SpeI and MluI. Control vector were generated by the

same procedure using the empty pCMV6-Entry vector (Origene, PS100001). Viral supernatants were concentrated using the Lenti-X™ Concentrator (Takara, PT4421-2) and resuspended to match a 40x concentrate. HUVECs were transduced with lentivirus matching a final concentration of 1x. Overexpression rates were determined experimentally by qPCR to ensure comparable expression of constructs between samples.

Image Analysis

Images were analysed using Volocity (Quorum Technologies), ImageJ and Microsoft Excel. Cell length was measured manually using ImageJ as the maximal extension of a cell. Cell width was determined as the maximal extension at angle of approx. 90° compared to cell length. Phalloidin stained fibers were measured manually using the line tool of ImageJ in 5 adjacent cells in at least 3 images per biological replicate. For PXN measurements, single cells excerpts were excised from whole images for further analysis. Positive signal was determined according to image thresholds and count and size of focal adhesions was determined employing the "Analyze Particle" Macro of ImageJ.

Plasmid mutagenesis, cloning and transfection

Plasmids used for expression of SDOS and control plasmids were purchased from Origene: pCMV6-Entry (Origene, #PS100001), SDOS-Myc tagged-pCMV6 (Origene, RC202638). The 4x Ala mutation was generated using the Q5® Site-Directed Mutagenesis Kit following the suppliers' instructions using the primers listed in the table below. The cZNF292 overexpression corresponds to the plasmid used in[5]. For CRISPR/Cas9 mediated deletions, we used the pX335_G2P (the pX335 was modified to express a GFP-T2A-Puromycin) plasmid encoding for a human codon-optimized SpCas9 nickase and chimeric guide RNA expression system. gRNAs were designed using http://crispr.mit.edu/ and cloned into BbpI-digested pX335_G2P vectors, 6 different gRNAs were used (see table for sequence). For overexpression of SDOS, $2*10^6$ HeLa cells were seeded in 10 cm cell culture dishes and cultured for 24h hours. The next day, plasmids were transfected with Lipofectamin 2000 following the

manufacturers' instructions in OptiMEM for 4h after which media were changed to supplemented DMEM.

Name	Primer
Q5_SDOS_H26A_fw	CCACTCGTGCGCCGCCATGCTGTACGC
Q5_SDOS_H26A_rv	CTCCAGCCCGGCCCTAGG
Q5_SDOS_F66A_fw	GGCTTCCCCGGGGGCGCAGTGGACCGGCGCTTCTGG
Q5_SDOS_R55A_rv	CAGCAGCCCGTCGAACTCCATCTGCATCAGCACCGAGAAG
Q5_SDOS_E138A_fw	CCACGGCCTGGCGGTGCTGGGCC
Q5_SDOS_E138A_rv	TCGCGCGAGTGCACCGCG
cZfp292 gRNA 1a fw	CACCGGAGACACAAACCTTCTCTA
cZfp292 gRNA 1a rv	AAACTAGAGAAGGTTTGTGTCTCC
cZfp292 gRNA 1b fw	CACCGTGTTTCATGTTGGTGTACCT
cZfp292 gRNA 1b rv	AAACAGGTACACCAACATGAAACAC
cZfp292 gRNA 2a fw	CACCGTATGGGGATAACAATAATC
cZfp292 gRNA 2a rv	AAACGATTATTGTTATCCCCATAC
cZfp292 gRNA 2b fw	CACCGTGAAGGGTTTTAGTGAGGTG
cZfp292 gRNA 2b rv	AAACCACCTCACTAAAACCCTTCAC
cZfp292 gRNA 3a fw	CACCGGTGGCATATTCCAGCCCCA
cZfp292 gRNA 3a rv	AAACTGGGGCTGGAATATGCCACC
cZfp292 gRNA 3b fw	CACCGTTGAGTATTGTGTTATGCA
cZfp292 gRNA 3b rv	AAACTGCATAACACAATACTCAAC

Animal models

mESC culturing and $cZfp292^{-/-}$ mice generation

The mESC were either cultured in feeder free 2i media or on feeder cells (mitomycin inactivated SWISS embryonic fibroblasts) containing LIF1 (1000 U/ml). 2i media: 1:1 Neurobasal:F12/DMEM, 2mM L-glutamine, 1x Penicillin/ Streptomycin (100x penicillin (5000 U/ml)/streptomycin (5000ug/ml), 2mM glutamine (100x GlutaMAX® Supplement), 1x non-essential amino acids (100x MEM NEAA), 1x Sodium pyruvate (100x, Gibco), 0.5x B-27 supplement, serum-free, 0.5x N-2 supplement, Glycogen synthase kinase 3 Inhibitor (GSK-Inhibitor), MAP-Kinase Inhibitor (MEK-

Inhibitor), ES-Serum media: Knockout Dulbecco's Modified Eagle's Medium, ES cell tested fetal calf serum (FCS), 2 mM glutamine, 1x Penicillin/Streptomycin, 1x non-essential amino acids, 110 nM ß-Mercaptoethanol, 1x nucleoside, 1000 U/ml LIF1. The cells were split with TrypLE and the reaction was stopped with the same amount of Phosphate-Buffered Saline (PBS) followed by centrifugation at 1000 rpm for 5min. The cells were frozen in the appropriate media containing 10% Dimethyl sulfoxide (DMSO). To minimize any effect of the 2i media on the developmental potential, mESC were only kept in 2i media for the antibiotic selection then the mESCs were maintained in ES-Serum media on feeder cells immediately. Wild-type F1G4 cells (2 million cells) were transfected with 1 µg CRISPR plasmid. After 48 hrs, 2mg/mL puromycin were applied for 2 days followed by 1mg/mL puromycin for one more day. Single mESC clones were picked up 7-8 days after transfection and plated onto 96-well Synthemax coated plates (Sigma, #CLS3535) and screened for genomic DNA deletion by PCR using primers listed above in the PCR section.

All animal procedures were conducted as approved by local authorities (RP Darmstadt) under the license numbers FU/1064 Life stock were generated by diploid morula aggregation of knock out transgenic cZfp292 mESC cells as described in [12]. SWISS mice (Janvier) were used as wild-type donor of morula stage embryos and as transgenic recipient host (as foster mothers for transgenic mutant embryos). Offspring was confirmed to be *cZfp292^{-/-}* by their fur color agouti and genotyping and backcrossed to C57Bl/6J mice (Janvier). Mice were housed in clear, plastic cages containing standard bedding and maintained on a 12-hour light and dark cycle at 23°C and 50% relative humidity. Mice were fed water and normal mouse chow diet which were available ad libitum.

Organ Harvest

All animal experiments were carried out in accordance with the principles of laboratory animal care as well as according to the German national laws. The studies have been approved by the local ethic committee (Regierungspräsidium Darmstadt, Hessen). For analysis of the aortic endothelium, aortas were dissected, en face prepared and stained according to the protocol published by Kyung et al. [13] though fixation was reduced to 2% formaldehyde/PBS for 3 min. Animals used for the analysis of the aortic endothelium were between 12-20 weeks of age and included animals of both genders,

assignment to experimental groups was based on genotype. For analysis of retinal blood vessel growth, retinas were prepared from postnatal d7 pups as described previously[14]. All animals were harvested and samples were processed and imaged randomly by personal unaware of the respective genotype.

Aortic Ring Assay

Thoracic segments of adult mice aorta were isolated, sectioned in HBSS/MgCl$_2$ and embedded in a collagen-gel matrix (1x M199 media, 1 mg/ml rat tail collagen) in 96-well plate. Aortic rings were cultured in DMEM/F12 media supplemented with 2.5% FCS and in the presence of 30ng/ml VEGF-A at 37°C degrees for 7 days. Media was changed every other day. For imaging, rings were washed with PBS/MgCl$_2$, fixed with 4% PFA for 30 min at RT, blocked with Dako protein blocking buffer and stained with biotinylated IsolectinB4 in PBLEC buffer overnight at 4°C. The next day, samples were washed thrice with PBS containing 0.1% TritonX-100, after which aortic rings were labelled with Strepatavidin-488 in PBS for 3h at room temperature. Samples were washed thrice with PBST and stored in PBS until imaging.

Statistical analysis

The ChemiDoc system (Biorad) was used for acquisition of gel and immunoblot data and ImageJ 1.52p was used for image processing. Data were analysed in Microsoft Excel 2011, GraphPad Prism 5 and Volocity. Data is shown as mean+SEM with individual data points. Data was checked for normality using the Shapiro-Wilk normality test with a threshold of 0.05. P-values were obtained using Student's two-tailed t-test or Kolmogorov-Smirnov test and are reported in the figure legends. Multiple-testing corrections were performed using the Bonferroni-Holm method as stated in the figure legends. Experiments shown in Online Figure IIIE are representative of one experiment. All other data were derived from more biological independent replicates, exact n are reported in the figure or the figure legends.
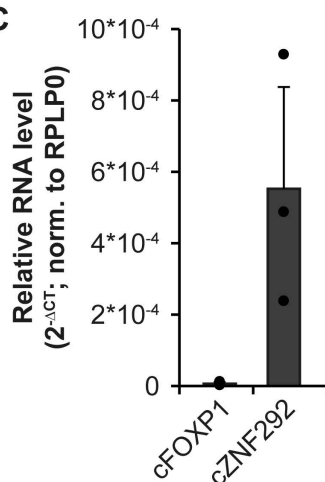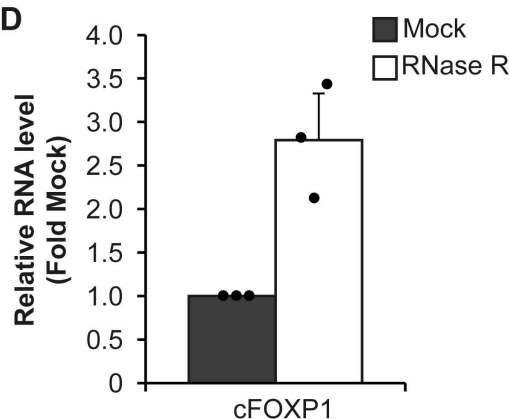
A
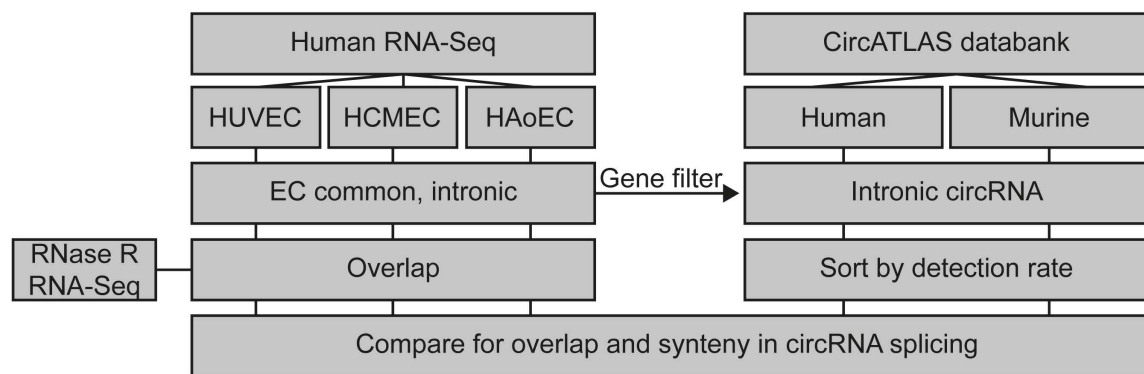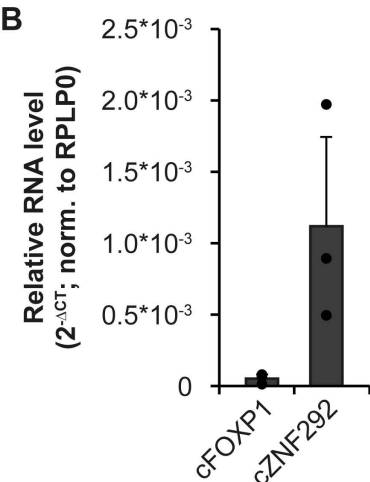
B **C** **D**

Online Figure I, Screening of locus conserved intronic circRNA. Refers to main figure 1. **A** Schematic representation of the screening process. Endothelial common intronic circRNA were identified from three different endothelial RNA-sequencing datasets and compared for checked for their resistance towards exonuclease digestion. CircRNA candidates were compared for the general detection rate among all intronic human or murine circRNA of the respective gene set as listed in the circATLAS database. Finally, circRNAs present in all datasets were analyzed manually for their synteny in circRNA splicing. **B/C,** Validation of circRNA expression in HUVECs (B) and HCMEC (C) by qPCR (n=3). **D,** Analysis of the RNA levels of chosen circRNA in RNase R treated HUVEC RNA vs Mock treated controls by qRT-PCR (n=3). Data is depicted as mean±SEM.
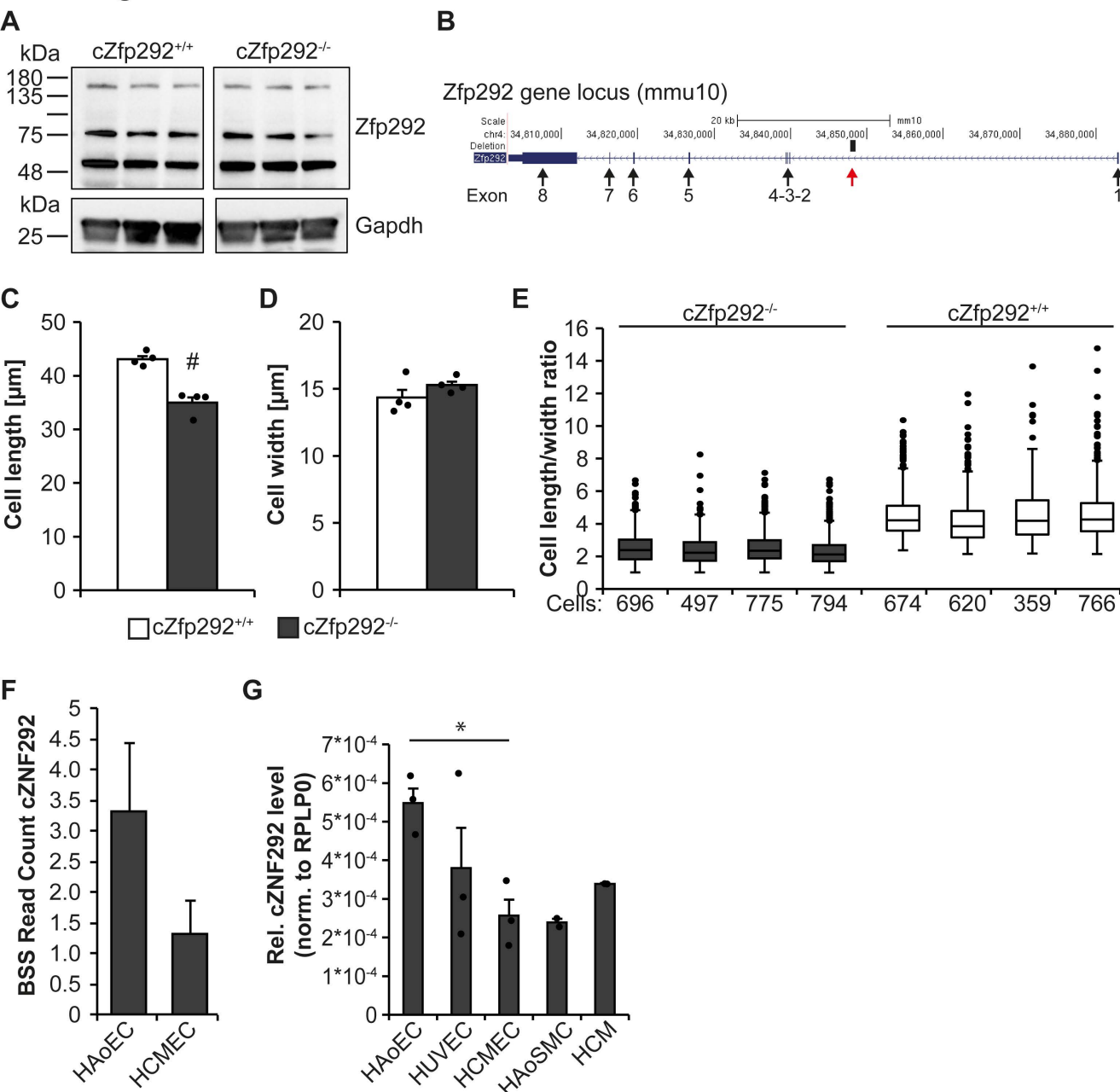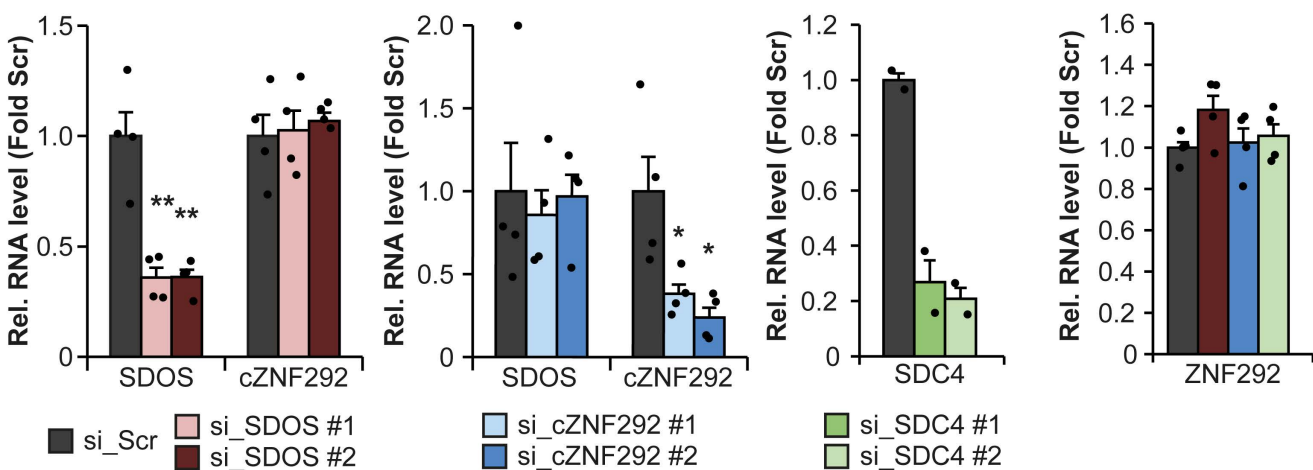
Online Figure II

Online Figure II, Endothelial cells of the aorta show morphological changes in cZfp292^{-/-} mice. Refers to main figure 2 and 3. **A** Immunoblot analysis of Zfp292 protein expression in muscle tissue of cZfp292 wildtype or mutant mice. **B,** Representation of the Zfp292 gene locus (GRCm38/mm10 assembly) as well as the size of the genomic deletion indicated by the red arrow. Image was generated using the UCSC genome browser. **C/D,** Assessment of cell length and width of aortic endothelial cells in cZfp292^{+/+} vs. cZfp292^{-/-} mice (n=4). **E,** Distribution of the cell length/width ratio of quantified cells depicted per animal, max. whiskers length is set to 1.5*IQR, outliers are shown separately. **F,** Detection of cZNF292 back-splice reads in human aortic endothelial cells (HAoEC) and human cardiac microvascular endothelial cells (HCMEC) RNA-sequencing analyzed using the DCC package(n=3). **G,** Quantification of cZNF292 levels in different HAoEC, human umbilical vein endothelial cells (HUVEC), HCMEC, human aortic smooth muscle cells (HAoSMC), human cardiomyocytes (HCM) by qRT-PCR after house-keeping normalization to RPLP0 levels (n=3). Data is depicted as mean±SEM, statistical analysis by two-sided unpaired student's t-test (*) or Kolmogorov-Smirnoff test (#), a value of p<0.05 is considered significant.
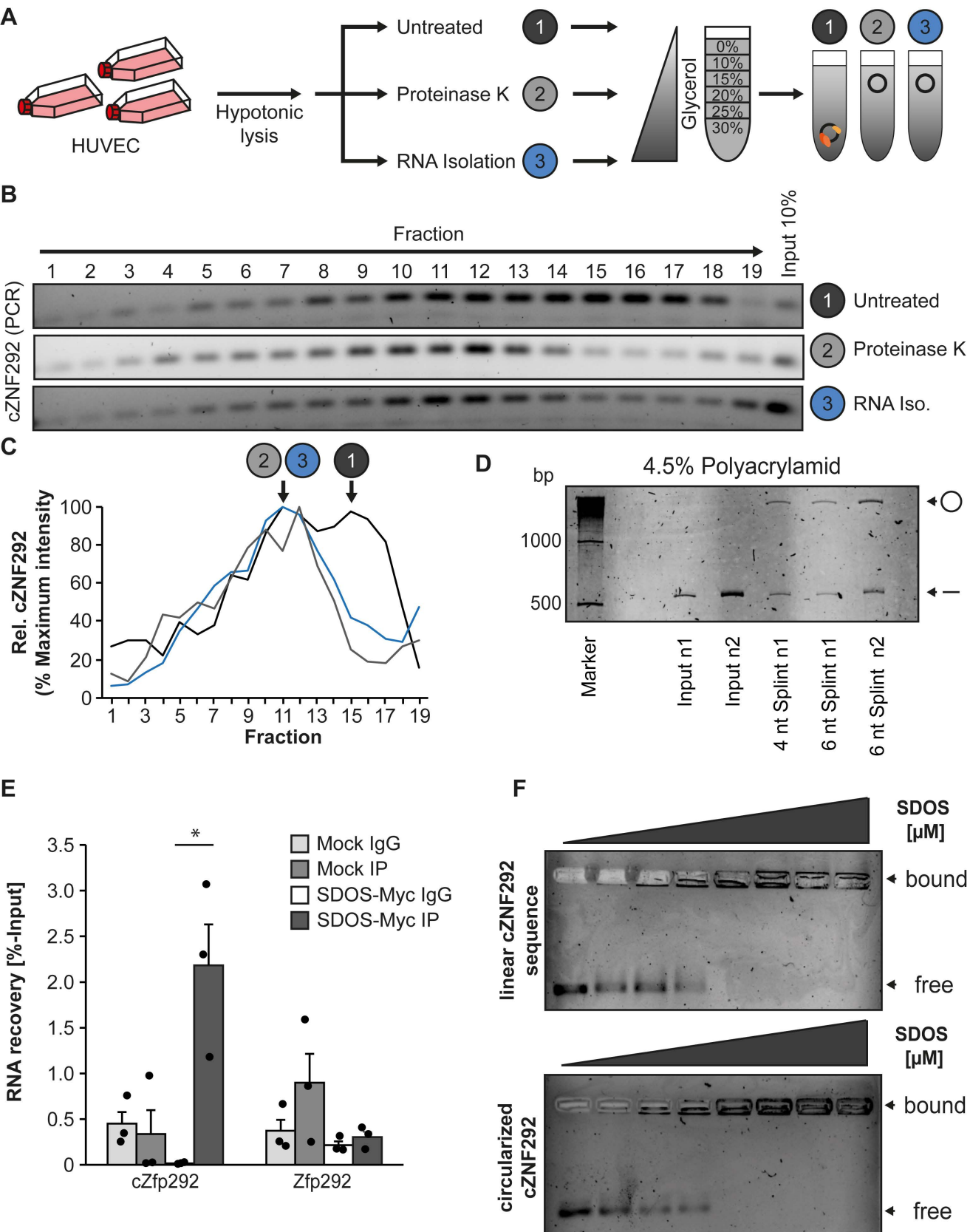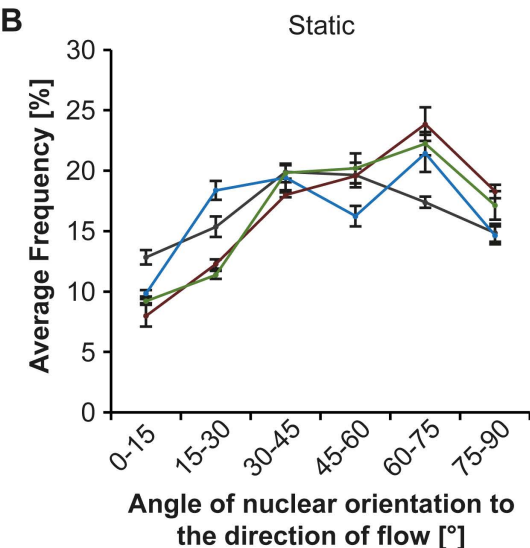
Online Figure III

Online Figure III, cZNF292 is protein associated and interacts with SDOS. Refers to main figure 4. **A,** Schematic experimental layout. **B,** Protein association was analyzed by glycerol-gradient centrifugation followed by PCR. **C,** Densiometric quantification of the images shown in panel B. **D,** Denaturing PAA-Urea gel showing in vitro transcribed cZNF292 RNA (518 nt: ex1a-ex4) before and after ligation using either a 4 nt or 6 nt splint. **E,** Quantification of murine RNA levels following the immunoprecipitation of overexpressed human SDOS-Myc in murine H5V cells (n=3). Human and murine SDOS protein are highly conserved with an amino acid identity of >95%. **F,** EMSA showing the interaction between SDOS and cZNF292 before and after ligation of in vitro transcribed cZNF292. Data is depicted as mean±SEM, statistical analysis by two-sided unpaired student's t-test, a value of $p < 0.05$ is considered significant.
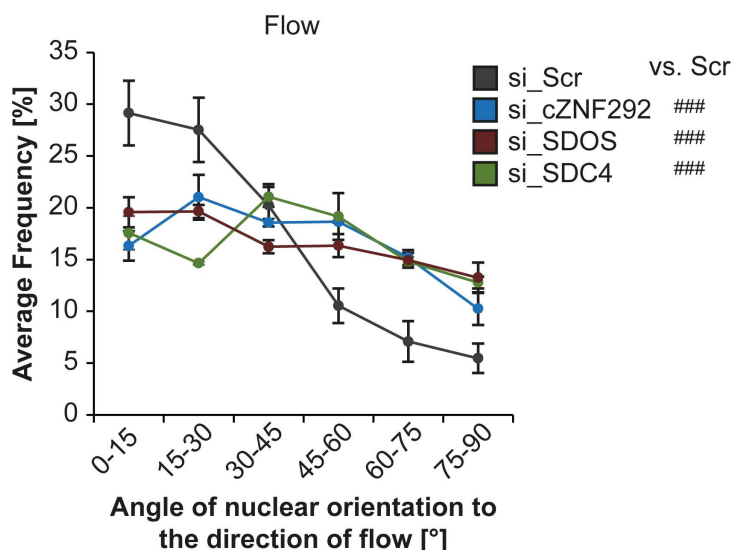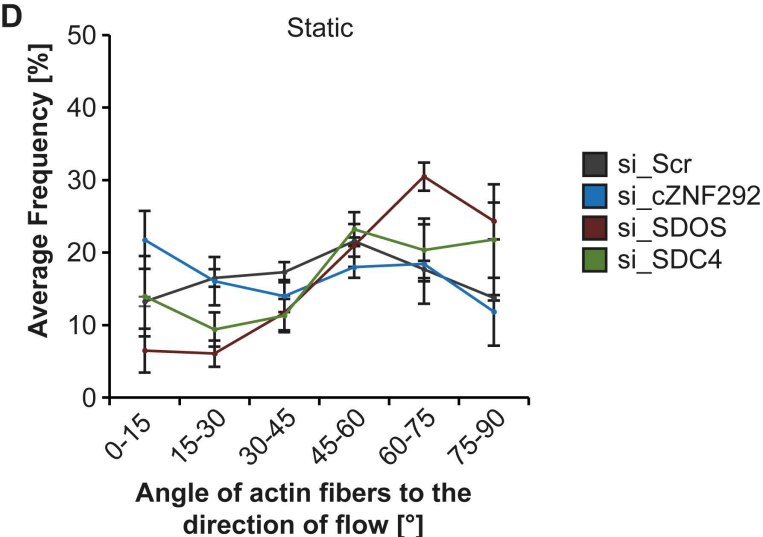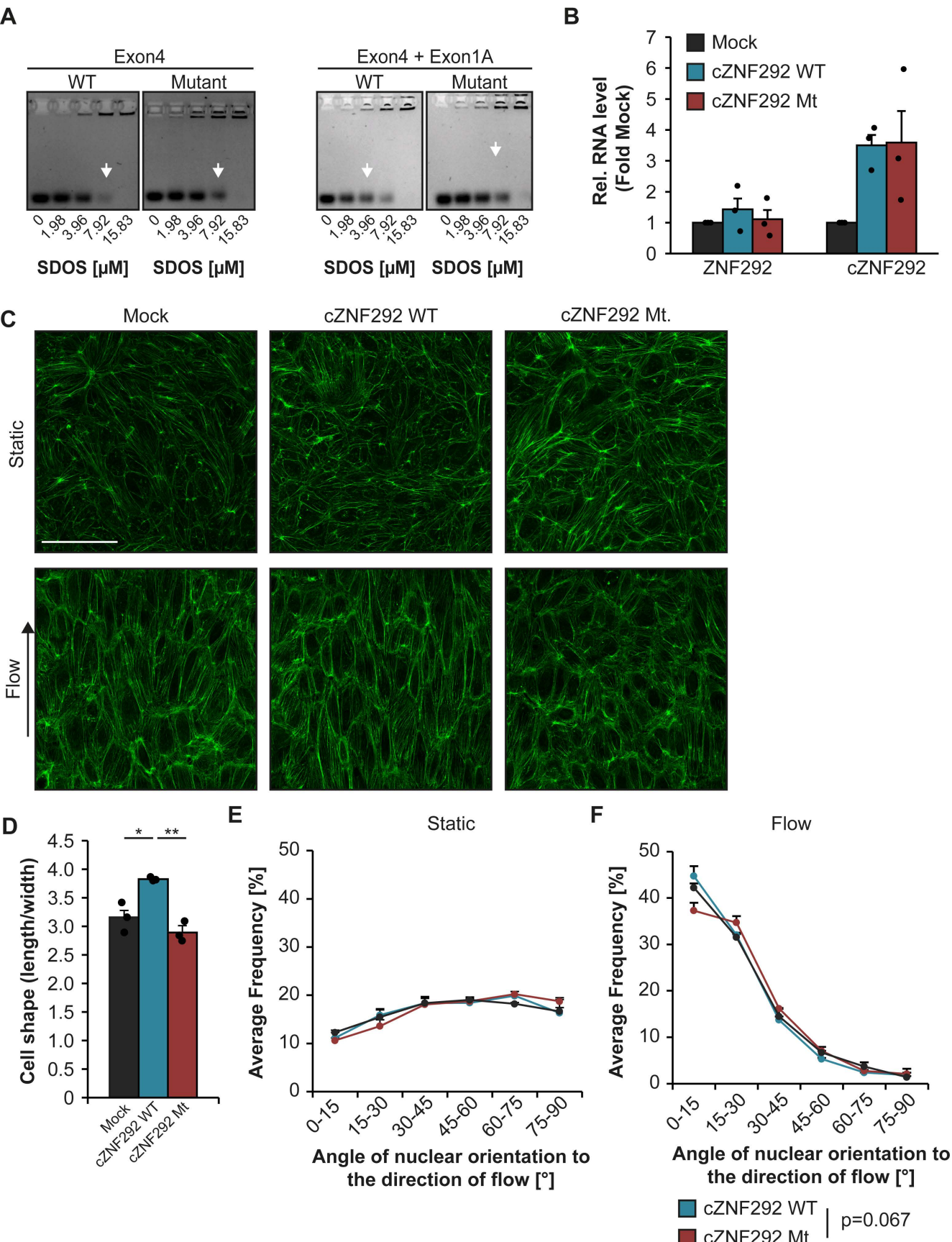
Online Figure IV

A

B Static

Angle of nuclear orientation to the direction of flow [°]

C Flow

Angle of nuclear orientation to the direction of flow [°]

D Static

Angle of actin fibers to the direction of flow [°]

Online Figure IV, cZNF292 /SDOS silencing affects the laminar flow response. Refers to main figure 5. **A,** Quantification of SDOS/cZNF292/SDC4/ZNF292 RNA levels after siRNA silencing in HUVEC (n=2-5). **B/C,** Distribution of the angle of orientation of nuclei in static controls slides (B) or after 48h exposure to 12 dyne laminar flow (n=3). **D,** Distribution of the angle of orientation of actin fibers compared to the direction of flow in HUVECs following siRNA silencing in static control slides (n=3). Data is depicted as mean±SEM, statistical analysis by two-sided unpaired student's t-test (*) or by two-sample Kolmogorov-Smirnov test, a value of p<0.05 is considered significant.

Online Figure V, Mutations in SDOS binding motifs reduced SDOS interaction. Refers to main figure 7. **A,** Representative agarose-gelelectrophoresis images on the interaction between SDOS and unlabelled RNA oligos comprising wildtype or mutated SDOS motifs of the sequence excerpts of exon4 or exon4+exon1A as analyzed in Fig.5C (n=3). White arrow heads highlight bands with visible difference in SDOS binding between wildtype and mutant oligos. **B,** Quantification of RNA levels by qRT-PCR 72h after overexpression of wildtype or cZNF292 with mutated SDOS binding sites by lentiviral transduction compared to mock treated controls (n=3). **C,** Representative phalloidin stainings of HUVECs following lentiviral overexpression of the cZNF292 wildtype or mutant and exposure to 48h of 12 dynes laminar flow. Static controls were treated identically but where not exposed to flow. **D,** Analysis of the cell shape of HUVEC following cZNF292 overexpression and exposure to flow (as above). Cell shape was estimated by the ratio of the longitudinal cell axis towards the cell's width (n=3). **E,F** Distribution of the angle of orientation of nuclei in static controls slides (E) or after 48h exposure to 12 dyne laminar flow (n=3). Data is depicted as mean±SEM, statistical analysis by two-sided unpaired student's t-test (*) with Bonferroni-Holm correction or by two-sample Kolmogorov-Smirnov test, a value of p<0.05 is considered significant. Scale bar in white equals 100 μm.
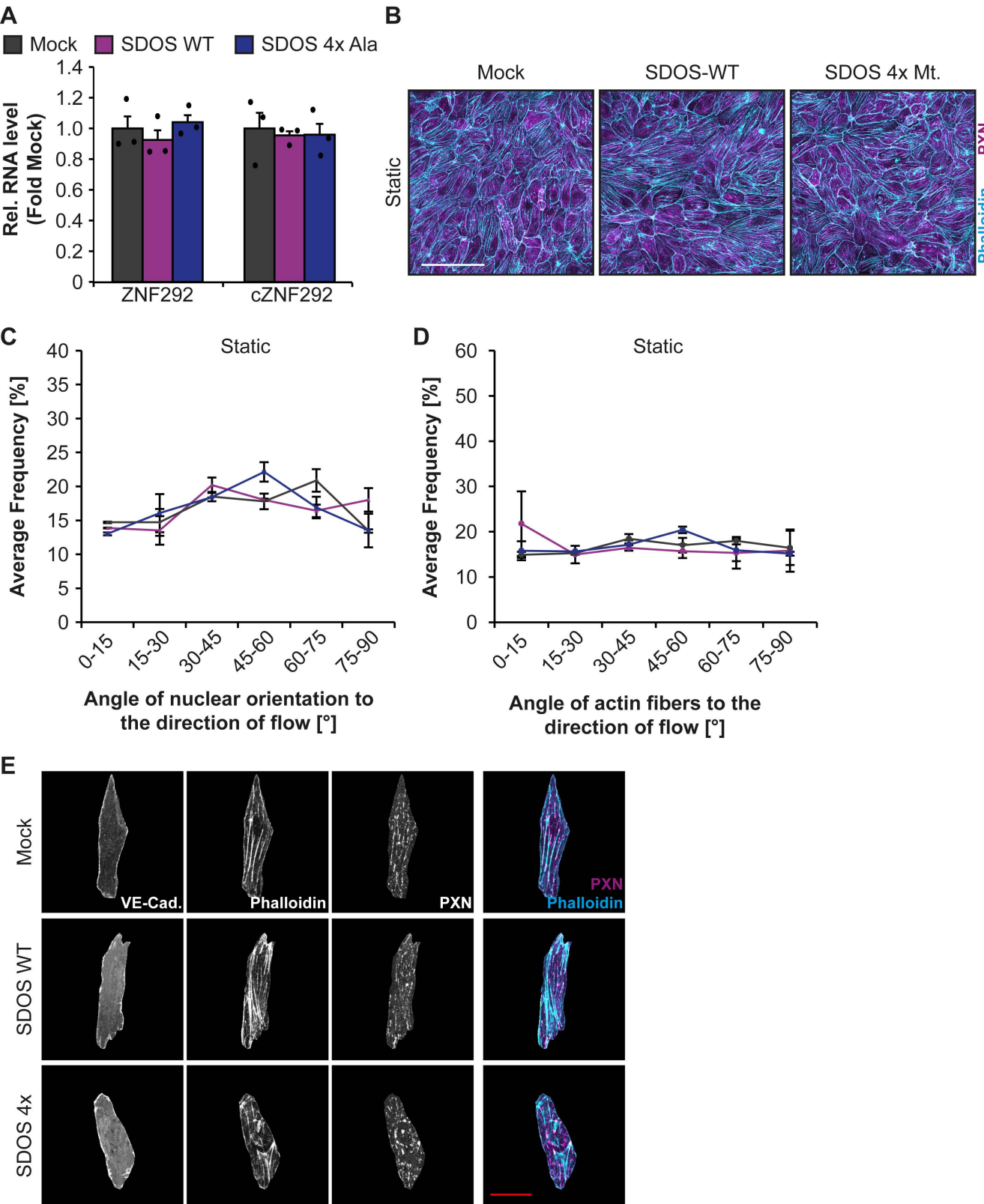
Online Figure VI

Online Figure VI, Overexpression of SDOS 4x Ala in laminar flow response. Refers to main figure 8. **A,** Quantification of linear and circular ZNF292 levels by qRT-PCR after lentiviral overexpression of wildtype or mutated SDOS (n=3). **B,** Representative immunofluorescence images showing HUVECs s following lentiviral overexpression of SDOS or the 4x Alanine mutant stained for F-Actin (Cyan by Phalloidin) and Paxillin (Magenta), Scale bar in white equals 100 µm. **C,** Distribution of the angle of orientation of nuclei or **D,** actin fibers compared to the direction of flow in HUVECs following lentiviral overexpression of SDOS variants in static control slides (n=2). **E,** Single cell excerpts of HUVECs after lentiviral overexpression of SDOS variants and 40h after exposure to 12 dyne laminar flow. HUVECs were stained for VE-Cadherin, Actin and Paxillin. Scale bar in red equals 25 µm. Data is depicted as mean±SEM.