SUPPLEMENTAL MATERIAL

Detailed Methods

Plasmids

TXNIP-GFP was obtained from Addgene (Addgene plasmid 18758). Human KLF2 cDNA was purchased from Origene (#SC127849, Rockville, MD). Both of the -924 bp and -157 bp KLF2 promoter reporter gene constructs were gifts from Dr Jerry Lingrel (University of Cincinnati). NF-κB luciferase reporter gene construct was a gift from Dr Chen Yan (University of Rochester).

Antibodies

Rabbit anti-TXNIP (VDUP1, C-term, #403700) and mouse anti-α-tubulin (#322500) were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-VCAM-1 (H-276, #SC-8304), mouse anti-ICAM-1 (G-5, #SC-8439), mouse anti-VE-Cadherin (F-8, #SC-9989), and goat anti-KLF2 (N-13, #SC-18690) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rat anti-VE-Cadherin (#322500) and rabbit anti-eNOS (#610298) were purchased from BD Biosciences Pharmingen (San Diego, CA). Rabbit anti-Lamin A/C (#2032) was purchased from Cell Signaling Technology (Beverly, MA).

Cell culture

HUVEC were isolated from human umbilical veins and seeded onto 0.2% gelatin-precoated culture dishes maintained in Medium 200 (Cascade Biologics, Portland, OR) with low-serum growth supplement (LSGS; Invitrogen, Carlsbad, CA), 5% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 IU/ml penicillin at 37°C in a humidified atmosphere of 95% air and 5% CO_2 as previously described.[1] HUVEC were used at passage 4 for all the experiments. THP-1 monocytes were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS, 100 μg/ml streptomycin and 100 IU/ml penicillin at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Plasmid and siRNA oligonucleotide transfection

HUVEC at greater than 90% confluence in 100-mm dishes were used for transfection. To overexpress TXNIP or KLF2 in EC, Lipofectamine 2000 (6 μl; Invitrogen, Carlsbad, CA) was mixed with Opti-MEM (500 μl; Invitrogen, Carlsbad, CA), and then cDNA (3 μg) diluted in 500 μl Opti-MEM was added to the solution, mixed gently, and incubated at room temperature for 20 min. A total of 1 ml of this mixture was added to HUVEC in 4 ml Opti-MEM and incubated for 3 h. Then medium was changed back to M200 medium and cells were harvested after 24 h. To knockdown TXNIP in HUVEC, we transfected cells with TXNIP-specific siRNA (#J-010814-05, Dharmacon, Lafayette, CO) by using Lipofectame 2000 and Opti-MEM following manufacturer's protocol.

Luciferase assay

HUVEC were transiently co-transfected with luciferase reporter gene constructs and β-galactosidase with Lipofectamine 2000 by using the similar transfection scheme as described above. For flow studies, transfected cells were incubated overnight at 37 ℃ for 18h and then exposed to s-flow (6 h) or d-flow (24 h) by using the cone and plate flow apparatus. To measure luciferase activity, transfected HUVEC were washed twice with phosphate buffered saline (PBS), lysed in Passive Lysis Buffer (#E194A, Promega, Madison, WI), and assayed by using a Luciferase Assay System (#E1501, Promega, Madison, WI) and a luminometer (BD Monolight 3010, BD Biosciences Pharmingen, San Diego, CA). Luciferase activity was normalized to β-galactosidase activity to correct for differences in transfection efficiency.

Immunofluorescence staining

Treated HUVEC in 35mm-dishes were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked with 10% normal goat serum in

PBS containing 0.5% Tween-20 for 1 h at room temperature. Then cells were stained with 5 μg/ml rabbit anti-TXNIP and mouse anti-VE-Cadherin overnight at 4°C in the blocking solution. Cells were then rinsed with 0.5% Tween-20 in PBS 3 times and incubated with the fluorescence-conjugated secondary antibodies (1:1000 dilution, Alex Fluor 546 and 488, respectively) for 1 h at room temperature. After another 3 rinses with the washing solution, images were acquired using an inverted epi-fluorescence microscope (IX50, Olympus) equipped with a charge-coupled device camera (Spot; Diagnostic Instruments, Inc) with Acroplan water 40× (N.A. 0.8) or 60× (N.A. 0.9) objective lens.

En face immunofluorescence staining

Immunofluorescence staining of mouse aortic EC was performed as described previously[2] with several modifications. Briefly, 12-week-old C57BL/6 and ApoE$^{-/-}$ mice were anesthetized with ketamine/xylazine cocktail (0.13/0.0088 mg/g body weight). Then we cut the jugular vein and perfused the arterial tree with saline containing 40 USPU/ml heparin from left ventricle for 5 min, followed by perfusion of pre-chilled 4% paraformaldehyde in PBS (pH 7.4) for 10 min. Subsequently, the whole aorta was dissected from iliac bifurcation to the heart, cut open longitudinally, permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 10% normal goat serum in Tris-buffered saline (TBS) containing 2.5% Tween-20 for 1 h at room temperature. Next, aortas were incubated with 5 μg/ml rabbit anti-TXNIP or 2 μg/ml rabbit anti-VCAM-1, and 5 μg/ml rat anti-VE-Cadherin in the blocking buffer overnight at 4°C. After rinsing with washing solution (TBS containing 2.5% Tween-20) 3 times, fluorescence-conjugated secondary antibodies (1:1000 dilution, Alexa Fluor 546 labeled anti-rabbit IgG and Alexa Fluor 488 labeled anti-rat IgG, respectively) were applied for 1 h at room temperature. Finally, after another 3 rinses in the washing solution, aortas were mounted in the ProLong antifade reagent (Invitrogen, Eugene, OR). Aortas were examined by a laser-scanning confocal microscope (FV-1000 mounted on IX81, Olympus) with UPlanSApo 20× or UPlanFL N 40x lens. Image analysis to quantify VCAM-1 membrane localization was performed in Image-Pro V6.2. Briefly, color intensities for both green (VE-Cadherin) and red (VCAM-1) channels from the desired region in the *en face* images were obtained by using Image-Pro. Then, the relative color intensity for each channel was plotted. Membrane-localized VCAM-1 was considered to be the overlapped area from both green and red channels.

In vitro THP-1 monocyte adhesion assay

Human monocyte leukemia cell line THP-1 was labeled for 30 min at 37°C with 10 μmol/L BCECF-AM (#B1170, Molecular Probes/Invitrogen, Eugene, OR) in RPMI 1640 medium, and washed three times with PBS. Labeled THP-1 monocytes (10^6 cells/ml) in M200 medium were added to monolayers of HUVEC and incubated for 40 min. Nonadherent THP-1 cells were removed by washing the culture 4 times with PBS. Cells were then stained with rhodamine-conjugated phalloidin (#R415, Invitrogen, Eugene, OR) and DAPI (#D1306, Invitrogen, Eugene, OR) to show the cell morphology and the nuclei. Attached cells were then observed by an inverted fluorescent microscope (IX50, Olympus) with an Acroplan 40× lens. EC-monocyte adhesion was quantified by calculating the percentage of EC associated THP-1 monocytes.

Blood pressure

Systolic blood pressure and heart rate were measured using a non-invasive tail-cuff method (plethysmography; Visitech System, Apex, NC).

Peripheral blood mononuclear cells (PBMC) isolation

Mouse PBMC were isolated by Optiprep density gradient medium (Nycomed, Oslo, Norway) following manufacturer's protocol. Briefly, diluted blood was layered on a 1.077 g/ml density barrier. After centrifugation at 700 g for 20 min, mononuclear cells at interphase were harvested using a pipette.

Subcellular fractionation

Nuclear and cytosolic protein was isolated as described previously.[3] In brief, treated HUVEC were washed and incubated with ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, and proteinase inhibitors) for 10 min. Cells were then scraped and homogenized with 20 strokes of a Dounce homogenizer. Homogenates were centrifuged at 700 g for 3 min to pellet nuclei. The supernatants were kept as cytosolic fraction. Nuclear pellets were resuspended in nuclei lysis buffer (50 mM Tris-Cl, 10 mM EDTA, 1% SDS, and proteinase inhibitors) as nuclear fraction.

Real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted using a TRIzol reagent (Invitrogen, Carlsbad, CA). For reverse transcription, 1 μg of total RNA was converted into first strand complementary DNA (cDNA) in a 20 μl reaction volume using a reverse transcription kit (#A3500, Promega, Madison, WI) following the manufacturer's instruction. Quantitative real-time PCR was then performed using an ABI 7900HT real-time PCR thermal cycler (Applied Biosystems, Foster City, CA). The comparative cycle threshold method was used to determine the relative mRNA expression of target genes after normalization to housekeeping gene GAPDH. Following primers were used: 5'-GAACCCAAACAGAGGCAGAG-3' for mouse VCAM-1-F and 5'-GTATCCCATCACTTGAGCAGG-3' for mouse VCAM-1-R; 5'-CCATCACCGTGTATTCGTTTC-3' for mouse ICAM-1-F and 5'-GCTGGCGGCTCAGTATCTC-3' for mouse ICAM-1-R; 5'-CACCTAAAGGCGCATCTGC-3' for mouse KLF2-F and 5'-TCGGTAGTGGCGGGTAAGC-3' for mouse KLF2-R; 5'-TACCCCCAATGTGTCCGTC-3' for mouse GAPDH-F and 5'-GGTCCTCAGTGTAGCCCAAG-3' for mouse GAPDH-R; 5'-AAGACCTACACCAAGAGTTC-3' for human KLF2-F and 5'-ACAGATGGCACTGGAATG-3' for human KLF2-R; 5'-ACGGATTTGGTCGTATTGGG-3' for human GAPDH-F and 5'-CGCTCCTGGAAGATGGTGAT-3' for human GAPDH-R.

Western blot analysis

HUVEC were harvested and lysed in ice-cold 1× lysis buffer (#9803, Cell Signaling Technology, Beverly, MA) supplemented with protease inhibitor cocktail (#P8340, Sigma-Aldrich, St. Louis, MO). Protein concentrations were determined by Bradford protein assay (#500-0006, Bio-Rad, Hercules, CA) using a spectrophotometer (DU-640B, Beckman Instruments Inc., Fullerton, CA). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred onto nitrocellulose membranes (Pall, East Hills, NY), and were subsequently blocked in 1× blocking buffer (#MB-070, Rockland Immunochemicals, Inc., Gilbertsville, PA) for 1 h. Then the blots were incubated overnight at 4°C with appropriate primary antibodies (1:1000 dilution except for anti-tubulin at 1:10,000). Then after being washed 3 times with 0.1% Tween-20, membranes were incubated with Alexa Fluor 568 or 680-conjugated secondary antibodies (1:10,000 dilution; Invitrogen, Eugene, OR). Images were acquired by using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Densitometric analysis of membranes was performed using Image J software (version 1.36b, National Institutes of Health).

Chromatin immunoprecipitation (ChIP) assay

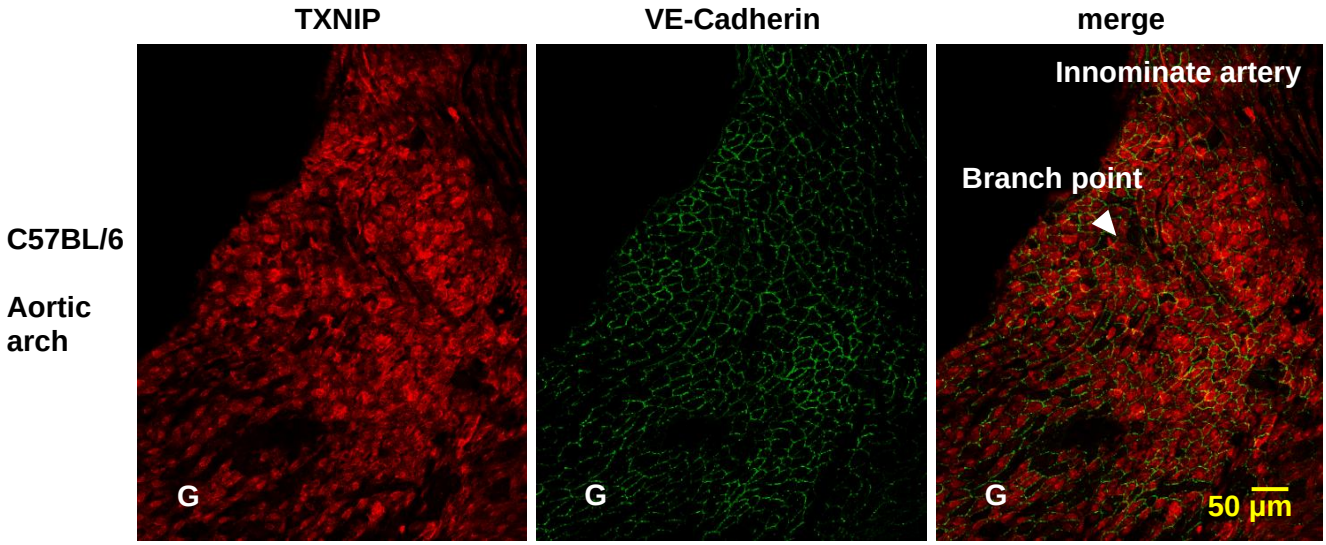
ChIP assays were performed as previously described.[4] In brief, HUVEC cultured in100-mm dishes were exposed to s- or d-flow from 0 to 24 h. Then cells were treated with 1% formaldehyde for 10 min at room temperature to cross-link the DNA-protein complex. Glycine (1.375 M, 1 ml) was added to quench formaldehyde and to terminate cross-linking reaction. Next, cells were washed twice with cold PBS and harvested by scraping using 10 ml PBS. After centrifugation at 500 g for 10 min at 4°C, cell pellets were resuspended in 10 ml lysis buffer (5 mM PIPES, 85 mM KCl, and 0.5% NP-40) and incubated on ice for 10 min. Nuclear lysates were obtained by centrifugation at 200 g for another 10 min and resuspended in nuclei lysis buffer (50 mM Tris-Cl, 10 mM EDTA, 1% SDS, and protease inhibitors). Nuclear lysates were sonicated on ice to shear DNA into fragments ranging 300~ 800 bp in size. Lysates were then centrifuged at 20,000 g for 15 min at 4°C to pellet the precipitated SDS, and pre-cleared by incubation

with 50 μl Protein A/G PLUS-Agarose beads (#SC-2003, Santa Cruz, Santa Cruz biotechnology, Santa Cruz, CA) at 4°C for 2 h. Then equal amounts of chromatin were incubated overnight with 1 μg rabbit anti-TXNIP or control rabbit IgG at 4°C. The protein A/G agarose beads (50 μl) were then added into the samples and incubated for 2 h at 4°C, followed by washing with high-salt wash buffer (50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 0.1% deoxycholate) 4 times and twice with TE buffer (1 mM EDTA, and 10 mM Tris-Cl). Proteins were then digested with 1 μl proteinase K (20 μg/μl) for 2 h at 55°C and the cross-linking was reversed by incubation of the samples overnight at 65°C. DNA samples were then purified with a PCR purification kit (QIAquick PCR Purification Kit, Qiagen, Valencia, CA). Finally, PCR or real-time PCR was performed by using a pair of primers (forward: 5'-TGTCAGCGCAAGGCCCAGGCCGCC-3' and reverse: 5'-CCGCCCAAGCCTTATAGGCGCG-3') encompassing the consensus sequence of the KLF2 -157 bp promoter. Chromatin samples that were kept before the immunoprecipitation were used as input control.

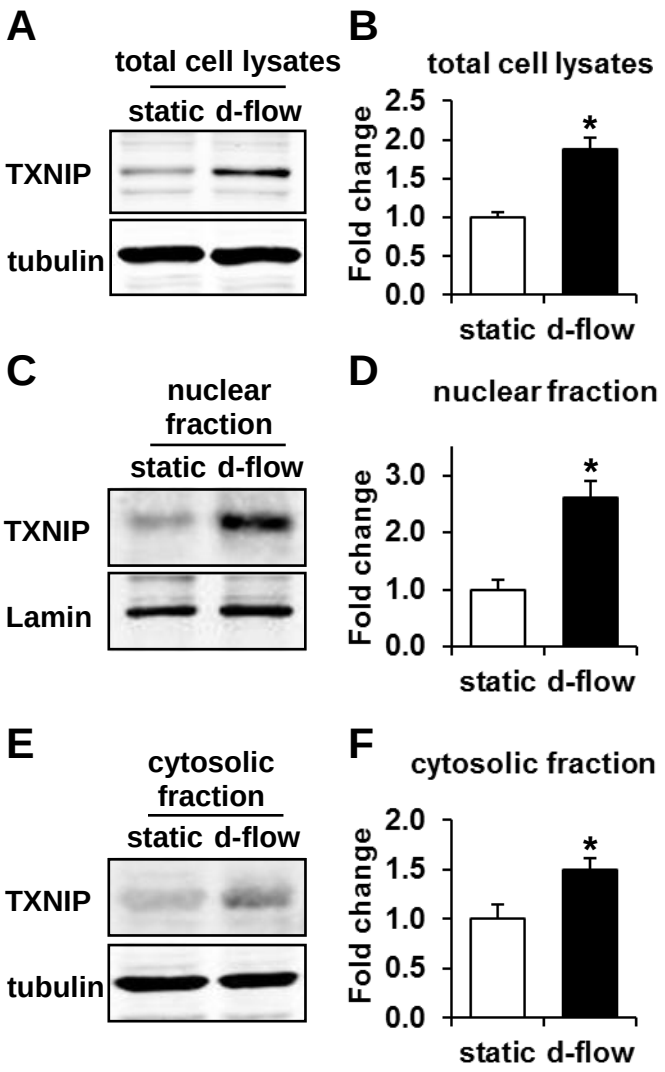
Supplemental References

1. Yamawaki H, Pan S, Lee RT, Berk BC. Fluid shear stress inhibits vascular inflammation by decreasing thioredoxin-interacting protein in endothelial cells. *J Clin Invest*. 2005;115:733-738
2. Heo KS, Lee H, Nigro P, Thomas T, Le NT, Chang E, McClain C, Reinhart-King CA, King MR, Berk BC, Fujiwara K, Woo CH, Abe J. Pkc{zeta} mediates disturbed flow-induced endothelial apoptosis via p53 sumoylation. *J Cell Biol*. 2011;193:867-884
3. Boulter E, Garcia-Mata R, Guilluy C, Dubash A, Rossi G, Brennwald PJ, Burridge K. Regulation of rho gtpase crosstalk, degradation and activity by rhogdi1. *Nature cell biology*. 2010;12:477-483
4. Carey MF, Peterson CL, Smale ST. Chromatin immunoprecipitation (chip). *Cold Spring Harb Protoc*. 2009;2009:pdb prot5279

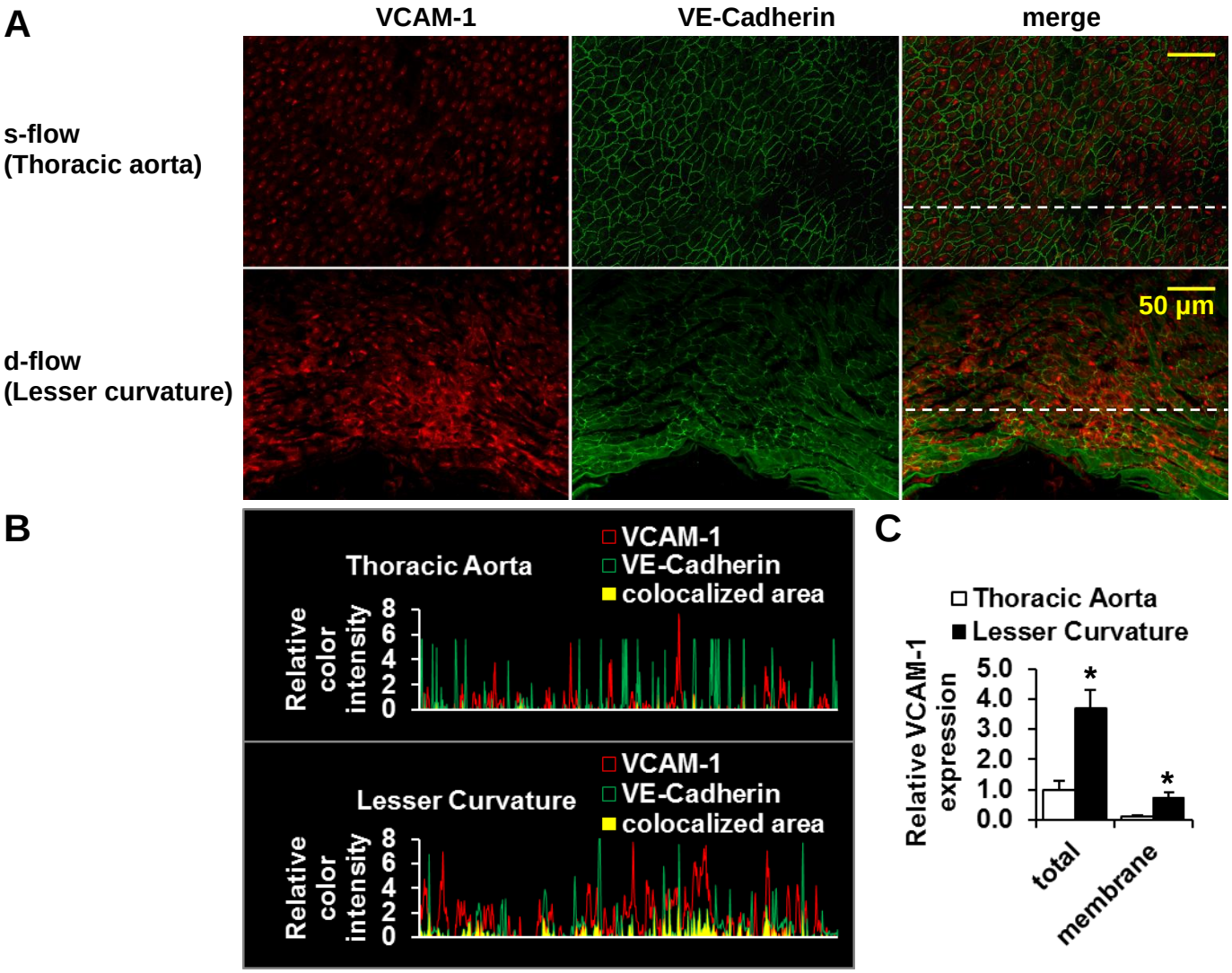
Supplemental Figures and Figure Legends



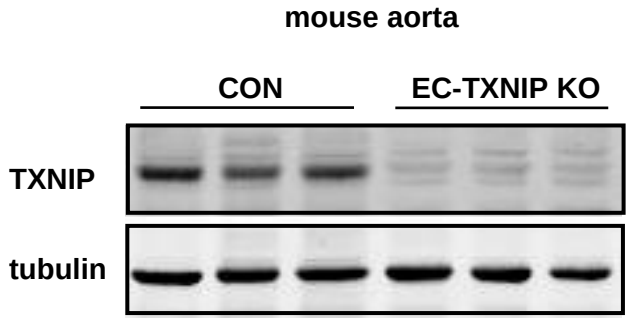
Online Figure I. TXNIP expression is increased at branch points. Shown is confocal immunofluorescence analysis of TXNIP expression at the branch point of the innominate artery from the aortic arch. Arrowhead points to the branch point. Region 'G' indicates the greater curvature. EC morphology is shown by VE-Cadherin staining.



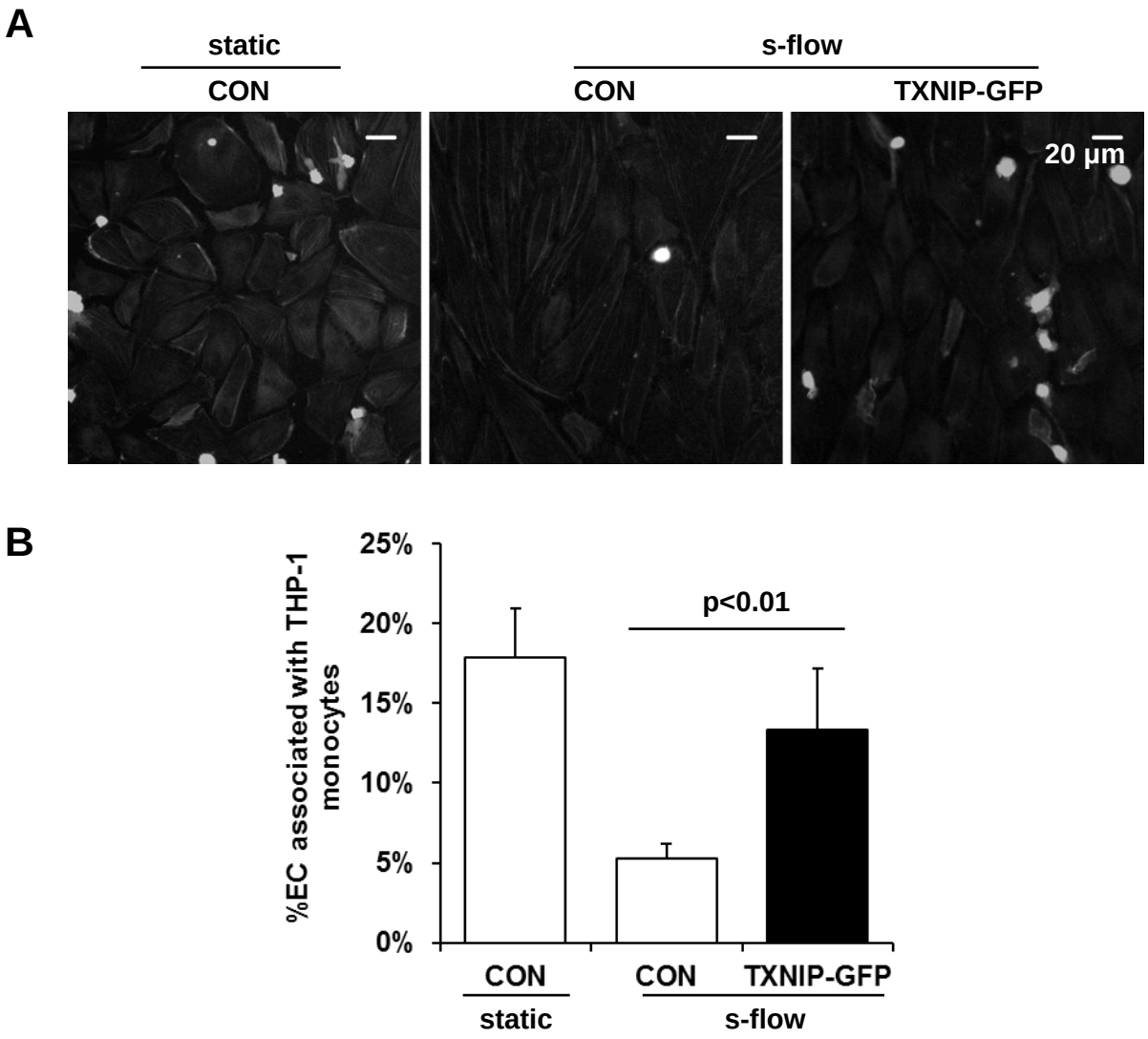
Online Figure II. D-flow significantly increases nuclear TXNIP accumulation. HUVEC were exposed to d-flow or kept at static condition for 24 h. Total cell lysates (A), nuclear (C) and cytosolic (E) fractions were then harvested for Western blot. (B, D, F) TXNIP expression levels in each fraction were analyzed by densitometric quantifications. Results are expressed as mean ± SD of 3 independent experiments. *P<0.05.



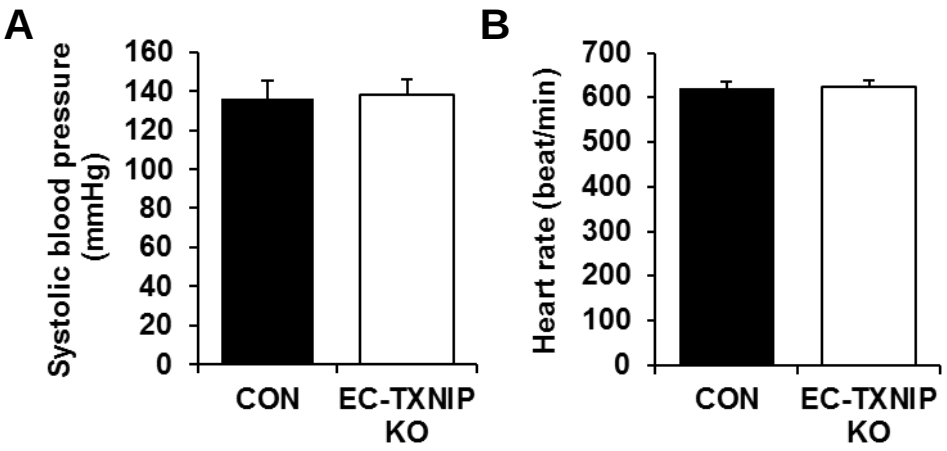
Online Figure III. Total and membrane VCAM-1 expression is increased in d-flow area. (A) Expression of VCAM-1 was analyzed by confocal immunofluorescence. Co-localization of red (anti-VCAM-1) and green (anti-VE-Cadherin) signals in merged images indicates the membrane VCAM-1 expression. (B and C) Quantifications were performed by randomly selecting 4 lines throughout the s-flow and d-flow area (as indicated by dashed lines in the merged images in A). (B) Relative color intensity through these lines was obtained by Image-Pro and was plotted. Yellow area indicates the overlapped region from both channels which is considered to be the membrane VCAM-1 expression. (C) Total and membrane VCAM-1 expression was then quantified by calculating the area under curve (AUC). *P<0.05.



Online Figure IV. TXNIP expression is reduced in mouse aorta from EC-TXNIP KO mice. Shown is Western blot analysis of aortic TXNIP expression from EC-TXNIP KO and control littermates, respectively.

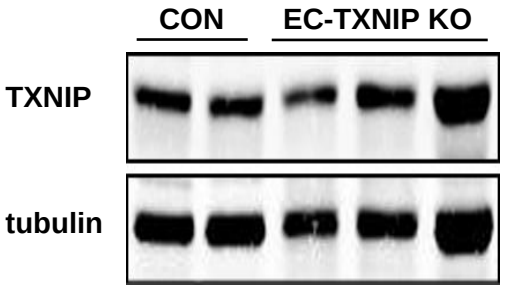


Online Figure V. Inhibition of EC-monocyte adhesion by s-flow is reversed by TXNIP overexpression. HUVEC were transfected with either TXNIP-GFP or control plasmid, kept at static condition or exposed to s-flow (12 dyn/cm^2) for 24 h, and then co-incubated with THP-1 monocytes. Shown are adherent monocytes labeled by BCECF-AM. EC morphology is shown by phalloidin staining. (B) Quantification data showing the percentage of EC associated with monocytes in the presence or absence of TXNIP overexpression, respectively. Results are expressed as mean ± SD of 3 independent experiments.

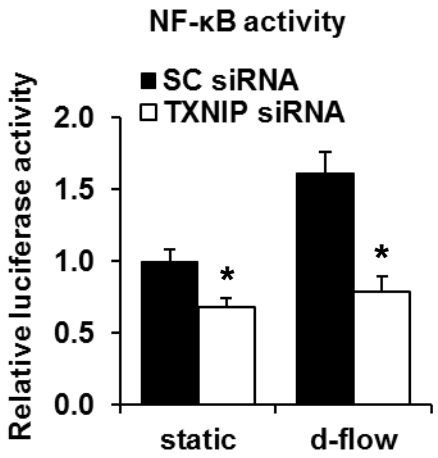


Online Figure VI. No significant differences in blood pressure and heart rate between control and EC-TXNIP knockout mice. Shown are systolic blood pressure (A) and heart rate (B) from EC-TXNIP knockout mice and control littermates. Data are expressed as mean ± SD from 5 mice in each group.

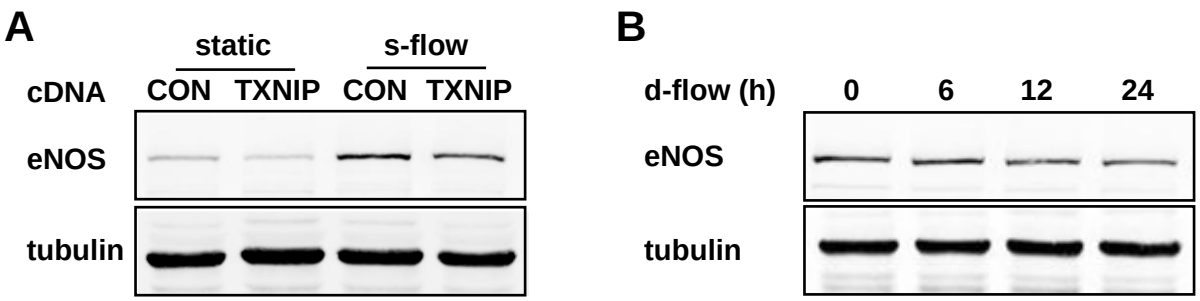
Mouse peripheral blood mononuclear cells (PBMC)



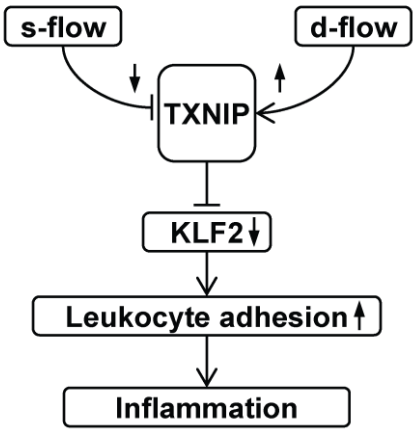
Online Figure VII. No differences in PBMC TXNIP expression between EC-TXNIP KO mice and control littermates. Mouse PBMC were freshly isolated by density-gradient centrifugation. Shown is Western blot analysis of PBMC TXNIP expression from control and EC-TXNIP KO mice.



Online Figure VIII. NF-κB activation in response to d-flow is reversed by TXNIP depletion. HUVEC were pretreated with scramble control or TXNIP-specific siRNA for 48 h, transfected with NF-κB luciferase reporter, and exposed to d-flow or kept at static condition for an additional 24 h. Cell were harvested and the luciferase activities were measured. Results are expressed as mean ± SD of 3 independent experiments. *P<0.05.



Online Figure IX. Expression of eNOS is repressed by TXNIP. (A) HUVEC in 100-mm dish were transfected with 3 μg TXNIP-GFP or control plasmid, and then exposed to s-flow or kept at static condition for 24 h. (B) HUVEC were exposed to d-flow for the indicated times. eNOS expression levels were analyzed by Western blot.



Online Figure X. A scheme describing the TXNIP-mediated cross talk between s-flow (anti-inflammatory) and d-flow (pro-inflammatory) pathways.