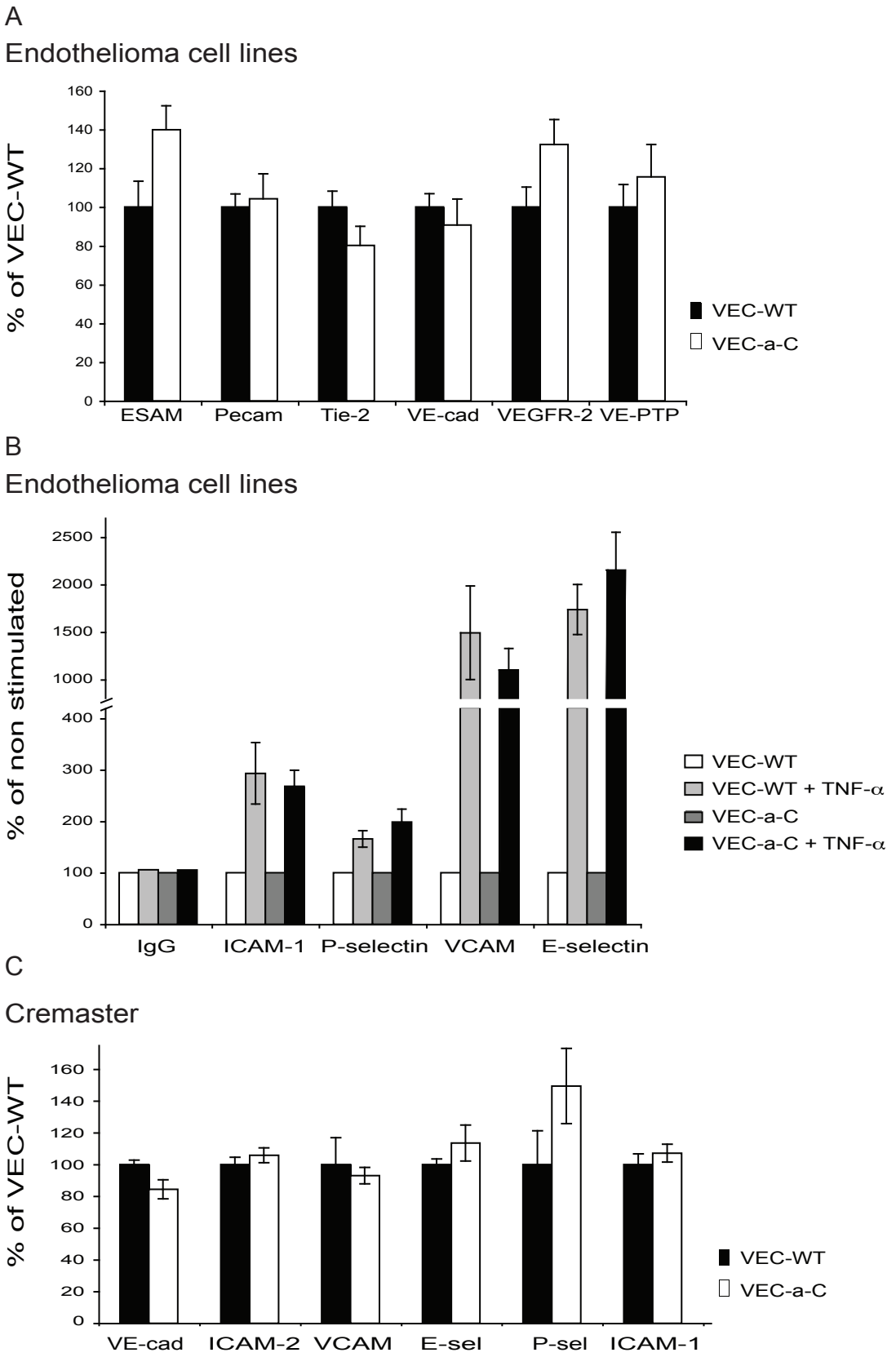
Supplementary figure S1



Supplemental Figure S1.
Expression of endothelial surface molecules is comparable in VEC-a-C and VEC-WT endothelial cells.
(A) Mean fluorescence intensity levels for the indicated surface molecules on VEC-WT and VEC-α-C endothelioma cells determined by flow cytometry. Levels of VEC-WT cells were set to 100 %. n = 3 to 5 (B) Mean fluorescence intensity signals for the indicated antigens after stimulation with TNF-α for 2 h (P-selectin and E-selectin) or 12 h (ICAM-1 and VCAM). Signals of untreated cells are set to 100 %. n =3 to 5. (C) Mean fluorescence intensity levels for the indicated surface molecules on endothelial cells from IL-1β stimulated cremaster muscle of VEC-WT and VEC-α-C mice determined by flow cytometry. Cells of cremaster muscles were dissociated using Collagenase A and and level of cell surface receptors of PECAM-1+CD45- negative cells were determined by in flow cytometry. Levels of cells from VEC-WT mice were set to 100 %. 4 mice were used for each group.

Supplementary figure S2

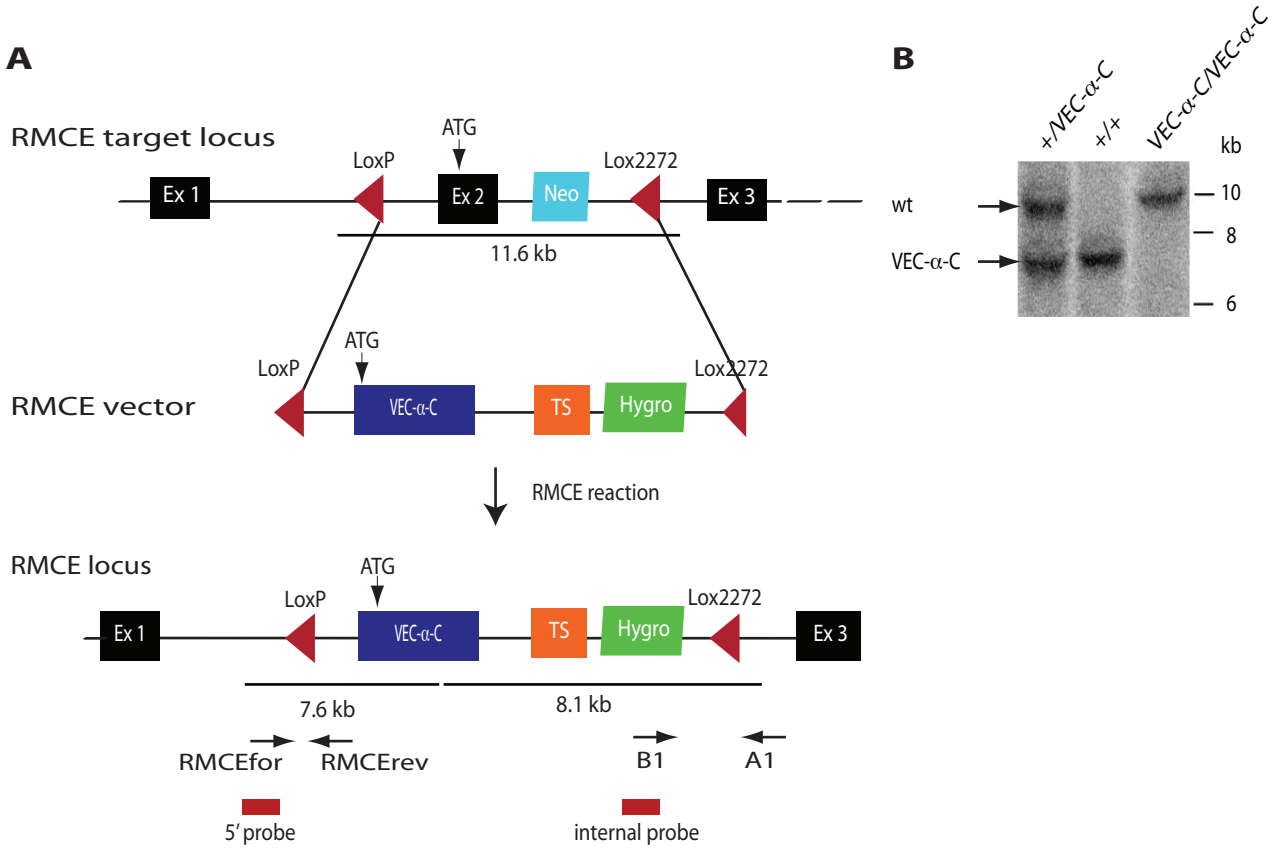


Figure S2. Generation of VEC-WT or VEC-α-C knock-in mice

(A) Schematic illustration of the targeting strategy for recombinase-mediated cassette exchange (RMCE) performed with ES cells (described elsewhere) harboring the two incompatible LoxP and Lox2272 sites flanking exon (Ex2) and a neomycin resistance cassette (Neo). These cells were co-transfected with a Cre recombinase expression vector and an RMCE vector containing the same two incompatible LoxP/Lox2272 sites flanking a replacement cassette. This cassette comprised the cDNA coding for VEC-α-C or VEC-WT followed by a transcriptional stop (TS) cassette and an FRT flanked hygromycin resistance cassette. Replacement of the LoxP/Lox2272 flanked genomic sequence by the replacement cassette resulted in inserting the VEC-α-C or VEC-WT cDNA in such a way that the starting codons of the cDNAs were in exactly the same position as the original starting exon of the genomic sequence. The size of DNA fragments expected in Southern blots upon BglII digestion of genomic DNA and hybridization with the 5' external probe and the internal probe are indicated as well as binding sites for primers used for genotyping. (**B**) Southern blot analysis of genomic DNA of a wild type (+/+), heterozygous knock-in mouse (+/VEC-α-C) and homozygous knock-in mouse (VEC-α-C/VEC-α-C) digested with BglII hybridized with the 5' probe generating a 7.6 kb fragment for the RMCE locus and a 9.9 kb fragment for the wild type locus.

Supplementary table S1

Genotypes from intercrosses of VEC-α-Cmice.

			no. of progeny			
mating	no. of litters	no. of offspring	+/+	+/VEC-α-C	VEC-α-C/VEC-α-C	% of VEC-α-C/VEC-α-C (% of expected)
+/VEC-α-C x +/VEC-α-C	25	180	66	107	7	4 (16)
+/VEC-α-C x VEC-α-C/VEC-α-C	15	83	0	72	11	14 (29)

mating	no. of litters	no. of offspring	no. of matings	no. of females	pups per female	% of VEC-α-C/VEC-α-C compared to VEC-WT
VEC-α-C/VEC-α-C x VEC-α-C/VEC-α-C	29	114	40	65	1,75	44 %
VEC-WT/VEC-WT x VEC-WT/VEC-WT	22	197	25	50	3,94	100 %

Supplementary table S2

Systemic leukocyte blood counts (cell number*106/mL blood, mean ± SEM) obtained from 7 mice per group.

	Total leukocytes	GR-1+ cells	CD4+ cells	CD8+ cells	CD19+ cells
VEC-WT	6.50 ± 0.48	1.34 ± 0.07	1,51 ± 0.13	1.07 ± 0.06	2,22 ± 0.27
VEC-α-C	9.72 ± 0.98	1.93 ± 0.12	1,96 ± 0.28	1.61 ± 0.17	4.21 ± 0.48

Supplementary table S3

Leukocyte counts (cell number*107/femur, mean ± SEM) in bone marrow of VEC-WT and VEC-α-C mice obtained from 6 mice per group.

	Total	B220+	CD4+	CD8+	Gr-1+	7/4+	c-kit+ lin-
VEC-WT	232.31 ± 18.58	51.17 ± 8.06	4.44 ± 0.49	3.69 ± 0.56	135.21 ± 7.45	119.38 ± 6.48	0.1 ± 0.02
VEC-α-C	218.14 ± 12.00	41.49 ± 5.00	4.44 ± 0.41	2.44 ± 0.26	133.42 ± 7.37	117.07 ± 6.77	0.13 ± 0.04

Supplementary table S4

Leukocyte counts (cell number*107/lymph nodes, mean ± SEM) in inguinal lymph nodes of VEC-WT and VEC-α-C mice obtained from 6 mice per group.

	Total	CD19+	CD4+	CD8+	Gr-1+	7/4+
VEC-WT	102.14 ± 8.79	32.47 ± 2.83	48.11 ± 5.33	25.03 ± 2.56	6.72 ± 0.56	0.54 ± 0.05
VEC-α-C	107.96 ± 14.24	40.92 ± 3.62	40.58 ± 6.55	31.26 ± 4.82	8.04 ± 1.26	0.54 ± 0.06

Supplementary table S5

Leukocyte counts (cell number*107, mean ± SEM) in spleen of VEC-WT and VEC-α-C mice obtained from 6 mice per group.

	Total	CD19+	CD4+	CD8+	Gr-1+	7/4+
VEC-WT	204.28 ± 17.40	116.64 ± 9.17	43.60 ± 3.59	22.57 ± 2.17	17.07 ± 1.13	4.73 ± 0.55
VEC-α-C	217.26 ± 28.41	136.65 ± 15.45	38.75 ± 5.51	23.85 ± 2.97	19.36 ± 2.89	4.73 ± 1.11

Supplementary table S6

Hemodynamic parameters (mean ± SEM of diameter, centerline velocity, shear rate, and WBC) of IL-1β treated cremaster muscle venules in VEC-WT and VEC-α-C mice

	Mice	Venules	WBC (x 106 cells/mL)	Gr-1+ cells (x 106 cells/mL)	Diameter (μm)	Blood velocity (μm/s)	Wall shear rate (103/s)
VEC-WT	4	27	6.95 ± 0.50	4.46± 0.44	29.00 ± 1.24	2.5 ± 0.03	1.60 ± 0.10
VEC-a-C	5	35	9.72 ± 1.05	4.77 ± 0.68	29.53 ±1.08	2.52 ± 0.03	1.51 ± 0.06

Supplemental methods:

Antibodies and reagents The following reagents and antibodies were used: VEGF164 (R&D), LPS (Sigma-Aldrich), IL-1ß (Abazyme), wnt3a (R&D), G-actin/F-actin in vivo assay kit (Cytoskeleton), collagenase A (Roche), mAbs against mouse VE-cadherin: BV13 (e-bioscience) and 11D4.1(Gotsch et al, 1997); mAbs 1G5.1 and 5D2.6 against mouse PECAM-1 and mAb αN-cad33 against mouse N-cadherin (our lab, unpublished); pAb VE19 against ESAM (Wegmann et al, 2006), pAb VE-PTP-C against VE-PTP (Nawroth et al, 2002), mAb 3G1 against Tie-2 (Koblizek et al, 1997), pAb S19 against p120 (Santa Cruz), mAb 9A9 against E-selectin (Norton et al, 1993) and mAb RB40.34 against P-selectin (Bosse & Vestweber, 1994), mAb 6C7 against VCAM-1 (Engelhardt et al, 1998), pAb against phospho-Erk1/2-Thr202/Tyr204 pAb and mAb against Erk1/2, mAb 4G10 against phosphotyrosine (Millipore), pAB C20 against VEGFR-2 (Santa Cruz), pAB against MLC2 and phospho-PLC2 (S19) (Cell Signaling), mAb YNI.1 against ICAM-1 (Takei, 1985), mAb R6-5-D6 against human ICAM-1 (BioX Cell), Alexa-488 conjugated monoclonal Claudin-5 antibody (4C3C2, Zymed), pAb against MRP-14 (R&D), mAB RV202 against vimentin, mAb against α-, β-and γ-catenin, FITC conjugated mAb RB6-8C5 against Ly-6G and Ly-6C (Gr-1); FITC-conjugated monoclonal anti-Ter119, anti-CD3, anti-CD4, anti-CD8, anti-CD19 , anti-CD45RB220, and anti-CD45 antibodies; PE-conjugated mAb Mec13.3 anti-PECAM-1 antibody, rabbit non-immune serum (IgG fraction), isotype-matched control mAbs (all from BD Biosciences). Alexa-568-conjucated phalloidin, and Alexa 488–, Alexa 633–, and Alexa 568–coupled secondary antibodies were purchased from Invitrogen, other secondary antibodies from Dianova.

Generation of VEC-α-C and VEC-WT knock-in mice

The VEC-α-C fusion construct was generated by fusing mouse VE-cadherin lacking the 75 C-terminal amino acids of the cytoplasmic tail to the C-terminal two thirds of mouse α-catenin (amino acids 301–906). To this end, a C to T mutation was inserted at bp 2130 (with bp 1 being the first of the start codon ATG) of the mouse VE-cadherin cDNA (Breier et al, 1996) giving rise to a new ClaI site. The ClaI-XbaI 3' fragment of VE-cadherin was replaced by a 2469-base pair Eco47III-XbaI fragment of mouse α-catenin thus creating a fusion molecule interface with base pairs ATG (Met, aa 708 of VE-cadherin) ATC (Ile aa 709 of VE-cadherin) GGC (Gly, additional aa) TTT (Phe, aa 301 of α-catenin).

The VEC-α-C, or VEC-WT cDNA were inserted into the endogenous VE-cadherin locus via Recombinase Mediated Cassette Exchange (RMCE) by using mouse ES cells (described elsewhere) where exon-2 of VE-cadherin containing the start codon had been flanked with two incompatible loxP sites (LoxP and Lox2272). These cells were transfected with the respective cassette exchange vector containing the desired cDNA, followed by a transcriptional stop cassette and an FRT flanked hygromycin cassette. The exchange cassette in this vector was flanked by LoxP and Lox2272, which allowed to replace exon 2 of VE-cadherin by co-transfecting above mentioned ES cells with this vector and a Cre-recombinase expression vector. Hygromycin-resistant colonies were screened by PCR and confirmed by Southern blotting. Binding sites of probes and primers as well as fragments generated by BglII digestion of genomic DNA are indicated in figure S2. Positive ES cell clones were injected into blastocysts of C57Bl/6 mice to generate chimeras, which were mated with C57Bl/6 mice. After confirmation of germline transmission of the RMCE locus by Southern blotting, these mice were intercrossed to generate homozygous mutant mice. Genotyping was performed using primers RMCEfor and RMCErev generating a 520 bp PCR product in wild type mice and a 565 bp PCR product in knock-in mice.

Cell culture

HUVEC were isolated and propagated as described (Baumeister et al, 2005). Polyoma middle T immortalization of embryonic endothelial cells was carried out as described previously (Reiss & Kiefer, 2004) using E9.5 mouse embryos, and cells were cultured as previously described (Baumer et al, 2006). For stimulation with VEGF, highly confluent endothelioma cells were starved over night with MCDB 131 medium (Invitrogen) containing 1 % BSA, and stimulated with 100 ng/ml recombinant VEGF164 in MCDB 131 medium with 1 % BSA for 5 min. HL60 cells were obtained from ATCC and maintained in RPMI supplemented with 10 % fetal bovine serum, 2 mM L glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C with 5 % CO_2. Differentiation into the granulocyte lineage was induced by 1.3 % DMSO in growth medium for three days. COS-7 cells were cultured and transiently transfected as described before (Nawroth et al, 2002).

Adenoviral transduction

Human VEC-α-C-GFP and full length VE-cadherin-α-catenin-GFP (VECfl-α-C-GFP) constructs were generated from a cDNA of human VEC-GFP that was a kind gift of F. E. Nwariaku (University of Texas, Dallas, USA) (Nwariaku et al, 2004). For the generation of human VECfl-α-C-GFP, the 3' two-thirds of the coding sequence for α-catenin were amplified using the primers ATATAGATCTGAGCGCTTTAGGCCGTCCC and ATATAGATCTTAAGATGCTGTCC ATGGCTTTG, resulting in a cDNA fragment containing BamH1 restriction sites at either end. This fragment was inserted into the BamH1 site between the full length coding regions of VE-Cadherin and GFP in VEC-WT-GFP. Human VEC-α-C-GFP cDNA was analogously constructed as mVEC-α-C (see above). To generate adenovirus vectors, cDNAs were cloned into pENTR 2B (Invitrogen) and the pAd-DEST vector was created using the LR Recombination Reaction (Gateway Technology;

Invitrogen). Viral lysates were prepared following the manufacturer's instruction (Invitrogen).

Cellular lysates, immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as described before (Winderlich et al, 2009). For SDS-Urea extracts, shock frozen lung tissues were lysed in 8 M Urea by sonication and subsequently 10 % SDS were added. Cell monolayers were lysed in 62.5 mM Tris/HCl (pH 6.8) with 8 M Urea and 2 % SDS. SDS sample buffer was added, followed by heating to 95 °C.

For analysis of phospho-MLC2, cells were lysed in hot Laemmli buffer containing 10 mM FaF, 1 mM Na_3VO_4, 10 mM Na-β-glycerophosphate, 0.5 mM PMSF and complete-EDTA free protease inhibitors.

For coimmunoprecipitations from lung tissue, lungs were homogenized and lysed in RIPA lysis buffer (1 % NP40, 1 % NaDeoxycholate, 0.1 % SDS, 0.01 M NH_2PH_4O, 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4) containing protease inhibitors for 4 h. Lysates were centrifuged at 4 °C for 1 h at 20000 g. For immunoprecipitation, lysates were first precleared using protein A-Sepharose for 2 h and then incubated with protein A-Sepharose and the indicated antibodies. Immunocomplexes were washed five times with RIPA buffer . Immunoprecipitated material and total cell lysates were analysed by SDS-PAGE and Western Blot as described previously (Winderlich et al, 2009).

Separation of cellular extracts into NP-40-soluble and insoluble fractions has been described previously (Navarro et al, 1995). Briefly, confluent cells were washed twice with PBS containing 1 mM $CaCl_2$ and extracted on ice with extraction buffer (10 mM Tris-HCl, 150 mM NaCl (TBS), pH 7.5 with 2 mM $CaCl_2$, 1x Complete EDTA-free protease inhibitor cocktail (Roche), 1% NP-40) for 30 min with gentle agitation. Cell extracts were collected

and centrifuged at 13,000 g for 5 min at 4 °C and the supernatant was defined as the NP-40-souble fraction. Remaining material (preserved nuclei and cytoskeletal fibres) were washed thrice with TBS containing proteinase inhibitors. This NP-40-insoluble fraction was then extracted with extraction buffer containing 1 % NP-40, 1 % Triton-X-100 and 0.5 % SDS. The extract was vigorously pipetted and centrifuged for 5 min at 14,000 g and 4 °C. The supernatant was defined as the NP-40-insoluble fraction.

Flow cytometry of endothelial cells from cremaster muscle:

Analysis of expression levels of endothelial surface antigens in the cremaster muscle was performed as described previously (Rossaint et al, 2011). 4 h after intrascrotal injection of IL-1β, cremaster muscles were minced with scissors and placed in a 60 mm dish containing PBS. Tissue was digested with collagenase A at 37 °C for 60 min. Cells were then incubated with primary antibodies, washed and incubated with anti-rat secondary APC-conjugated antibodies. After washing five times, cells were stained with FITC-conjugated CD45 and PE-conjugated PECAM-1 antibodies. Endothelial cells were determined as CD45 negative and PECAM-1 positive.

VE-Cadherin internalization assay

VE-Cadherin internalization assays were carried out as previously described (Xiao et al, 2003). In brief, HUVECs were seeded at confluency and infected with adenoviral VEC-WT-GFP or VEC-α-Cat -GFP 16 hrs prior to the assay. The cells were incubated for 1 hr at 4 °C with mAb Cadherin 5 against VE-cadherin, washed twice to remove unbound antibody and subsequently transferred to 37 °C in the presence of 150 µm chloroquine for either 15, 30, or 60 min. After fixation (2%PFA, 20 min), cells were permeabilized with 0.5 % Triton X-100 for 10 min at RT and blocked with 3 % BSA for 1 hr at RT. For immunostainings, a

secondary antibody conjugated to Alexa-568 (Molecular Probes) was used. Quantification of endocytosis was based on Image J software determining intracellular VE-cadherin antibody-staining. Maesurements were standardized to different expression levels of VEC-WT-GFP and VEC-α-C-GFP expression levels. Intracellular staining intensity at the 0 min time point was subtracted from values at other time points.

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