SUPPLEMENTAL MATERIALS AND METHODS

Fluorescence-activated Cell Sorting (FACS) Analysis

HUVEC were washed with PBS, detached using 1X accutase (Sigma; St. Louis, MO) and then fixed in FACS buffer containing 1% PFA. Cells were analyzed by flow cytometry using a BD Accuri™ C6 flow cytometer and CFlow Plus Analysis software (BD Biosciences; San Jose, CA). Samples were manually gated and analyzed using the FloJo v10 software package.

mRNA Preparation and Quantification

RNA was collected 48h post-treatment using TRIzol® (Life Technologies; Grand Island, NY), according to the manufacturer's protocol, and converted to cDNA using iScript (BioRad; Berkeley, CA), according to the manufacturer's protocol. qRT-PCR ΔΔCT analysis was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems; Grand Island, NY). Primers for Flt1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to quantify mRNA (**Table S3**). Data are reported normalized to GAPDH.

Table S1: Antibodies and Nuclear Stain.

Primary Antibody	Company	CATALOG #	Use	Western Blot Dilution	Immunofluorescence Dilution/Time
β-actin	Cell Signaling	3700P	HUVEC	1:10,000	
GFP	Abcam	ab6556	HUVEC	1:7,500	
VEGFR2	Cell Signaling	2479S	HUVEC	1:2,000	
pVEGFR2-Y1175	Cell Signaling	2478S	HUVEC	1:1,000	
Flt1	Abcam	ab32152	HUVEC	1:2,000	1:1,000; 48h
PECAM	Cell Signaling	3528S	HUVEC		1:1,500; 48h
Conjugated phalloidin	Life Technologies	ab109202	HUVEC		1:50; 48h
Alexa Fluor® 488	Life Technologies	A-21206	HUVEC		1:750; overnight
Alexa Fluor® 555	Life Technologies	A-21428	HUVEC		1:750; overnight
Alexa Fluor® 647	Life Technologies	A-21235	HUVEC		1:750; overnight
DRAQ-7	Abcam	ab109202	HUVEC		1:1,000; 1h
Isolectin conjugated Alexa488	Life Technologies	I21411	Mouse retina		1:100; overnight
DAPI	Life Technologies	D1306	Mouse retina		1:1,000; 30 mins

Table S2: siRNAs for Flt1 Isoforms.

Targeted Isoform	Sequence (5'-3')
sFlt1	GAGCACTGCAACAAAAAGGCTGTTTTCTCTCGGATCTC
mFlt1	GGAAATAGTGGGTTTACATAC

Table S3: qPCR Primers.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
GAPDH	CCTCAAGATCATCAGCAATGCCTCCT	TTGGTATCGTGGAAGGACTCATGACC
Flt1	AGGGCCTCTGATGGTGATTGTTGA	ATGCAGCACTACACATGGAGCCTA

SUPPLEMENTAL FIGURES

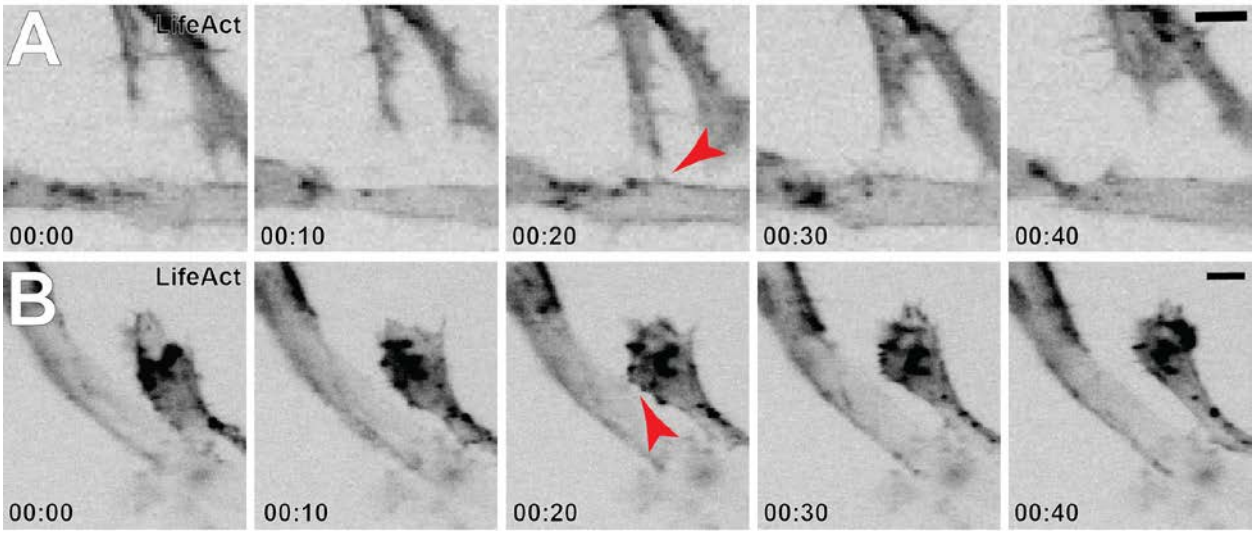


Figure S1: High Magnification Images of Transient Contacts.

(A-B) Representative time-lapse images of LifeAct infected d3-5 HUVEC sprouts. Transient contact, red arrowhead. Scale bar, 10 μm; time (lower left) hrs:mins.

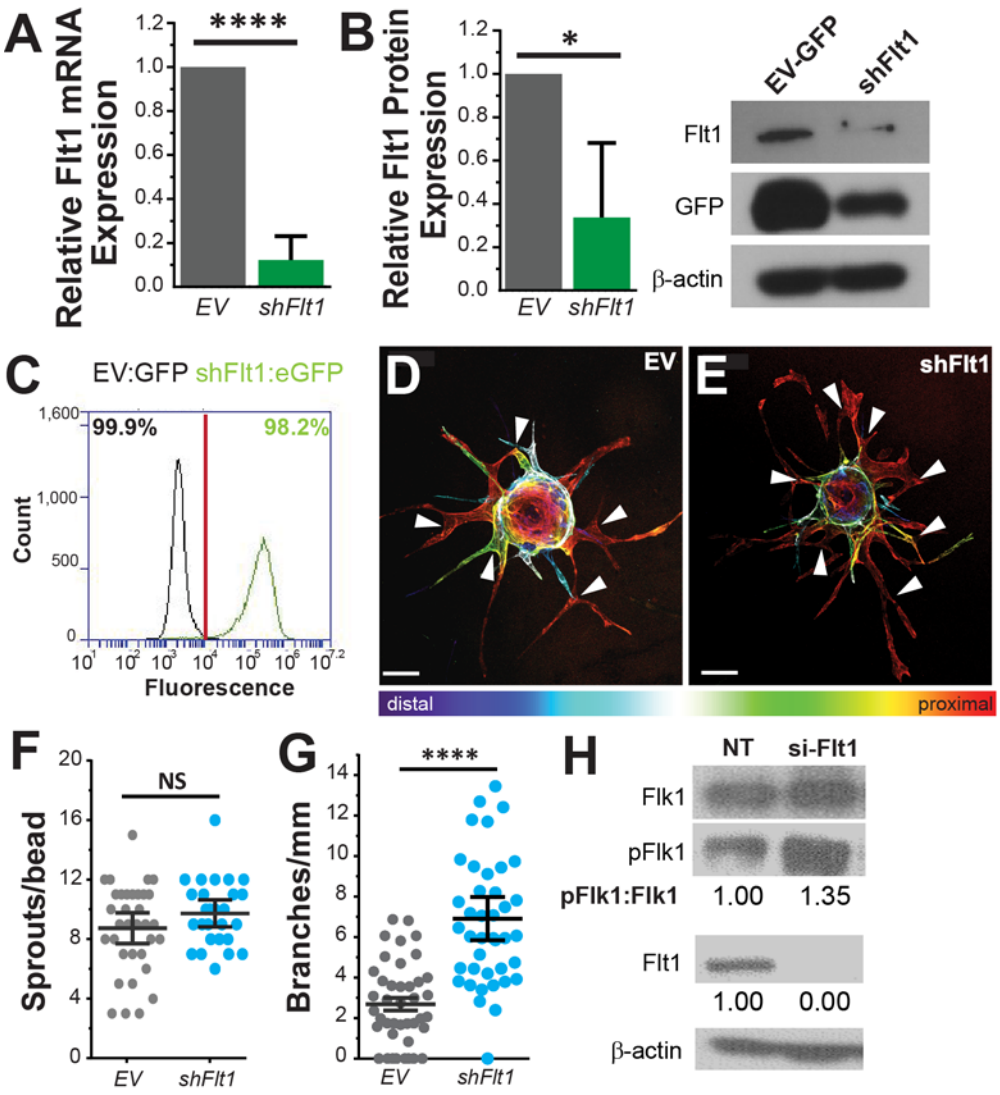


Figure S2: Flt shRNA Validation.

(A-C) HUVEC collected 48h post-infection with indicated lentivirus and analyzed by qRT-PCR (A), Western blot (B), or FACS sorted for infection efficiency (via GFP signal) (C). Flt1 mRNA expression was normalized to GAPDH and relative to control. Flt1 protein expression was normalized to β-actin and relative to EV-GFP. **(D-E)** Representative images of HUVEC sprouts with indicated treatments at d5, stained with phalloidin and depth-encoded. Arrowheads indicate branch points. Scale bar, 50 μm. **(F-G)** Quantification of indicated parameters at d5 (control, n=41 beads; shFlt1, n=41 beads). **(H)** Western blot of HUVEC lysates collected 48h after indicated treatments. Protein expression was normalized to β-actin and relative to NT. Fold change from NT shown below lanes. Statistics, Two-tailed Student's t-test (*, $p<0.05$; ****, $p<0.0001$; NS, not significant). Error bars, mean ± 95% CI.

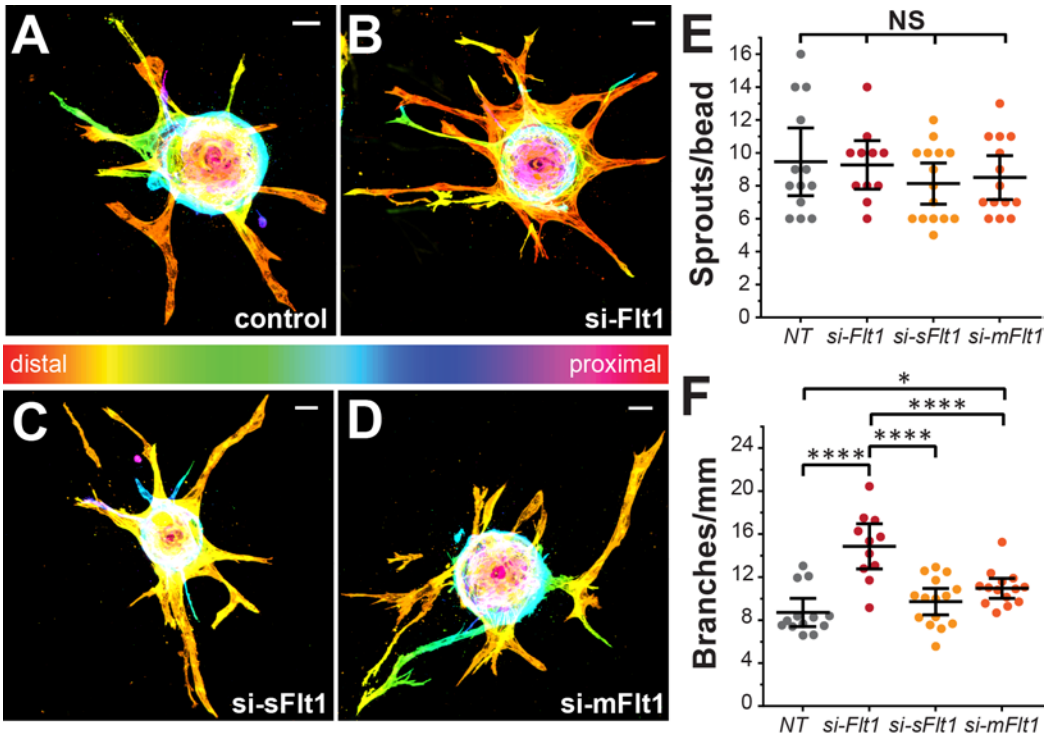


Figure S3: Isoform Selective Knockdown Validation.

(A-D) Representative images of d5 HUVEC sprouts with indicated treatments, stained for phalloidin and depth-encoded. Scale bar, 50 μm. **(E-F)** Quantification of indicated parameters at d5. Control (n=15 beads); total si-Flt1 (n=12 beads); si-sFlt1 (n=15 beads); si-mFlt1 (n=14 beads). Statistics, One-way ANOVA with Bonferroni's post-hoc correction (*, $p<0.05$; ****, $p<0.0001$; NS, not significant). Error bars, mean ± 95% CI.

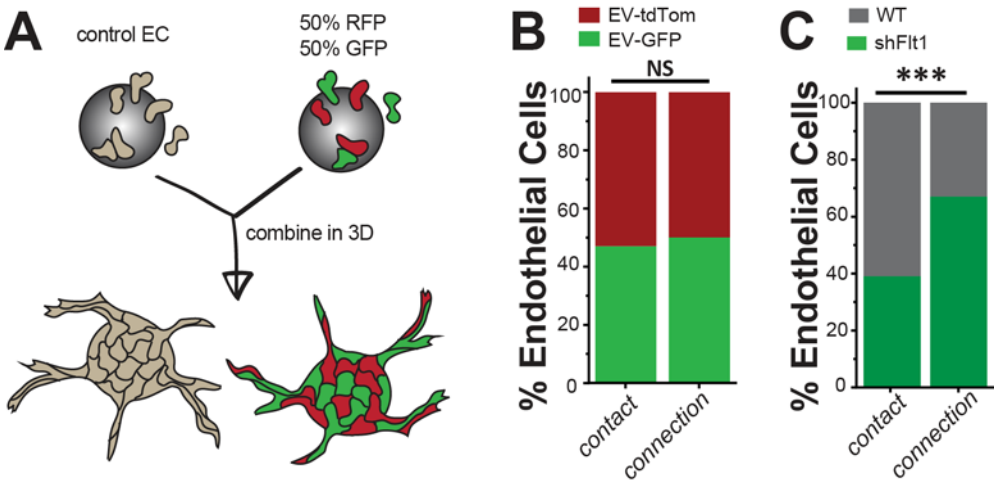
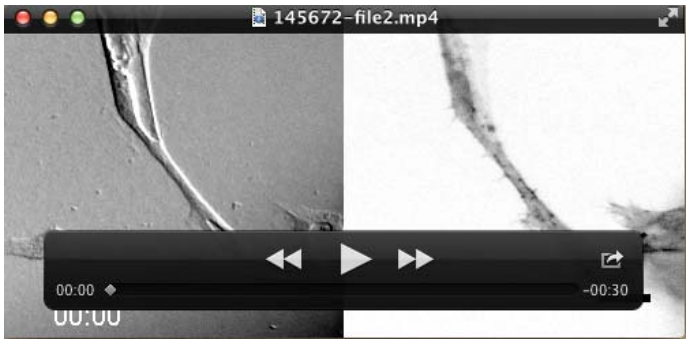
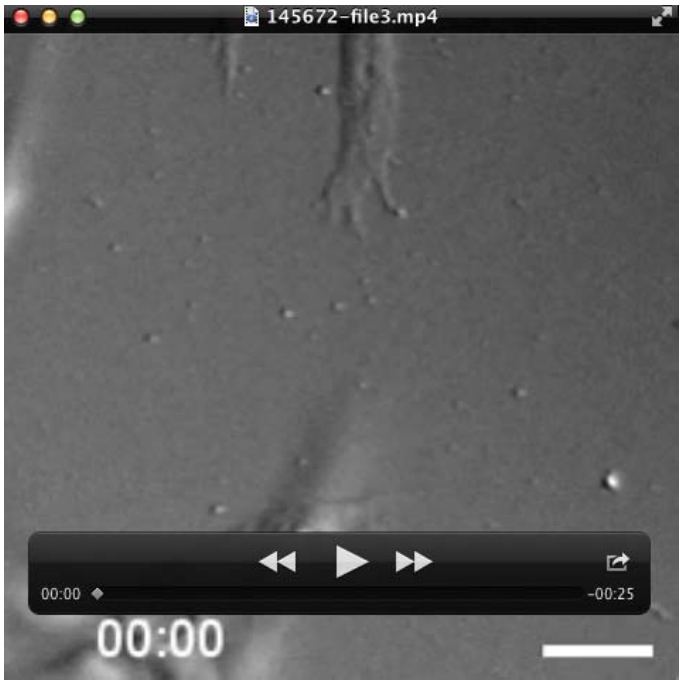


Figure S4: Transient Contacts and Stable Connections in Mosaic Target Vessels.

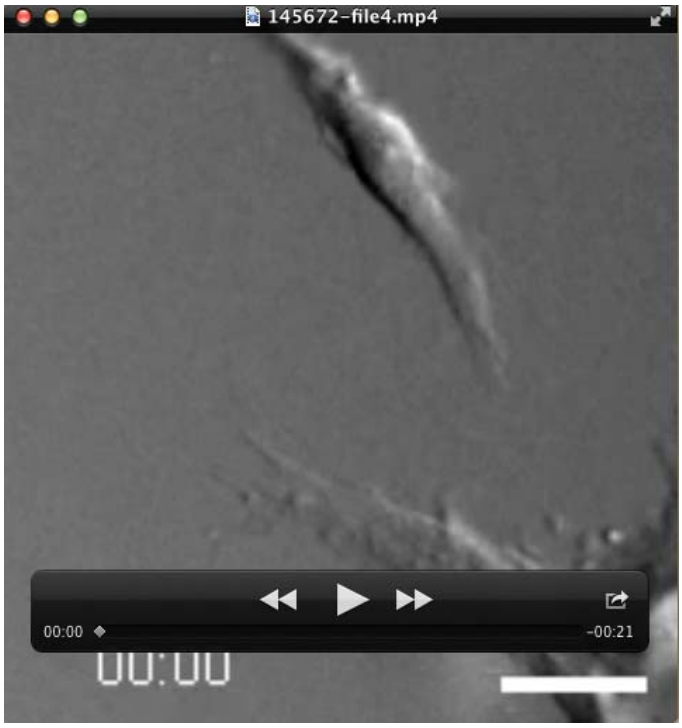
(A) Schematic of experimental design for mosaic vessel live-imaging analysis. **(B-C)** Percentage of transient contacts and stable connections with WT sprouts and endothelial cells with indicated manipulations in mosaic target vessels. (EV-tdTomato vs. EV-GFP, n=19 contacts, n=8 connections; shFlt1 vs. WT, n=95 contacts, n=36 connections). Statistics, Observed vs. Expected Binomial Test (***, $p<0.001$; NS, not significant).



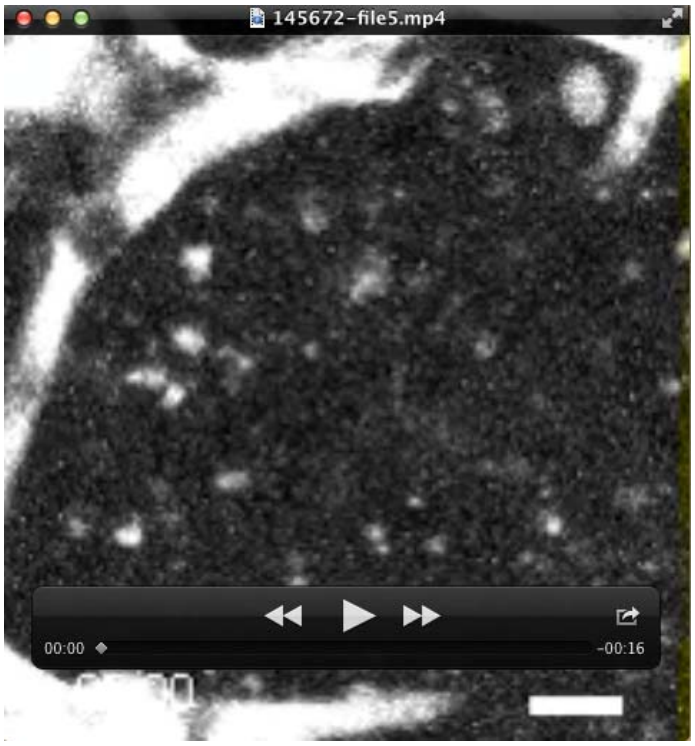
Movie 1. Connections were examined by time-lapse microscopy using a fluorescent LifeAct lentivirus, allowing simultaneous observation of both Differential Interference Contrast optics (DIC, left) and polymerized actin (right). Transient contacts (arrowheads) and stable connection (arrow) are noted. Insets, high magnification time frames for transient contacts and stable connections. For transient contacts, the frame showing the contact and the frame immediately following (and lacking the contact) are shown for clarity; for stable connections the first frame showing the connection is shown. Scale bar, 25 µm (lower right); time, hrs:mins (lower left).



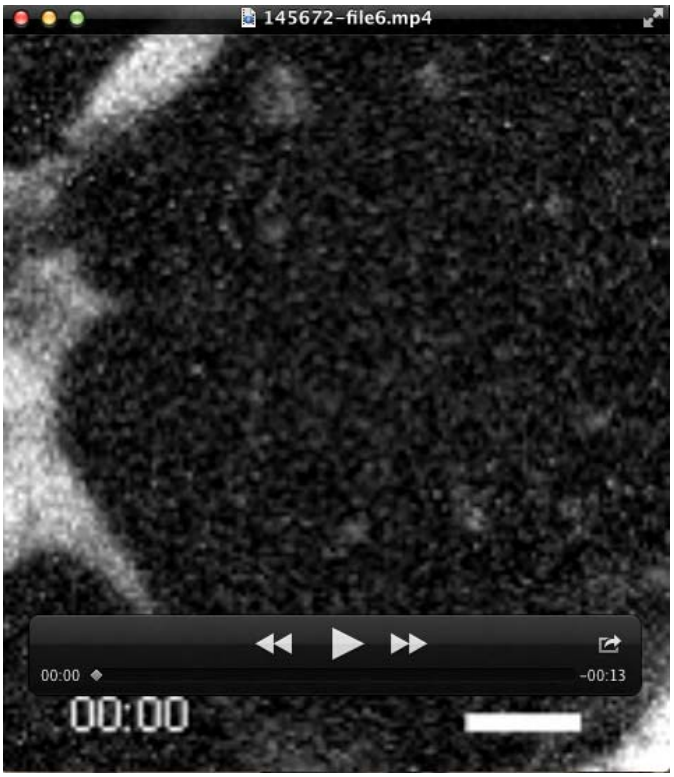
Movie 2. Wildtype HUVEC vessels were examined by time-lapse microscopy. Transient contacts (arrowhead) and stable connection (arrow) are noted. Insets, high magnification time frames for transient contacts and stable connections. For transient contacts, the frame showing the contact and the frame immediately following (and lacking the contact) are shown for clarity; for stable connections the first frame showing the connection is shown. An additional vessel is present outside the focal plane that was not scored. Scale bar, 25 µm (lower right); time, hrs:mins (lower left).



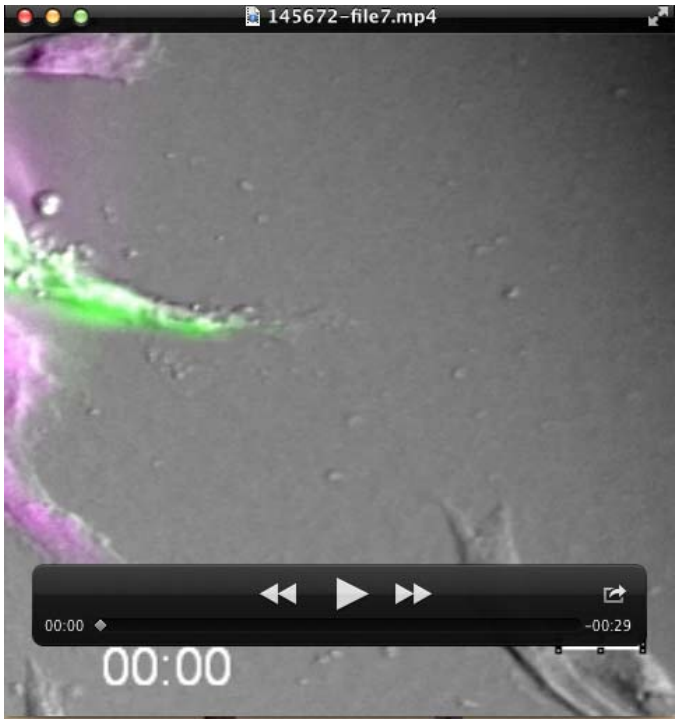
Movie 3. HUVEC vessels after lentivirus infection with shRNA against Flt1 were examined by time-lapse microscopy. Transient contacts (arrowhead) and stable connection (arrow) are noted. Insets, high magnification time frames for transient contacts and stable connections. For transient contacts, the frame showing the contact and the frame immediately following (and lacking the contact) are shown for clarity; for stable connections the first frame showing the connection is shown. Scale bar, 25 µm (lower right); time, hrs:mins (lower left).



Movie 4. Wildtype mouse ES cell-derived vessels expressing PECAM-eGFP were examined by time-lapse microscopy. Transient contacts (arrowheads) and stable connection (arrow) are noted. Insets, high magnification time frames for transient contacts and stable connections. For transient contacts, the frame showing the contact and the frame immediately following (and lacking the contact) are shown for clarity; for stable connections the first frame showing the connection is shown. Scale bar, 25 µm (lower right); time, hrs:mins (lower left).



Movie 5. *flt1*$^{-/-}$ mutant mouse ES cell-derived vessels expressing PECAM-eGFP were examined by time-lapse microscopy. Stable connection (arrow) is noted. Inset, high magnification of first frame showing the stable connection. Scale bar, 25 µm (lower right); time, hrs:mins (lower left).



Movie 6. HUVEC vessels with either wildtype (WT, no cytoplasmic reporter) or a 50/50 mixture of untransfected cytoplasmic-tdTomato and cytoplasmic-eGFP transfected with si-Flt1 were examined by time-lapse microscopy. Stable connection (arrow) is noted. Inset, high magnification first frame showing a stable connection. Scale bar, 25 µm (lower right); time, hrs:mins (lower left).