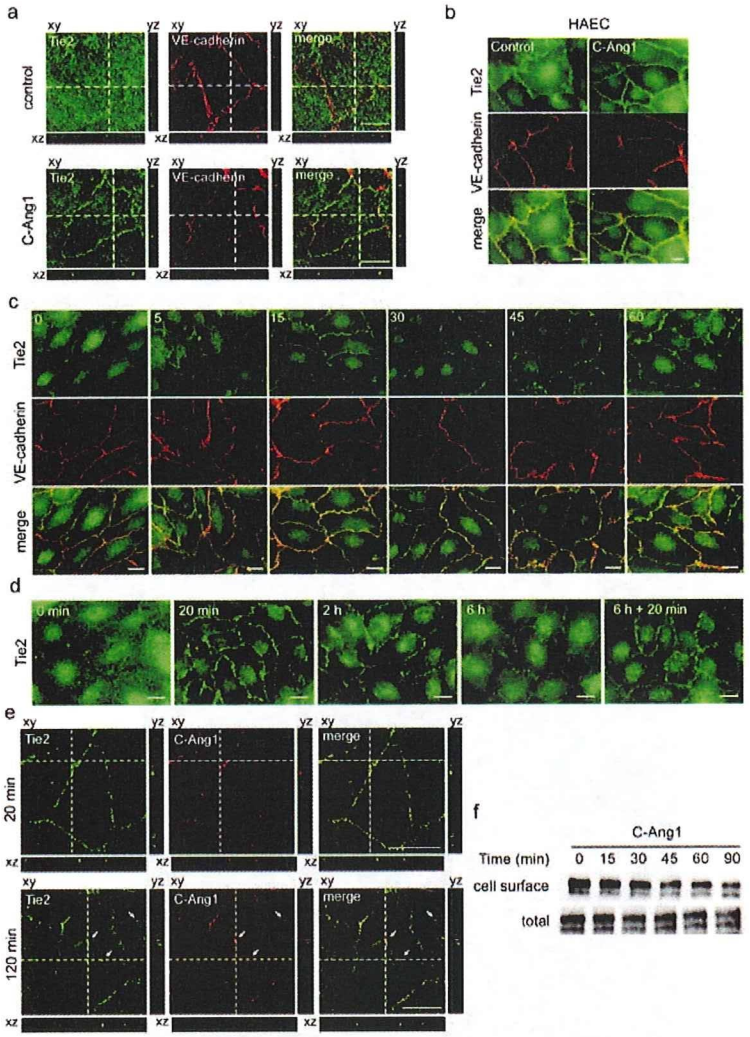


Figure S1 Tie2 accumulate at endothelial cell-cell contacts. **(a)** Confluent HUVECs plated on collagen-coated glass-base dish were starved in medium 199 containing 0.5% BSA for 3 h and stimulated with vehicle (control) or 200 ng ml^{-1} COMP-Ang1 (C-Ang1) for 20 min. The cells were then fixed, immunostained with anti-Tie2 and anti-VE-cadherin antibodies, and visualised with Alexa 488- and Alexa 546-conjugated secondary antibodies. Optical sections of Alexa 488 (green) and Alexa 546 (red) images were obtained by an FV-1000 confocal laser scanning microscope (Olympus). The bottom and the right panels of each image show *xz* section and *yz* section at x line and y line (white broken lines) of the *xy* image. Images for immunostaining of anti-Tie2 and anti-VE-cadherin antibodies are merged (merge). **(b)** Confluent human aortic endothelial cells (HAECs) were stimulated with vehicle (control) or COMP-Ang1 (C-Ang1) and imaged similar to **a** using an Olympus IX-81 epifluorescent microscope instead of a confocal microscope. **(c)** Confluent HUVECs stimulated with COMP-Ang1 for the time (min) indicated on the top panels were immunostained with anti-Tie2 and anti-VE-cadherin antibodies, and visualised similarly to **b**. **(d)** Confluent HUVECs were stimulated with COMP-Ang1 for the time indicated on the top panels, and stained with anti-Tie2 antibody similarly to **b**. In right panel, the cells exposed to COMP-Ang1 for 6 h were re-challenged with COMP-Ang1 for 20 min (6 h + 20 min). **(e)** Confluent HUVECs stimulated with COMP-Ang1 for 20 min (top panels) and 120 min (bottom panels) were stained with anti-Tie2 and anti-Flag antibodies, and visualised with Alexa 488- and Alexa 546-conjugated secondary antibodies, respectively. Optical sections of Alexa 488 (green: Tie2) and Alexa 546 (red: C-Ang1) images were obtained as described in the legend of **a**. The bottom and the right panels of each image show *xz* section and *yz* section at x line and y line (white broken lines) of the *xy* image. Images for immunostaining of anti-Tie2 and anti-Flag antibodies are merged (right column: merge). **(f)** Confluent HUVECs were stimulated with COMP-Ang1 for the time (min) indicated at the top. After the stimulation, cell-surface (upper panel) and total (bottom panel) expression level of Tie2 was quantified by biotinylation assay as described in Supplementary Methods. **(g)** Confluent HUVECs plated on collagen-coated glass-base dish were transfected with the plasmid encoding Tie2-GFP and that encoding HcRed-p120 catenin, starved for 3 h, and stimulated with 200 ng ml^{-1} COMP-Ang1. Tie2-GFP and HcRed-p120 catenin were time-lapse imaged on Olympus IX-81 inverted fluorescence microscope. A series of GFP and HcRed images were collected by MetaMorph 6.1 software. Images of GFP (top) and HcRed (middle) at the time points (min) indicated on the top panels after COMP-Ang1 stimulation were shown. Merged images are also shown at the bottom. **(h)** Confluent HUVECs transfected with the plasmid encoding Tie2-GFP was starved and stimulated with either vehicle (control; top panels) or COMP-Ang1 (C-Ang1; bottom panels) for 30 min. After the stimulation, the cells were stained with anti-VE-cadherin antibody and visualised by Alexa 546-conjugated secondary antibody as described in the legend of **b**. Images of GFP (green), Alexa 546 (red), and the merged images are shown at the left, middle, and right columns, respectively. The boxed areas are enlarged beneath each image. The scale bars represent 20 μm (a-e, g, h).

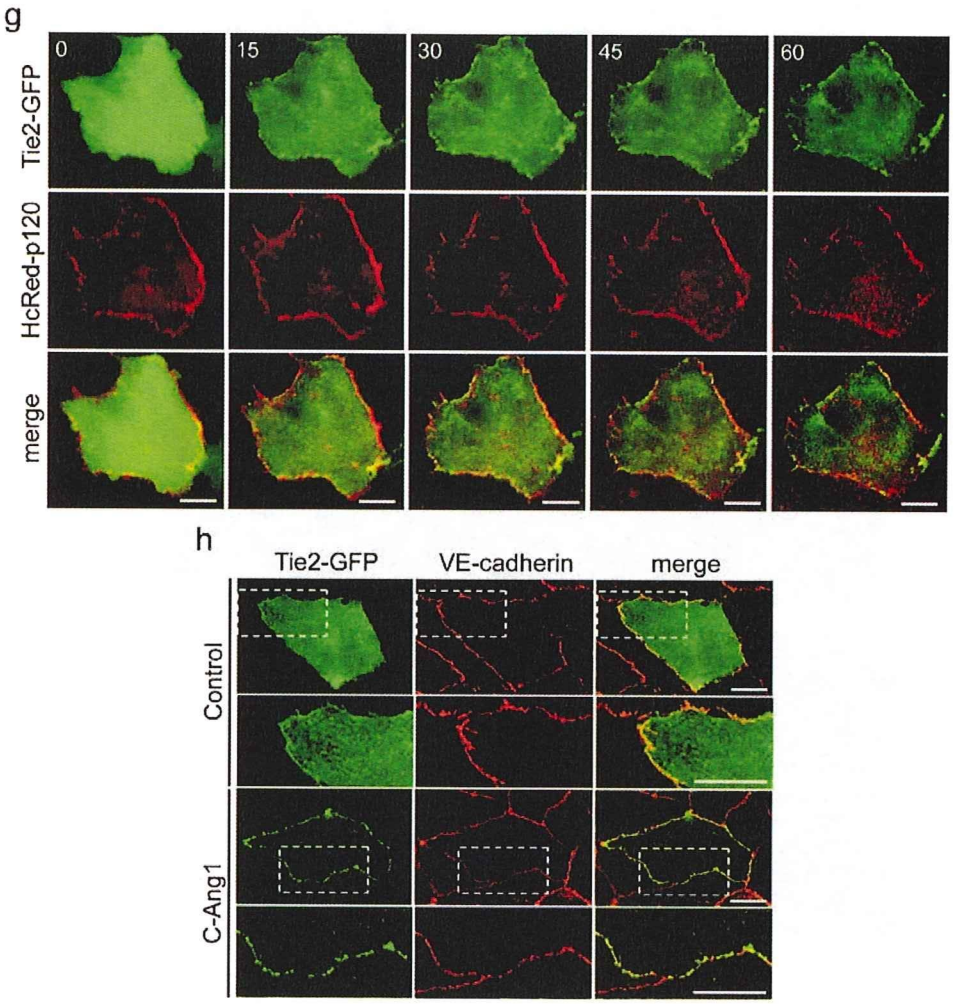


Figure S1 Continued

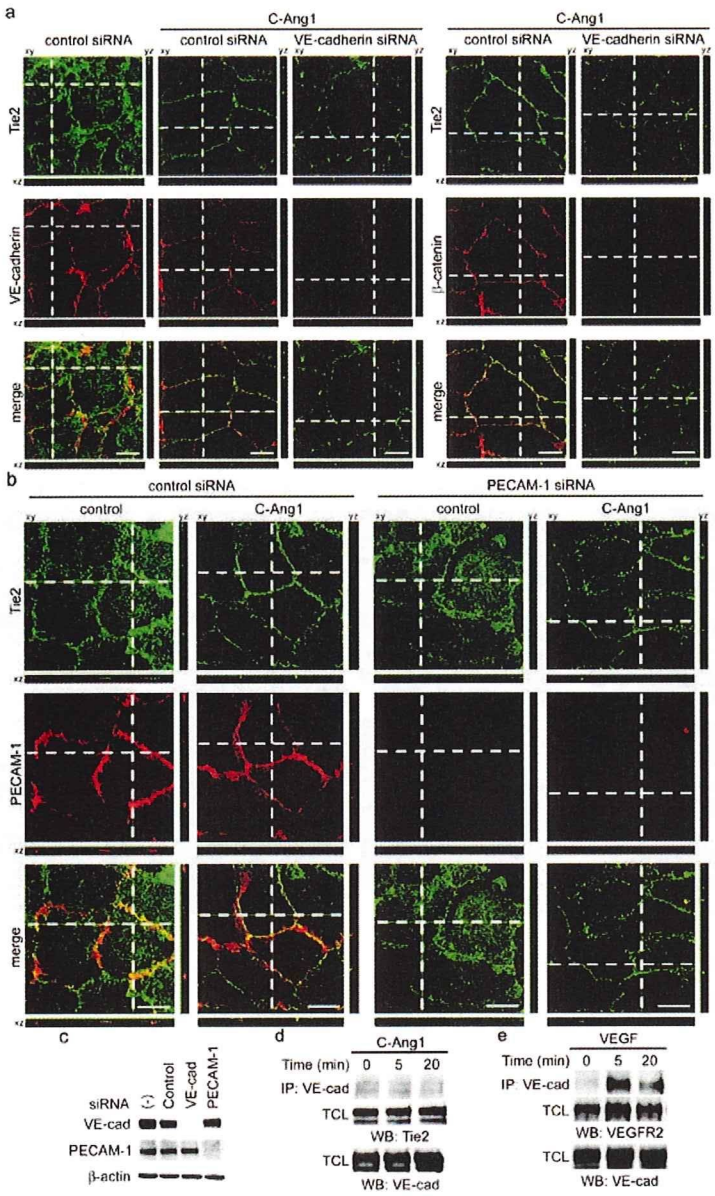


Figure S2 Translocation of Tie2 at cell-cell contacts does not require VE-cadherin and PECAM-1, but depends upon the expression of Tie2 on the neighbouring cells. **(a)** HUVECs transfected with control small interfering RNAs (siRNAs) or VE-cadherin siRNAs as indicated at the top were replated on the collagen-coated glass-base dish. The cells were stimulated with vehicle (most left column) or 200 ng ml^{-1} COMP-Ang1 (C-Ang1: from second column to fifth column), fixed, and immunostained with anti-Tie2 (top) and anti-VE-cadherin (middle) antibodies (from the first column to third column) and with anti-Tie2 (top) and anti-β-catenin (middle) antibodies (the fourth and fifth column, respectively), similarly to **Fig. S1a**. Merged images are shown at the bottom (merge). Note that depletion of VE-cadherin inhibits the accumulation of β-cateinin but not Tie2 at cell-cell contacts. **(b)** Similarly to **a**, HUVECs transfected with either control siRNAs or platelet and endothelial adhesion molecule-1 (PECAM-1) siRNAs were stimulated with vehicle (control) or COMP-Ang1 (C-Ang1), and immunostained with anti-Tie2 (top) and anti-PECAM-1 (middle) antibodies. Merged images are shown at the bottom (merge). Note that accumulation of Tie2 at cell-cell contacts was not affected by depletion of PECAM-1. **(c)** The cell lysates from HUVECs transfected without (-) or with control, VE-cadherin (VE-cad), or PECAM-1 siRNAs were subjected to SDS-PAGE followed by immunoblotting with antibodies as indicated at the left. We used two kinds of siRNAs for VE-cadherin and PECAM-1. Representative results obtained from one siRNA for each target are shown (a, b, and c), as described in the Supplementary Methods. **(d, e)** VEGFR2 but not Tie2 associates with VE-cadherin upon ligand stimulation. The cell lysates from HUVECs stimulated with COMP-Ang1 (d) or VEGF (e) for the time (min) indicated at the top were immunoprecipitaed with anti-VE-cadherin antibody. Total cell lysates (TCL) and immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with antibodies indicated at the bottom. **(f)** CHO cells expressing Tie2-GFP were stimulated with vehicle (control) or COMP-Ang1 (C-Ang1) for 20 min and optically sectioned for GFP images using a confocal microscope similarly to **Fig. S1a**. Note that Tie2-GFP, homogenously expressed on the plasma membrane before stimulation, was targeted to cell-cell contacts by COMP-Ang1 stimulation, as revealed in *xz* and *yz* sections. **(g)** CHO cells expressing either Tie2-GFP, Tie2Δcyto-GFP, or Tie2KD-GFP surrounded by wild type CHO cells were stimulated with vehicle (control) or COMP-Ang1 (C-Ang1). Note that Tie-GFP but not Tie2Δcyto-GFP and Tie2KD-GFP was internalised upon COMP-Ang1 stimulation, suggesting that *trans*-association of Tie2 at cell-cell contacts prevents Tie2 from internalisation and that kinase activity of Tie2 is required for internalisation of Tie2. The scale bars represent 20 μm (a, b, f, g).

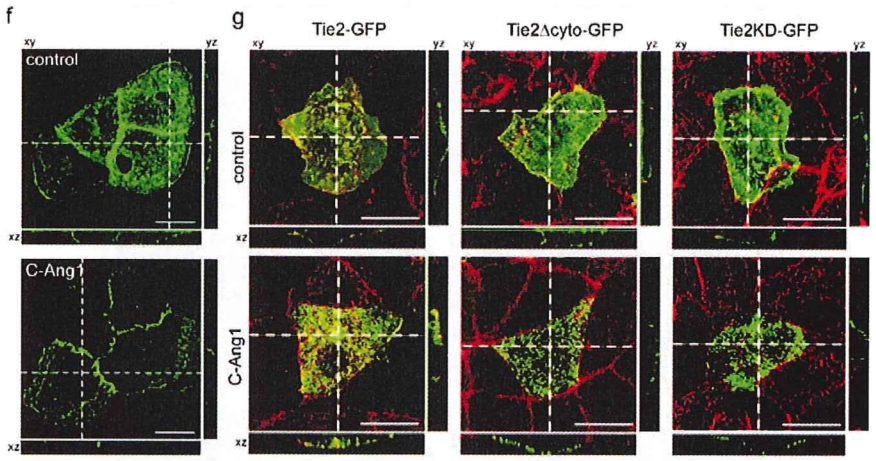


Figure S2 Continued

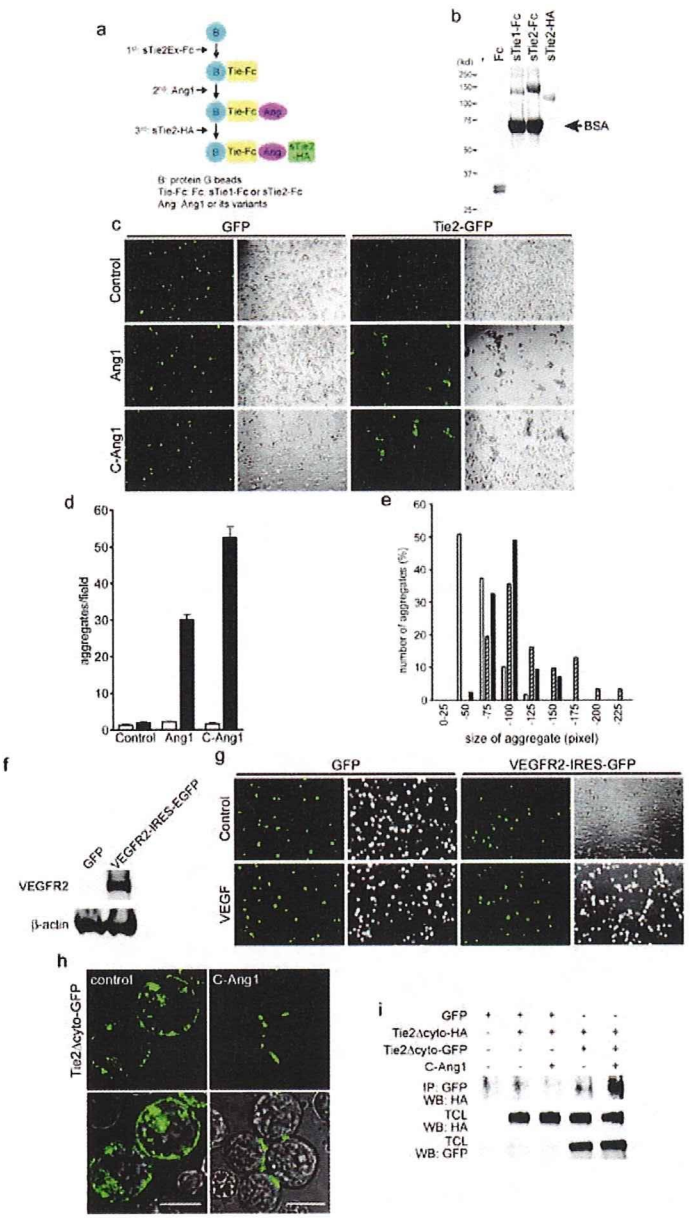


Figure S3 Ang1 induces *trans*-association of Tie2 at cell-cell contacts. **(a)** Schematic illustration of the experiment for in vitro Ang1-bridged Tie2 association assay. Detail of the assay protocol is described in Methods. **(b)** SDS-PAGE and coomassie staining confirmed the purity of the proteins used for Ang1-bridged Tie2 association assay. Arrow indicates BSA. **(c)** Aggregation of suspension 293F cells expressing GFP (left panels) and Tie2-GFP (right panels) was induced by either vehicle (control; top panels), Ang1 (Ang1; middle panels), and COMP-Ang1 (C-Ang1; bottom panels), as described in Methods. Left and right images of the panels show the GFP and the phase-contrast images, respectively. **(d)** To quantify the cell aggregation observed in **c**, the number of cell aggregate per field of microscopic view was counted. Aggregate was defined as cell mass consisting of more than 4 cells. The number of aggregate of the cells expressing GFP and Tie2-GFP was shown as white and black columns, respectively. Data are expressed as mean number ± standard deviation of, at least, ten different fields. **(e)** In the 293F cell aggregation assay described in **Fig. 2d**, the size of aggregate of the cells stimulated with COMP-Ang1 was measured using MetaMorph 6.1 software. Open, hatched and closed columns indicate the number of aggregate of the cells expressing Tie2-GFP, Tie2Δcyto-GFP and Tie2KD-GFP, respectively. **(f)** The lysates from cells expressing GFP and cells expressing both VEGFR2 and IRES-driven GFP were subjected to immunoblot analysis with antibodies indicated at the left. **(g)** Aggregation of 293F cells expressing GFP (left panels) and those expressing both VEGFR2 and IRES-driven GFP (right panels) was induced by either vehicle (control; top panels) and VEGF (VEGF; bottom panels), as described in the legend of **c**. Note that neither 293F cells expressing GFP nor 293F cells expressing VEGFR2 and IRES-driven GFP did not aggregate upon VEGF stimulation. **(h)** BaF3 cells expressing Tie2Δcyto-GFP (BaF-Tie2Δcyto-GFP) were incubated with vehicle (control; left column) and COMP-Ang1 (C-Ang1; right column) in suspension, as described in the legend of **Fig. 2f**. GFP and DIC images were obtained through a confocal microscope. Upper and lower panels show the GFP images merged without or with the DIC images, respectively. Note that Tie2Δcyto-GFP clearly localises at the site of cell-cell contacts only when stimulated with COMP-Ang1. The scale bars represent 10 μm. **(i)** BaF-Tie2Δcyto-HA cells (Tie2Δcyto-HA) were mixed-cultured with either BaF3 cells or BaF-Tie2Δcyto-GFP cells (Tie2Δcyto-GFP), and stimulated with 400 ng ml^{-1} of COMP-Ang1 for 5 h. Cell lysates were immunoprecipitated with anti-GFP antibody. Immunoprecipitates (IP) and aliquots of cell lysate (TCL) were subjected to Western blot analysis (WB) with anti-HA and anti-GFP antibodies as indicated at the left of each panel. Note that Tie2Δcyto-HA is coimmunoprecipitated with Tie2Δcyto-GFP upon COMP-Ang1 stimulation.

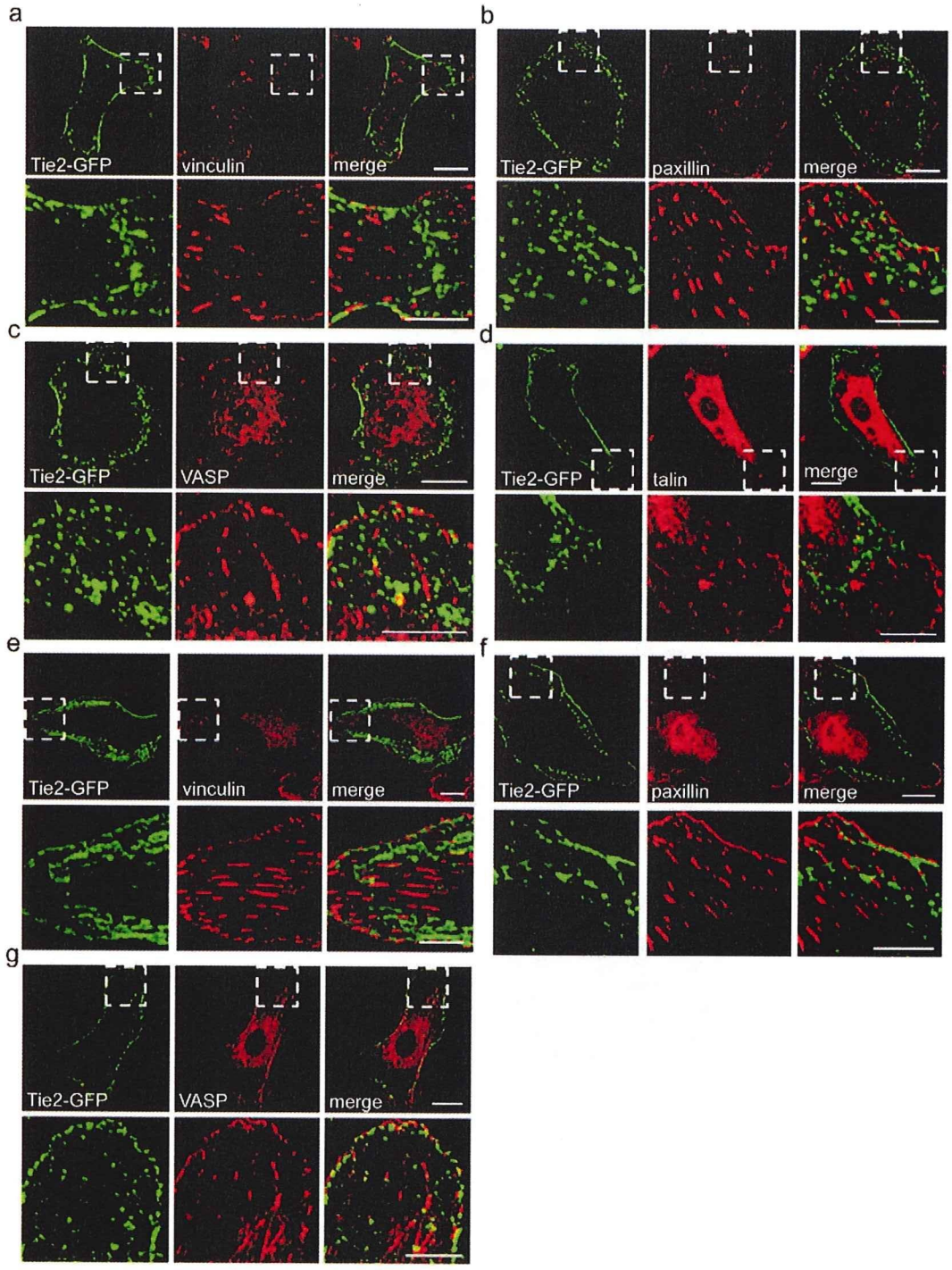


Figure S4 Tie2 accumulates to cell-substratum contacts upon Ang1 stimulation. **(a)** Subconfluent HUVECs were stimulated with vehicle (control) or COMP-Ang1 (C-Ang1) for 20 min, immunostained after fixation, and imaged similarly to **Fig. S1b**. As far as cells contact neighboring cells, Tie2 accumulates at cell-cell contacts upon Ang1 stimulation. **(b)** Isolated HUVECs stimulated with either vehicle (control; top panels), COMP-Ang1 (C-Ang1; middle panels) or native Ang1 (Ang1; bottom panels) for 20 min were fixed, immunostained with anti-Tie2 and anti-paxillin antibodies, and optically sectioned by a laser confocal microscope as in the legend of **Fig. S1a**. Merged images of Tie2 and paxillin are stacked and shown at the left column (stacked image). The sections at the bottom of the cell are shown (basal surface). The boxed region is enlarged at the right (enlargement). Note that Tie2 diffusely expressed on the bottom is relocated to the cell periphery at the bottom after COMP-Ang1 and native Ang1 stimulation. In addition, the peripheral accumulation at the bottom of the cells does not colocalise to paxillin-positive focal complexes, although Tie2 and paxillin expression partially overlap. **(c)** Images at the time point (30 min) of **Fig. 3b** are shown again and the boxed regions are enlarged at the bottom. Note that Tie2-GFP expressed in CHO cells does not colocalise with vinculin-positive focal complexes and focal adhesions. The scale bars represent 20 μm (a-c). The scale bars in the enlarged images represent 5 μm (a-c).

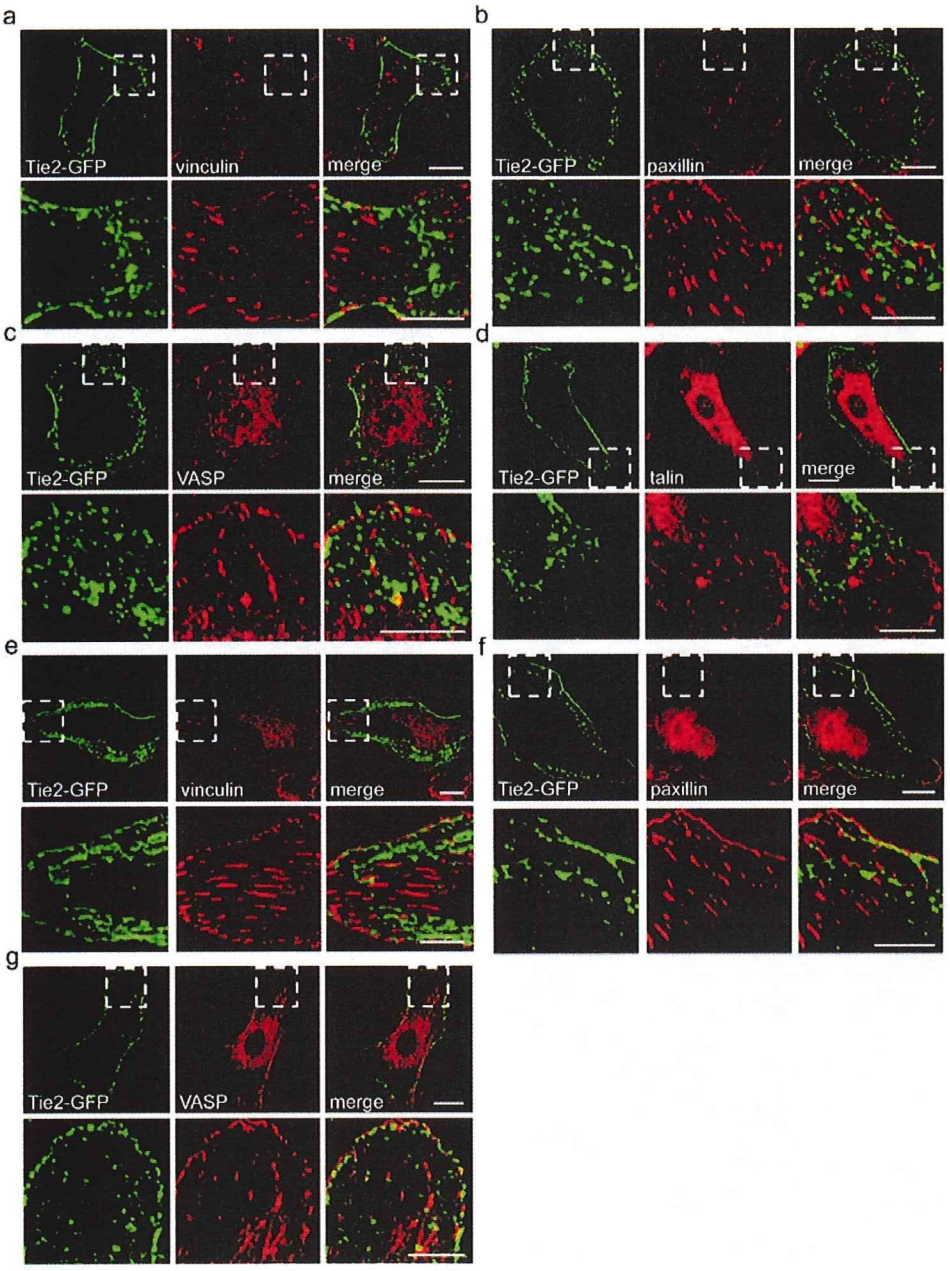


Figure S5 Tie2 accumulates to cell-substratum contacts distinct from focal complexes, focal adhesions, and fibrillar adhesions upon Ang1 stimulation. **(a-d)** Sparse HUVECs plated on fibronectin-coated dish were transfected with the plasmid encoding Tie2-GFP, and stimulated with COMP-Ang1 for 20 min. After fixation, the cells were stained with either anti-vinculin (**a**), anti-paxillin (**b**), anti-VASP (**c**), or anti-talin (**d**) antibodies, and visualized with Alexa546-labeled secondary antibodies. GFP (left panels) and Alexa546 (middle panels) images at cell-substratum interface were obtained by an FV1000 confocal laser scanning microscope. Merged images are shown at the right panels. The boxed regions are enlarged at the bottom of each image. **(e-g)** Tie2-GFP-expressing HUVECs plated on collagen-coated dish were stimulated as described in the legend of **a**, and stained with either anti-vinculin (**e**), anti-paxillin (**f**) or anti-VASP (**g**) antibodies, and visualized with Alexa546-labeled secondary antibodies. GFP and Alexa546 images at cell-substratum interface were obtained and shown similarly to **a**. **(h, i)** Sparse HUVECs plated on fibronectin-coated dish were transfected with the plasmid encoding Tie2-GFP, and stimulated with vehicle (control; left columns) or COMP-Ang1 (C-Ang1; middle and right columns) for 20 min. After fixation, the cells were stained with either anti-α5 integrin (**h**) or anti-fibronectin antibody (**i**), and visualized with Alexa546-conjugated secondary antibody. GFP (Tie2-GFP; top panels) and Alexa546 (α5 integrin or fibronectin; middle panels) images at cell-substratum interface were obtained by an FV1000 confocal laser scanning microscope. Merged images are shown at the bottom. The boxed regions are enlarged at the right of each image. **(j)** Sparse HUVECs plated on fibronectin-coated dish were co-transfected with the plasmid encoding Tie2-HA and that expressing GFP-tensin, and stimulated similarly to the legend of **h**. After fixation, the cells were stained with anti-HA antibody, and visualized with Alexa546-conjugated secondary antibody. Alexa546 (Tie2-HA; top panels) and GFP (GFP-tensin; middle panels) images at cell-substratum interface were recorded and shown as described in the legend of **h**. The scale bars represent 20 μm (a-j). The scale bars in the enlarged images represent 10 μm (a-j). Note that Tie2-GFP or Tie2-HA at cell-substratum contacts does not colocalise with markers for focal complexes, focal adhesions, and fibrillar adhesions.

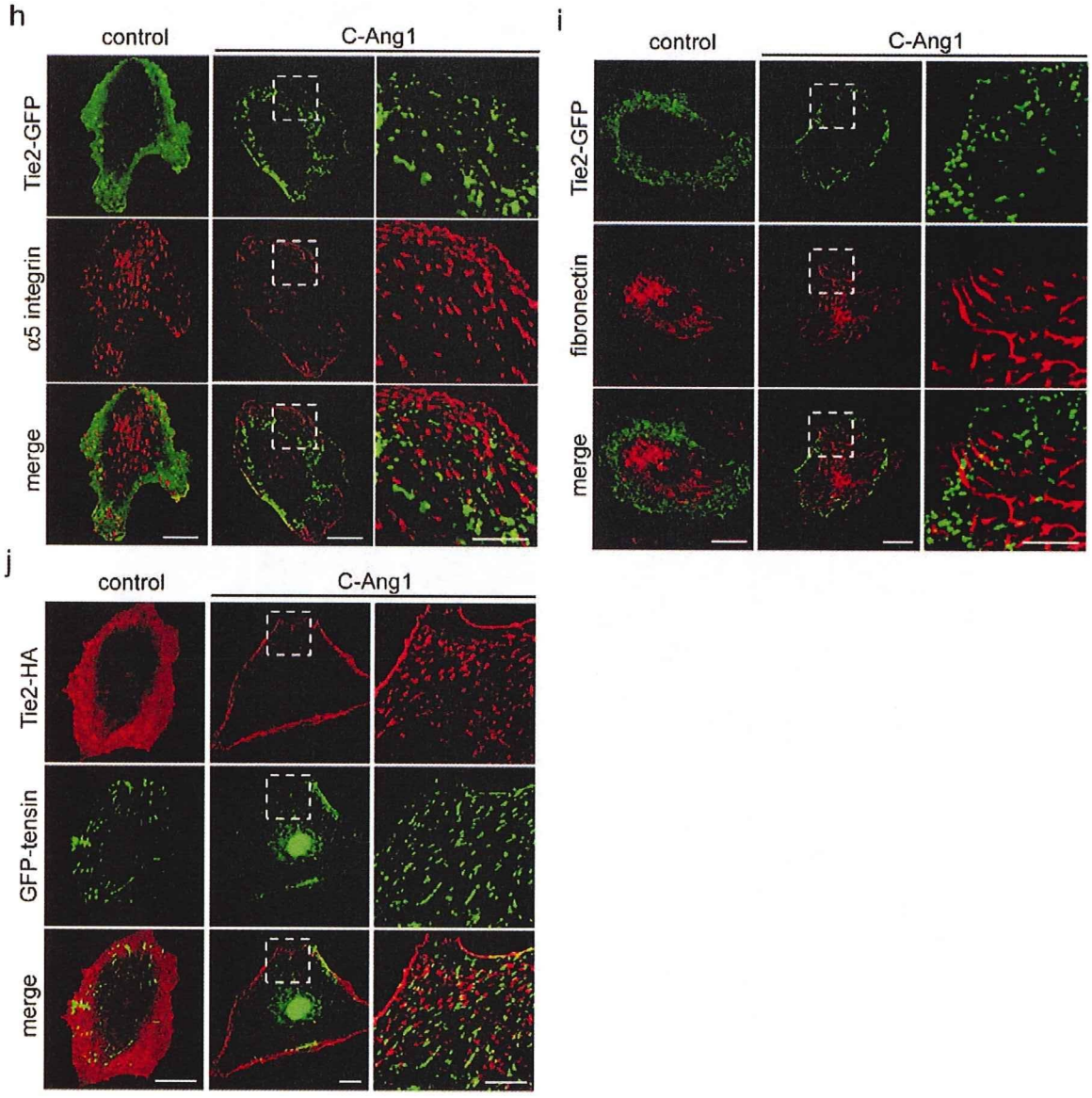


Figure S5 Continued

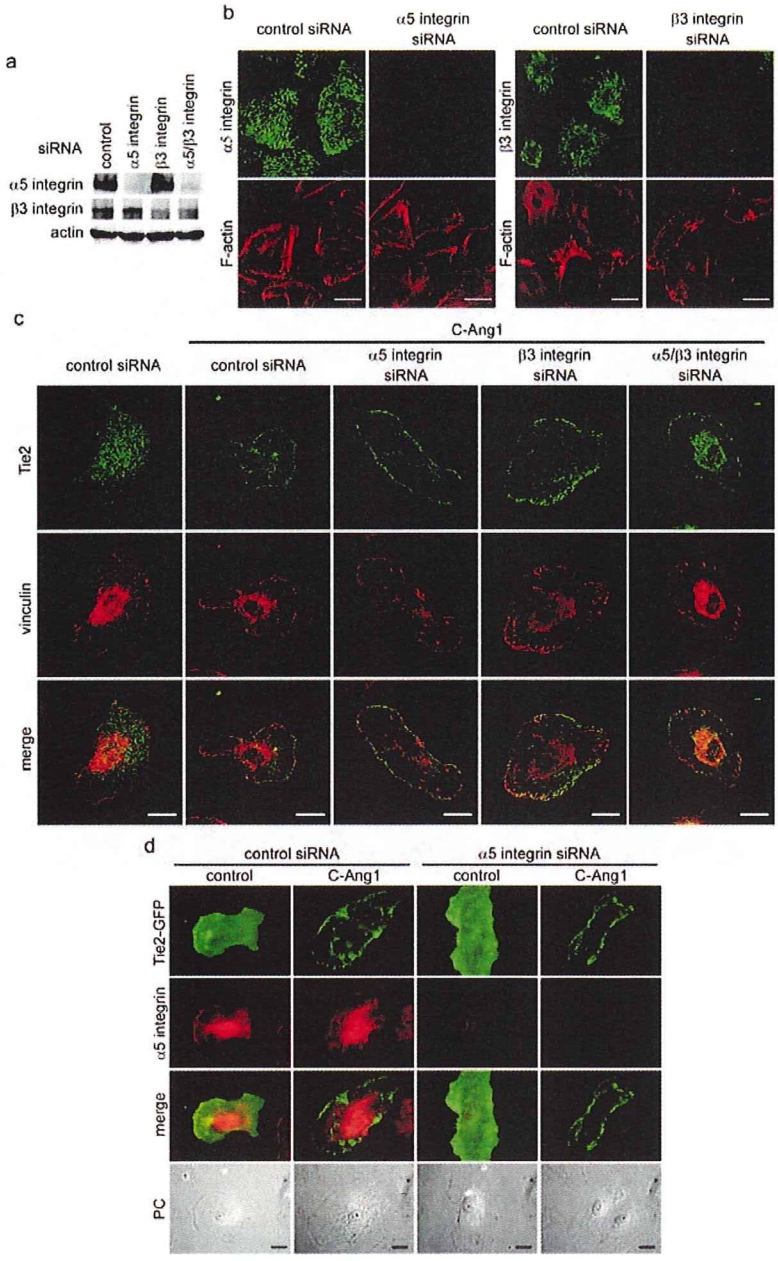
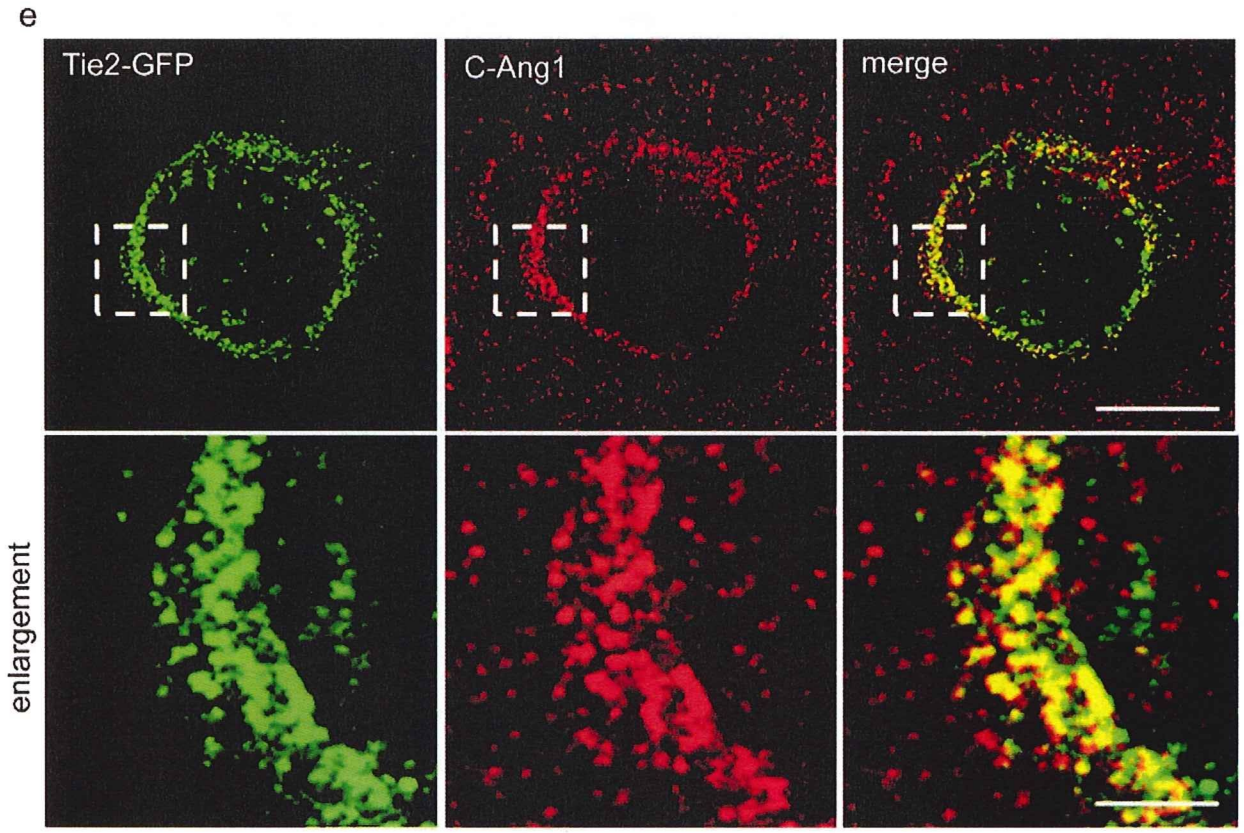


Figure S6. Depletion of α5 or β3 integrin does not affect the anchoring of Tie2 to cell-substratum contacts. **(a)** Cell lysates from HUVECs transfected with siRNAs indicated at the top for 24 h were subjected to immunoblot analysis using antibodies indicated at the left. **(b)** HUVECs transfected with siRNAs indicated at the top were immunostained with anti-α5 integrin (left) and anti-β3 integrin (right) and optically sectioned similarly to **Fig. S1a.** The sections of the bottom of the cells were shown. Cells were identified by F-actin staining using rhodamine-phalloidin. A representative result from more than 100 cells observed at one of three independent experiments is shown. **(c)** Isolated HUVECs transfected with siRNAs indicated at the top were unstimulated (control; first column) or stimulated with COMP-Ang1 (C-Ang1), followed by the immunostaining with anti-Tie2 (top) and anti-vinculin (middle) antibodies, and optically sectioned similarly to **Fig. S1a**. The sections at the bottom of the cells are shown. Merged images are shown at bottom. Note that COMP-Ang1-induced accumulation of Tie2 at cell-substratum contacts of the cell periphery was not affected by depletion of α5 and β3 integrins. **(d)** HUVECs transfected with control or α5 integrin siRNAs cultured for 24 h were replated on collagen-coated glass base dish. Cells were further transfected with the plasmid expressing Tie2-GFP, starved for 3 h, and stimulated with vehicle (control) and 200 ng ml^{-1} COMP-Ang1 (C-Ang1) for 20 min. After fixation, the cells were immunostained with anti-α5 integrin antibody and visualised with Alexa 546-conjugated secondary antibody. Images of GFP (green) and Alexa 546 (red) are shown at the top and the second panels, respectively. Merged images of GFP and Alexa 546 and the phase-contrast images are shown on the third and the bottom panels, respectively. We used two kinds of siRNAs for α5 and β3. The results obtained from one siRNA for each target are shown (a, b, c, and d). **(e)** Images at the time point (30 min) of **Fig. 3c** are enlarged. The boxed regions are enlarged at the bottom. Note that Tie2-GFP clearly colocalises with Flag-tagged COMP-Ang1 at the bottom of the cells. The scale bar represents 20 μm (b-e). The scale bar in the enlarged image represents 5 μm (e). **(f)** To estimate non-specific binding of Flag-tagged proteins to ECM, binding of Flag-tagged bacterial alkaline phosphatase (BAP) protein to ECM was examined by immunofluorescence analysis as described in the legend of **Fig. 3d**.



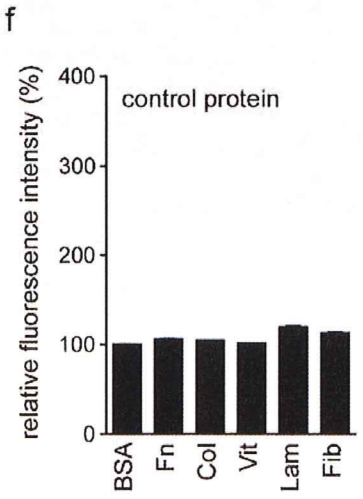


Figure S6 Continued

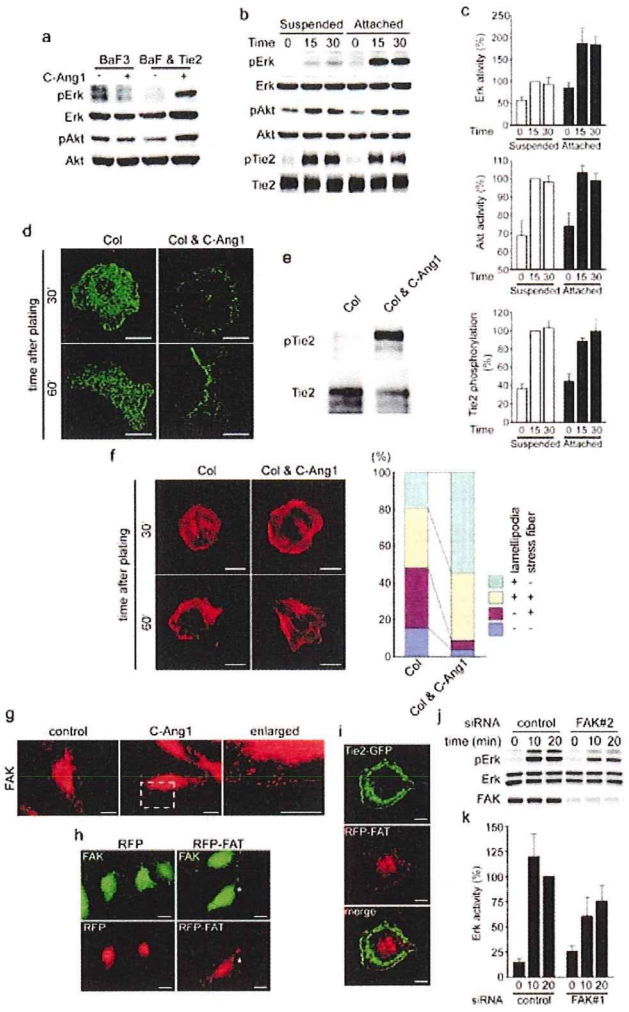


Figure S7 ECM-anchored Ang1 induces Tie2 signalling. **(a)** BaF3 and BaF-Tie2 cells placed on a fibronectin-coated dish were stimulated with vehicle (-) and COMP-Ang1 (+) for 15 min as described in Supplementary Methods. Erk and Akt activation was analyzed similarly to the legend of **Fig. 4d**. Phosphorylated and total Erk2, but not Erk1, were shown in the top and the second panels, since phosphorylated Erk1 was overlapped with non-specific bands. **(b)** Suspended BaF-Tie2 cells and the cells placed on a fibronectin-coated dish were stimulated with COMP-Ang1 for the time indicated on the top (min) as described in Supplementary Methods. Phosphorylation of Tie2, Erk, and Akt were analyzed as described in the legend of **Fig. 4b** and **4d,** respectively. **(c)** Phosphorylation of Erk, Akt and Tie2 observed in **b** was quantified as described in the legend of **Fig. 4c** and **4e,** and shown at top, middle, and bottom panels, respectively. Values are expressed as means ± standard deviations from five independent experiments. **(d)** COMP-Ang1 was bound to collagen-coated glass base dish as described in Supplementary Methods. HUVECs were placed on the COMP-Ang1-unbound collagen coated dish (Col) or COMP-Ang1-bound collagen-coated dish (Col & C-Ang1), for 30 (upper panels) and 60 min (bottom panels), and immunostained with anti-Tie2 antibody. Tie2 reacted with the primary antibody was visualized by Alexa 488-conjugated secondary antibody. Alexa 488 images of cell-substratum interface were obtained through a confocal microscope. **(e)** To examine whether ECM-bound COMP-Ang1 induces Tie2 signaling, starved HUVECs were placed on the COMP-Ang1-bound (Col & C-Ang1) or COMP-Ang1-unbound dish (Col) for 20 min as described in **d**. Cell lysates were immunoprecipitated with anti-Tie2 antibody. Immunoprecipitates and aliquots of cell lysate were subjected to Western blot analysis with anti-phosphotyrosine (pTie2) and anti-Tie2 (Tie2) antibodies, respectively. **(f)** HUVECs were stimulated as described in **d**, and stained with rhodamine-phalloidin. Rhodamine images were obtained through an epifluorescence microscope, and shown in left panel. The number of cells that produce lamellipodia and stress fibers identified by F-actin staining was counted. Accordingly, the cells were classified into four groups as follows; the cells that exhibit only lamellipodia, those that produce both lamellipodia and stress fibers, and those that exhibit only stress fibers, those that do not exhibit both lamellipodia and stress fibers (Col, n=102; Col & C-Ang1, n= 101). **(g)** Sparse HUVECs were stimulated with vehicle (left panel: control) or COMP-Ang1 (middle panel: C-Ang1) and immunostained with anti-FAK antibodies as described in the legend of **Fig. 3a**. FAK reacted with the primary antibody was visualized with Alexa 546-conjugated secondary antibody. The boxed area in the middle panel is enlarged (right panel). **(h)** HUVECs were transfected with either the vector encoding RFP or that encoding RFP-FAT, and stained with anti-FAK antibody. FAK reacted with the antibody was visualized with Alexa 488-conjugated secondary antibody. Alexa 488 and RFP images are shown in the upper and the lower panels, respectively. Asterisks indicate the cells expressing RFP-FAT. Note that FAK is replaced with RFP-FAT at the focal complexes and focal adhesions in the cell indicated by the asterisk. **(i)** Sparse HUVECs transfected with the plasmids encoding Tie2-GFP and RFP-FAT were starved for 3 h, and stimulated with COMP-Ang1 for 30 min. GFP, RFP and the merged images were shown at the top, the middle and the bottom panels, respectively. **(j)** HUVECs were transfected with control siRNA (control) or with FAK siRNA (FAK#2), which recognizes different sequence of FAK from that targeted by FAK#1 siRNA used in Fig. 5i. The cells were then starved and stimulated with COMP-Ang1 for the time (min) as indicated at the top similarly to the legend of **Fig. 4b**. Cell lysates were subjected to immunoblot analysis using anti-phospho-Erk (pErk) and anti-Erk (Erk) antibodies to assess Erk activity and with FAK antibody (FAK). **(k)** Erk phosphorylation induced by COMP-Ang1 stimulation observed in **j** was quantified as described in the legend of **Fig. 4e**. Values are expressed as means ± standard deviations from six independent experiments. The scale bars represent 20 μm (d, f, g, h, and i) and 5 μm (enlarged image in g), respectively.

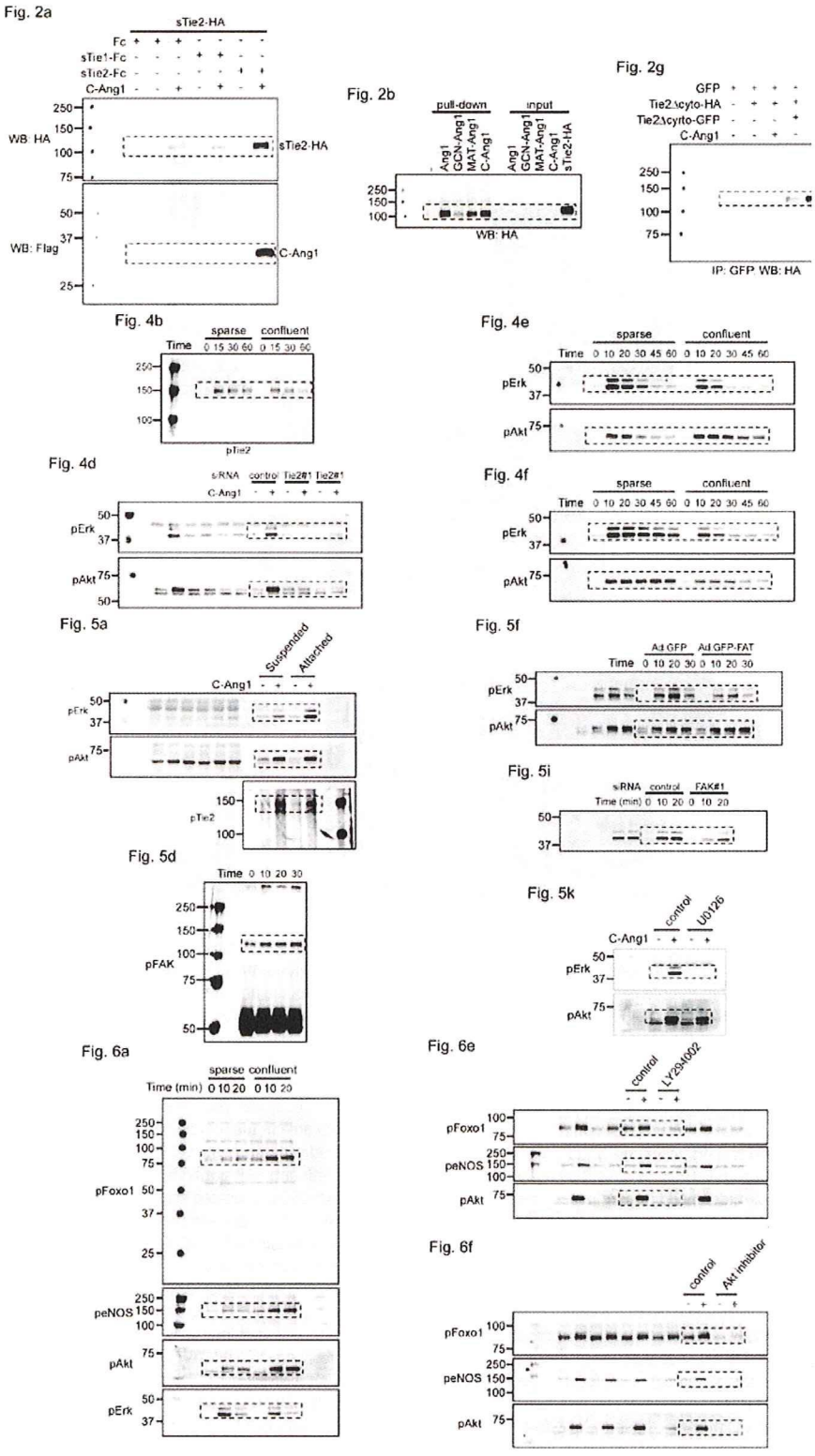


Figure S8 Full-scan images of key Western blots.

Supplementary Movie Legends

Movie S1 Recruitment of Tie2 at cell-cell contacts upon COMP-Ang1 stimulation. Confluent HUVECs plated on collagen-coated glass base dish were transfected with the plasmid encoding Tie2-GFP and that encoding HcRed-p120 catenin, starved for 3 h, and stimulated with COMP-Ang1 (200 ng ml^{-1}). A series of GFP image (left) and HcRed (right) images were saved as a stack file, which was converted to a video file. Elapsed time is indicated as h : min.

Movie S2 Internalisation of ectopic Tie2 upon COMP-Ang1 stimulation in the absence of cell-cell contacts between the cells ectopically expressing Tie2. CHO cells transfected with the plasmid expressing Tie2-GFP were plated on collagen-coated dish, starved for 3 h, and stimulated with COMP-Ang1 (200 ng ml^{-1}). A series of GFP images (left) and phase-contrast images (right) were saved as a stack file, which was converted to a video file. Elapsed time is indicated as h: min. Note that Tie2-GFP-expressing cells are surrounded by those that do not express Tie2-GFP.

Movie S3 *Trans*-association of Tie2 at cell-cell contacts upon COMP-Ang1 stimulation. CHO cells transfected with the plasmid expressing Tie2-GFP were stimulated with COMP-Ang1 and time-lapse imaged similarly to the legend of Movie S2. Note that Tie2-GFP-expressing cells contact each other.

Movie S4 *Trans*-association of Tie2 at cell-cell contacts does not need the cytoplasmic domain of Tie2. CHO cells transfected with the plasmid expressing Tie2Δcyto-GFP were stimulated with COMP-Ang1 and time-lapse imaged similarly to the legend of Movie S2.

Movie S5 Sparse HUVECs plated on a collagen-coated dish were transfected with the plasmid encoding Tie2-GFP and that encoding RFP-Crk, starved for 3 h, and stimulated with COMP-Ang1 (200 ng ml^{-1}). Fluorescence images of GFP and RFP simultaneously recorded were merged at each time point. A series of GFP (top) and merged images (bottom) were saved as stack files, which were converted to video files. The boxed areas in the left movies are enlarged on the right side. Note that Tie2-GFP is accumulated at the extended membrane close to RFP-Crk-marked focal complexes as indicated by arrow. Tie2-GFP remains at the initial accumulation site at cell-substratum even after RFP-Crk detaches from initial focal complexes. Elapsed time is indicated as h: min.

Movie S6 Sparse CHO cells plated on a collagen-coated dish were transfected with the plasmid encoding Tie2-GFP and that encoding RFP-Crk, starved for 3 h, and stimulated with COMP-Ang1 (200 ng ml^{-1}). Fluorescence images of GFP and RFP and phase-contrast images were simultaneously recorded. GFP and RFP images were merged at each time point. A series of GFP (top left), RFP (top right), merged (bottom left) and phase-contract (bottom right) images were saved as stack files, which were converted to video files. Note that Tie2-GFP is accumulated at cell periphery close to RFP-Crk-marked focal complexes, and remains at the initial accumulation site even after RFP-Crk detaches from initial focal complexes. Elapsed time is indicated as h: min.

Movie S7 Sparse CHO cells expressing Tie2Δcyto-GFP and RFP-Crk were stimulated with COMP-Ang1 and time-lapse imaged similar to that described in the legend of Movie S6. Note that similar to Tie2-GFP, Tie2Δcyto-GFP is also targeted to cell periphery close to RFP-Crk-marked focal complexes, and stabilised at initial accumulation site.

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Reagents and antibodies. COMP-Ang1, Ang1, GCN4-Ang1 and MAT-Ang1 were prepared as N-terminally Flag-tagged proteins as described before [1]. VEGF, sTie2-Fc and sTie1-Fc were purchased from R & D systems (Minneapolis, MN). Anti-GFP antibody was generated as described previously [2]. Other antibodies used here were purchased as follows: anti-Tie2 and anti-VE-cadherin from Santa Cruz Biotechnology (Santa Cruz, CA); anti-VE-cadherin, anti-PECAM-1, anti-β-catenin, anti-paxillin, anti-β3 integrin, anti-FAK, anti-VASP and anti-eNOS from BD bioscience (San Jose, CA); anti-HA from Roche Applied Science; anti-Flag (M2), anti-vinculin, anti-β-actin and anti-talin from Sigma-Aldrich; anti-α5 integrin and anti-fibronectin from Chemicon International Inc. (Temecula, CA); anti-phosphotyrosine (PY100), anti-Erk, anti-phospho-Erk, anti-Akt, anti-phospho-Akt, anti-VEGFR2, anti-Foxo1, anti-phospho-Foxo1 and anti-phospho-eNOS from Cell Signaling Technology (Beverly, MA); anti-phosphoTie2 ($pTyr^{1102/1108}$) from Calbiochem (San Diego, CA); anti-Tie2 (TEK4) from eBioscience (San Diego, CA); horseradish peroxidase-coupled sheep anti-mouse, anti-rabbit and anti-goat IgG from GE Healthcare Life Science; Alexa 488- or Alexa 546-labeled secondary antibodies from Molecular Probes (Eugene, OR). Rhodamine-phalloidin was purchased from Molecular Probes.

Plasmids and adenoviruses. A pcDNA-Tie2 plasmid encoding full-length murine Tie2 was kindly provided by T. Suda (Keio University, Tokyo, Japan). cDNA fragments encoding full-length mTie2 and its deletion mutant lacking the cytoplasmic region (amino acids 1-810) amplified by PCR using pcDNA-Tie2 as a template were inserted into pEGFP-N1 vector (Clontech, Mountain View, CA), namely pEGFP-N1-Tie2 and pEGFP-N1-Tie2Δcyto plasmids. A cDNA fragment encoding Tie2 lacking the cytoplasmic region tagged with GFP and that encoding Tie2 lacking cytoplasmic domain tagged with HA was inserted into pIRESneo vector (Clontech) to construct pIRESneo-Tie2Δcyto-GFP plasmid and pIRESneo-Tie2Δcyto-HA plasmid, respectively. pEGFP-N1-Tie2KD vector encoding a kinase-deficient mutant of Tie2 (K854R) was generated

using QuickChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). To generate the plasmid expressing the secreted form of the extracellular domain of Tie2 (amino acids 1-744) fused with HA tag followed by a six-His tag (sTie2-HA), a DNA fragment encoding sTie2-HA was amplified by PCR and inserted to pcDNA3.1 vector (Invitrogen Corp.). To construct pCMV-Tie2-HA and pCMV-Tie2Δcyto-HA vectors, cDNA fragment encoding EGFP was replaced with cDNA encoding HA tag in pEGFP-N1-Tie2 and pEGFP-N1-Tie2Δcyto vectors, respectively. To construct pVEGFR2-IRES-EGFP vector, a cDNA fragment encoding full-length VEGFR2 was amplified by PCR using BCMGSneo-KDR as a template [3] and inserted into pIRES2-EGFP vector (Clontech). For the preparation of a plasmid expressing RFP-FAT, cDNA fragment encoding RFP and that expressing FAT region of FAK (amino acids 885-1052) were PCR-amplified and sequentially inserted into pCXN2 vector [4]. pHcRed-p120 catenin and pCA-RFP-CrkI expressed HcRed tagged-p120 catenin and RFP-tagged CrkI, respectively [2,4]. pGFP-tensin encoding GFP-tagged chicken tensin was generously provided by K.M. Yamada (National Institute of Health)[5]. To generate pEGFP-C1-Foxo1 vector, a cDNA encoding Foxo1 was amplified by PCR using a Foxo1-expressing plasmid, kindly provided by A. Fukamizu (University of Tsukuba, Tsukuba, Japan)[6], as a template, and inserted into pEGFP-C1 vector (Clontech). A cDNA fragment encoding GFP-tagged FAT amplified by PCR was subcloned into pAdeno-X vector (Clontech), and the adenovirus was produced by using the Adeno-X system according to the manufacturer's protocol (Clontech). A recombinant adenovirus vector encoding constitutively active form of Foxo1was kindly provided by J. Nakae (Kobe University Graduate School of Medicine, Kobe, Japan)[7].

Cell culture, transfection, siRNA-mediated protein knockdown, and adenovirus infection. HUVECs and HAECs were purchased from Kurabo, and maintained as described previously [8]. CHO cells were cultured in nutrient mixture F-12 HAM media (Sigma) supplemented with 10% FCS. 293F cells were cultured in Free Style 293 expression media (Invitrogen Corp.) according to manufacturer's protocol. 293T cells were maintained in DMEM (Nissui) supplemented with 10% FCS. BaF3 cells and BaF-Tie2 cells (BaF3 cells stably expressing Tie2) were maintained in RPMI1640

supplemented with 10% FCS and 2 ng ml^{-1} murine IL3 as described before [9]. HUVECs were transfected with Lipofectamine 2000 reagent (Invitrogen Corp.), and both CHO and 293T cells were transfected with Lipofectamine Plus reagents (Invitrogen Corp.). 293F cells were transfected by using 293 fectin reagent (Invitrogen Corp.). Stealth siRNAs targeted to human α5-integrin (HSS105535, HSS105536), human β3 integrin (HSS105565, HSS105567), human VE-cadherin (HSS 101681, HSS101682), human PECAM-1 (HSS107803, HSS107804), human Tie2 (HSS110623, HSS110624) and human FAK (HSS108799, RNAi Duplex1 (Oligo ID12938-040)) were purchased from Invitrogen Corp. Tie2 siRNA (sc-36677) was obtained from Santa Cruz Biotechnology. As a control, siRNA duplex with irrelevant sequences was used. HUVECs were transfected with 20 nM siRNA duplexes using Lipofectamine 2000 reagent according to the manufacturer's instructions. After incubation for 48 h, the cells were used for the experiments. HUVECs were infected with adenoviruses at the appropriated multiplicities of infection for 12 h, and replaced with virus-free culture media. After additional culture for 24-48 h, the cells were used for the experiments.

Generation of BaF3 transfectant. To establish BaF3 cells stably expressing Tie2Δcyto-GFP (BaF-Tie2Δcyto-GFP) and the cells stably expressing Tie2Δcyto-HA (BaF-Tie2Δcyto-HA), a pIRESneo-Tie2Δcyto-GFP plasmid and a pIRESneo-Tie2Δcyto-HA plasmid were transfected into BaF3 cells by electroporation. Transfectants were selected with 600 μg ml^{-1} G418 (Invitrogen Corp.) for 2 weeks, then stained with anti-Tie2 antibody, and sorted by fluorescence-activated cell sorting analysis using FACSAria Cell-Sorting System (BD bioscience) to obtain the cells expressing comparable level of Tie2. Sorted cells were maintained in the presence of 400 μg ml^{-1} G418.

Ang1-ECM binding assay. Glass-base dishes were coated with 20 μg ml^{-1} BSA, fibronectin, vitronectin, laminin, and fibrinogen in PBS, or 0.15 mg ml^{-1} collagen type-I (Nitta Gelatin Inc., Osaka, Japan) in 1 mM HCl at 4 °C overnight. After washing with PBS, dishes were blocked with Block Ace (Dainippon Pharmaceuticals, Osaka, Japan) for 2 h at RT, and subsequently with 1% heat-inactivated BSA (at 85 °C for 12 min) in PBS for 2 h at 37 °C. The dishes were then incubated with 400 ng ml^{-1} Ang1, COMP-

Ang1, and Flag-tagged bacterial alkaline phosphatase (BAP) protein (Sigma-Aldrich) as a negative control in medium 199 containing 1% BSA for 1 h at 37 °C, and washed five times with PBS. Ang1, COMP-Ang1, and BAP protein bound to the dishes were detected by staining with anti-Flag antibody, and visualized with Alexa 546-labeled secondary antibody as described above. Average fluorescence intensity of ten random fields per dish was measured using MetaMorph 6.1 software.

Detection of Tie2, Erk, Akt, Foxo1 and eNOS phosphorylation. HUVECs placed on collagen-coated dish at density of 2,000 cells cm^{-2} and 40,000 cells cm^{-2} were cultured for 24 h to obtain sparse and confluent cell cultures, respectively. After starvation in medium 199 containing 1% BSA for 6 h, the cells were stimulated with COMP-Ang1 and growth media (HuMedia-EG2 supplemented with a growth additive set (Kurabo)) as described in the figures. When indicated in the figures, the cells were stimulated in the presence of 20 μM U0126 (Cell Signaling Technology), 20 μM LY294002 (Cell Signaling Technology), or 8 μM Akt inhibitor IV (Calbiochem). Cells washed twice with ice-cold PBS were lysed at 4 °C in RIPA buffer containing 50 mM Tris-HCl at pH7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 20 mM sodium fluoride, 1 mM sodium vanadate and 1 x protease inhibitor cocktail and centrifuged at 15,000*g* for 20 min at 4 °C. The supernatant was used as precleared cell lysate. To quantify the phosphorylated Tie2, Tie2 was immunoprecipitated with anti-Tie2 antibody from the precleared lysates. Immunoprecipitated Tie2 and aliquots of cell lysate were subjected to SDS-PAGE and Western blot analysis with anti-phosphotyrosine and anti-Tie2 antibodies, respectively. To evaluate the phosphorylation of Erk, Akt, Foxo1 and eNOS, aliquots of total cell lysate were subjected to Western blot analysis with anti-phospho-Erk, anti-phospho-Akt, anti-phospho-Foxo1 and anti-phospho-eNOS antibodies. The total contents of Erk, Akt, Foxo1 and eNOS were also assayed in a parallel run using corresponding antibodies.

To stimulate BaF3 and BaF-Tie2 cells in either floating or adhering condition, the cells were starved for 4 h in RPMI1640 media. The cells were then either allowed to attach to fibronectin-coated dish (attached cells) or kept in 15 ml-conical tube (suspended cells) for 30 min at 37 °C, and stimulated as described in the figures. For the cells

stimulated on fibronectin coated-dish, the supernatants containing floating cells were transferred to 15 ml-conical tubes including ice-cold PBS, collected by centrifugation, and lysed in RIPA buffer. The adhering cells were lysed in RIPA buffer, and both lysates were combined. For the suspended cells, the reactions were stopped by adding ice-cold PBS into the tubes, and the cells were collected by centrifugation and lysed in RIPA buffer. To stimulate floating and adhering HUVECs, the cells starved in 1% BSA for 6 h were detached from the culture dish using Cell dissociation buffer (CDB: Invitrogen Corp.). The cells either allowed to adhere on collagen-coated dish or kept in 15 ml-conical tube for 1 h at 37°C were stimulated with COMP-Ang1 for 15 min. The floating and adhering cells were lysed as described for BaF3 and BaF-Tie2 cells. Phosphorylation of Tie2, Erk, and Akt was analyzed as described above.

Stimulation of HUVECs with ECM-bound COMP-Ang1. To stimulate HUVECs with ECM-bound COMP-Ang1, collagen-coated glass-base dishes were incubated with 3 μg ml^{-1} COMP-Ang1 in PBS for 2 h at 37 °C, and washed four times with PBS. HUVECs starved in medium 199 containing 1% BSA for 3 h were detached from the culture dish using CDB, and resuspended in medium 199 containing 0.5% BSA. The cells were placed on the COMP-Ang1-bound dish and incubated for the periods indicated in the figure. After washing with PBS, the cells were fixed and stained with anti-Tie2, anti-vinculin, anti-FAK antibodies and rhodamine-phalloidin as described above. To quantify the formation of focal complexes, fluorescence intensity relative to vinculin at the cell periphery was measured by line intensity scanning using MetaMorph 6.1 software.

To examine Tie2 activation by ECM-bound COMP-Ang1, 10 cm-collagen dish was incubated with 3 μg ml^{-1} COMP-Ang1 in PBS for 2 h at 37 °C, and washed four times with PBS. HUVECs starved in medium 199 containing 1% BSA for 6 h were detached using CDB, and resuspended in medium 199. The cells were then placed on the COMP-Ang1-bound dish for 20 min, washed twice with ice-cold PBS, and lysed at 4 °C in RIPA buffer. Then, lysates were centrifuged at 15,000 rpm for 20 min at 4 °C. The phosphorylation of Tie2 was examined as described above.

To examine the effects of ECM-bound COMP-Ang1 and MEK inhibitor U0126 on endothelial migration, both lower and upper sides of transwell membrane filters (6.5-

mm diameter, 8.0-μm pore size polycarbonate filter, Corning Coster Corporation, Tokyo, Japan) were coated with 0.3 mg ml^{-1} collagen type I in 1 mM HCl at 4 °C overnight, washed with PBS and air-dried. Subsequently, lower side of the membranes were coated with or without 3 μg ml^{-1} COMP-Ang1 in PBS at 37 °C for 2 h, and washed three times with PBS. Before experiments, HUVECs were starved in medium 199 containing 1% FCS for 48 h, detached from the dish using CDB, and resuspended in medium 199 containing 1% FCS at a density of 3.0 x 10^5 cells ml^{-1}. The cells were preincubated with or without 20 μM U0126 for 1 h at 37 °C and then seeded into the precoated transwell inserts placed in a 24-well plate containing 600 μl medium 199 containing 1% FCS (3 x 10^4 cells $well^{-1}$). After 5 h, the upper membrane of the inserts was swabbed to remove nonmigrated cells. Then, the inserts were washed four times with PBS, fixed in 100% methanol for 2 min at -20 °C, and stained with Hoechst 33342 (Sigma-Aldrich). Migration of HUVEC was quantified by counting the number of the cells in six random fields per insert.

Detection of subcellular localization of GFP-Foxo1. Sparse and confluent HUVECs grown on collagen-coated glass-base dish were transfected with a pEGFP-C1-Foxo1 vector encoding GFP-Foxo1. Next day, the cells were starved for 6 h in medium 199 containing 0.5% BSA and stimulated with or without COMP-Ang1 for the time periods as indicated in figure. After the stimulation, the cells were washed with PBS and fixed in 100% methanol for 2 min at -20 °C. Fluorescence images of GFP were recorded with an Olympus IX-81 inverted fluorescence microscope, and the number of cells with nuclear localization of GFP-Foxo1 was counted. At least 100 GFP-positive cells were scored for each treatment, and three independent experiments were carried out.

Real-time reverse transcription-PCR. Confluent and sparse HUVECs on collagen-coated dish were starved in HuMedia-EB2 medium containing 0.5% FCS for 15 h, and stimulated with COMP-Ang1 (200 ng ml^{-1}) for 1 h. After the stimulation, total RNA was purified using RNeasy Mini Kit (Qiagen, Valencia, CA). Quantitative real-time reverse transcription-PCR was carried out using QuantiFast SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's instruction. For each reaction, 100 ng of total RNA was

transcribed for 10 min at 50 °C, followed by a denaturing step at 95 °C for 5 min and 40 cycles of 10 s at 95 °C and 30 s at 60 °C. Fluorescence data were collected and analyzed using Mastercycler ep realplex (Eppendorf, Hamburg, Germany). The specificity of amplification was confirmed by melting curve analysis. The primers used for amplification were as follows: for human *KLF10*, 5'-TTCCGGGAACACCTGATTTTC-3' and 5'-GCAATGTGAGGTTTGGCAGTA-3'; for human *SOCS3*, 5'-TGCGCCTCAAGACCTTCAG-3' and 5'-GAGCTGTCGCGGATCAGAAA-3'; for human *KLF2*, 5'-CTACACCAAGAGTTCGCATCTG-3' and 5'-CCGTGTGCTTTCGGTAGTG-3'; for human *TIS11d*, 5'-TGTGCAAGACAGAGAAATCCC-3' and 5'-GAGTGCCGTCGGAGGAATC-3'; for *Cx40*, 5'-TCCTGGAGGAAGTACACAAGC-3' and 5'-ATCACACCGGAAATCAGCCTG-3'; for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), 5'-ATGGGGAAGGTGAAGGTCG-3' and 5'-GGGGTCATTGATGGCAACAATA-3'. For normalization, expression of human *GAPDH* was determined in parallel as an endogenous control.

Biotinylation of cell surface Tie2. Confluent HUVECs plated on collagen-coated plates were serum-starved in medium 199 containing 1% BSA for 6 h, and stimulated with COMP-Ang1. After the stimulation, the cells were washed with ice-cold PBS, and incubated with 0.5 mg ml^{-1} of EZ Link Sulfo-NHS-Biotin (Pierce Biotechnology, Rockford, IL) for 60 min at 4 °C. The reaction was quenched by treating the cells with 50 mM NH_4Cl in PBS for 10 min 4 °C. The cells were then lysed in RIPA buffer and centrifuged at 15,000 *g* for 20 min at 4 °C. The supernatant was used as precleared cell lysate. Biotinylated proteins were isolated from the lysate with avidin-immobilized agarose beads (Pierce Biotechnology). Precipitated biotinylated proteins and aliquots of cell lysate were subjected to Western blot analysis with anti-Tie2 antibody.