Cell cultures

HUVECs were grown in M199 medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal calf serum (FCS, Invitrogen), endothelial cell growth factor (100 μg/mL) (Sigma Chemical Co. St. Louis, MO) and porcine heparin (100 μg/mL, Sigma). HUVEC were used at early passages (I-IV) and grown on plastic surface coated with porcine gelatin (Sigma).

Fetal bovine aortic endothelial GM 7373 cells, corresponding to the BFA-1c multilayered transformed clone[2], were grown in Eagle's minimal essential medium (MEM, Gibco Life Technologies) containing 10% FCS, vitamins, essential and non essential amino acids.

Immortalized Balb/c murine aortic endothelial cells (MAECs) were obtained from R. Auerbach (University of Wisconsin, Madison, WI) and grown in Dulbecco's modified minimal essential medium (DMEM, Invitrogen) added with 10% fetal calf serum (FCS).

Bovine aortic endothelial cells (BAECs, provided by A. Vecchi, Istituto Clinico Humanitas IRCCS, Milan) were cultured in MEM-Eagle's medium (Gibco Life Technologies) supplemented with 10% FCS, 2% essential amino acids and 2% vitamins [3].

MAECs were transfected with a pcDNA3.1 expression vector harboring the mouse VEGFR2 cDNA (provided by G. Breier, Max Planck Institute for Physiological and Clinical Research, Bad Nauhein, Germany) or with the empty vector to generate stable transfectants (VEGFR2-MAECs and mock-MAECs, respectively) according to standard protocols.

BIAcore analysis

A BIAcore X apparatus (BIAcore Inc., Piscataway, NJ) was used to analyze VEGF-A and gremlin (both from R&D Systems, Minneapolis, MN) interaction with $sVEGFR2_{D1-7}$ (sVEGFR2, Calbiochem, San Diego, CA) immobilized to the sensorchip. To this purpose, recombinant human sVEGFR2 (40 μg/mL in 10 mM sodium acetate, pH 5.3) was allowed to react with a flow cell of a CM5 sensorchip that was previously activated with a mixture of 0.2 M N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 0.05 M N-hydroxysuccinimide (35 μL, flow rate 10 μL/minute). These experimental conditions allowed the immobilization of approx. 0.083 pmol/mm^2 of sVEGFR2. After ligand immobilization, matrix neutralization was performed with 1.0 M ethanolamine (pH 8.5) (35 μL, flow rate 10 μL/minute). Activated/deactivated sensorchip was used as a negative control and for blank subtraction.

To allow the association with immobilized sVEGFR2, increasing concentrations of VEGF-A or gremlin were injected in HBS-EP buffer (0.01 M Hepes pH 7.4 *plus* 0.005% surfactant P20, 0.15 M NaCl, 3 mM EDTA) over the sVEGFR2 surface for 4 minutes (VEGF-A: sample volume 40 μL, flow rate 10 μL/minute and dissociation time 120 seconds; gremlin: sample volume 40 μL, flow rate 5 μL/minute, and dissociation time 240 seconds). Binding parameters were calculated by the nonlinear-curve-fitting software package BIAevaluation 3.2 (Biacore), using a single-site model with drifting baseline.

In parallel experiments, 25 nM VEGF-A or gremlin were injected for 4 minutes on the sVEGFR2 surface in the absence or in the presence of 314 nM $sVEGFR1_{D1-7}$-Fc (sVEGFR1-Fc), $sVEGFR2_{D1-7}$-Fc (sVEGFR2-Fc), sVEGFR3-Fc or sFGFR1a(IIIc)-Fc chimeras (all from RELIATech GmbH, Braunschweig, Germany). The response (in RU) was recorded at the end of injection and binding data were plotted as percentage of maximal bound analyte.

VEGF-A/VEGFR competitive ELISA-based assay

96-well plates were coated for 16 hours at room temperature with 250 ng/mL of sVEGFR1-Fc or sVEGFR2-Fc in saline phosphate buffer (PBS) at 100 μL per well followed by a 3 hours blocking step with 1% bovine serum albumin (BSA). Then, VEGF-A [20 ng/mL dissolved in PBS containing 0.1% BSA, 5 mM EDTA, 0.004% Tween 20 (PBET buffer)] was added in presence of different

competitors and incubated for 1 hour at 37 °C followed by 1 hour incubation at room temperature. An anti-human VEGF monoclonal antibody (R&D System) diluted in PBET buffer at 300 ng/mL was added to the wells and incubated for 1 hour at 37 °C followed by 1 hour incubation at room temperature. Finally, wells were incubated for 1 hour at room temperature with a secondary donkey anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology).

Western blotting and receptor cross-linking assays

Confluent ECs were made quiescent by a 20 hour-starvation in 5% FCS. After stimulation with gremlin, cells were lysed and 50-100 μg aliquots were analyzed by 6% SDS-PAGE followed by Western blotting with antibodies against pVEGFR2 (pTyr1175, Cell Signaling Technology, Boston, MA; pTyr951, Santa Cruz Biotechnology), pTyr (clone 4G10, Millipore, Bedford, MA), focal adhesion kinase (FAK9, or src (Santa Cruz Biotechnology).

For cross-linking experiments, gremlin was first conjugated with 5.0 nM of the bi-functional photoactivable biotin-label transfer cross-linker Sulfo-SBED Biotin Label transfer reagent [4] according to manufacturer's instructions (Pierce, Rockford, IL) to generate sulfo-gremlin. Then, confluent ECs were added with sulfo-gremlin dissolved in PBS or with linker reagent alone in the absence or presence of a molar excess of unlabelled gremlin or VEGF-A. After 120 minute incubation at 4°C, ECs were UV irradiated to photo-activate the free aryl azide group and lysed in lysis buffer [50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1% Triton X-100, 1.0 mM Na_3VO_4, and protease and phosphatase inhibitors (all from Sigma)]. Then, samples (1.0 mg of protein) were immunoprecipitated with anti-VEGFR2 antibody and separated on SDS-PAGE under reducing conditions to allow the transfer of the biotin moiety from sulfo-gremlin to the interacting protein receptor. Analysis of the immunocomplexes were performed using Streptavidin-HRP and visualized using the ECL Western blotting kit (GE Health Care, Piscataway, NJ).

Immunofluorescence analysis

Cells were seeded on gelatin-coated glass coverslips in DMEM added with 2% FCS. After overnight incubation, cells were treated with gremlin or VEGF-A for 0-30 minutes at 37°C, washed, fixed in 3% paraformaldehyde/2% sucrose in PBS, permeabilized with 0.5% Triton-X100, and saturated with goat serum in PBS. Then, cells were incubated with a rabbit monoclonal anti-pVEGFR2 antibody (pTyr1175; Cell Signaling Technology) followed by Alexa Fluor 488 anti-rabbit IgG (Invitrogen). Cells were photographed using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective.

Fluorescence resonance energy transfer (FRET) analysis

Vectors for the expression of the FRET pair VEGFR2-ECFP and VEGFR2-EYFP where obtained from Mauro Giacca (Trieste, Italy). BAECs were transiently transfected with both plasmids and seeded on NUNC multi-well chambers 48 hours after transfection. After adhesion, cells were starved overnight in serum-free medium without phenol red added with 20 mM HEPES to stabilize its pH. Cells with similar expression of ECFP and EYFP proteins were stimulated with 50 ng/mL gremlin, 30 ng/mL VEGF-A, 30 ng/mL FGF2, or vehicle. Cells were observed for 10 minutes before and 30 minutes after stimulation under the Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective. Time lapse files were analyzed by Axiovision "FRET module" software (Zeiss) using Youvan's method [1].

EC motility

Cell motility was assessed by time lapse videomicroscopy . ECs were seeded at 150 cells per mm^2 in 24 well-plates for 2 hours and then stimulated with gremlin or VGEF-A dissolved in DMEM *plus* 0.5% FCS in the absence or in the presence of different VEGFR2 inhibitors [5μM SU5416 [5], 3 μM cyclo-VEGI [6] (both from Calbiochem)]. Constant temperature (37°C) and pCO_2 (5%) were maintained throughout the experimental period by means of an heatable stage and climate chamber.

Cells were observed under an inverted photomicroscope (Zeiss Axiovert 200M) and phase-contrast snap photographs (one frame every 5 minutes) were digitally recorded for 180 minutes. Cell paths (15-20 cells per experimental point) were generated from centroid positions and migration parameters were analyzed with the "Chemotaxis and Migration Tool" of ImageJ software (http://rsbweb.nih.gov/ij).

EC chemotaxis

HUVEC were seeded at 1.0 x 10^6 cells/ml in the upper compartment of a Boyden chamber containing gelatin-coated PVP-free polycarbonate filters (5 μm pore size, Costar, Cambridge, MA). VEGF-A or gremlin dissolved in M199 with 1% FCS was placed in the lower compartment. 1% FCS medium was used as negative control. After 5 h of incubation at 37°C, cells migrated to the lower side of the filter were stained with Diff-Quik (Dade-Behring, Milan, Italy). Five random fields were counted for each triplicate sample.

Rac activation assay

HUVEC (4.0 x 10^4 cells per mm^2) were made quiescent by a 20 hour-starvation in 5% FCS. After stimulation with gremlin (100 ng/mL) or VEGF-A (50 ng/mL) cells were lysed and 5-10 μg aliquots were analyzed with the Rac1,2,3 G-LISA™ Activation Assay kit according to manufacturer's instructions (Cytoskeleton Inc., Denver, CO).

EC sprouting

Type I collagen gel invasion assay were performed on HUVEC aggregates as described [7]. Briefly, spheroids were prepared in 20% methylcellulose medium, embedded in type I collagen gel, and stimulated with gremlin or VEGF-A in the absence or in the presence of 5 μM SU5416 or 3 μM cyclo-VEGI. Formation of radially growing cell sprouts was observed during the next 48 hours. Sprouts were photographed at 200x magnification using an Axiovert 200M microscope equipped with a 20x objective (LD A PLAN 20X/0,30PH1, Zeiss).

Artery ring assay

One-millimetre human umbilical artery rings were embedded in fibrin gel [8] and cultured in human EC medium SFM (Gibco Life Technologies) with gremlin or VEGF-A in the absence or in the presence of 0.1 μM SU5416 or 20 nM VEGFR2 Kinase Inhibitor I [9] (Calbiochem). After 3 days, EC sprouts, morphologically distinguishable from scattering fibroblasts/smooth muscle cells, were counted under an inverted microscope at 100x magnification and photographed at day 6 of incubation.

Chick embryo chorioallantoic membrane (CAM) assay

Alginate beads (5 μL) containing vehicle, gremlin, or VEGF-A (both at 100 ng per embryo) in the absence or in the presence of 150 ng of sVEGFR2 or 5.0 μm SU5416 were placed on the CAM of fertilized White Leghorn chicken eggs at day 11 of incubation [7]. After 72 hours, newly formed blood vessels converging towards the implant were counted at 5x magnification using a STEMI SR stereomicroscope equipped with an objective f equal to 100 mm with adapter ring 475070 (Zeiss).

REFERENCES

1. Youvan, D.C. et al. Calibration of Fluorescence Resonance Energy Transfer in microscopy using genetically engineered GFP derivates on nickel chelating beads. *Biotechnology* **3**, 1-18 (1997).
2. Grinspan, J.B., Mueller, S.N. & Levine, E.M. Bovine endothelial cells transformed in vitro by benzo(a)pyrene. *J Cell Physiol* **114**, 328-338 (1983).

3. Giuliani, R., Bastaki, M., Coltrini, D. & Presta, M. Role of endothelial cell extracellular signal-regulated kinase1/2 in urokinase-type plasminogen activator upregulation and in vitro angiogenesis by fibroblast growth factor-2. *J Cell Sci* **112 (Pt 15)**, 2597-2606 (1999).
4. Gupta, N. et al. Identification and characterization of p63 (CKAP4/ERGIC-63/CLIMP-63), a surfactant protein A binding protein, on type II pneumocytes. *Am J Physiol Lung Cell Mol Physiol* **291**, L436-446 (2006).
5. Fong, T.A. et al. SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res* **59**, 99-106 (1999).
6. Zilberberg, L. et al. Structure and inhibitory effects on angiogenesis and tumor development of a new vascular endothelial growth inhibitor. *J Biol Chem* **278**, 35564-35573 (2003).
7. Mitola, S. et al. Angiopoietin-1 mediates the proangiogenic activity of the bone morphogenic protein antagonist Drm. *Blood* **112**, 1154-1157 (2008).
8. Belleri, M. et al. Antiangiogenic and vascular-targeting activity of the microtubule-destabilizing trans-resveratrol derivative 3,5,4'-trimethoxystilbene. *Mol Pharmacol* **67**, 1451-1459 (2005).
9. Sun, L. et al. Synthesis and biological evaluations of 3-substituted indolin-2-ones: a novel class of tyrosine kinase inhibitors that exhibit selectivity toward particular receptor tyrosine kinases. *J Med Chem* **41**, 2588-2603 (1998).

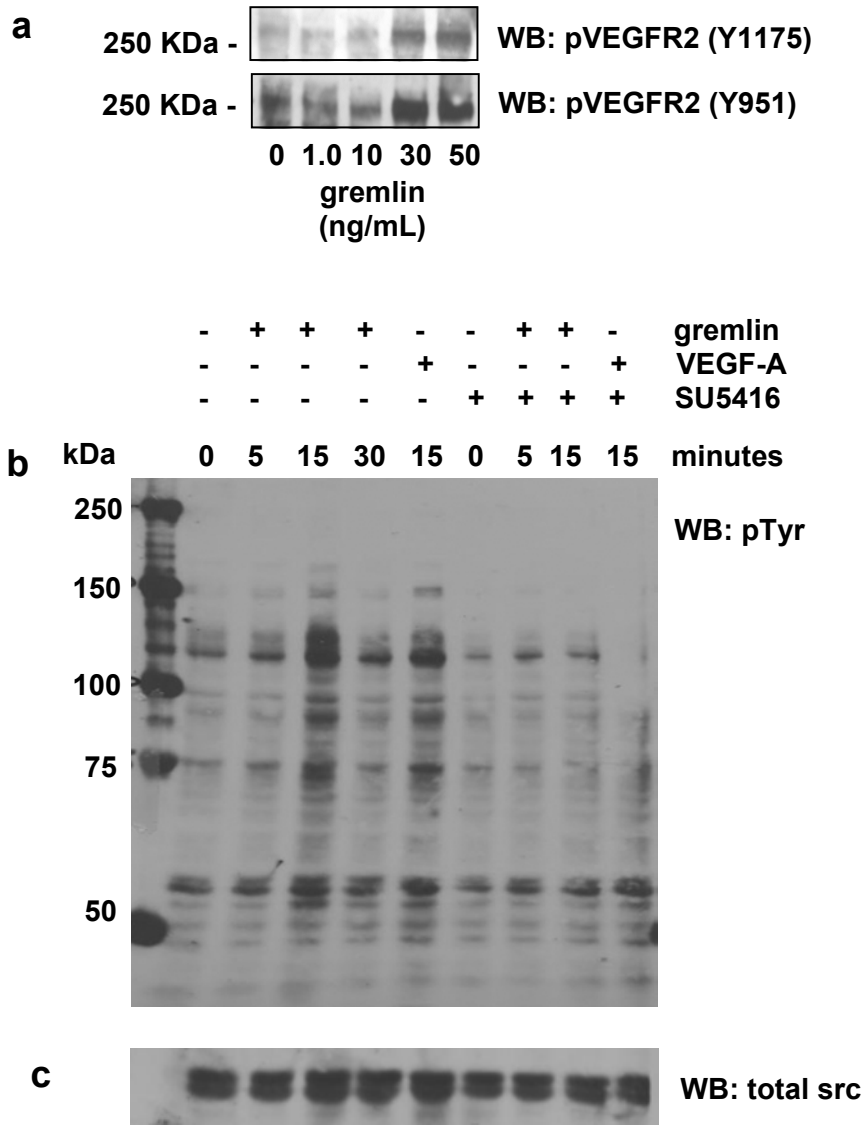

Figure S1. Gremlin induces VEGFR2 autophosphorylation and TK-VEGFR2-dependent tyrosine phosphorylation in HUVECs. a) Serum-starved HUVECs were stimulated for 15 minutes with the indicated concentrations of gremlin or VEGF-A. At the end of incubation, 50 μg of cell extracts were probed by Western blotting with monoclonal antibodies against phosphoTyr1175 and phosphoTyr951 VEGFR2 residues (Cell Signaling Technology). **b**) Serum-starved HUVECs were stimulated for 0-30 minutes with 50 ng/mL gremlin or for 15 minutes with 30 ng/mL VEGF-A in the absence or in the presence of 5.0 μM SU5416 (Calbiochem). At the end of incubation, 100 μg of cell extracts were probed by Western blotting with a monoclonal anti-pTyr antibody (clone 4G10, Millipore). Uniform loading of the gel was confirmed by incubation of the membranes with an anti-src antibody (Santa Cruz Biotechnology) (**c**).

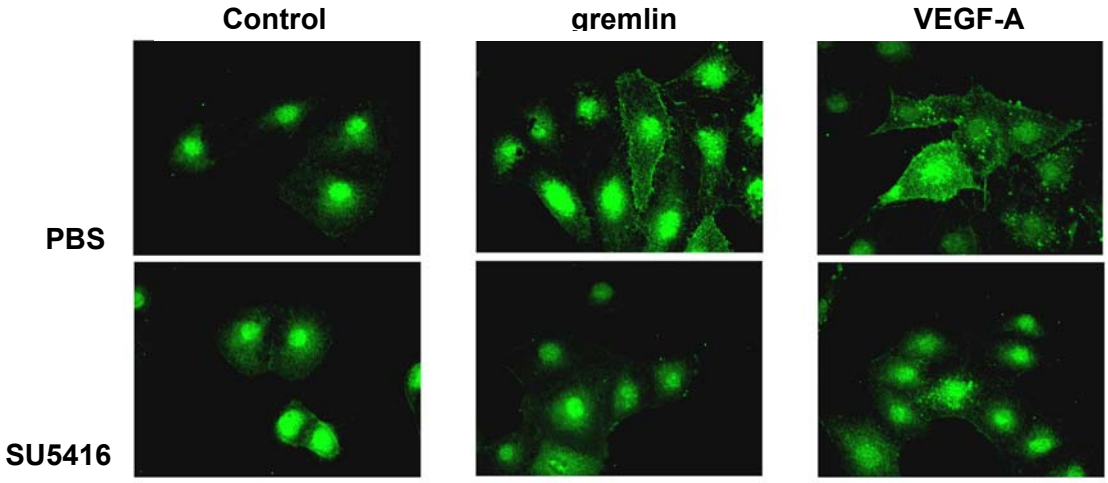

Figure S2. VEGFR2 phosphorylation induced by gremlin in VEGFR2 over-expressing ECs. Serum-starved VEGFR2-GM7373 ECs were stimulated for 0 or 5 minutes with 50 ng/mL of VEGF-A or gremlin in the absence or in the presence of 5.0 μM SU5416 and immunostained with rabbit monoclonal anti-phosphoVEGFR2 antibody (pTyr1175) followed by Alexa Fluor 488 anti-rabbit IgG (Invitrogen, Carlsbad, CA). Analysis was performed using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective (Carl Zeiss, Gottingen, Germany).

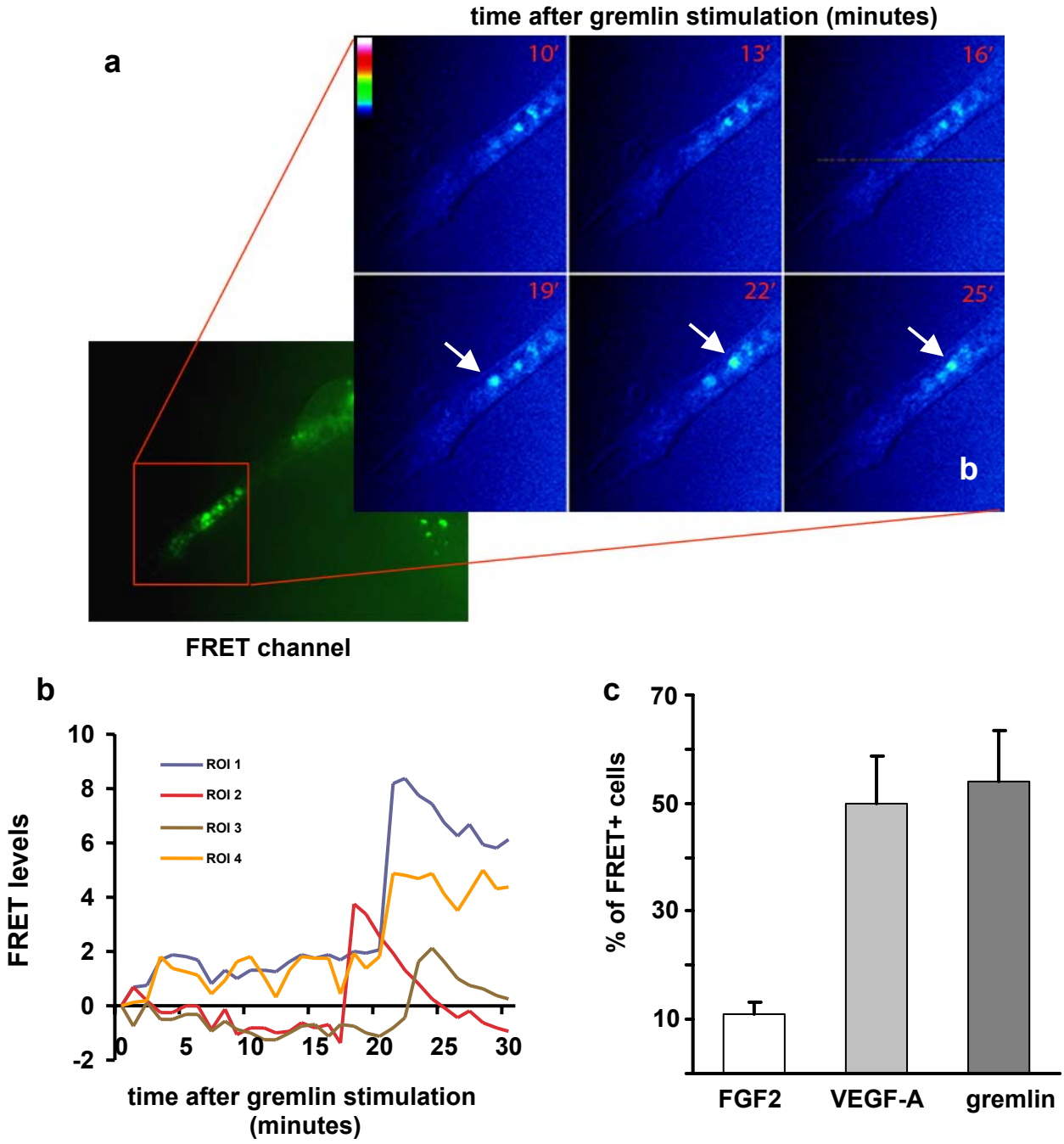

Figure S3. Gremlin induces VEGFR2 internalization and dimerization. BAECs were transiently co-transfected with the FRET pair VEGFR2-EYFP and VEGFR2-ECFP. After 48 hours, 50 cells with similar expression of ECFP and EYFP proteins were analyzed for 10 minutes before and 30 minutes after stimulation with 30 ng/mL FGF2, 30 ng/mL VEGF-A, or 50 ng/mL gremlin. Analysis was performed using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective. Constant temperature (37°C) and pCO_2 (5%) were maintained throughout the experimental period by means of heatable stage and climate chamber (incubator model S, Zeiss, Gottingen, Germany). Time-lapse series of images were analyzed by the Axiovison software "FRET module" using the Youvan's method [1]. **a**) Detail of one gremlin-stimulated cell (as acquired in the FRET channel) in which the kinetics of appearance of intracellular FRET events (arrows) are shown by color pixel scoring for FRET levels from 0 (dark blue) to 100 (white) in panel **b** and quantified in the regions of interest (ROI) after subtraction of FRET levels at T_0 of stimulation in panel **c**; similar results were obtained for VEGF-A-stimulated cells (data not shown). **c**) Percentage of FRET-positive cells during the 30 minutes stimulation.

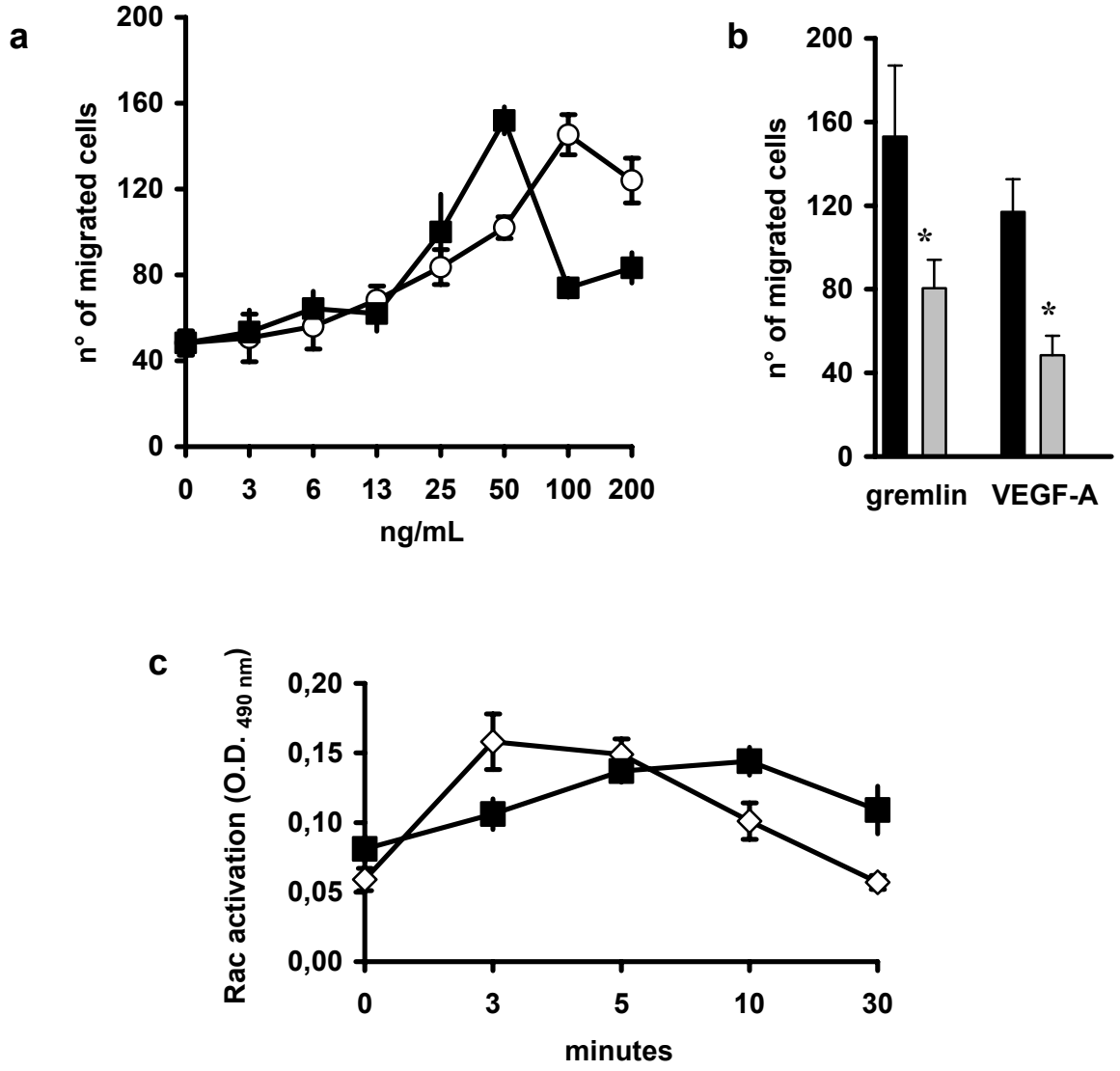

Figure S4. Both gremlin and VEGF-A are chemotactic for HUVECs. a) HUVECs were assessed for their capacity to migrate in response to increasing concentrations of gremlin (◇) or VEGF-A (■) in a Boyden chamber assay. After 5 hours, cells migrated to the lower side of the filter were counted. **b**) HUVEC migration in response to an optimal dose of gremlin (100 ng/mL) or VEGF-A (50 ng/mL) in absence (black bars) or in presence (gray bars) of 20 μg/mL of neutralizing anti-VEGFR2 antibodies (kindly provided by H.A. Weich, Max Plank Institute, Germany). **c**) Serum-starved HUVECs were stimulated with 100 ng/mL of gremlin (◇) or 50 ng/mL of VEGF-A (■). At different times after stimulation, 5 μg of cell extracts were analyzed for Rac activation by G-LISA™. *, $p < 0.01$, Student's *t* test.

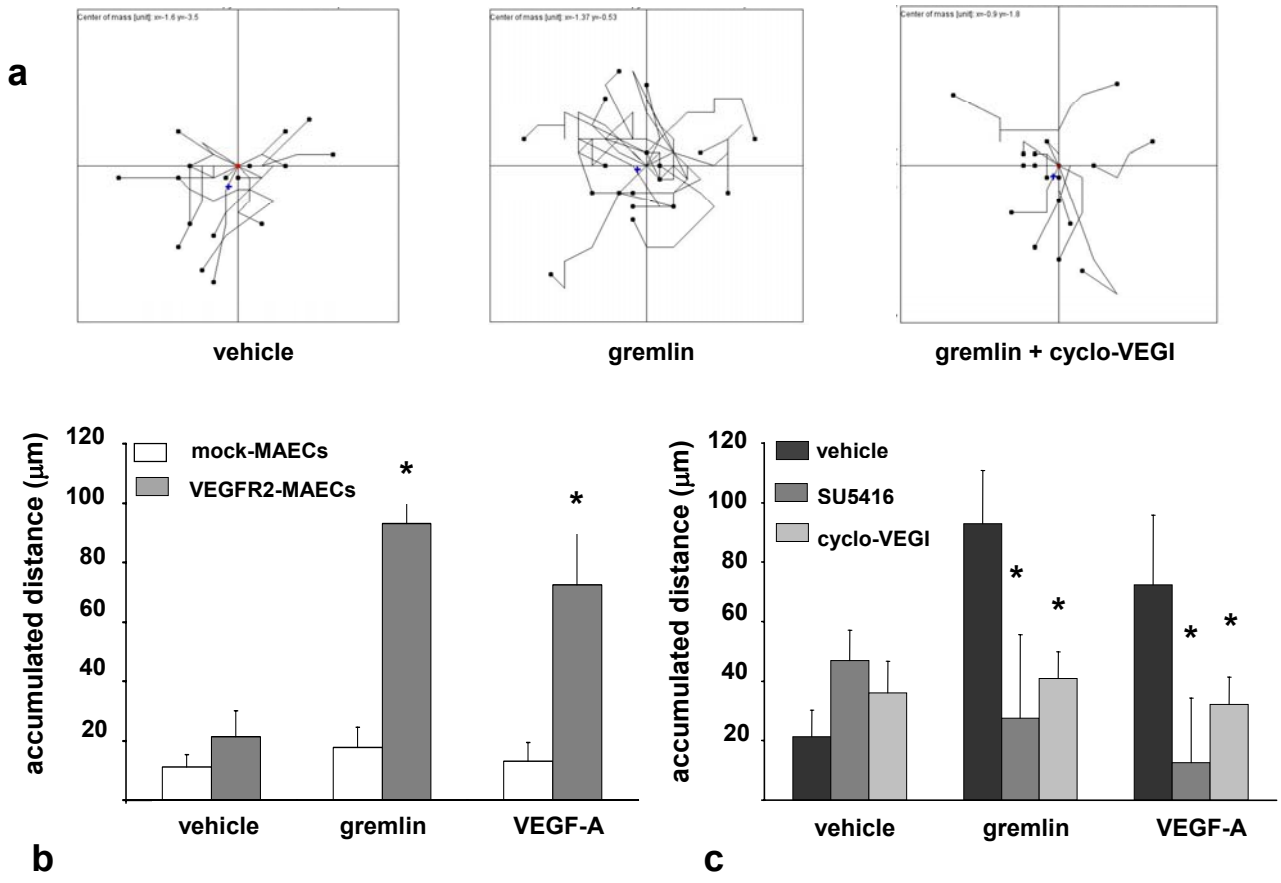

Figure S5. Gremlin induces VEGFR2-dependent EC motility. MAECs were transfected with the pcDNA3.1 expression vector harbouring the mouse VEGFR2 cDNA or with the empty vector to generate stable transfectants (VEGFR2-MAECs and mock-MAECs, respectively). Two hours after plating (150 cells per mm^2), cells were stimulated with 50 ng/mL gremlin or 30 ng/mL VEGF-A dissolved in DMEM *plus* 0.5% FCS in the absence or in the presence of the VEGFR2 specific inhibitors SU5416 (5.0 µM) or cyclo-VEGI (3.0 µM). Cell motility was assessed by time lapse videomicroscopy using an inverted photomicroscope (Zeiss Axiovert 200M). Constant temperature (37°C) and pCO_2 (5%) were maintained throughout the experimental period by means of heatable stage and climate chamber. Phase-contrast snap photographs (one frame every 5 minutes) were digitally recorded for 180 minutes. Cell paths (15-20 cells per experimental point) were generated from centroid positions and migration parameters were analyzed with the "Chemotaxis and Migration Tool" of ImageJ Software (http://rsbweb.nih.gov/ij). **a**) Representative tracked paths of VEGFR2-MAE cells stimulated by vehicle, gremlin alone or in presence of cyclo-VEGI. **b)** Accumulated distances (in µm) of mock-MAE and VEGFR2-MAE cells stimulated by gremlin or VEGF-A. *, statistically different from mock-MAE cells ($p< 0.01$, Student's *t* test). **c**) Accumulated distances (in µm) by VEGFR2-MAE cells stimulated by gremlin or VEGF-A in the absence or in the in presence of VEGFR2 inhibitors. *, statistically different from VEGFR2-MAE cells stimulated in the absence of any inhibitor ($p< 0.01$, Student's *t* test).

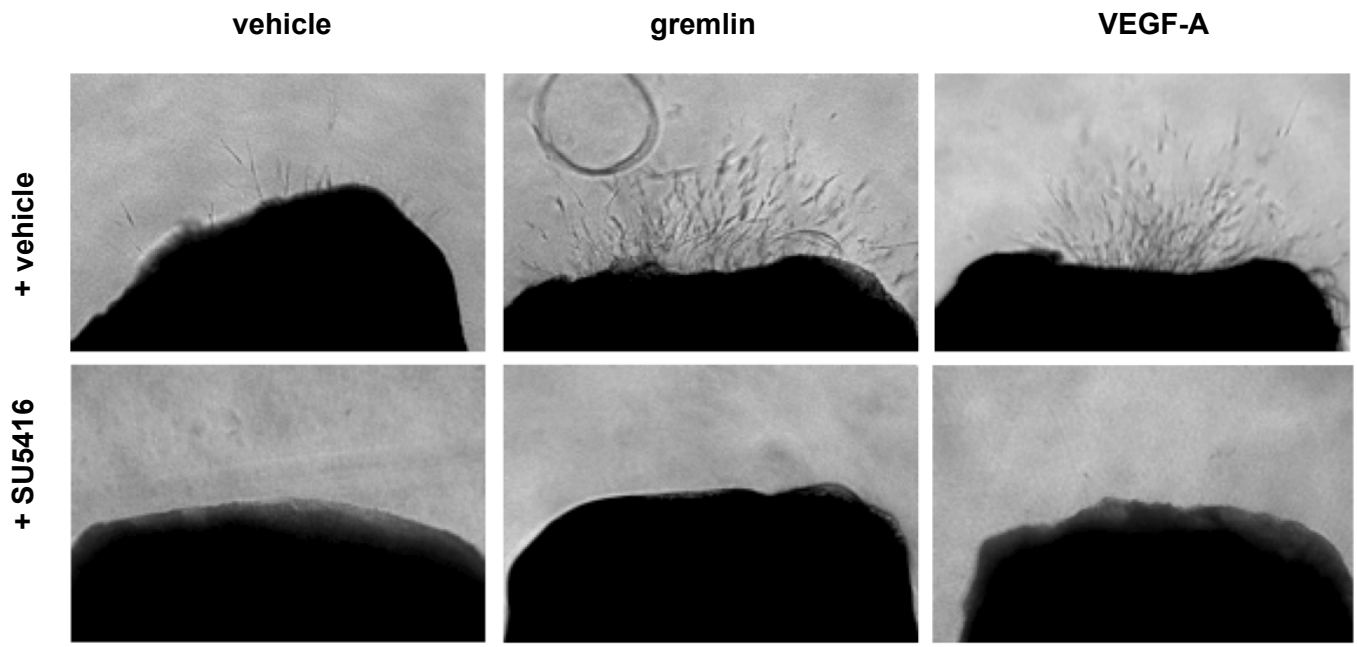

Figure S6. Gremlin induces VEGFR2-dependent EC sprouting from human umbilical artery rings. Human umbilical artery rings were embedded in fibrin gel and stimulated with 50 ng/mL gremlin or 30 ng/mL VEGF-A in the absence or in the presence of the VEGFR2 inhibitor SU5416 (0.1 μM). After 6 days, rings were photographed at 200x magnification using an Axiovert 200M microscope equipped with a 20x objective (LD A PLAN 20X/0,30PH1, Zeiss). Note the presence of numerous EC sprouts in gremlin- and VEGF-A-treated artery rings whose formation is potently inhibited by the VEGFR2 TK inhibitor SU5416.

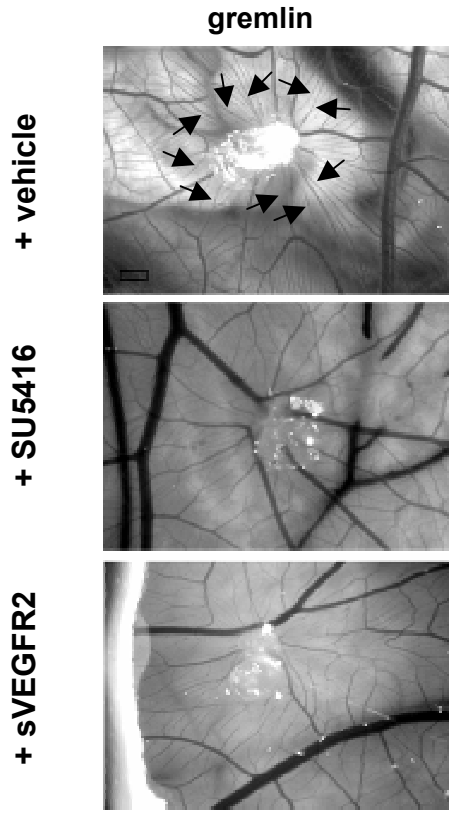

Figure S7. VEGFR2 mediates the angiogenic activity of gremlin in the chick embryo CAM. Alginate pellets containing 100 ng per pellet of gremlin alone or added with sVEGFR2 (150 ng per pellet) or 5.0 μM SU5416 were implanted on the top of chick embryo CAMs at day 11 of development. After 3 days, CAM were photographed *in ovo* at 5x magnification under a stereomicroscope. Note the numerous newly-formed microvessels converging versus the gremlin implant (arrows) that were significantly reduced in the presence of SU5416 or sVEGFR2.

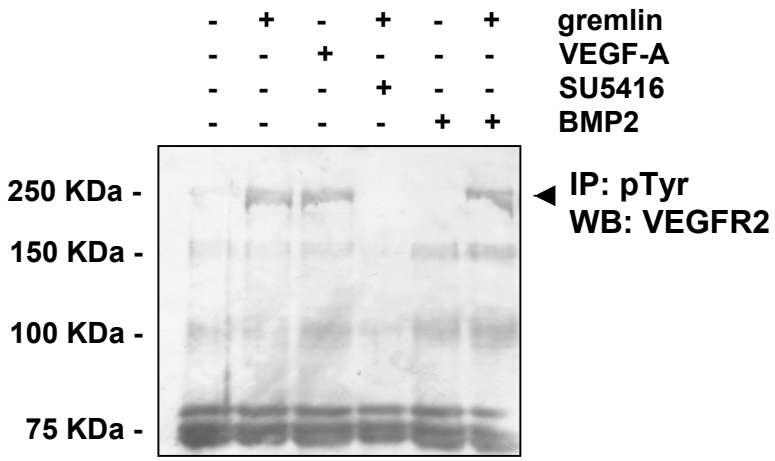

Figure S8. Gremlin activates VEGFR2 in a BMP-independent manner. a) Serum-starved murine microvascular ECs were stimulated for 15 minutes with 50 ng/mL of BMP2, 20 ng/mL of VEGF-A or 50 ng/mL of gremlin alone or in the presence of 5.0 μM SU5416 or BMP2. At the end of incubation, 1.0 mg of cell extracts were immunoprecipitated with anti-pTyr antibody and probed by Western blotting with antibodies against VEGFR2. Arrowhead points to the 250 kDa tyrosine-phosphorylated VEGFR2.