SUPPLEMENTAL FIGURES

Beta3 integrin promotes long-lasting activation and polarization of Vascular Endothelial Growth Factor Receptor 2 by immobilized ligand

C. Ravelli et al.

FIGURE S. I

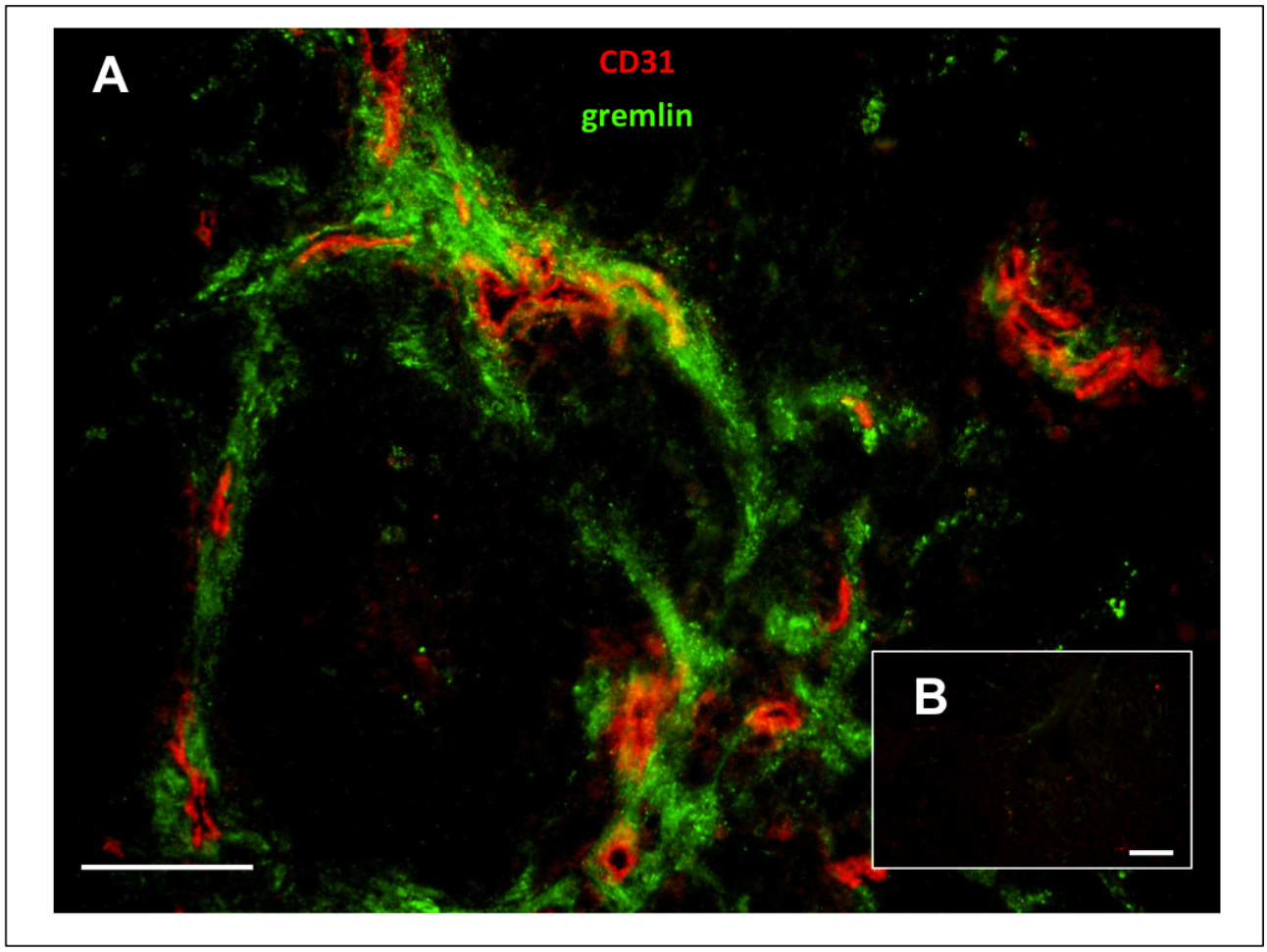


Figure S. I: Gremlin accumulates in the stroma surrounding CD31$^+$ vessels of human adenocarcinoma xenografts. Human endometrial adenocarcinoma HEC-1-B-derived Tet-FGF2 cells were injected s.c. in nude mice. (A) Sections from 8 weeks tumors were double-immunostained with the rat monoclonal anti-mouse CD31 antibody MEC 13.3 and with goat anti-murine gremlin antibody (R&D Systems) followed by biotin-conjugated rabbit anti-rat secondary antibody (DAKO) and Alexa-fluor 488 rabbit anti-goat secondary antibody (Molecular Probes) followed by incubation with Texas red Avidin D (Vector). Samples were analysed using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective (Bar, 100 μm). (B) Image shows secondary antibodies only staining.

FIGURE S. II

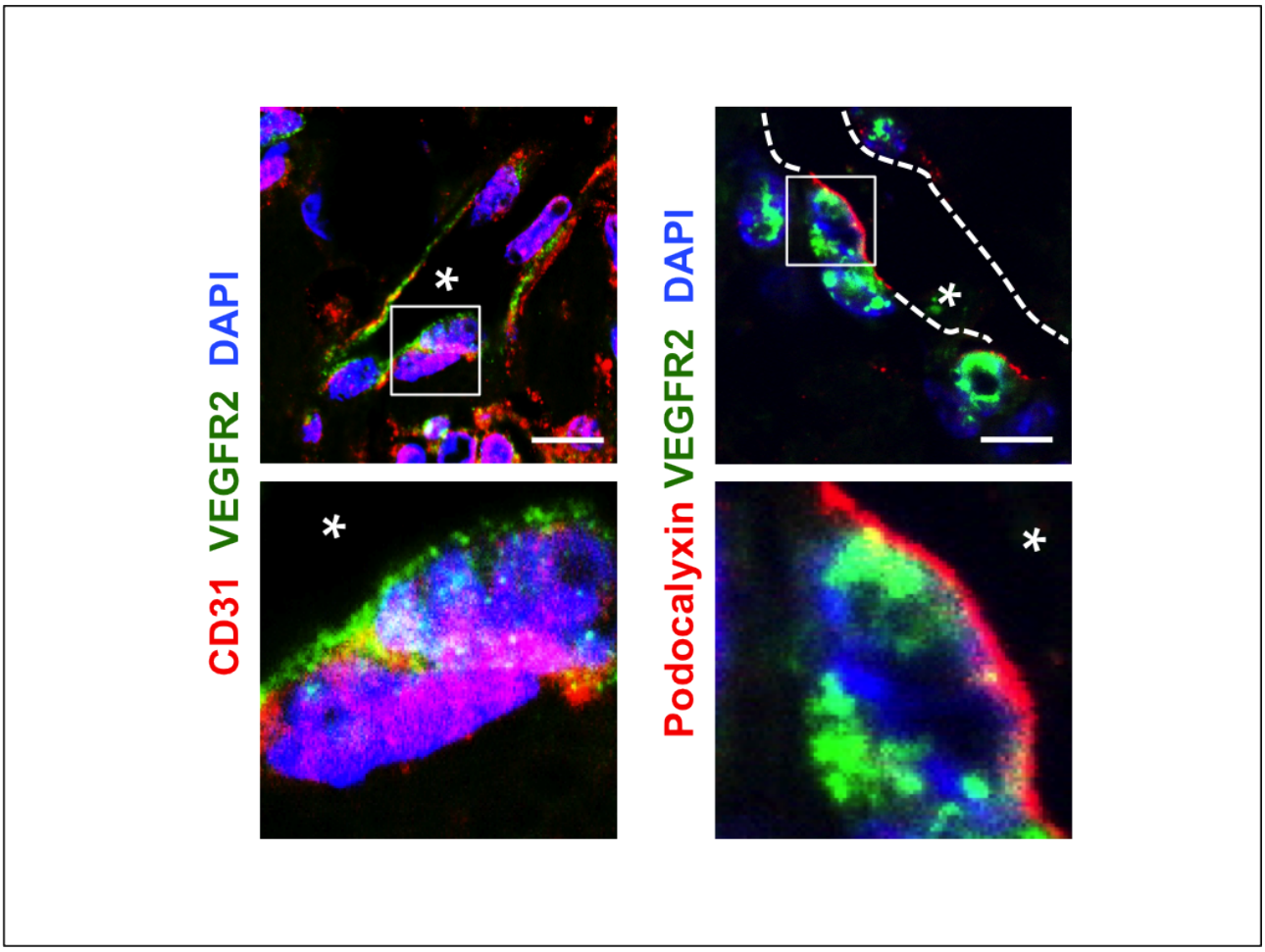


Figure S. II: VEGFR2 is equally distributed between apical and basal aspects of ECs in vivo vessel formation induced by ECM-bound FGF2. Matrigel plugs containing 1.0 μg/mL of FGF2 were implanted s.c. in C57BL/6 mice. After 1 week, plugs were stained for CD31 or podocalyxin (red), VEGFR2 (green) and nuclei (blue) and analyzed. Images of 0.3 μm sections were collected using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective and ApoTome system (Zeiss). Asterisks indicate vessel lumens (Bar, 10 μm).

FIGURE S. III

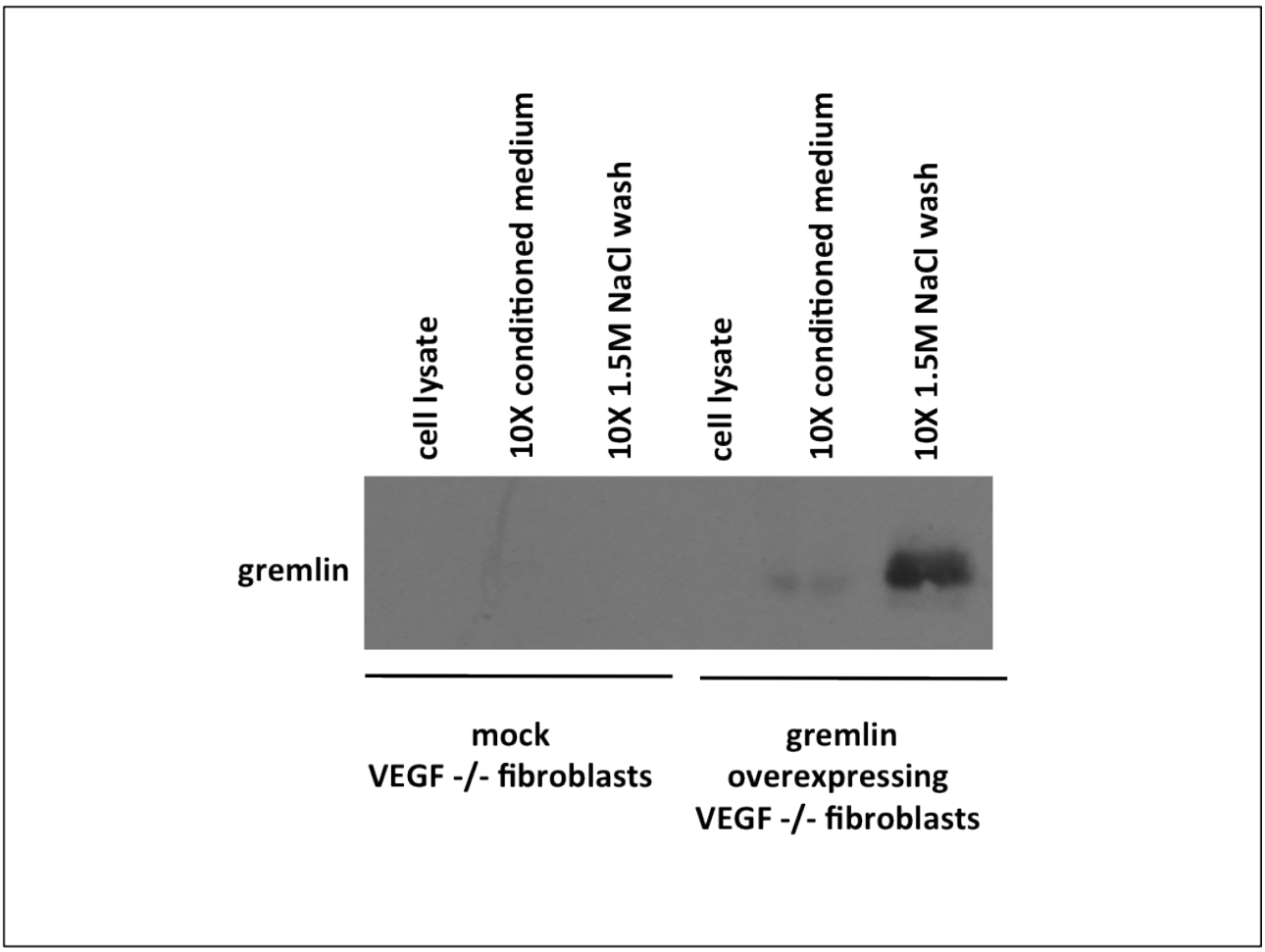


Figure S. III: Gremlin produced by gremlin-overexpressing VEGF$^{-/-}$ fibroblast is bound to extracellular ECM. 80% confluent mock- and gremlin-overexpressing VEGF$^{-/-}$ fibroblasts were starved in DMEM without FCS. After 24 hours the conditioned medium was harvested and cells were washed once with PBS and once with PBS containing 1.5 mol/L NaCl. Cells were then lysed. Conditioned medium and 1.5 mol/L NaCl wash were concentrated 10 times using Centricon devices (Millipore). Finally, lysate and 20 μL of concentrated conditioned medium and concentrated 1.5 mol/L NaCl wash were analysed by Western blotting using a specific anti-gremlin antibody (R&D System).

FIGURE S. IV

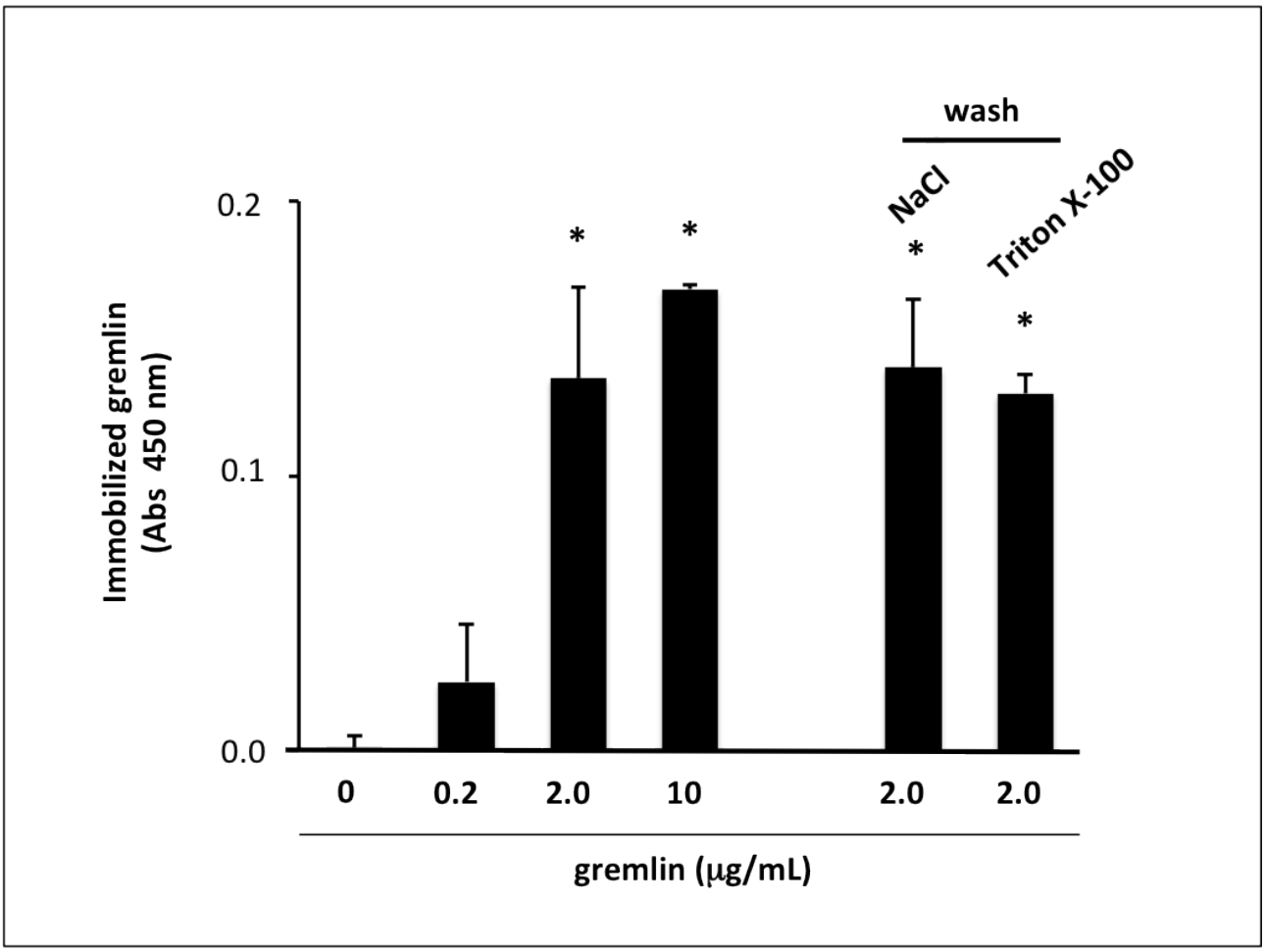


Figure S. IV: Substrate-bound gremlin does not elute from tissue culture plastic. Aliquots (100 μL) of sterile PBS containing gremlin (0.2-2-10 μg/mL) were added to polystyrene tissue culture plates. After 16 hours of incubation at 4°C wells were washed 3 times with cold PBS. Uncovered plastic was blocked with 1 mg/mL BSA for 1 hour at room temperature and immobilized gremlin was measured by ELISA. Plastic-immobilized gremlin resists extraction with 2 mol/L NaCl and detergent treatment following incubation for 1 hour at 37°C with 0.2% Triton X-100.

FIGURE S. V

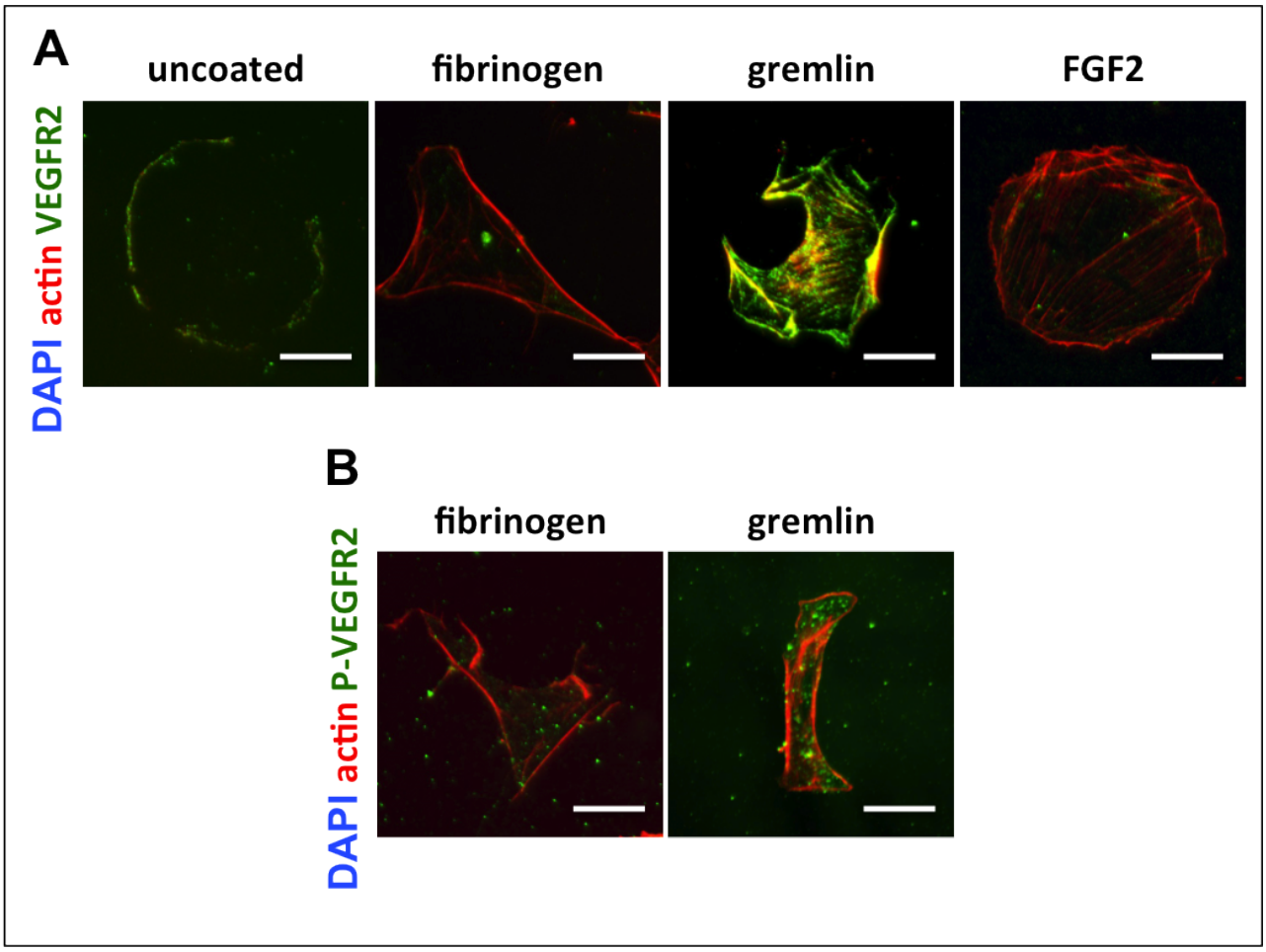


Figure S. V: Substrate-bound gremlin recruits and activates VEGFR2 in EC VPMs. (A) Ventral plasma membranes (VPMs) from VEGFR2-GM7373 cells seeded on uncoated coverslips, immobilized FG, gremlin or FGF2 were stained for VEGFR2, actin and nuclei. (B) VPMs from VEGFR2-GM7373 cells seeded on immobilized FG or gremlin and stained for phospho-VEGFR2 (pTyr1175), actin and nuclei. Samples were analyzed with epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective epifluorescence microscope (Bar, 10 μm). Note the absence of nuclear DAPI staining and the persistence of actin filaments used to unequivocally identify the VPM remnants bound to the substratum.

FIGURE S. VI

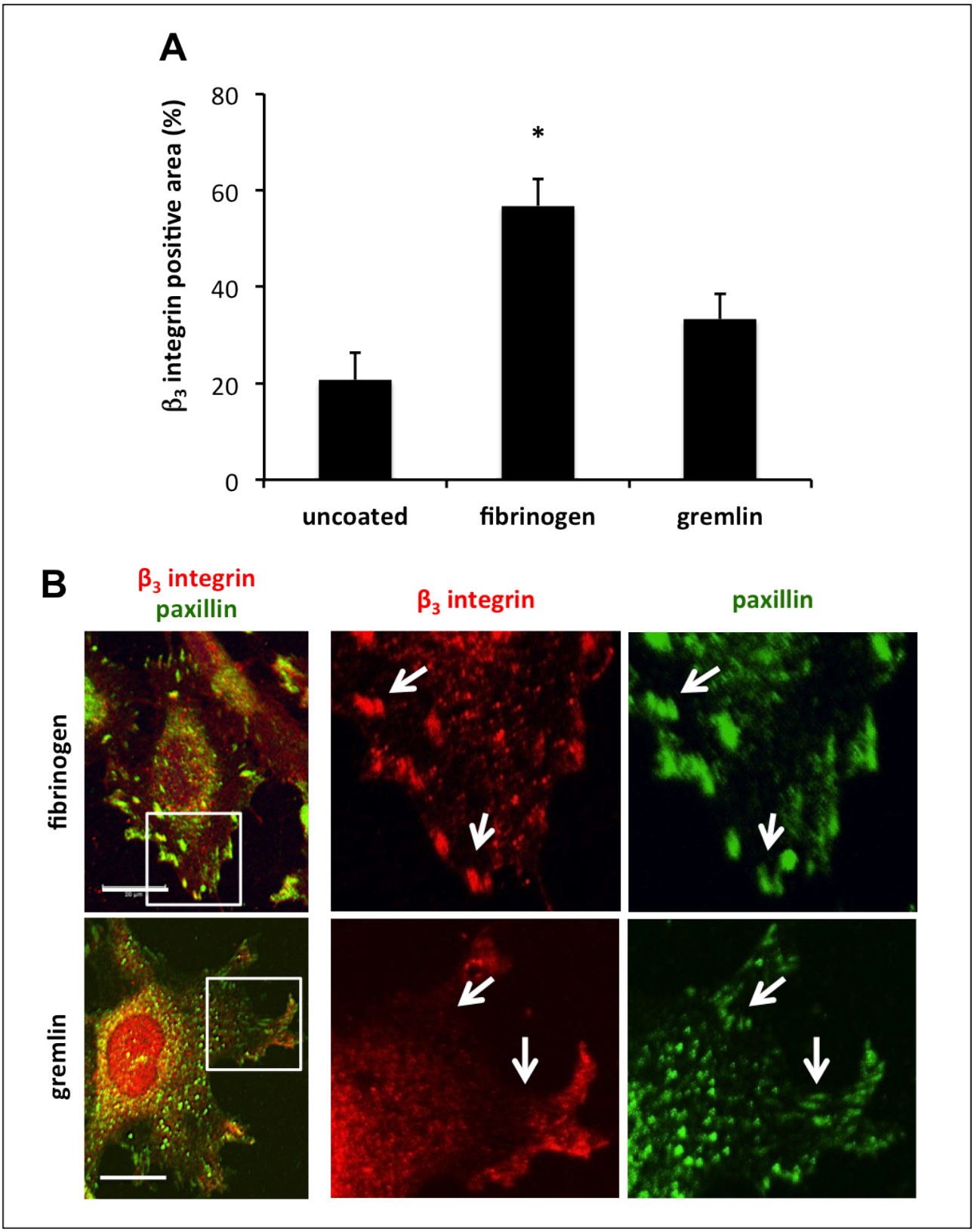
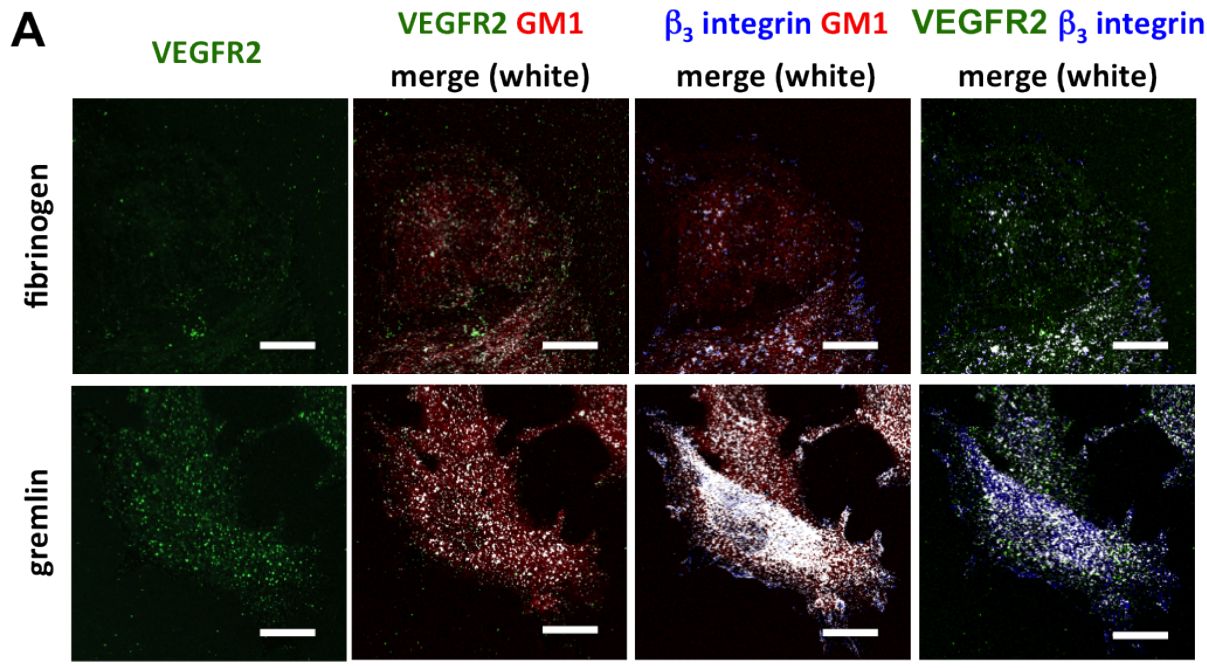


Figure S. VI: Immobilized gremlin does not recruit β_3 integrin in EC VPMs. (A) β_3-EGFP-overexpressing GM7373 cells were seeded on substrate-bound gremlin, FG or uncoated coverslips. A similar cell adhesion was observed under all the experimental conditions (not shown). After 4 hours VPMs were prepared from ECs, fixed and stained with TRITC-phalloidin. Then, cells were photographed under a Zeiss Axiovert 200M epifluorescence microscope equipped with a

Plan-Apochromat 63x/1.4 NA oil objective and ApoTome system (Bar 20 μm). β_3-EGFP was quantified in 30 cells/sample using Image-Pro Plus software. Data are expressed as percentage ± SEM of β_3 integrin positive area in respect to the total VPM area, as defined by actin staining (*, $P<0.01$, Student's t test). (B) HUVECs were seeded on substrate-bound FG or gremlin. After 2 hours cells were fixed and immunostained with anti-paxillin and anti-β_3 integrin antibodies followed by AlexaFluor 488-conjugated anti-mouse IgG and Cy5-conjugated anti-rabbit IgG. Samples were analysed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63x/1.4 NA oil objective (Zeiss). Panels show the double immunostaining for paxillin and β_3 integrin at the basal portion of adherent cells (Bar, 20 μm). Areas highlighted by white boxes are shown at higher magnification for β_3 integrin and paxillin immunolocalization (arrows indicate paxillin-positive focal adhesions).

FIGURE S. VII



B **% DOUBLE COLOCALIZATION**

	GM1 with VEGFR2	VEGFR2 with GM1	GM1 with β_3 integrin	β_3 integrin with GM1	VEGFR2 with β_3 integrin	β_3 integrin with VEGFR2	*n*
fibrinogen	4.25±2.01	22.15±7.29	11.18±6.81	33.38±12.91	10.21±7.56	5.95±3.80	10
gremlin	10.53±2.83 *	49.42±8.95 *	36.03±5.38 *	66.21±7.76 *	33.51±6.39 *	13.28±4.06 *	12

C **% TRIPLE COLOCALIZATION (molecule triplet)**

	GM1-VEGFR2-β_3 integrin	VEGFR2-GM1-β_3 integrin	β_3 integrin-GM1-VEGFR2	*n*
fibrinogen	0.29±0.36	1.58±1.89	0.62±0.71	10
gremlin	1.99±0.71 *	10.21±3.33 *	3.78±1.42 *	12

Figure S. VII: Recruited VEGFR2 co-localizes with β_3 integrin and GM1 ganglioside. HUVECs were seeded on immobilized FG or gremlin, stained for VEGFR2 (green), β_3 integrin (blue) and GM1 ganglioside (red) and acquired using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63x/1.4 NA oil objective (Zeiss). Images show the basal portion of adherent ECs (Bar, 10 μm). Pictures were analyzed using BlobProb ImageJ plugin [10]: white blobs represent areas in which respectively VEGFR2 co-localizes with GM1 co-localizes with GM1 and VEGFR2 co-localizes with β_3 integrin. (B) Double co-localization values are the number of voxels in the co-localizing objects divided by the total number of voxels for that molecule (*, P<0.001, n=10). (C) Co-localization between VEGFR2, β_3 integrin and GM1 ganglioside was calculated three times,

each one with respect to the first molecule listed. Values are number of voxels in the colocalizing objects divided by the total number of voxels for that molecule (*, $P<0.001$, $n=10$).

FIGURE S. VIII

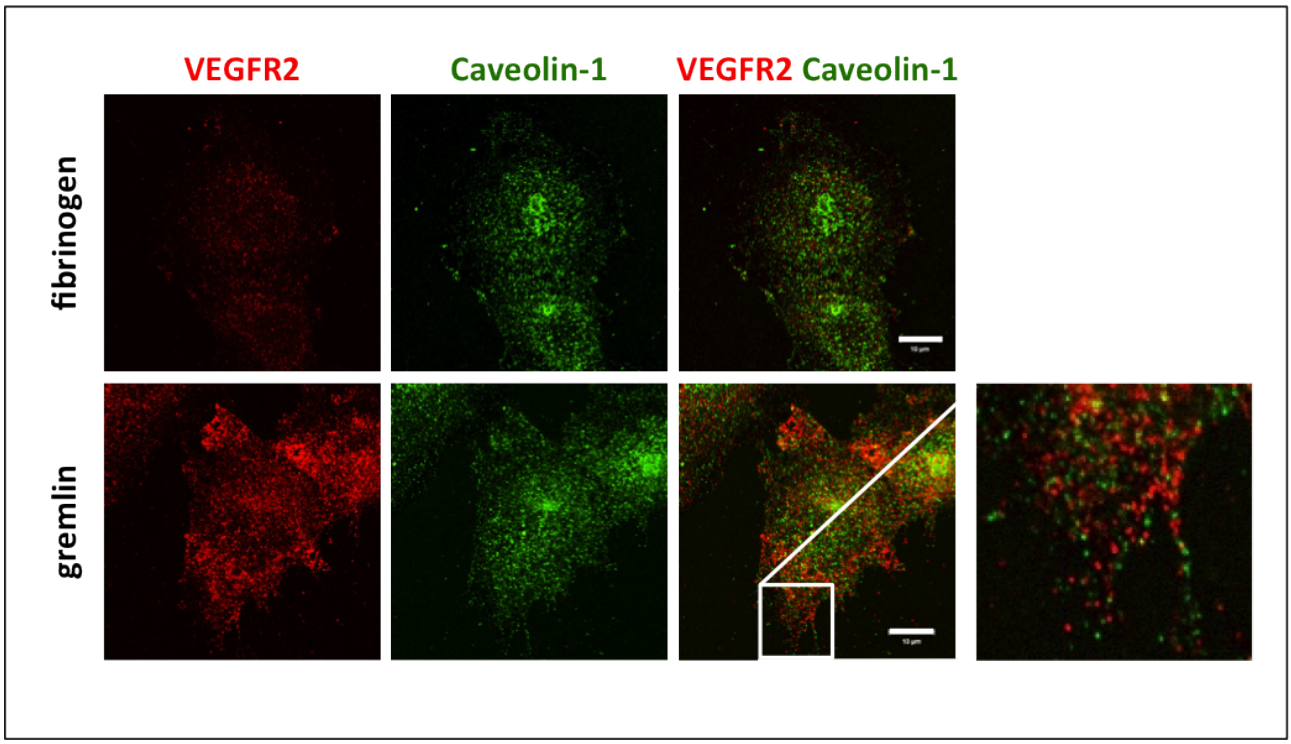


Figure S. VIII: Recruited VEGFR2 does not co-localize with Caveolin 1. HUVECs were seeded on substrate-bound FG or gremlin. After 2 hours cells were fixed and immunostained with anti-VEGFR2 and anti-Caveolin 1 antibodies followed by AlexaFluor 594-conjugated anti-rabbit IgG and AlexaFluor 488-conjugated anti-mouse IgG. Samples were analysed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63x/1.4 NA oil objective. Panels show the double immunostaining for VEGFR2 and Caveolin 1 at the basal portion of adherent cells (Bar, 10 μm). Area highlighted by white box is shown at higher magnification. Co-localization coefficients were calculated for both FG and gremlin and they do not significantly differ (FG: 0.17±0.11; gremlin: 0.19±0.14).

FIGURE S. IX

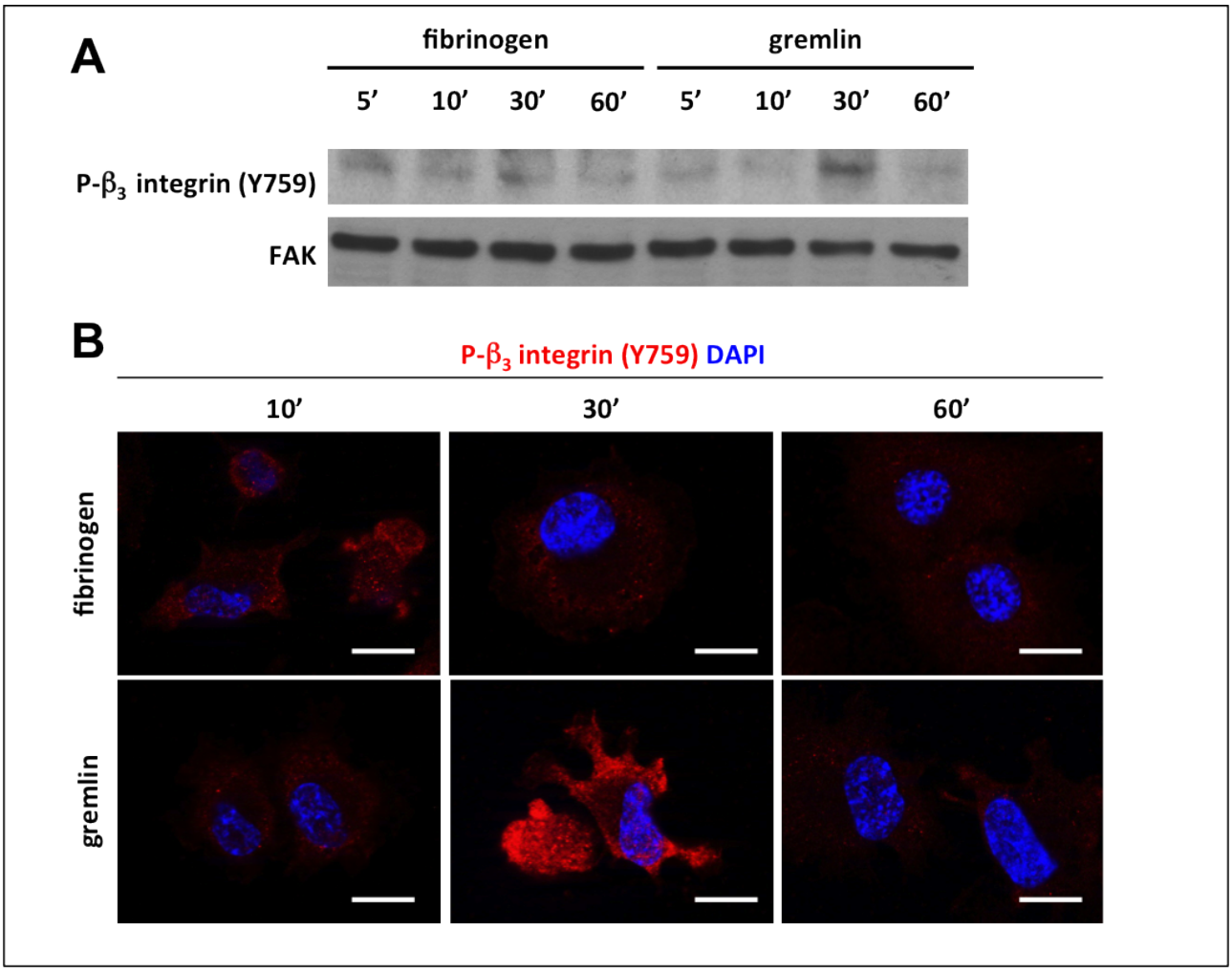


Figure S. IX: Immobilized gremlin induces β_3 integrin phosphorylation. (A) 50 µg of cell extracts of HUVECs seeded for the indicated time on substrate-bound FG or gremlin were probed by phospho-β_3 integrin (pTyr759) Western blotting. Uniform loading was confirmed by FAK Western blotting. (B) HUVECs were seeded on substrate-bound FG or gremlin for the indicated time. Cells were the fixed and immunostained with anti-phospho-β_3 integrin (pTyr759) followed by AlexaFluor 594-conjugated anti-mouse IgG and DAPI staining. Samples were analysed using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63X/1.4 NA oil objective and ApoTome system (Zeiss. Bar, 10 µm).