Subcellular fractionation

HUVECs were detached from confluent cultures grown in 10-cm dishes by treatment with 20 mM EDTA in ice-cold phosphate-buffered saline. Cells were pelleted and resuspended in homogenization buffer (10 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, 0.25 M sucrose, supplemented with a protease inhibitor mixture), and then disrupted by 15 passages through a 25-gauge needle. Nuclei and unbroken cells were pelleted by centrifugation at 3000 *g* for 10 minutes. Postnuclear supernatants were centrifuged at 200,000 × *g* for 1 hour in a Sorvall Micro-ultracentrifuge (Discovery M150), and the membrane pellet was re-suspended in 200 µl of homogenization buffer. All steps were carried out at 4°C. For fractionation, OptiPrep (60%, wt/vol), a ready-made solution of iodixanol (5,5′-[(2-hydroxy-1–3-propanediyl)-bis(acetylamino)]bis[*N*,*N*′-bis(2,3-dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide]), was used. Separation of different membrane compartments was achieved by centrifugation in three-step (10–20–30%; wt/vol) iodixanol gradients as described previously[1]. Briefly, one-third of the post-nuclear supernatant was mixed with Opti-Prep (60%, wt/vol) iodixanol and homogenization buffer to generate solutions of 10, 20, or 30%

iodixanol. Equal volumes of these three solutions were layered into centrifuge tubes and samples were centrifuged at 353,000 *g* for 3 hours at 4°C in a Sorvall Micro-ultracentrifuge swinging bucket S55S rotor. Sequential 100µl fractions were then collected from the top of the gradient, and proteins were resolved by SDS-PAGE and immunoblotted.

Immunoblotting and immunoprecipitation

For immunoblotting studies, cells were washed twice in ice-cold PBS and lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with inhibitors of proteases and phosphatases. Cells were scraped from the dish with a rubber policeman. Lysates were passed 3 times through a 27-gauge needle and clarified by centrifugation at 3000 *g* for 5 minutes. Supernatants were corrected to equivalent protein concentrations using a Bradford protein estimation kit (Bio-Rad). Proteins were resolved on SDS-PAGE followed by immunoblotting and visualization of antibody binding with the ECL reagent. For immunoprecipitation studies, cells were lysed in RIPA buffer containing a protease and phosphatase inhibitor cocktail. Insoluble materials were removed by centrifugation (5 minutes at 3000 *g*). Lysates were precleared with uncoupled protein A or G beads and incubated for 3 hours with Ab-coupled beads at 4 °C. Immune complexes were washed with lysis buffer and then subjected to SDS-PAGE and immunoblotting.

^{35}S-metabolic labeling

HUVECs were left uninfected or were infected with syntaxin 6-cyto or 16-cyto or uninfected for 16 hours. They were then starved for 2 hours with DMEM Met-Cys-free medium supplemented with 2% dialyzed FCS. Met-Cys-starved cells were pulsed with 200 µCi/mL of both ^{35}S-methionine and cysteine (EasyTag Express ^{35}S Protein labeling mix, PerkinElmer, MA, USA) for 20 minutes. After a chase period (several time points tested) during which cells were incubated with medium containing a 100-fold excess of cold methionine- and cysteine, cells were lysed in RIPA buffer. VEGFR2 was immunoprecipitated using a rabbit MAb against VEGFR2 and protein G sepharose beads. Immunoprecipitated samples were resolved by SDS-PAGE. Gels were subsequently fixed for 30 minutes in isopropanol: water: acetic acid (25:65:10) and were treated for 30 minutes with Amplify-fluorographic Reagent before being subjected to autoradiography.

Cell-surface biotinylation

To measure the surface pool of VEGFR2, we covalently labeled cell-surface proteins using a membrane impermeant biotinylation reagent (NHS-SS-biotin; Pierce, Rockford, IL). All steps were performed at 4°C. Cells were washed 3 times in PBS and then incubated with 0.15 mg/mL sulfo-NHS-SS-biotin in PBS for 10 minutes. The unreacted biotinylation reagent was quenched by washing once with a buffer containing 25 mM Tris, pH 8; 137 mM NaCl; 5 mM KCl; 2.3 mM $CaCl_2$; 0.5 mM $MgCl_2$; and 1 mM Na_2 HPO_4. The cells were then again washed 3 times with PBS, and resuspended in RIPA buffer containing protease inhibitor cocktail. The resulting lysates were centrifuged at 14,000 *g* for 10

minutes at 4°C. A sample taken from the supernatant at this point represented total cellular VEGFR2. Streptavidin-sepharose high-performance beads (GE Healthcare) were added to the remaining supernatant (100 µl packed beads per 500 µl lysate) and left on a rotator at 4°C for 2 hours. Beads were collected by centrifugation at 14,000 *g* for 10 seconds at 4°C, and supernatant was removed. The beads were then washed 3 times in lysis buffer at 4°C, and protein was extracted from the beads by heating at 95°C with SDS-PAGE sample buffer.

Degradation of surface biotinylated VEGFR2

Measurement of degradation of the cell surface pool of VEGFR2 was performed using methods described previously[2], with some modification. HUVECs that had been serum-starved overnight were surface labeled with 0.2 mg/mL NHS-SS-biotin for 30 minutes at 4°C. Cells were washed twice in ice-cold PBS and transferred to serum-free medium containing VEGF at 4ºC for 30 minutes. Samples were then incubated at 16°C for an additional 30 minutes to allow internalized VEGF-VEGFR2 complexes to accumulate in early endosomes. In a previously published study, we showed that cell surface receptors (EGF or transferrin receptors) accumulate in early endosomes upon a 16ºC temperature block (which allows endocytosis but not receptor degradation or recycling)[3]. Biotin remaining at the cell surface was removed by sodium 2-mercaptoethanesulphonate (MesNa; Sigma) reduction. Briefly, a solution of 20 mM MesNa in 50 mM Tris, 100 mM NaCl was adjusted to pH 8.6 with 10 M NaOH and immediately added to HUVEC monolayers. Reduction was allowed to

proceed for 15 minutes on ice at 4°C with gentle rocking. MesNa was quenched by the addition of 20 mM iodoacetamide (IAA) for 10 minutes. The cells were washed twice in ice-cold PBS. Intracellular, biotin-labeled receptors were further chased at 37°C with serum-free medium containing 50 ng/mL VEGF, to allow them to reach lysosomes for degradation. After each time point of the 37ºC chase, the medium was aspirated and the dishes were rapidly transferred to ice and washed twice with ice-cold PBS. To remove any intracellular biotinylated-VEGFR2 that may have been recycled to the surface during the chase, we subjected samples to MesNa treatment, followed by quenching with IAA as described above, and then by washing with ice-cold PBS. Cells were lysed in RIPA buffer and the biotinylated proteins were precipitated with Streptavidin-sepharose beads. After affinity pull down, biotinylated proteins were resolved by SDS-PAGE and immunoblotting.

Microscopy

For immunofluorescence studies, cells were grown on acid-washed glass coverslips. Cells were fixed in 4% paraformaldehyde (PFA) in Dulbecco's modified phosphate buffered saline (PBS) for 25 minutes at room temperature, quenched with 100 mM glycine in PBS for 15 minutes at RT, and washed with PBS. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 2 minutes at RT, blocked with PBS containing 5% glycine and 5% normal goat or donkey serum for 60 minutes, and incubated overnight at 4°C with primary antibodies. Slides were incubated for 1 hour in a 1:100 dilution of either Alexa

Fluor 488- or Alexa Fluor 594-conjugated secondary Ab, and mounted using Vecta Shield mounting medium containing DAPI.

Fluorescence images were acquired using a Leica spinning-disk confocal microscope equipped with a Hamamatsu EM-CCD digital camera (Hamamatsu Photonics) and the “Metamorph” image acquisition and processing software (Molecular Devices Corporation, Downingtown, PA). All images were acquired using a 63×, 1.3 NA objective. In any given experiment, all photomicrographs were exposed and processed identically for a given fluorophore. Images were corrected for background fluorescence using unlabeled specimens. For double-labeling experiments, control samples were labeled identically with the individual fluorophores and exposed identically to the dual-labeled samples at each wavelength to verify that there was no crossover of signal from different emission channels at the exposure settings used.

Cell proliferation assay

HUVECs (2×10^4) were seeded in 96-well plates. After 24 hours, the cells were either left untreated or infected with syntaxin 6-cyto or syntaxin 16-cyto. After 6 hours of infection, cells were serum (0.1%)-starved for 6 hours and then treated with VEGF at 50 ng/mL for 24 hours. Proliferation was measured using the thiazolyl blue tetrazolium bromide (MTT) colorimetric assay according to the manufacturer’s recommendations (Promega). The absorbance at 490 nm was determined using Spectra Fluor PLUS (Molecular Devices, Sunnyvale, CA). For

each group, samples were prepared in triplicate. The data are presented as fold increase over levels in controls.

Wound closure assay

An *in vitro* wound closure assay was used to assess cell migration according to previously published methods.[4,5] HUVECs were grown on collagen-coated 6-well plates and were either left untreated or infected with syntaxin 6-cyto or syntaxin 16-cyto. Subsequently, cell monolayers were serum starved for 16 hours, and then scratched with a 10 µl pipette tip. Thymidine (10 mM; Sigma, St Louis, MO) was included during the incubation to inhibit cell proliferation. Samples were allowed to heal in the presence or absence of VEGF. Migration of cells into the wounded area was followed by time-lapse video microscopy using an Axiovert 200M microscope (5X objective) from Carl-Zeiss Microimaging, GmbH (Munich, Germany. Images were taken every 15 minutes for 24 hours, along 3 defined but separate regions of the scratch in each well. We counted the number of cells migrating into the wounded area after 24 hours of wounding, in the presence or absence of VEGF.

Directional cell invasion assay

Cell invasion was measured using a modified Boyden chamber with Costar Transwell inserts (8 µm pore size; Corning). Uninfected controls and cells infected with syntaxin 6-cyto or syntaxin 16-cyto were harvested after 8h of infection, seeded ($5 \times 10^5/cm^2$) on the 0.1% collagen-coated upper surface of the

filter membrane, and serum starved for 16 hours. Serum-free medium containing VEGF-A (50 ng/mL) was added to the lower chamber to stimulate chemotaxis. After 4 hours of incubation at 37°C, cells on both sides of the membrane were fixed and stained with the DiffQuik staining kit. Cells on the upper surface of the membrane were removed using a cotton swab. Cells that had penetrated the lower surface of the membrane were imaged under the microscope, and random fields from each image were counted to calculate the difference in migration between control and test samples.

Tube formation

Growth factor-reduced Matrigel (BD Biosciences) was applied to the wells of 8-well chamber slides (Lab-Tek, Nalge Nunc International, NY) and left to adhere at 37°C for 30 minutes. Control uninfected and syntaxin 6-cyto- or syntaxin 16-cyto-infected cells were harvested with trypsin after 20 hours of infection, followed by 12 hours of serum starvation. Cells were resuspended at 5 $X10^5$ cells/mL in serum-free medium, and 200 µl of this cell suspension was added to each well. After 45 minutes at 37ºC (5% CO_2), serum-free medium was removed and replaced with medium containing 0.1% FCS and VEGF-A (50 ng/mL). Tube formation was assessed beginning 6 hours later. Images of representative fields were taken using a Leica spinning-disk confocal microscope, with a 5X objective and under the DIC mode. Endothelial tubes were quantified following their identification by counting the branches using Image J software (NIH). Experiments were repeated three times independently.

Animal (Nu/Nu mice) care and angiogenesis assay

All animal studies were approved by The University of Iowa Animal Care Committee and The Mayo Clinic and Foundation Institutional Animal Care Use Committee, and were carried out in accordance with the principles and procedures outlined in the NIH guidelines for the care and use of experimental animals. Mice (Nu/Nu; 6–8 wks old) under anesthesia were injected with the indicated recombinant adenoviruses, which had been purified using Quick Spin High Capacity G-50 Sephadex columns (Roche) and then diluted in PBS+3% glycerol. Syntaxin 6-cyto [5×10^6 plaque-forming units (PFU) in a volume of 10-15 µl] was injected intradermally into the dorsal ear skin of mice. Two days later, Ad-$VEGF_{164}$ (10^8 PFU/10 µl) was injected intradermally at the syntaxin-cyto-injection site. Ad-$VEGF_{164}$ expresses the predominant (164 amino acid) murine isoform of VEGF-A (Ad-$VEGF_{164}$).[6] Mice were either injected once with the indicated syntaxin-cyto form or mock injected 2 days prior to Ad-$VEGF_{164}$ injection. Animals were euthanized on day 5 post-Ad-VEGF administration, at which time ears were photographed or excised for further analysis.

Mouse ear extracts

The angiogenic areas were removed from mouse ears using an 8 mm biopsy punch as described before.[6] Ear tissues were snap frozen in liquid nitrogen, pulverized in the presence of liquid nitrogen, and resuspended in lysis buffer: 50 mM Tris-Hcl, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.1% sodium

deoxycholate, 0.1% SDS, 100 mM NaCl, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM Pefabloc SC, and 2 mg/mL protease inhibitor cocktail. Protein concentrations were determined using the DC Protein Assay kit (Bio-Rad). Lysates containing 30 µg of protein were analyzed by SDS-PAGE, and Western blotting was performed with antiserum against either syntaxin 6-cyto or syntaxin 16-cyto.

For quantitation of tissue VEGF164 by ELISA (Quantikine Mouse VEGF Immunoassay Kit; R&D Systems, Minneapolis, MN, USA), 8-mm punch biopsy samples were homogenized in T-PER (Pierce, Rockford, IL). Insoluble materials were removed and lysates were diluted in lysis buffer and further processed following the manufacturer's instructions.

Immunohistochemistry

Immunohistochemical staining of mouse tissue sections was carried out as described previously, with slight modification.[7] Briefly, slides with paraffin-embedded tissue sections (5 µm) were hydrated through xylene and graded alcohol. Antigen retrieval was performed with 20 µg/mL proteinase K at 37°C for 20 minutes. After blocking for 1 hour with 2% normal goat serum containing 1% BSA and 0.1% Triton X-100 at RT, slides were incubated for 2 hours with a rat anti-mouse monoclonal antibody for CD31/PECAM-1 at a 1:50 dilution at RT. Following three washes in PBS with 0.5% Tween 20, tissue sections were incubated with a 1:500 dilution of biotin-conjugated goat anti-rat IgG for 30

minutes at RT. Samples were then subjected to a 30-minute incubation with streptavidin-HRP (1:500), and endogenous peroxidases were subsequently quenched by adding 3% H_2O_2 in PBS. Immunoreactivity was detected using the DAB substrate kit for peroxidase in accordance with the manufacturer's recommendations, (Vector Laboratories, Burlingame, CA). Sections were counterstained with Hematoxylin, dehydrated through graded alcohol into xylene, and glass coverslips were applied.

Ear vessel vascular permeability assay

Mice were injected with syntaxin 6-cyto or syntaxin 16-cyto prior to the administration of Ad-VEGF, as described before for the mouse ear angiogenesis assay.[6] Angiogenesis was monitored, and at day 5 post application of Ad-$VEGF_{164}$, the mice were injected intravenously with 100 µl 0.5% Evan's Blue dye (Sigma, St Louis, MO, USA) in saline. After 30 minutes, the mouse ears were photographed. For quantification of dye extravasation, another group of mice was euthanized, and the ears were removed, oven-dried at 55°C, and weighed. Evans Blue dye was then extracted from the ears, by incubation in 500 µl formamide for 24 hours at 55°C. Evans Blue extravasation into the ear was measured spectrophotometrically at 620 nm, using a standard curve of Evans Blue in formamide.

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Figure S1. Schematic summary of findings
(A) In endothelial cells, the plasma membrane, endosomes and Golgi apparatus are enriched for VEGFR2. These VEGFR2 pools are maintained by endocytic and secretory transport pathways. This subcellular localization of VEGFR2 is essential for VEGF signaling and angiogenesis, and is regulated by syntaxin 6, which colocalizes with VEGFR2 at the Golgi apparatus and endosomes. (B) When syntaxin 6 function is inhibited, the cellular pool of VEGFR2 is depleted as a consequene of enhanced degradation in lysosomes. Our data suggest that syntaxin 6 inhibition impairs delivery of the Golgi-localized pool of VEGFR2, (which most likely represents newly synthesized VEGFR2) to the PM, and that in this context this pool of VEGRF2 is diverted to lysosomes for degradation. We propose that by regulating trafficking of VEGFR2 from the Golgi and/or endosomes, syntaxin 6 contributes to the maintenance of proper levels of this receptor in different subcellular compartments, and that this syntaxin 6 function is required for efficient receptor signaling and angiogenesis.

Figure S2. Syntaxin 6, syntaxin 10 and, syntaxin 16 are enriched in the trans-Golgi network
Colocalization of syntaxin 6, syntaxin 16 and EGFPsyntaxin 10 with TGN46. HUVECs were incubated with PAbs against endogenous syntaxin 6 or syntaxin 16 and a MAb against TGN46, followed by incubation with appropriate fluorescently tagged secondary antibodies. Fluorescence images were acquired and representative images are shown. Scale bar represents 5 μm.

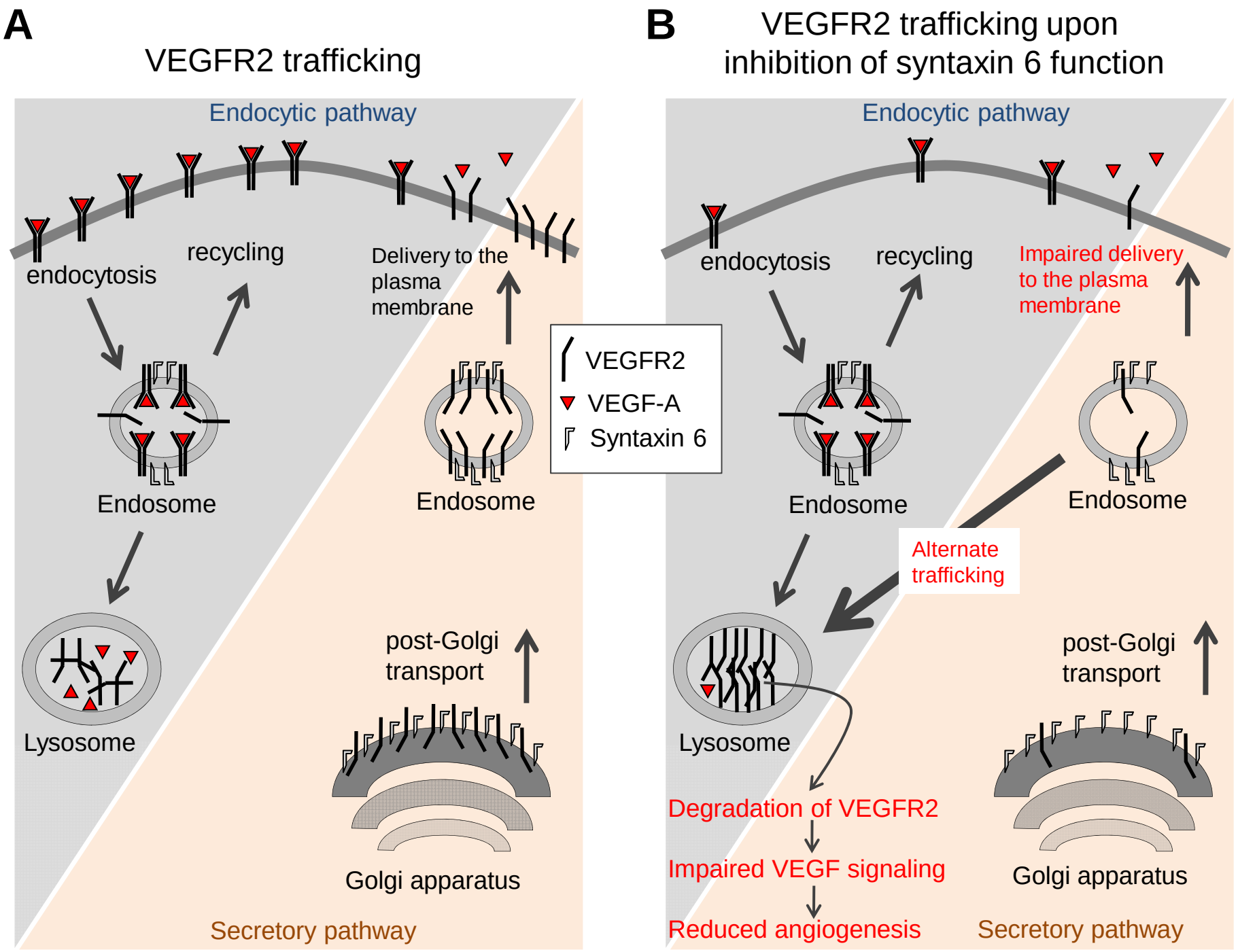
Figure S3. Stimulation with VEGF decreases colocalization between syntaxin 6 and VEGFR2 at the Golgi and increases colocalization at the endosomes. (A) Serum-starved HUVECs were treated with VEGF-A (30 minutes) and labeled with monoclonal antibodies against VEGFR2 and syntaxin 6. (B,C) Golgi- and endosome-localized fluorescence intensities of syntaxin 6 and VEGFR2 were quantified by image analysis. Values are expressed as a fraction of the total syntaxin 6 in the Golgi apparatus, or endosomes colocalizing with VEGFR2. Values in B, C represent mean ± SD (n=50 cells for each condition from 3 separate experiments; $p \leq 0.05$). Scale bar represents 5 μm.

Figure S4. Adenoviral expression of syntaxin 6-cyto and syntaxin 16-cyto in HUVECs
Uninfected control HUVECs, and syntaxin 6-cyto or syntaxin 16-cyto treated HUVECs after 20 hours of infection were fixed, permeabilized, and processed for immunofluorescence microscopy. (A) Uninfected control cells and syntaxin 6-cyto infected cells were incubated with anti-syntaxin 6 MAb, and syntaxin 16-cyto infected cells were stained with anti-syntaxin 16 MAb, followed by incubation with appropriate fluorescently tagged secondary Ab and immunofluorescence microscopy. Quantitation of signals in images revealed that ≥95% of the cells in each field expressed syntaxin 6-cyto or syntaxin 16-cyto. (B) TGN46 expression in control uninfected, syntaxin 6-cyto or syntaxin 16-cyto infected cells, 24 hours after initiation of infection. Note that infection with syntaxin 6-cyto or syntaxin 16-cyto did not alter the integrity of the Golgi. Scale bar represents 5 μm.

Figure S5. siRNA-mediated knockdown of syntaxin 6 and syntaxin 16
HUVECs were transfected with siRNAs against syntaxin 6 or syntaxin 16. After 72 hours of transfection, cells were fixed and immunostained using anti-syntaxin 6 or -syntaxin 16 MAbs. Fluorescence images of randomly chosen fields of cells were acquired at low magnification (40×), and fluorescence (excluding nuclei) was quantified and used to calculate the percentage of

cells showing ≥ 90% reduction in syntaxin 6 or syntaxin 16 relative to untreated (control) cells. Values are the mean ± SD (n=90 cells from 3 independent experiments; $p \leq 0.003$).

Figure S6. Loss of syntaxin 10 function does not affect cellular distribution of VEGFR2
(A) HUVECs were transfected (by nucleofection) with plasmid encoding EGFP-syntaxin 10-cyto. 36 hours later, the samples were fixed and labeled with rabbit MAb against VEGFR2, and fluorescence images were acquired. Note that EGFP-syntaxin 10-cyto (pseudocolored red) expression did not change the cellular distribution of VEGFR2. (B) 293T cells were infected with recombinant lentiviruses containing shRNA against *syntaxin 10*. After 48 hours of infection, doxycycline (Dox) was added to induce expression of the shRNA, after which the cells were incubated with Dox for a further 72 hours. Cells were harvested for the preparation of cell lysates at both the 48- and 72-hr time points. Equal amounts of protein were resolved by SDS-PAGE and then immunoblotted with anti-syntaxin 10 Ab. Note that syntaxin 10 knockdown was significant after 72 hours of shRNA treatment. (C) HUVECs were treated with recombinant lentiviruses encoding an shRNA against syntaxin 10. shRNA expression from these lentiviruses is associated with IRES-driven expression of turbo-RFP, which acts as a reporter, and was induced as discussed in "B." After 72 hours of shRNA expression, samples were fixed and labeled with a rabbit MAb against VEGFR2 and fluorescently tagged secondary Abs, and images were acquired by fluorescence microscopy. Note that VEGFR2 localization was unaffected in cells expressing an shRNA against syntaxin 10 (turbo-RFP positive cells). Scale bar represents 5 μm.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Golgi apparatus
Secretory pathway
VEGFR2
VEGF-A
Syntaxin 6
B
VEGFR2 trafficking upon inhibition of syntaxin 6 function
Endocytic pathway
endocytosis
recycling
Impaired delivery to the plasma membrane
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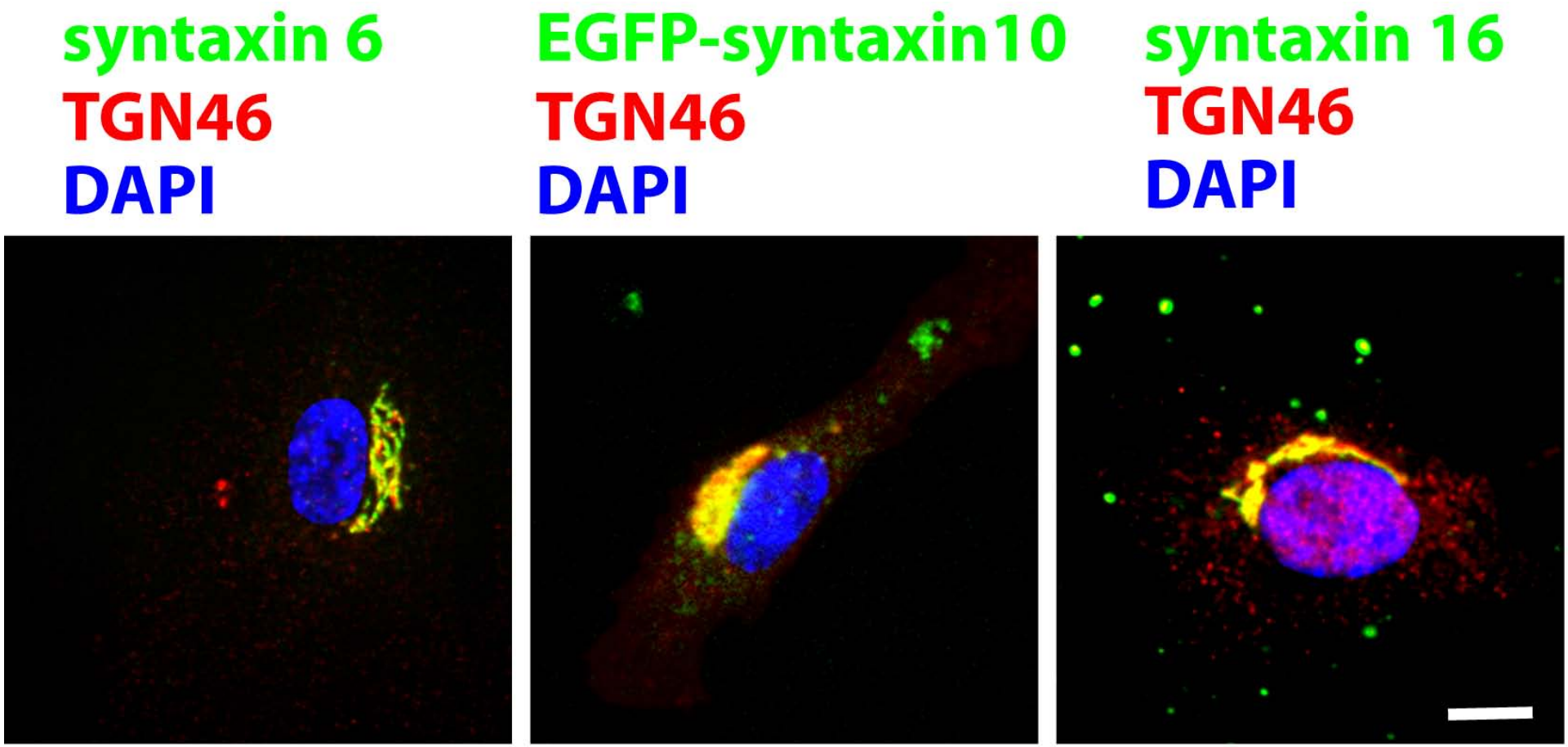
Reduced angiogenesis
Golgi apparatus
Secretory pathway

Figure S1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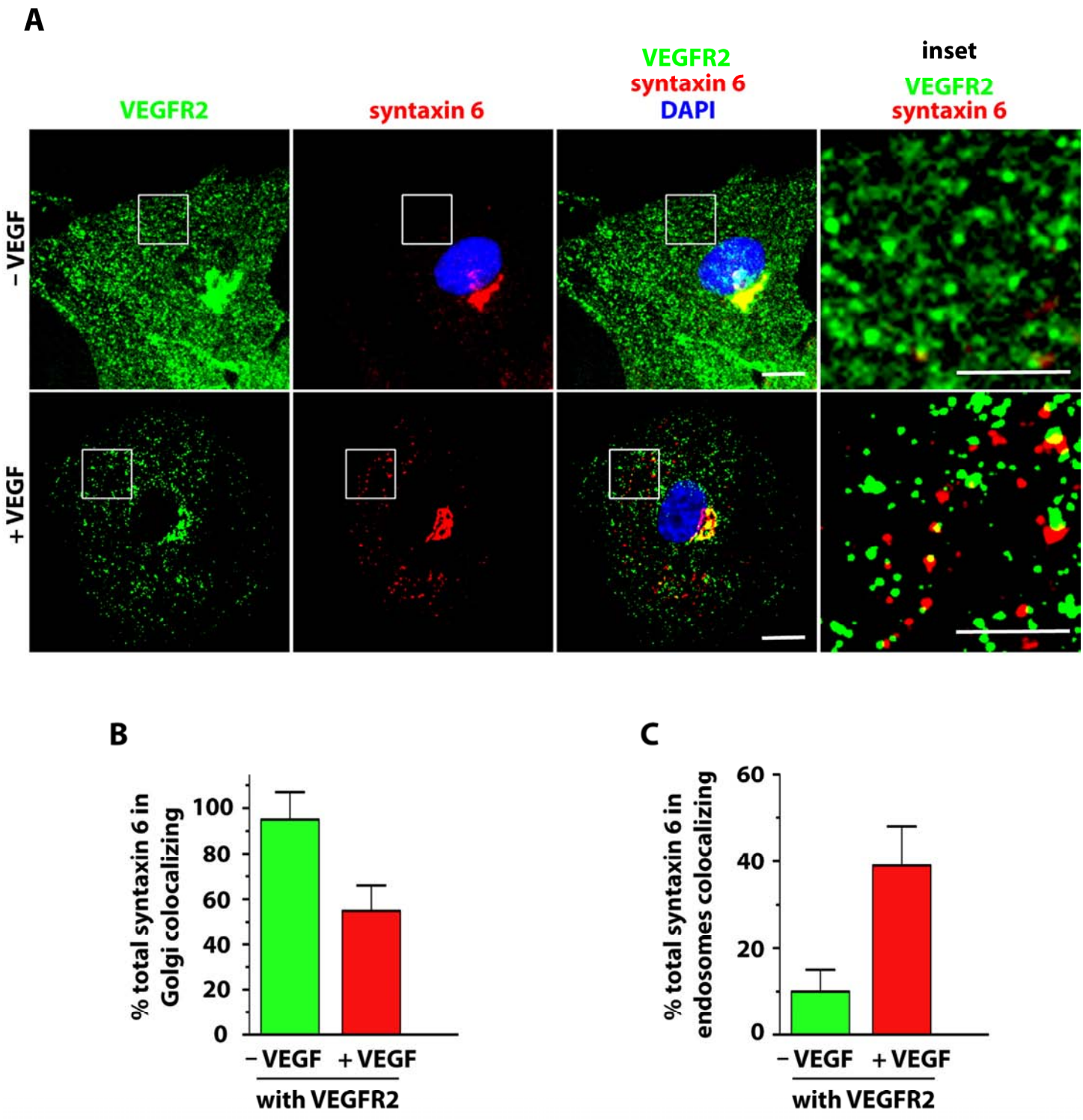
FigureS2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VEGFR2
syntaxin 6
DAPI
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VEGFR2
syntaxin 6
– VEGF
+ VEGF
B
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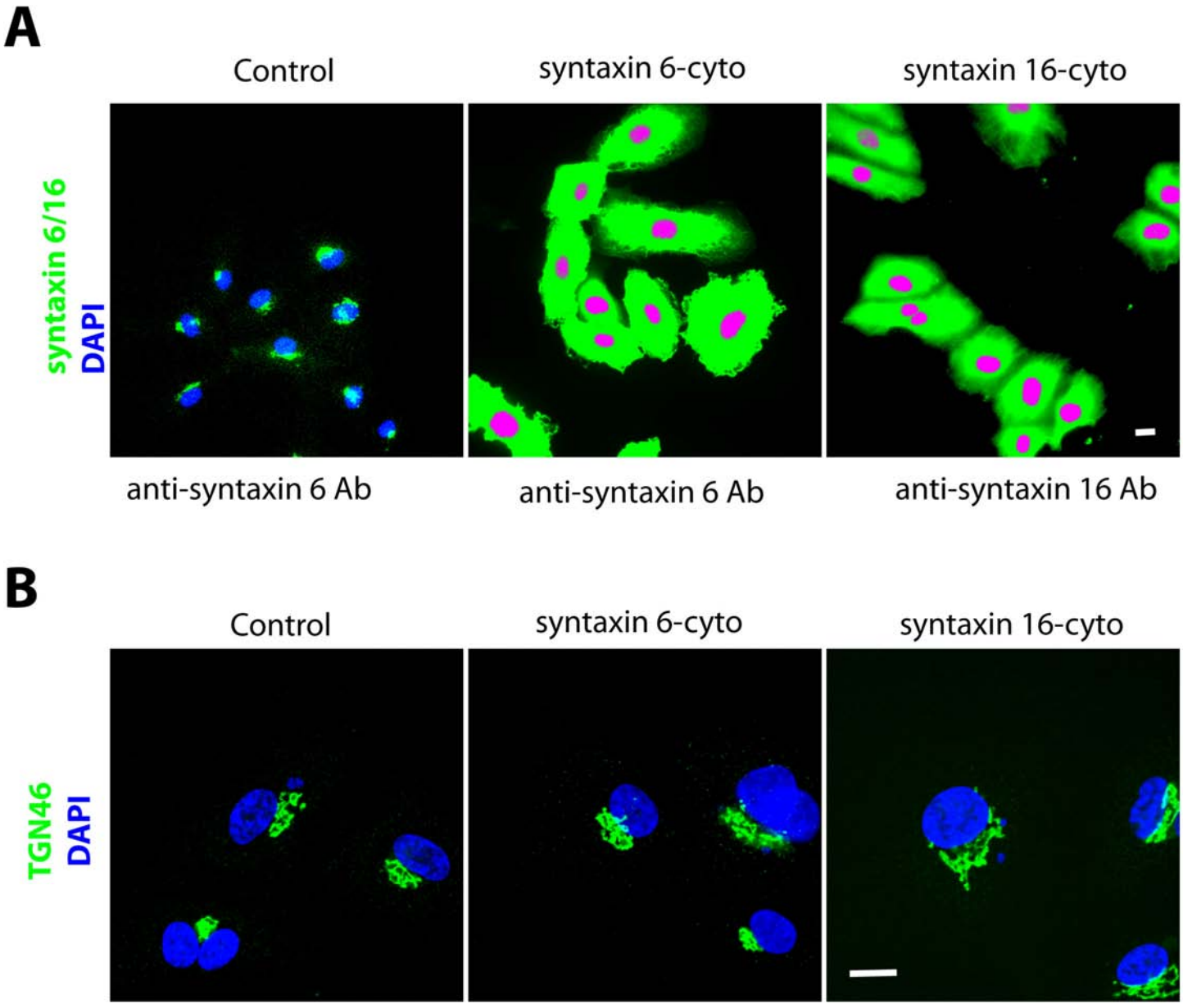
100
80
60
40
20
0
– VEGF
+ VEGF
with VEGFR2
C
% total syntaxin 6 in endosomes colocalizing
60
40
20
0
– VEGF
+ VEGF
with VEGFR2

FigureS3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syntaxin 16-cyto
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syntaxin 16-cyto
TGN46
DAPI

FigureS4

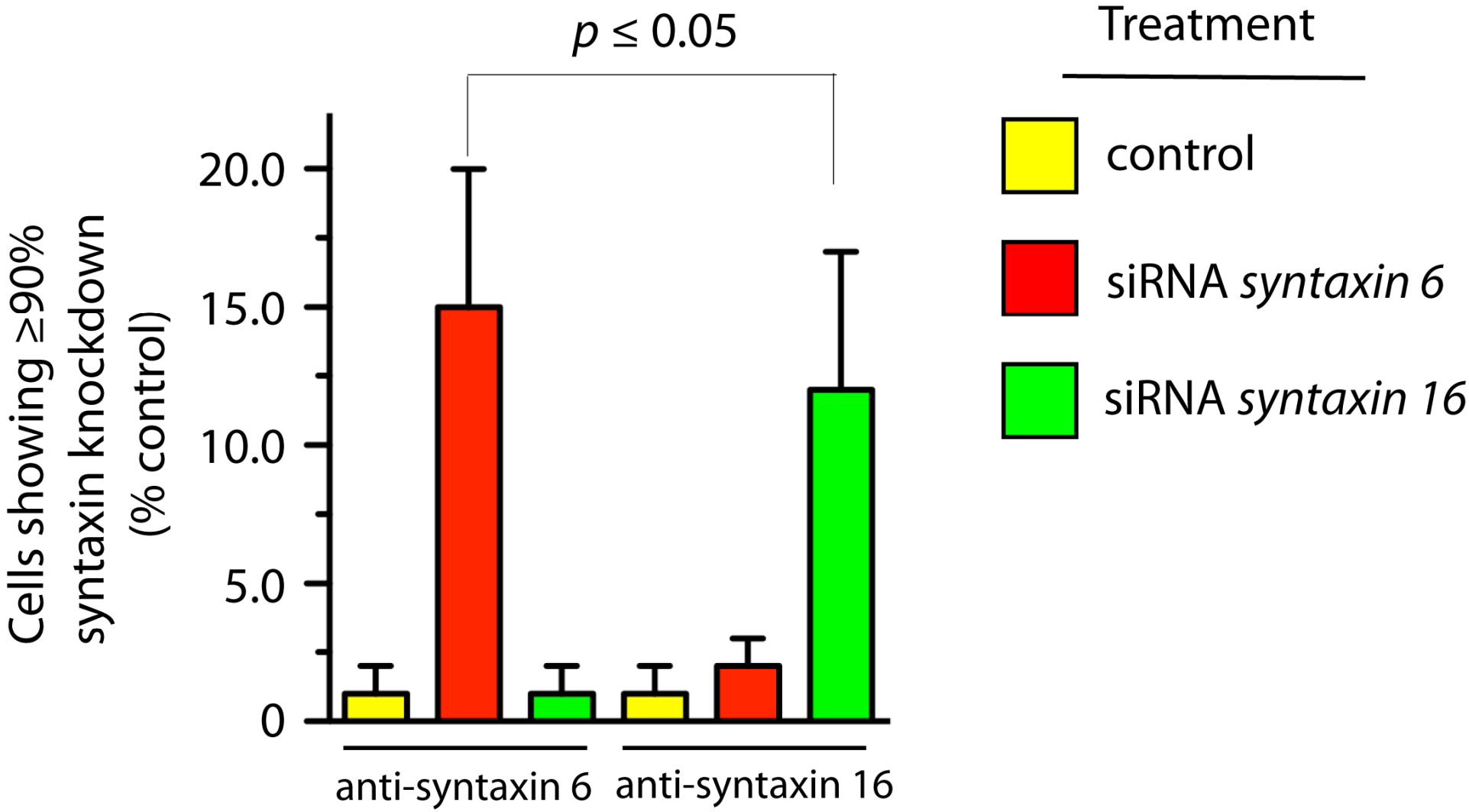
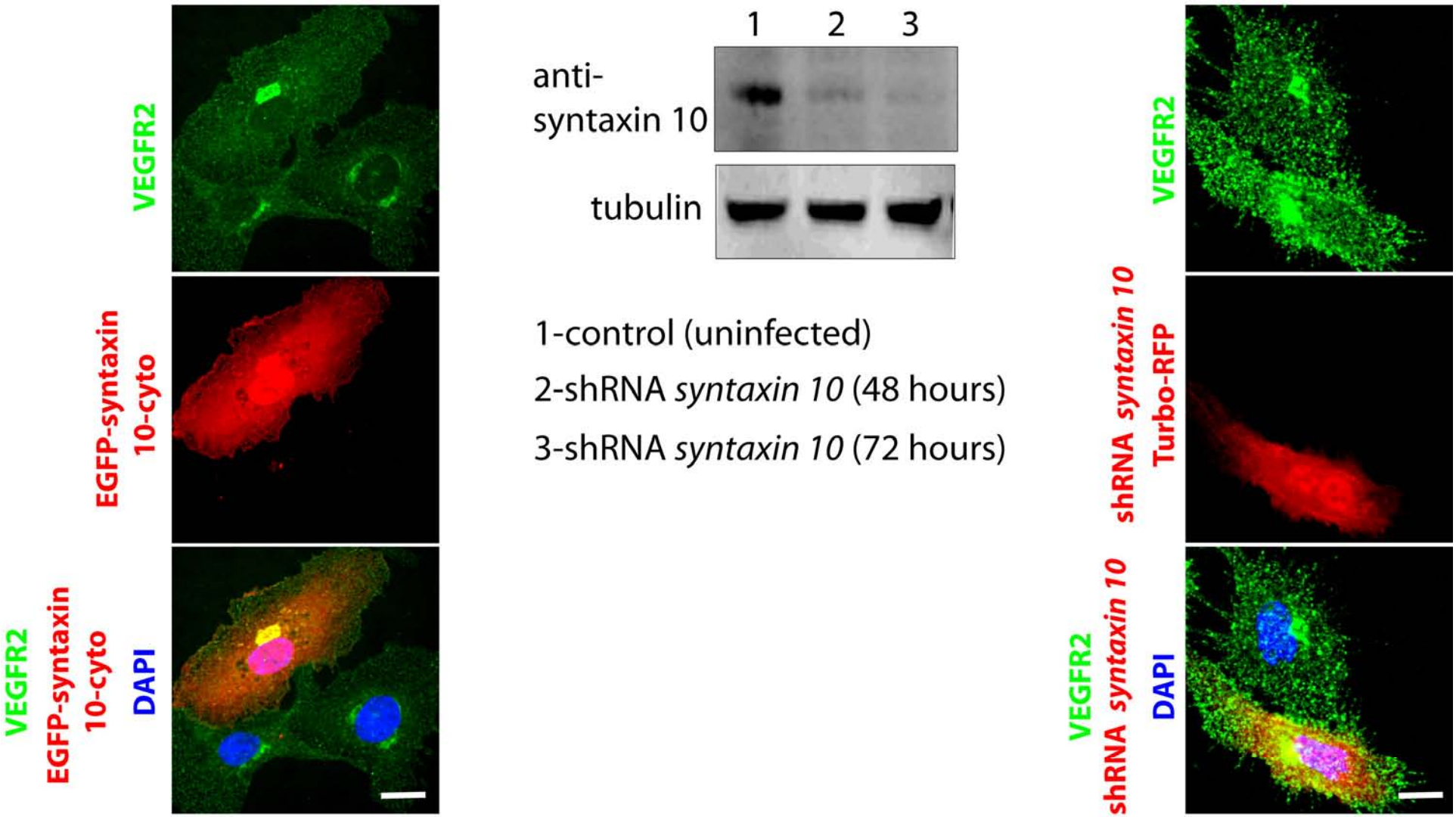

$p \leq 0.05$
Treatment
control
siRNA *syntaxin 6*
siRNA *syntaxin 16*
Cells showing ≥90% syntaxin knockdown (% control)
20.0
15.0
10.0
5.0
0
anti-syntaxin 6
anti-syntaxin 16

Figure S5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2-shRNA syntaxin 10 (48 hours)
3-shRNA syntaxin 10 (72 hours)
C
VEGFR2
shRNA syntaxin 10
Turbo-RFP
VEGFR2
shRNA syntaxin 10
DAPI

Figure S6