Tumor growth inhibition and anti-metastatic activity of a mutated furin-resistant Semaphorin-3E isoform

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Supplemental Movie 1 ***(Caption)***

HUVEC were grown in glass-bottomed dishes (WillCo-dish; Willcowells) and analyzed by time-lapse video-microscopy within a cell culture incubator chamber (see Suppl. Methods for details), immediately after addition of 7mM Uncl-Sema3E into the culture medium and for approx. 25 minutes. Phase contrast images were captured with a 10× objective. Suppl. Movie 1A shows the entire captured field, while Movies 1B and 1C show a close up of two representative fields. The pace of the process is sped up 15-folds in each of the movies.

SUPPLEMENTAL METHODS

Antibodies and other reagents. Anti-phosphotyrosine (4G10; Upstate); biotinylated anti-human PlexinD1 (aa 47-1271, by R&D); anti-ErbB2 (29D8 from Cell Signaling and e2-4000 from Invitrogen); anti-Phospho-Tyr_{397}-FAK (Millipore); anti-Phospho-Thr202/Tyr204-MAPK-Erk1/2 (20G11, Cell Signaling); anti-MAPK-Erk1/2 (137F5, Cell Signaling); anti-Vinculin (V4505, Sigma); anti-Paxillin (clone Y113, Chemicon); anti-Integrinβ1 active conformation specific (clone 9EG7, BD Pharmingen); anti-activated-Caspase3 (#9661, Cell Signaling); anti-mouse-E-Cadherin (cat. 13-1900, Invitrogen); anti-mouse-N-Cadherin (ab18203, Abcam); anti-Vimentin (clone V9, MAB3400-Millipore or V6630, Sigma-Aldrich); anti-mouse-E-cadherin (cat. 610181, BD Biosciences); anti-mouse-CD31 (MEC 13.3, BD-Biosciences); anti-αSMA (Sigma), anti Mouse Endoglin/CD105 (R&D); anti Ki-67 (DAKO); anti-Sema3E (AF3239, R&D Systems). Recombinant human Heregulin1-β1 was from R&D Systems, while recombinant human TGF-β1 was from Peprotech.

Cell lines. Tumor cell lines were from ATCC and our batches. Human umbilical vascular endothelial cells (HUVECs) were from Clonetics. Primary human brain vascular pericytes (HBVP) were from ScienCell Research Laboratories (cat. #1200). Tumor cell lines were grown in standard medium supplemented with with L-glutamine and 10% FBS (Sigma). HUVECs were grown on gelatin-coated dishes in complete Endothelial Cell Basal Medium (EBM-2, from Lonza). Primary pericytes were grown in formulated pericytes medium (cat. #1201, ScienCell, Research Laboratories).

Real-time quantitiative PCR analysis of gene expression. Total RNA from tumor cell lines or tissues was isolated by using RNeasy Protect Mini Kit (Qiagen) according to manufacturer's instructions. cDNA preparation was performed according to standard procedures, using M-MLV Reverse Transcriptase ImpromII (Promega) and oligo-dT 15mer and random primers (Sigma-Genosys). Q-PCR analysis was performed by using the Applied Biosystems 7900HT Fast Real-time PCR System and SYBRgreen PCR primers or Taqman probes listed below.

Gene symbol	**Ref. Seq.**	**Fw Sequence (5'-3')**	**Rev Sequence (5'-3')**
h-SEMA3E	NM_012431	CGGGGCACATTATCACCT	CCAGCAGCATTGTATGGAGA
h-ACTB	NM_001101.2	GGAGGAGCTGGAAGCAGCC	GCTGTGCTACGTCGCCCTG
m-sema3e	NM_011348.2	CTGTGCCTTCATCAGAGTCG	GGAGGAGTTGGGGTCAAAA
m-actb	NM_007393.3	GGAGGAAGAGGATGCGGCA	GAAGCTGTGCTATGTTGCTCTA
h-PLXND1	NM_015103.2	TCCTGGTGAACGACACAGAC	ACCGAGCAGTGGATGATTCT
h-CDH1	NM_004360.3	TTGAAAGAGAAACAGGATGGCT	TCATTCTGATCGGTTACCGTGA
h-VIM	NM_003380	AAGAAATGGCTCGTCAACTTCG	GGAGTGTCGGTTGTTAAGAACT

Gene symbol	**Ref. Seq.**	**Assay ID**
PDGFB	NM_002608.2 NM_033016.2	Hs00966522_m1
SNAI1	NM_005985.3	Hs00195591_m1
SNAI2	NM_003068.4	Hs00161904_m1
ACTB	NM_001101.3	Hs99999903_m1
PLXND1	NM_015103.2	Hs00892410_m1

Gene expression was quantified as relative number of transcripts compared to β-actin (for Sema3E levels in different cell lines), or as fold-changes (upon experimental treatments) compared to respective controls.

Knock down of gene expression by RNA-interference. The expression of PlexinD1 was silenced in tumor and/or endothelial cells by expressing puromycin-selectable constructs carrying targeted small hairpin RNA (TRCN0000061550 Mission shRNA from Sigma). Rnd2 expression was knocked down in HUVEC by transfection with siRNA duplexes ON-Target Plus SMART pool (L-009727-00-0010; Dharmacon-Thermo Scientific) or siCONTROL nontargeting siRNA pool (D-001206-13), using Lipofectamine 2000 (Invitrogen), according to manufacturer's protocols and previous reports (Valdembri et al 2009). Briefly, the cells were transfected twice (at 0 and 24 h) with 200 pmol of siRNA duplexes; after 24 hours since the second oligofection, cells were used in functional assays and gene knock down was verified in parallel by Real Time Q-PCR. PlexinD1 expression was similarly knocked down in primary pericytes by transfecting Silencer select pre-designed siRNA (P/N: 4392420, Ambion, Life Technologies) according to manufacturer's protocols. In all cases, gene expression knock-down was validated by Q-PCR.

Ectopic gene expression in mammalian cells. mSema3E was mutated in the furin cleavage site (R_{556}RFRR) in order to protect it from the proteolytic activity of pro-protein convertases (Uncleavable-Sema3E, see Christensen et al 2005). Expression constructs encoding mouse wt, p61-Sema3E (Casazza et al 2010) or Uncl-Sema3E (fused with a polyHis-Myc-epitope tag at C'-term) were subcloned into the lentiviral transfer plasmid pRRLsin.cPPT.hCMV.GFP.Wpre and gene transfer was perfomed as previously described (Follenzi et al 2002) This method ensured stable gene transfer with very high efficiency, without need to select individual cell clones. Control cells were similarly obtained upon gene transfer with viral particles containing an empty vector (EV). In addition, we assayed multiple independent batches of cells transduced with each construct.

Sema3E-receptor binding assays. Uncl-Sema3E or p61-Sema3E cDNAs were subcloned in frame with Secreted Placental Alkaline Phosphatase (AP); the resulting expression constructs were transfected in COS cells and the secreted proteins were harvested from conditioned media for later use in binding assays. In situ binding assays were performed as described previously (Tamagnone et al 1999). Briefly, Plexin D1-expressing COS cells were incubated for 1 h with recombinant secreted Uncl-Sema3E fused to AP. After five washes, cells were fixed, heated for 10 min at

65°C to inactivate endogenous phosphatases, and incubated with NBT–BCIP (nitro blue tetrazolium–5-bromo-4-chloro-3-indolyl-phosphate) AP substrate (Promega). Binding competition assay was performed on overexpressing plexinD1 COS cells treated with p61-Sema3E fused to SeAP plus increasing amount of Uncl-Sema3E (3nM, 7nM, 15nM, 60nM). Cells were then washed three times, fixed with acetone-formaldehyde, and incubated at 65°C for 15 min to inactivate endogenous phosphatases. Cell-bound p61-Sema3E was quantified by measuring SeAP activity on p-nitrophenylphosphate hydrolysis at 37°C in a reaction buffer containing 1M Tris-HCl pH 9.5, 1% bovine serum albumin, 1 mM $MgCl_2$. Absorbance values were measured at a wavelength of 405 nm and corrected for background absorbance.

Purified Uncl-Sema3E production. Affinity-purified Uncl-Sema3E, applied for treatment in all *in vitro* and *in vivo* experiments (except those based on gene transfer, systemic naked DNA hydroporation or lentiviral vector delivery), was obtained as follows. We subcloned murine Sema3E cDNA mutated in the furin cleavage site into a lentiviral expression vector (NSPI) including a human immunoglobulin Fc tag fused at the C'-terminal. HEK293 cells transfected to express the protein were grown in DMEM high glucose (4.5 g/l) medium containing 10% FCS. The conditioned medium collected from the culture was filtered to remove debris and Uncl-Sema3E-Fc protein was affinity purified by using protein G sepharose beads (Sigma). After sample loading, the column was extensively washed, including in the presence of 300mM NaCl to remove non-specific binding proteins. The purified protein was finally eluted in presence of 100mM Glycine pH 3, subsequently neutralized, snap frozen and stored at -80°C. For systemic in vivo delivery experiments, tumor-bearing mice were randomized (four days after transplant) and received either 30mg/kg of purified Uncl-Sema3E or the same amount of bovine albumin (as control), injected intraperitoneally twice a week.

Immunoprecipitation, SDS-PAGE and Western Immunoblotting. Cellular proteins were solubilized in EB-extraction buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% Glycerol, 1% TritonX-100; additionated with 1 mM Na_3VO_4, 100 mM NaF, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin) for immunoprecipitation experiments. Boiling Laemmli-SDS 2.5% lysis buffer was used for total protein extracts. When appropriate, the total protein concentration in cell lysates was determined by BCA assay (Pierce). Protein immunoprecipitation was performed by incubation with ProteinA-Sepharose beads and appropriate antibodies. Immunopurified proteins or equal amounts of total cellular lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked in phosphate-buffered saline, 0.1% Tween, 10 % BSA. The membrane was then incubated with primary antibodies, followed by the appropriate peroxidase-conjugated secondary antibody (Bio-Rad). Final detection was done by enhanced chemiluminescence (ECL, Amersham Biosciences).

Cell migration assays. Cell motility was assayed using Transwell® chamber inserts (Costar, Cambridge, MA) with a porous polycarbonate membrane (8 μM pore size) as described previously (Barberis et al 2004). To allow haptotactic migration, the lower side of the filter was coated with 10 μg/ml fibronectin. Cells were harvested from culture dishes by treatment with 1mM EDTA and resuspended in 0.2% BSA-containing medium. Approximately $1x10^5$ cells were added in the upper chamber, and allowed to migrate through the filter towards the lower chamber (including regulatory molecules, when indicated) for 6-8 hours, in a cell culture incubator. For experiments with primary pericytes we included $3.5x10^4$ cells in the upper chamber and the assay was performed in 0.1% FBS-containing medium. Eventually, the cells adherent to the upper side of the filter were mechanically removed, while those migrated to the lower side were fixed with 11% glutaraldehyde and stained with crystal violet. The dye was then solubilized in 10% acetic acid to measure absorbance at 595nm in a microplate reader (OD values are shown in the graphs as arbitrary units).

Immunofluorescence analysis of endothelial cells. HUVECs were cultured overnight on glass coverslips, previously coated with 10µg/µl fibronectin (Sigma). After the indicated treatments, cells were fixed with 4% paraformaldehyde (PAF) in PBS for 20 min on ice. Cells were then permeabilized for 5 min on ice with 0.2% Triton X-100 in PBS, washed and incubated with blocking buffer (PBS supplemented with 2% goat serum, Vector Laboratories Inc.) for 30 min at room temperature. Primary antibodies were incubated with cells for 30 min; secondary antibodies were conjugated with AlexaFluor dyes (Molecular Probes). F-actin was stained with Phalloidin-FITC (Sigma) and nuclei with DAPI (Roche). Immunofluorescence images were captured using a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica

Microsystems), by maintaining constant laser power, gain and offset settings. Digital images were evaluated by computer assisted analysis employing Image-ProPlus 6.2 software. For the quantification of active-β1-Integrin, we quantified mean fluorescence intensity in the respective channel in at least four independent broad microscopic fields (over several adjacent focal levels) per each experimental condition by means of the Leica Confocal Software Histogram Quantification Tool.

Cytofluorimetric analysis of Uncl-Sema3E induced endothelial cell apoptosis. HUVECs grown for 24 hours in gelatin-coated dishes were treated with 20 nM Uncl-Sema3E for different times. Cells were then fixed and permeabilized with FIX & PERM reagent (Invitrogen, Carlsbad, CA), according to manufacturer's instructions, and subsequently incubated at room temperature for 30 min with anti-active-Caspase-3 mAb (#559565 BD PharMingen) and Goat Anti-Rabbit APC-coniugated antibody (4050-11S SouthernBiotech). Stained cells were finally analyzed by CyAn ADP Analyzer (Beckman Coulter, Brea, CA) and the resulting data analyzed by Summit 4.3 software (Beckman Coulter, Brea, CA).

Time-lapse microscopy analysis of living cells. For the dynamic analysis of endothelial cell collapse induced by Uncl-Sema3E, time-lapse video-microscopy analysis was performed using a Leica AF6000LX workstation. HUVEC were plated into glass-bottomed dishes (WillCo-dish; Willcowells) and placed onto a sample stage within an incubator chamber set at 37 °C in an atmosphere of 5% CO2 and 20% humidity. Phase contrast images were captured with a 10× objective every 2 minutes, keeping lamp intensity at minimum to avoid phototoxicity. Movies were generated by employing the LAS AF Leica Application Suite software (Leica).

Cell proliferation analysis. Tumor cells were seeded in multiple 96-well dishes (Costar) at an initial density of 2×10^3 cells per well. After 16 hours, culture medium was changed and the cells were grown in presence of 0.5% FBS for the following days. Every 24 hours, one multiwell dish was fixed with 11% glutheraldehyde, stained with crystal violet, and the absorbance was read using a standard colorimetric system at 562nm.

Histological analysis. OCT-embedded frozen samples were cut in 10 µm thick sections and probed with primary antibodies (according to standard methods). Subsequent detection was done by HRP- or fluorescent-conjugated secondary antibodies (Alexa 488 or 546, Molecular Probes). Immunofluorescence images were captured using a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems), by maintaining constant laser power, gain and offset settings. In certain experiments, a Leica DM IRBM microscope was also used. Digital images were evaluated by computer assisted analysis employing Image-ProPlus 6.2 or Metamorph software. Unless otherwise indicated, quantification was done by analyzing at least 5 sections and 10 fields per tumor (considering 6 tumors per experimental condition). The extent of pericyte coverage was quantified by drawing a region of interest (ROI) close to each $CD31^+$ blood vessel, and calculating the percentage of co-staining in the two channels detecting CD31 and αSMA markers. The statistical significance of results was verified by calculating p values with Student's t-test.

Assessment of tumor vessel perfusion, vessel permeability and tissue hypoxia. Vessel perfusion was revealed upon intravenous injection of 0.05 mg/mouse FITC-labeled lectin (*Lycopersicon esculentum*; Vector Laboratories) 10 minutes before sacrifice and tumor excision. Vessel permeability was analyzed by intravenous co-injection of 0.25 mg Texas Red-conjugated Dextran 70 kD (Molecular Probes) and 0.05 mg FITC-labeled lectin (as above) in anesthetized mice; ten minutes later, the animals were perfused with saline and 2% PAF; the tumors were then harvested and frozen in optimum cutting temperature (OCT) compound. Tumor hypoxia was revealed upon injection of 60 mg/kg pimonidazole hydrochloride into tumor-bearing mice 2 hours before sacrifice. To detect the formation of pimonidazole adducts, tumor sections were immunostained with hypoxyprobe-1-Mab1 (Chemicon) following the manufacturer's instructions.

Experiments in RIP-Tag2 mouse model. Generation of RIP-Tag2 mice as a model of pancreatic β-cell carcinogenesis has been previously reported (Hanahan 1985). RipTag2 mice were maintained in the C57Bl/6J background (The Jackson Laboratory). We analyzed at least eight tumors per each condition, in multiple experiments. Alzet osmotic minipumps (2002 model, Charles River Laboratories) were used to accomplish local delivery of Uncl-Sema3E in the pancreas of experimental mice. Osmotic minipumps were incubated in physiological saline for 2 h at 37 C° before implantation, and then filled with saline or a solution of purified Uncl-Sema3E calculated to deliver to the mice a dose of 1 mg/Kg/day over a period of 14 days (between 12-14 week of age). Osmotic minipumps were connected to a silastic tube (25 mm length, 1.65 mm external diameter, 0.7 mm inner diameter) and implanted subcutaneously in the right side of abdomen, under anesthesia with isofluorane 1.5%. The attached tube was passed into the peritoneal cavity and sutured to the abdominal wall to reach directly the pancreas. The proximity of tube tip to the pancreas was re-assessed at the time of sacrifice. Sunitinib L-malate (Axon Medchem BV) was administered to mice daily by oral gavage, at a dose of 40 mg/Kg. Total tumor burden was quantified by measuring with a caliper and estimating the volume of individually excised macroscopic tumors (> 1 mm^3) with the formula: $V = a \times b^2 \times 0.52$, where a and b represent the longer and shorter diameter of the tumor, respectively. In order to assess the local invasiveness of primary RT2 tumors, we applied recognized criteria based on histo-pathological analysis of H&E stained sections, and classified them into “encapsulated” non-invasive (IT), microinvasive (IC1) and highly invasive (IC2)(Lopez and Hanahan, 2002; Paez-Ribes et al., 2009)**.** Moreover, the presence or absence of invasive tumor cells into loco-regional lymph nodes, and of liver metastasis, was assessed by immunohistochemistry on serial sections (at least 20 per each mice) using a rabbit anti-SV40 T-antigen (sc-20800; Santa Cruz), as previously described (Pàez-Ribes et al 2009).

Lymph nodes metastases analysis. Fresh frozen sections of pancreas and lymph nodes (LN) from each group of treatment were serially cut (10 μm). The presence or the absence of LN metastases was first evaluated by H&E-staining and confirmed by rabbit anti-SV40 T-antigen (sc-20800, 1:50 Santa Cruz) immunofluorescence, as previously described (Pàez-Ribes et al 2009). The size of the metastasis was measured as SV40-T-antigen positive regions in ten images for each mouse per group of treatment. All immunofluorescence images were captured by using a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems) and by maintaining the same laser power, gain and offset settings. In order to quantify the tumor burden in

LN, we employed the Leica Confocal Software Histogram Quantification Tool to measure, in each picture, the width and length of the tumor region and estimate its volume by the following formula for spheroids: $v = 0.52 \times (\text{width})^2 \times (\text{length})$.

Systemic delivery of lentiviral vectors**.** Concentrated lentiviral particles (20μg of p24-HIV equivalents/mouse) encoding Uncl-Sema3E (myc-tagged) or an empty vector (as control) were injected intravenously into immunodeficient $nu^{-/-}$ CD1 mice, as previously described (30). Four weeks after vector delivery, persistent Uncl-Sema3E expression in hepatocytes was confirmed by immunohistochemistry, and the mice were injected s.c. into the right posterior flank with A549 cells (8×10^6 per mouse). Tumor growth and lung metastatsis were evaluated as described in main manuscript.

Blood tests. Blood tests in mice were performed according to standard analytical methods. Briefly, blood samples were collected via intra-cardiac puncture, in presence of Na Citrate to avoid blood clotting, and analyzed using photometric method (Modular Roche diagnostic).

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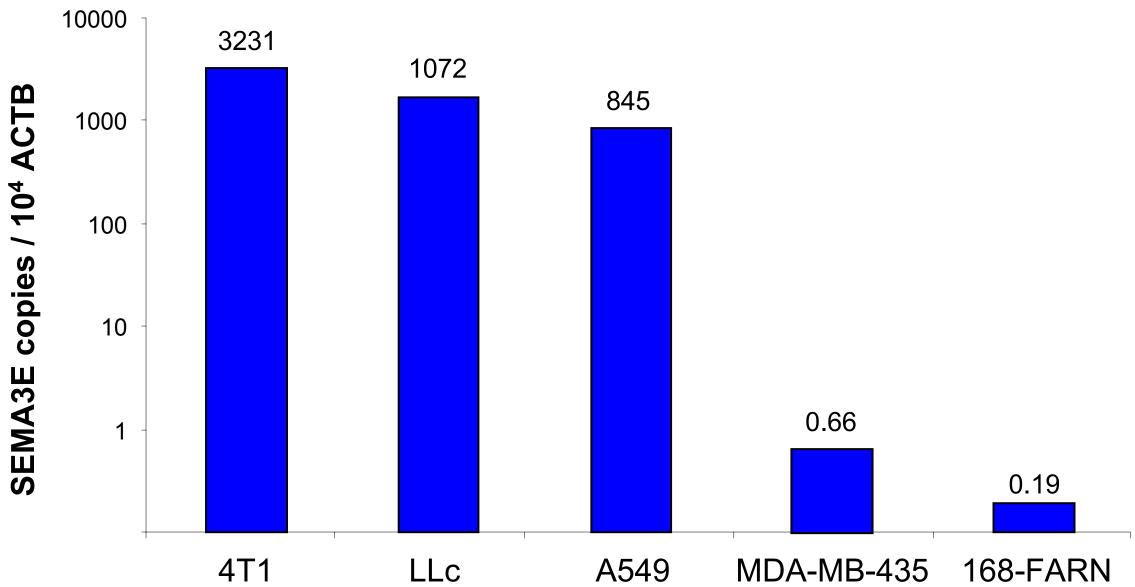
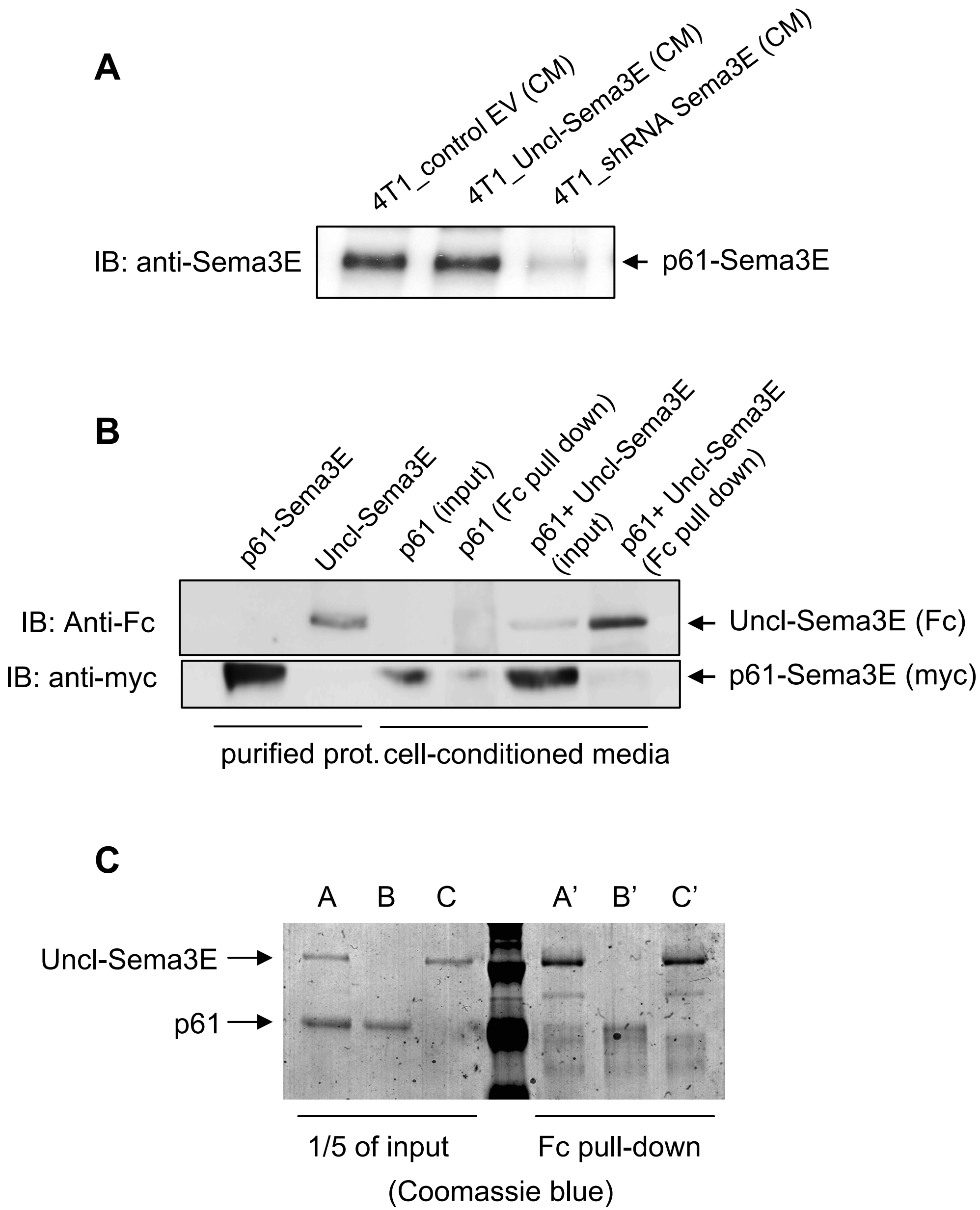
Supplemental Figure 1



Quantitative RT-PCR expression analysis to assess the levels of Sema3E transcripts in the different cancer cell lines used in our study. The graph indicates the estimated number of SEMA3E transcripts relative to 10^4 beta-Actin transcripts in each cell line. Y-axis is in logarithmic scale. Actual values are indicated on the top of individual bars.

Supplemental Figure 2

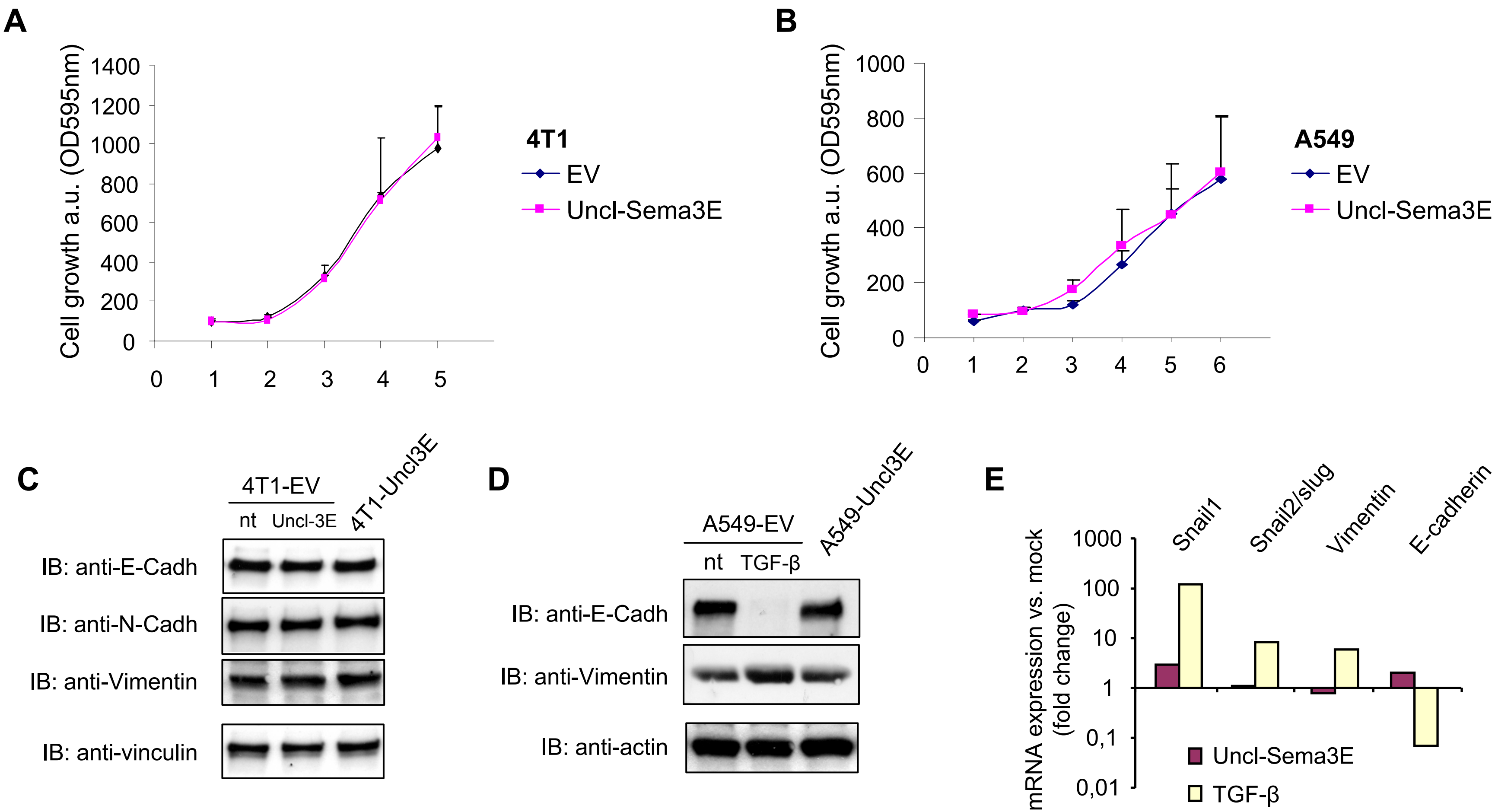


A. The conditioned medium (CM) of 4T1 tumor cells either control-EV, overexpressing Uncl-Sema3E, or knocked down for Sema3E expression by RNAi, was harvested and concentrated 1000-times, and eventually analyzed by immunoblotting with an anti-Sema3E antibody to reveal endogenous p61 levels. The CM of Sema3E-depleted cells provided a control of band specificity.

B. The conditioned medium of MDA-MB435 cells expressing p61-Sema3E (myc-tagged), or co-expressing p61 and Uncl-Sema3E (Fc-tagged), were subjected to pull-down experiments using protein-G beads in order to capture Uncl-Sema3E. Immunoblotting analysis revealed that, although p61 was abundantly present in the conditioned media of co-expressing cells (see "input" lanes), it was not specifically found in association with Uncl-Sema3E ("pull-down lanes"). The first two lanes on the left were loaded with purified proteins to provide reference bands.

C. Affinity purified Uncl-Sema3E (Fc-tagged, lane A) and p61-Sema3E (lane B) were allowed to interact in cell culture medium (lane C), and the mix was subjected to to pull-down experiments using protein-G beads in order to capture Uncl-Sema3E (lanes A', B', C'). Coomassie blue protein staining revealed that p61 did not specifically associate with Uncl-Sema3E. Legend: A, A' = Uncl-Sema3E + p61-Sema3E; B, B' = p61 alone; C, C' = Uncl-Sema3E alone.

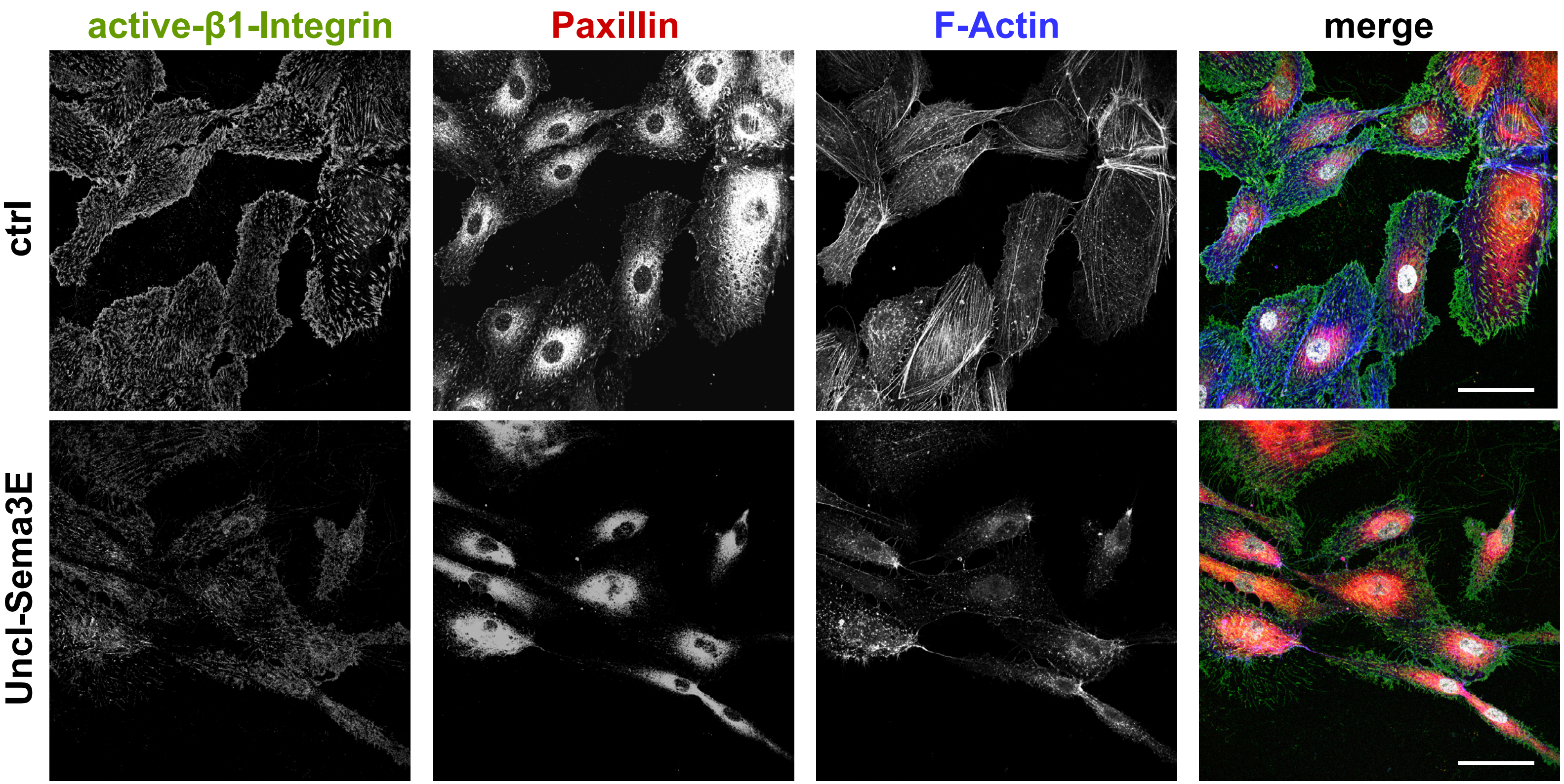
Supplemental Figure 3

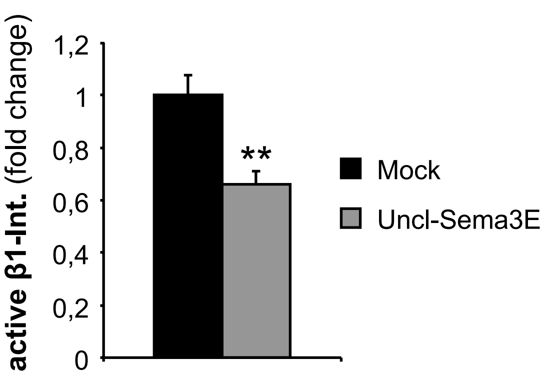


A-B: 4T1 and A549 tumor cells overexpressing Uncl-Sema3E (and respective controls transduced with empty vector, EV) were analyzed in vitro to reveal potential growth rate changes in culture medium containing 0.5% FBS.

C-E: Uncl-Sema3E overexpressing 4T1 cells (or EV cells treated with purified Uncl-Sema3E for 24 hours) did not show significant changes in the expression of typical markers of epithelial-mesenchymal transition (EMT) compared to controls, as revealed by western blotting **(C)**. The same was true in A549 cells, as shown by expression analysis with western blotting **(D)** or Q-PCR **(E)**; notably, cells treated with 5 ng/ml TGF-β (for 24h) underwent major phenotypic changes, and were included as internal positive controls of EMT.

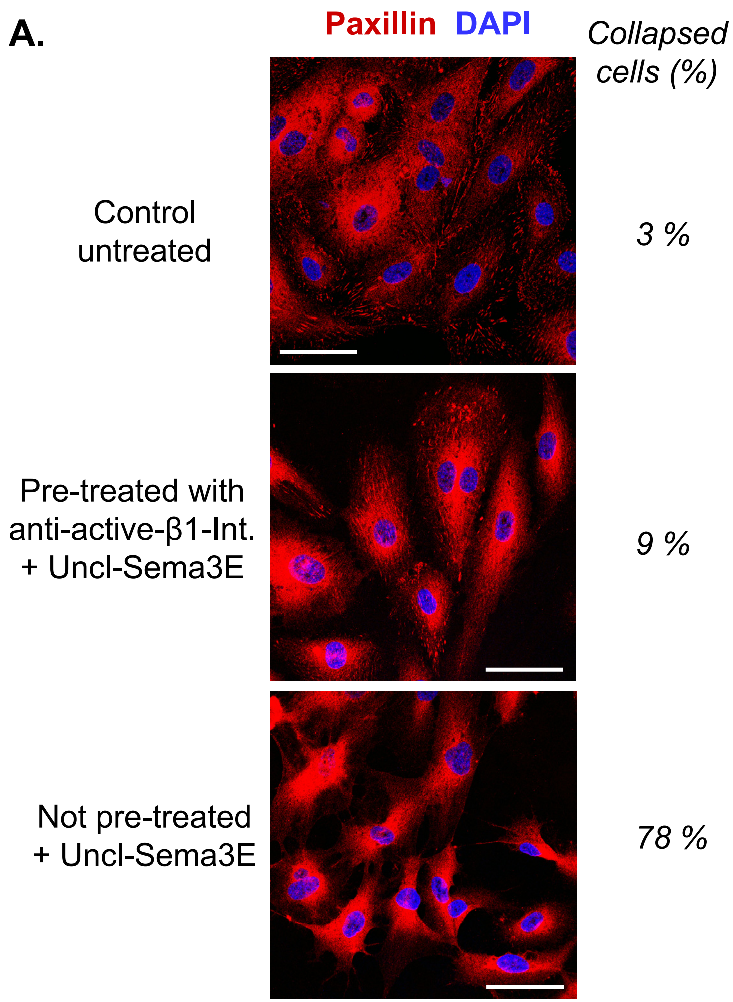
Supplemental Figure 4



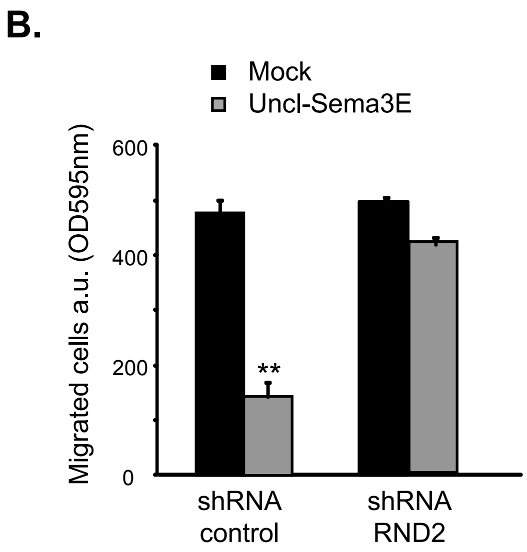


Uncl-Sema3E downregulates active integrin-β1 in endothelial cells.
HUVEC cells were treated (or not) with 7nM Uncl-Sema3E for 5' and then incubated with antibodies detecting integrin-β1 molecules in the active conformation on the cell surface (green channel); signal quantification is shown in the graph (see Methods for details); **$p<0.002$. After fixation, the cells were furthermore stained to reveal Paxillin (red), F-actin (blue) and the nuclei with DAPI (shown in white). Scale bars: 50 µm.

Supplemental Figure 5

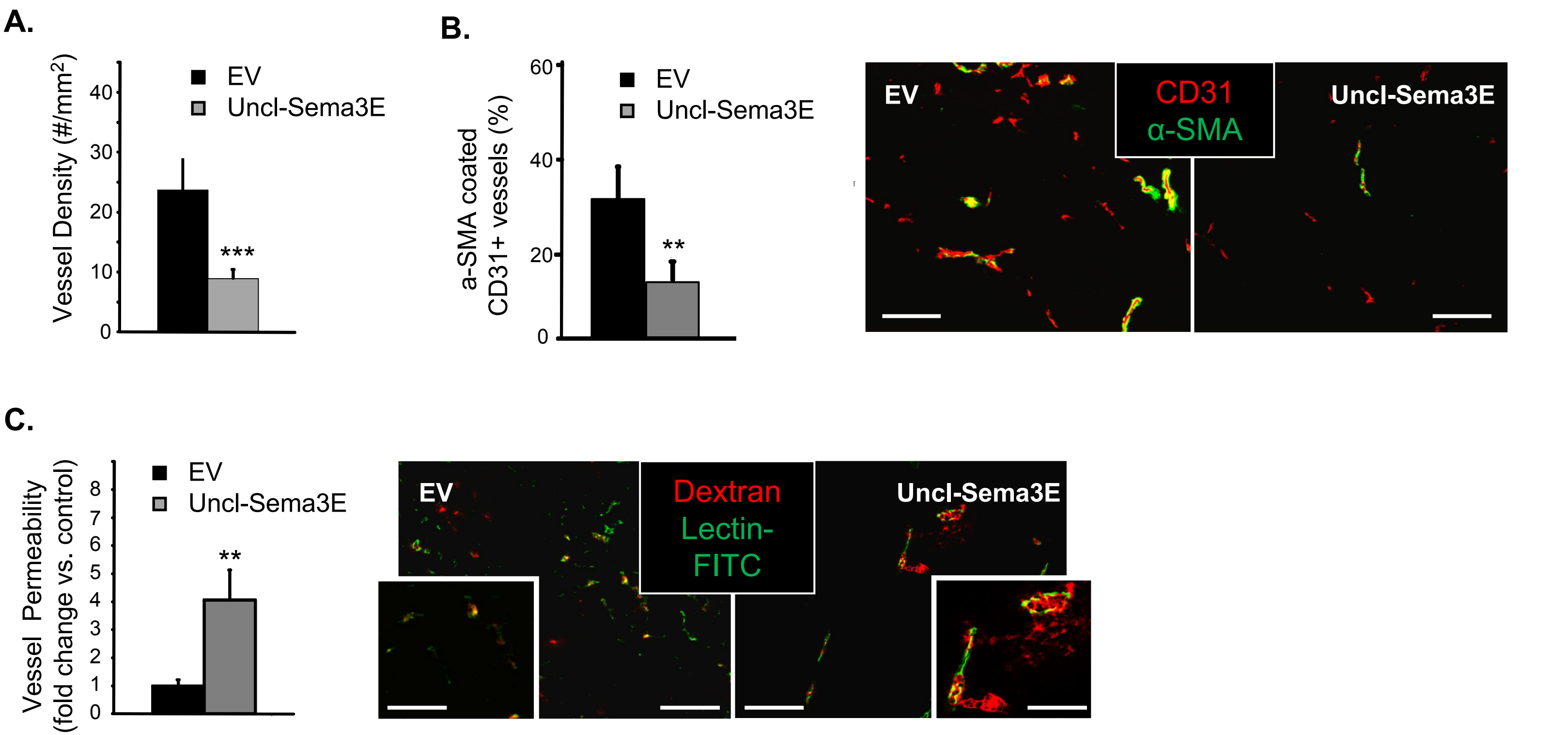


HUVEC cells were preincubated with an antibody directed against the active conformation of Integrin-β1 in order to stabilize cell adhesion to the substrate, before adding 7nM Uncl-Sema3E for 10 minutes. The collapsing response (quantified on the left as the fraction of cells with diameter ≤ 30 μm) is dramatically reduced by preventing de-adhesion from the substrate. Scale bars: 50 μm.



Rnd2 expression was knocked down in HUVEC by siRNA transfection (verified by Q-PCR, not shown) and cell migration in presence of Uncl-Sema3E was assayed as shown in main Fig. 3C; **p<0.005.

Supplemental Figure 6

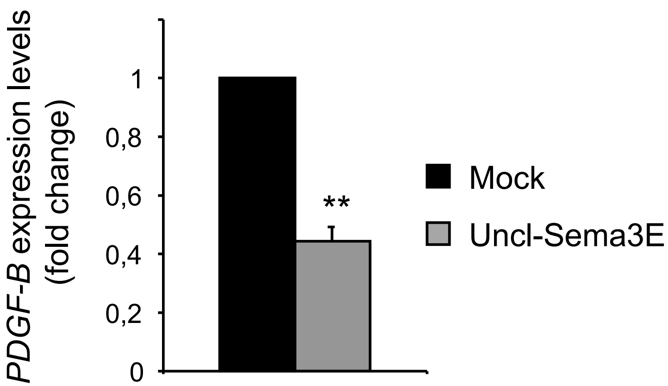


Uncl-Sema3E disrupts tumor vessels in mice

A. Tissue sections from EV and Uncl-Sema3E MDA-MB-435 tumors of comparable size (excised at d17 of experiments shown in main Fig. 5B) were immunostained for the endothelial cell marker CD31 and vessel density was quantified; $^{**}p<0.005$. **B.** Double-staining for CD31 (red) and aSMA (green) of tumor sections above further revealed a reduced number of pericyte-covered tumor vessels in Uncl-Sema3E tumors compared to controls; $^{**}p<0.002$. Scale bars: 100µm. **C.** Vessel permeability was increased in Uncl-Sema3E tumors compared to controls, as revealed by systemic injection of Texas Red-conjugated Dextran (70kDa) and FITC-conjugated Lectin (green); $^{**}p<0.001$. Scale bars: 100µm (50µm in high magnification insets).

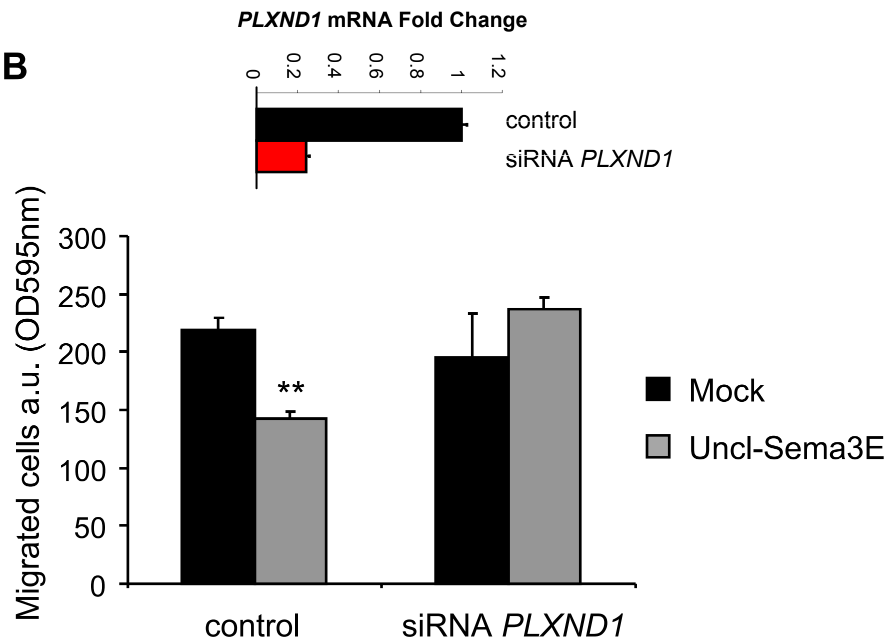
Supplemental Figure 7

A



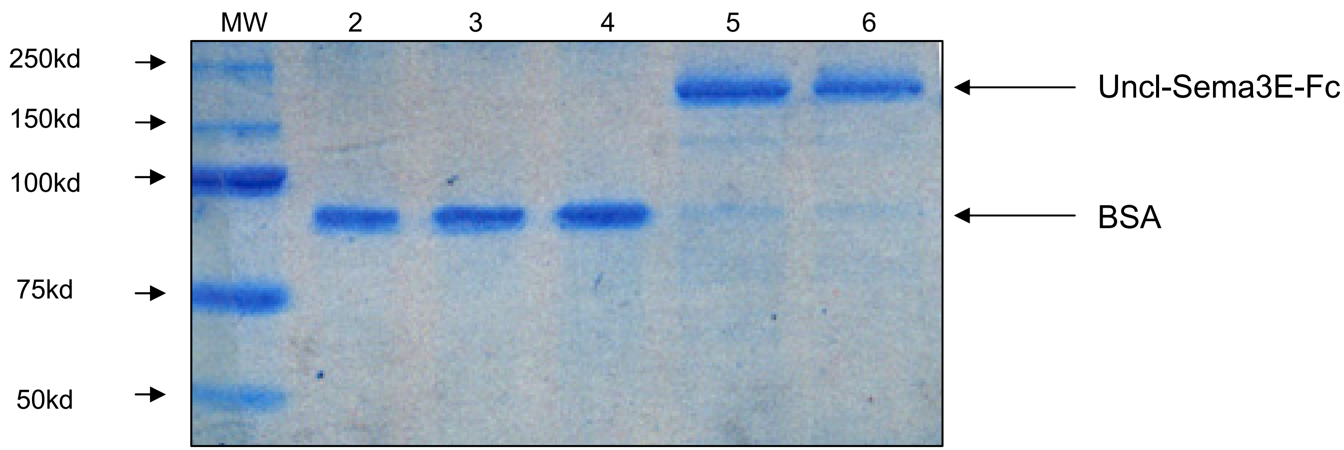
PDGF-B mRNA expression in endothelial cells treated with 7nM Uncl-Sema3E for 8 hours was measured by Q-PCR. The graph shows the average±SD of fold-change values (vs. controls) obtained in two independent experiments performed in triplicate; **p <0.005

B



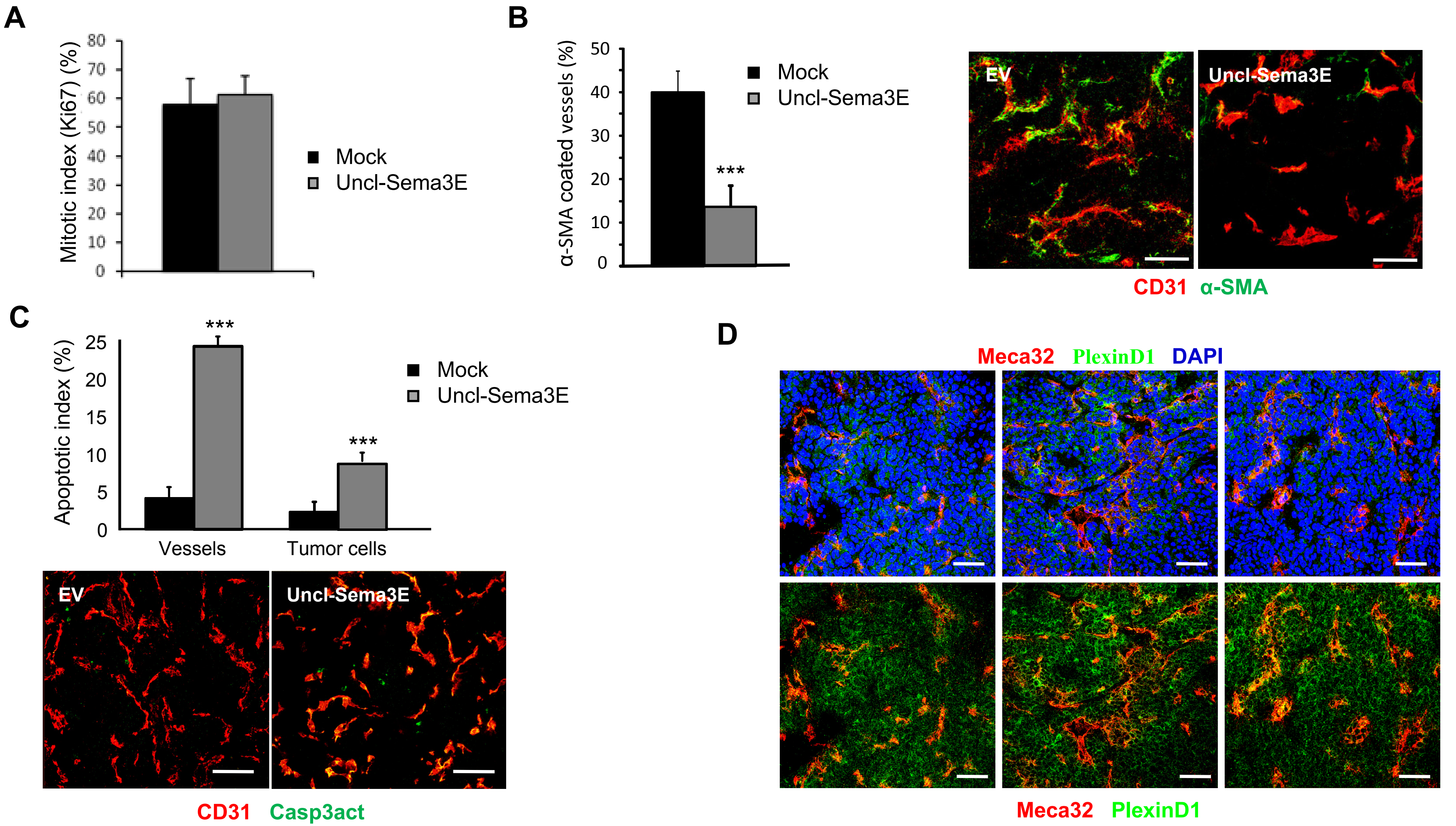
The spontaneous migration of human primary pericytes in presence or absence of 7nM Uncl-Sema3E, was assessed in Transwell inserts (see Suppl. Methods for details). Pericytes subjected to PlexinD1 knock-down by siRNA (expression analysis by Q-PCR on top) were insensitive to the inhibitory activity of Uncl-Sema3E; **p <0.005.

Supplemental Figure 8



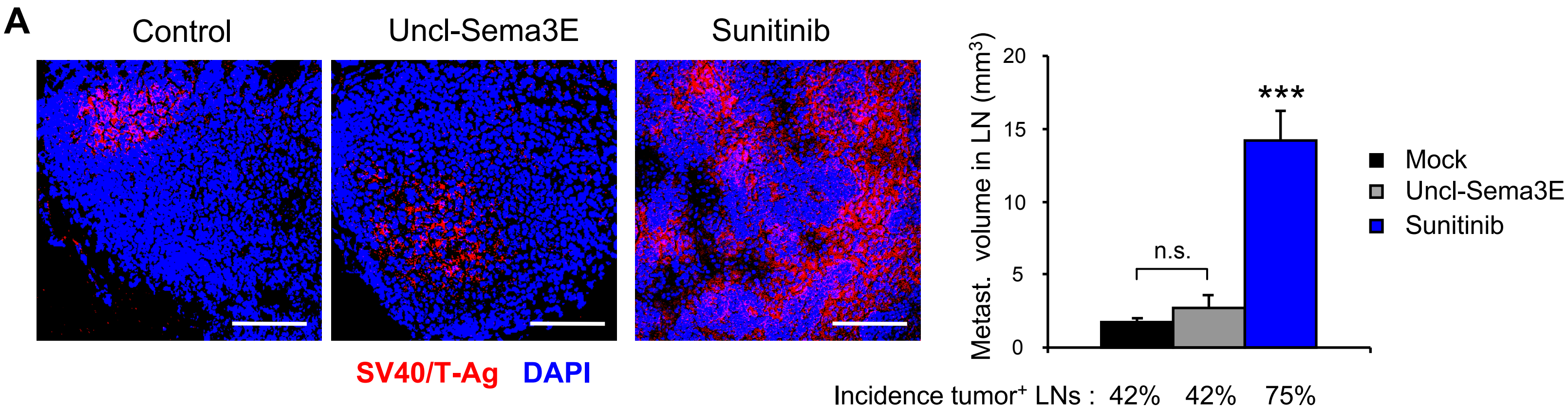
Coomassie blue stained PAGE analyzing affinity purified Uncl-Sema3E-Fc.
Lane 1: MW Standards. Lanes 2-4: Bovine Serum Albumine.
Lanes 5-6: Uncl-Sema3E eluated fractions from affinity column.

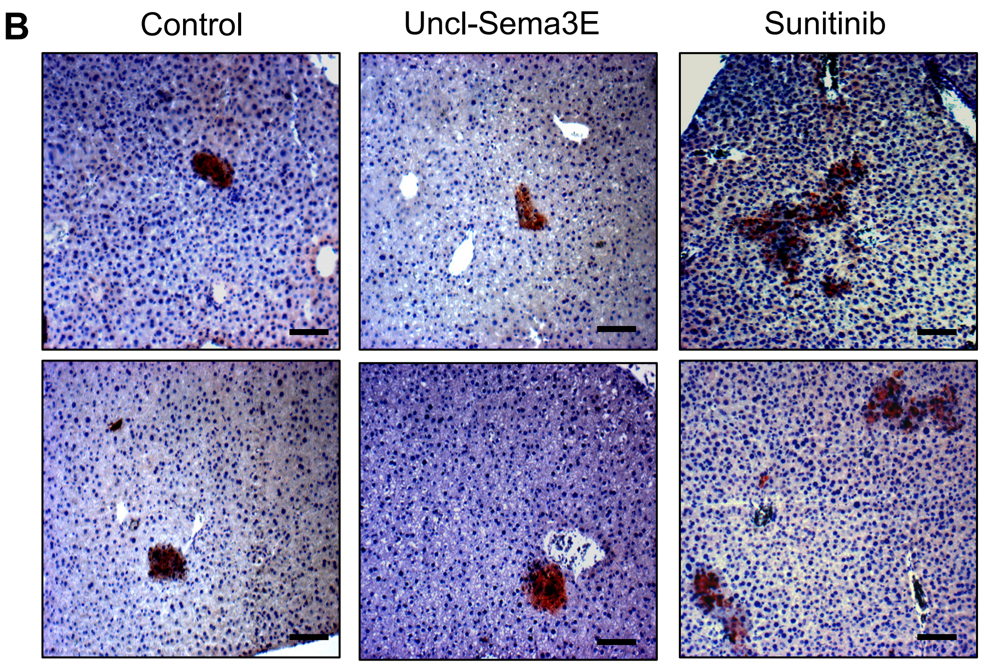
Supplemental Figure 9



Histological analysis of pancreatic tumors from RT2 mice (14 wk) either untreated or treated with locally-delivered Uncl-Sema3E. **A.** The mitotic index was determined by immunostaining Ki67^{+} cells and counterstain all nuclei with DAPI. **B.** Vessel coverage with mural cells was assessed by double staining for α-SMA and CD31 markers. **C.** Endothelial cell apoptosis in tumor vessels was assessed by double staining for CD31 and activated-caspase3 markers. In addition, the fraction of act-casp3+ apoptotic cancer cells (vs. DAPI staining, not shown) was increased in Uncl-Sema3E treated tumors; ***p<0.0005. **D.** Confocal images of PlexinD1-expressing cells (green) in untreated tumors. Endothelial cells were labelled by Meca32 (red); DAPI was used to stain nuclei. Scale bars throughout the figure: 100μm.

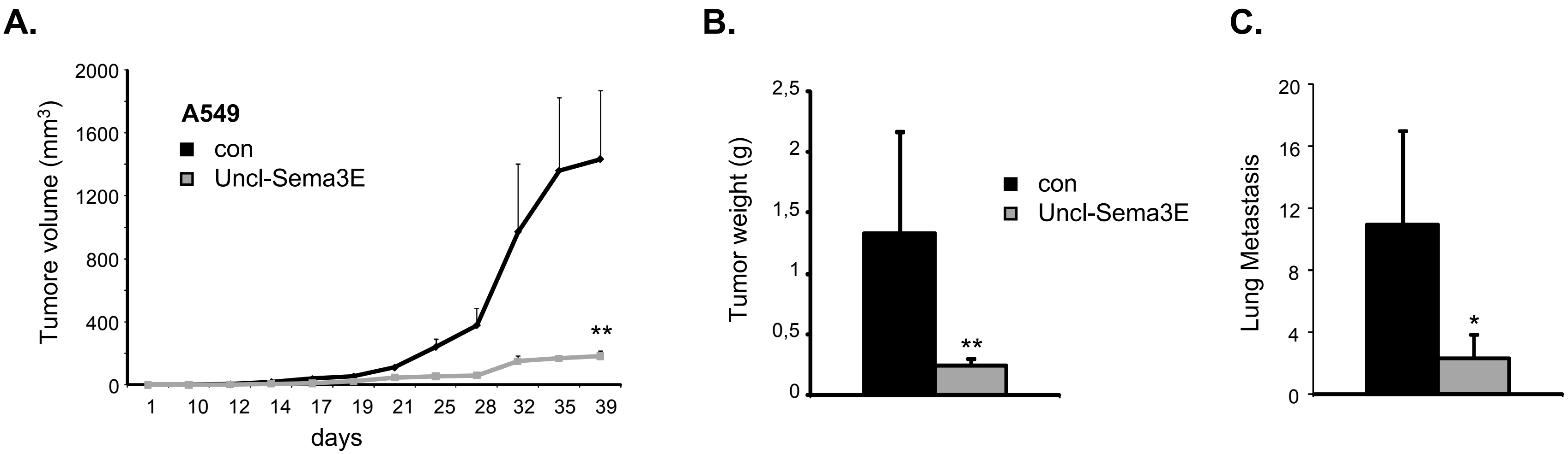
Supplemental Figure 10





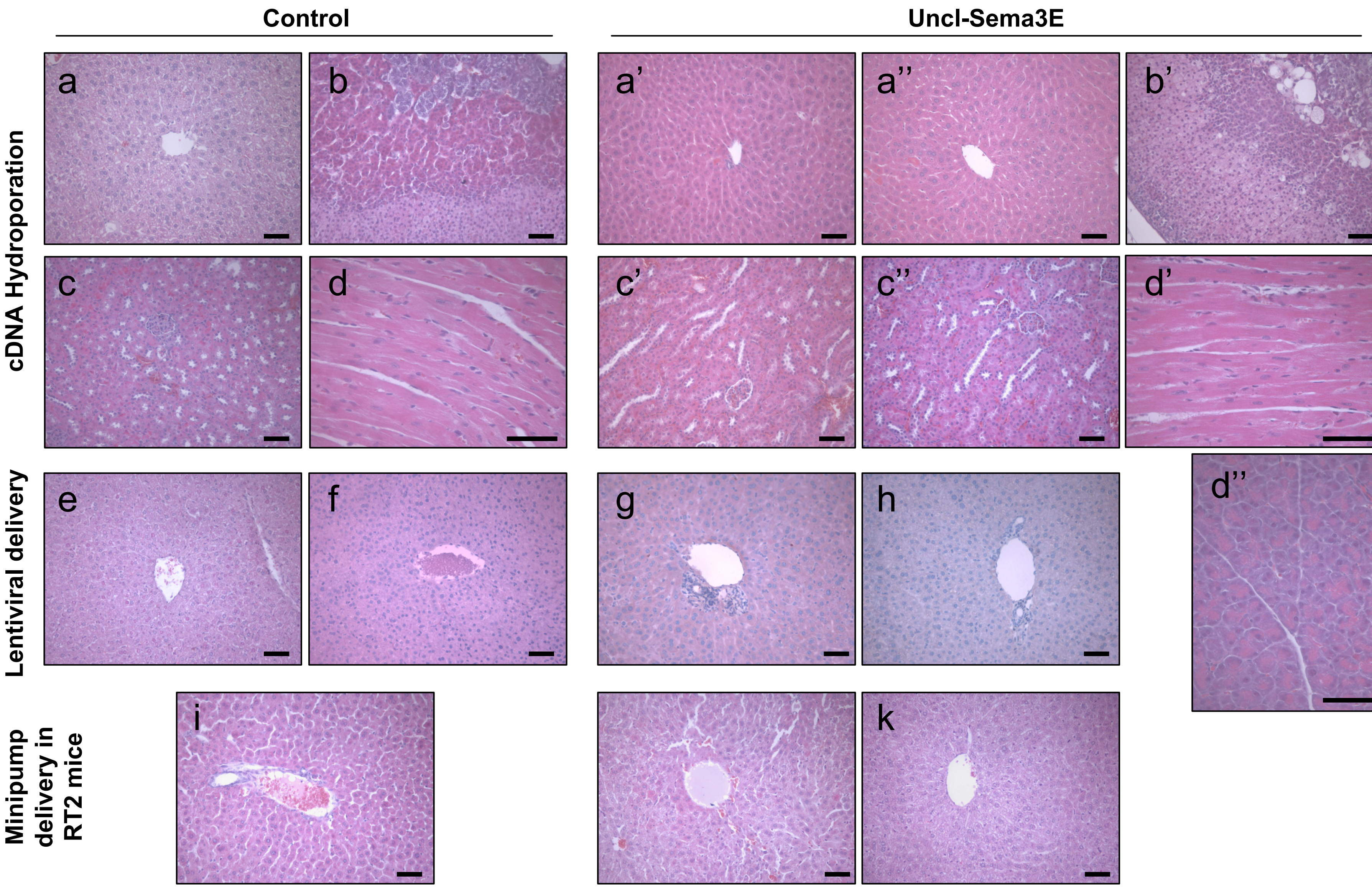
Uncl-Sema3E anti-angiogenic activity did not induce increased metastatic spreading. **A.** The presence of tumor cells in loco-regional lymph nodes (LN) of RT2 mice treated with Uncl-Sema3E (n=14) or Sunitinib (n=8) or mock untreated (n=12) was assessed by immunostaining for SV40 T-antigen (representative images are shown on the left; scale bars: 200µm). The graph on the right indicates the mean tumor burden present in LN (±SD); ***$p<0.0001$. Below the graph is indicated the overall incidence of tumor-infiltrated LN in the different conditions; the difference vs. controls is significant for the Sunitinib treated arm (by the chi-square test: $p<0.05$). **B.** Liver tissue sections of RT2 mice treated with Uncl-Sema3E or Sunitinib or mock untreated (as above) were immunostained for SV40 T-antigen to detect the presence of metastatic tumor cells. We occasionally found isolated micrometastatic foci in control RT2 mice (2 out of 12) and Uncl-Sema3E-treated mice (2 out of 14). In contrast, the anti-angiogenic activity of Sunitinib was associated with remarkably increased incidence of distant metastasis in the liver (6 out 8 analyzed mice; $p<0.0005$, by the chi-square test). Microscopic images display the few metastatic cases in control and Uncl-Sema3E treated mice, as well as representative images of the liver of Sunitinib-treated mice; scale bars: 200µm).

Supplemental Figure 11



Lentiviral-mediated systemic delivery of Uncl-Sema3E into nude mice suppressed the growth of A549 human tumor xenografts in nude mice (**A-B**). Moreover, systemic Uncl-Sema3E inhibited the spontaneous metastatic dissemination of A549 cancer cells to the lungs (**C**); *p<0.03; **p<0.005. See Suppl. Methods for experimental details.

Supplemental Figure 12



Tissue sections from organs of mice treated with Uncl-Sema3E (via multiple delivery approaches, as indicated on the left), and respective mock-treated controls, were stained with H&E. Legend: panels a, a', a'', e-h, i-k = liver; panels b, b' = adrenal gland; panels c, c', c'' = kidney; panels d, d' d'' = heart (higher magnification). Scale bars: 200µm.

Supplemental Figure 13

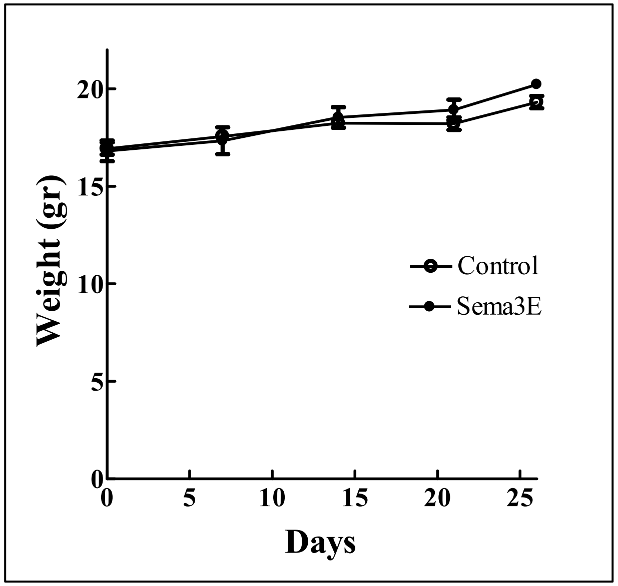
A.

	RBC	WBC	PLT	GOT	GPT	Creat	Urea
	10^6/µl	10^3/µl	10^3/µl	U/l	U/l	mg/dl	mg/dl
Control	**8.77** ± 0.29	**4.87** ± 1.00	**530** ± 70	**16.7** ± 1.2	**20.0** ± 4.0	**0.15** ± 0.01	**17.3** ± 1.5
Uncl-Sema3E	**8.43** ± 0.41	**4.88** ± 0.96	**505** ± 31	**14.8** ± 2.5	**19.0** ± 0.8	**0.17** ± 0.05	**16.2** ± 2.1

Blood values in mice.

Legend:
RBC: erythrocyte counts (red blood cells)
WBC: leucocyte counts (white blood cells)
PLT: platelet counts
GOT: glutamic oxalacetic transaminase
GPT: glutamic piruvic transaminase

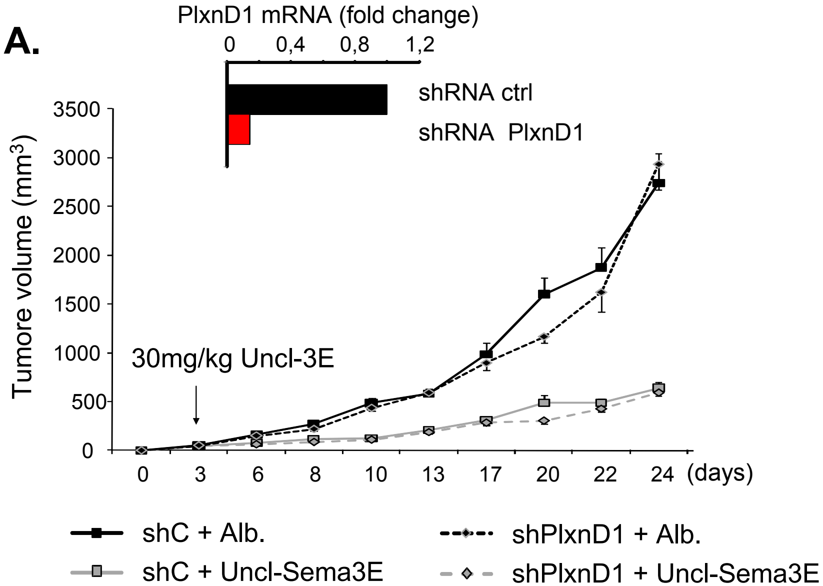
B.



Assessment of body weight changes of immunodeficient nude mice, not transplanted with tumor cells, upon i.p. administration of purified Uncl-Sema3E (or mock control), similarly to experiments shown in main Fig. 10 and Suppl. Fig. 14.

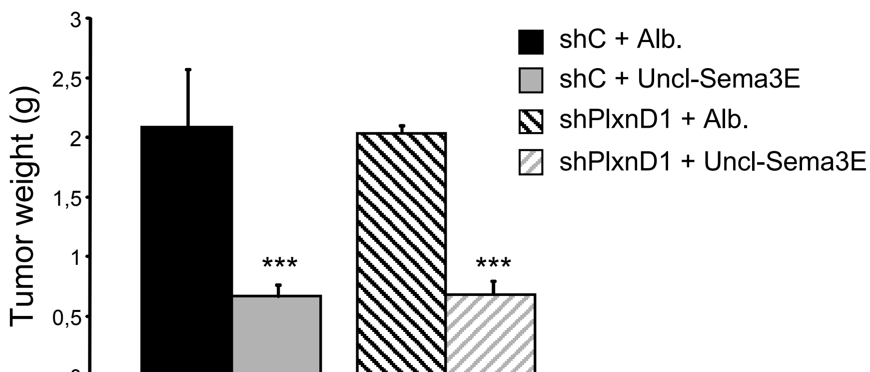
Supplemental Figure 14

A.



4T1 cells were transduced to stably express shRNAs targeting PlexinD1 (or unrelated control sequences), and the expression knock down was verified by Q-PCR (shown on top left). PlexinD1-deficient and control cells were then transplanted into nude mice to establish orthotopic tumors and the mice were subjected to systemic treatment with purified Uncl-Sema3E-Fc or control (similarly to experiments shown in main Fig. 9). The graphs show the growth of primary tumors (**A**), the total tumor burden (**B**) and the number of lung metastasis (**C**) at the end of the experiment. Data are given as average ±SD of six mice for each experimental group; ***p<0.0005.

B.



C.