CCBE1 Enhances Lymphangiogenesis via A Disintegrin and Metalloprotease With Thrombospondin Motifs-3–Mediated Vascular Endothelial Growth Factor-C Activation

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Background—Hennekam lymphangiectasia–lymphedema syndrome (Online Mendelian Inheritance in Man 235510) is a rare autosomal recessive disease, which is associated with mutations in the *CCBE1* gene. Because of the striking phenotypic similarity of embryos lacking either the *Ccbe1* gene or the lymphangiogenic growth factor *Vegfc* gene, we searched for collagen- and calcium-binding epidermal growth factor domains 1 (CCBE1) interactions with the vascular endothelial growth factor-C (VEGF-C) growth factor signaling pathway, which is critical in embryonic and adult lymphangiogenesis.

Methods and Results—By analyzing VEGF-C produced by CCBE1-transfected cells, we found that, whereas CCBE1 itself does not process VEGF-C, it promotes proteolytic cleavage of the otherwise poorly active 29/31-kDa form of VEGF-C by the A disintegrin and metalloprotease with thrombospondin motifs-3 protease, resulting in the mature 21/23-kDa form of VEGF-C, which induces increased VEGF-C receptor signaling. Adeno-associated viral vector–mediated transduction of CCBE1 into mouse skeletal muscle enhanced lymphangiogenesis and angiogenesis induced by adeno-associated viral vector–VEGF-C.

Conclusions—These results identify A disintegrin and metalloprotease with thrombospondin motifs-3 as a VEGF-C–activating protease and reveal a novel type of regulation of a vascular growth factor by a protein that enhances its proteolytic cleavage and activation. The results suggest that CCBE1 is a potential therapeutic tool for the modulation of lymphangiogenesis and angiogenesis in a variety of diseases that involve the lymphatic system, such as lymphedema or lymphatic metastasis. **(*Circulation*. 2014;129:1962-1971.)**

Key Words: angiogenesis effect ■ endothelium ■ growth substances ■ metalloproteinases

Vascular endothelial growth factor (VEGF) -C is the main driver of lymphangiogenesis in embryonic development and in various lymphangiogenic processes in adults.[1] It acts via VEGF receptor (VEGFR) -3 and, in its proteolytically processed mature form, also via VEGFR-2. Deletion of the *Vegfc* gene in mice results in failure of lymphatic development because of the inability of newly differentiated lymphatic endothelial cells to migrate from the cardinal veins to sites where the first lymphatic structures form.[2,3] This phenotype could be rescued by the application of recombinant VEGF-C.[2] For the rescue, a mature form of VEGF-C was used, which lacked the N- and C-terminal propeptides. In cells secreting endogenous VEGF-C, these propeptides need to be proteolytically cleaved off from the central VEGF homology domain in order for VEGF-C to reach its full signaling potential.[4] VEGF-C can also activate the main angiogenic receptor VEGFR-2 when both propeptides are cleaved off.[4] Hence, the mature VEGF-C can stimulate also angiogenesis.

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Mutations in VEGF-C and VEGFR-3 have been shown to result in hereditary lymphedema.[5–7] Another hereditary condition with lymphedema as a cardinal symptom is Hennekam lymphangiectasia–lymphedema syndrome.[8] In a subset of clinically diagnosed patients, mutations in the *CCBE1* gene were found responsible for the disease,[9,10] but it has been unclear how the mutant collagen- and calcium-binding epidermal growth factor domains 1 (CCBE1) causes the lymphatic phenotype. CCBE1 is a 2-domain protein with an N-terminal potential Ca-binding domain with epidermal growth factor–like repeats and a C-terminal domain with collagen-like repeats. Most of the known mutations in the *CCBE1* gene are point mutations affecting its N-terminal domain; only 2 of all

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the identified mutations affect the collagen-like domain. All of the human mutations are expected to result in a functionally impaired CCBE1 protein but not in complete lack of CCBE1, which is likely incompatible with survival on the basis of gene deletion studies.[11]

In *Vegfc*-deficient embryos the differentiation of lymphatic endothelial cells from blood vascular endothelial cells in the cardinal veins appears unaffected, but they fail to egress from the cardinal veins.[2,3] Compared with *Vegfc*-deficient embryos, the migration deficiency of nascent lymphatic endothelial cells in *Ccbe1*-deficient embryos is only partial. They form abnormal sprouts that fail to segregate from the cardinal veins, and the egressing lymphatic endothelial cells are unable to coalesce into discrete lymphatic structures.[3,11] Hence, the earlier developmental block in the *Vegfc*-deficient embryos was attributed to the lack of a migration signal provided by VEGF-C, whereas the later block in *Ccbe1*-deficient embryos was attributed to a defect in endothelial cell migration, perhaps because of the lack of migratory cues from the extracellular matrix, of which CCBE1 is thought to be a component.[12]

Both *Ccbe1*+/− and *Vegfc*+/− heterozygous embryos show a reduction of Prox1-positive endothelial cells emigrating from the cardinal veins.[3] The double heterozygous *Ccbe1*+/−;*Vegfc*+/− embryos have an aggravated version of this phenotype, suggesting that CCBE1 and VEGF-C participate synergistically in the lymphatic separation,[3] which is also supported by data from zebrafish.[12]

In the present study, we have explored the link between CCBE1 and VEGF-C using both in vitro and in vivo assays and report that CCBE1 affects lymphangiogenesis by enhancing the cleavage of VEGF-C by the A disintegrin and metalloprotease with thrombospondin motifs-3 (ADAMTS3) metalloprotease, which removes the N-terminal propeptide from pro–VEGF-C, resulting in the mature, fully active VEGF-C.

Methods

Transfections, Metabolic Labeling, and Protein Analysis

293T and 293S GnTI$^-$ cells were (co)transfected with expression constructs coding for the indicated proteins. Twenty-four hours after the transfection, the cells were metabolically labeled with [^{35}S]-cysteine/[^{35}S]-methionine (PerkinElmer, Waltham, MA), and 48 hours later, conditioned cell culture medium and lysates were harvested. For the short-term labeling experiments, harvesting was performed after 24 hours. To produce unlabeled protein, the culture media were exchanged and supernatants and lysates harvested 48 hours later. After immunoprecipitation, the samples were electrophorated in 4% to 20% SDS-PAGE. For autoradiography, gels were dried and exposed to phosphoimager plates or x-ray film. For the immunodetection, the proteins were transferred to nitrocellulose. Specific signals were detected by enhanced chemiluminescence. Quantitation of the autoradiographies and Western blots was performed from the laser scanner readouts or scanned x-ray film using the ImageJ software (National Institutes of Health, Bethesda, MD).

Ba/F3-VEGFR/EpoR Assays

The Ba/F3-hVEGFR-3/EpoR,[13] Ba/F3-mVEGFR-2/EpoR[14] and Ba/F3-hVEGFR-2/EpoR bioassays were performed with conditioned cell culture medium as described.[15]

Stimulation of VEGFR-3 Phosphorylation

Near confluence porcine aortic endothelial cells expressing VEGFR-3 or VEGFR-3 plus neuropilin-2 were washed with PBS and starved over night in DMEM 0.2% BSA. ΔNΔC–VEGF-C, pro–VEGF-C, and CCBE1Δ175 were diluted to 0.02, 0.40, and 5.00 μg/mL in 1 mL of DMEM/0.1% BSA and incubated at 37°C for 30 minutes. The cells were stimulated for 10 or 30 minutes to detect phosphorylation of VEGFR-3 or downstream signaling proteins and then washed with ice-cold PBS. To cross-link proteins, the cells were washed twice with PBS, and purified proteins were applied in PBS (ΔNΔC–VEGF-C 100 ng/mL, pro–VEGF-C 1000 ng/mL, and CCBE1Δ175 at 25–50 μg/mL). After 3.5 minutes DTSSP (ThermoScientific, Waltham, MA) was added to a final concentration of 2 mmol/L, and cross-linking was performed for 6.5 minutes at 37°C. Cells were washed once with ice-cold TBS, lysed with 1% Triton X-100, and the immunoprecipitated fraction or the total lysate analyzed by SDS-PAGE/Western blot.

VEGFR-3 Trafficking in Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells stably transfected with the pMXs–VEGFR-3–green fluorescent protein vector[16] were grown on glass-bottom microwells (MatTek Co, Ashland, MA) for 24 hours. The FCS concentration was reduced to 0.5%, and 12 hours thereafter, the cells were placed in an incubator (36°C and 5% CO_2) on a Zeiss LSM 5 DUO Confocal microscope and treated with pro–VEGF-C or ΔNΔC–VEGF-C (100 ng/mL). The green fluorescent protein signal was recorded at the 488-nm wavelength. The human VEGFR-3 blocking antibody hF4-3C5 was used at 5 μg/mL.

Recombinant Adeno-Associated Viral Vector Production

Adeno-associated viral vector (AAV) 9 vectors were made by a 3-plasmid transfection method and purified by ultracentrifugation using discontinuous iodixanol gradient, as described,[17] except that we used the serotype-determining helper plasmid p5E18-VP2/9 instead of p5E18-VP2/8.[18]

In Vivo Experiments

Tibialis anterior muscles of FVB/N male mice were injected with 1:1 mixed solutions of AAV9s encoding mouse (m)CCBE1-V5, mVEGF-C, or HSA. The AAV9-HSA and AAV9–ΔNΔC–mVEGF-C single vectors were used as negative and positive controls, correspondingly. The total concentration of the vector particles in a single injected dose was 6×10^{10}. Three weeks after transduction, the mice were euthanized by CO_2 overdose. The tibialis anterior muscles were isolated, embedded into O.C.T. (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands), sectioned (10-μm thickness), and stained for the lymphatic (Lyve-1, Prox-1) and blood vascular markers (platelet endothelial cell adhesion molecule-1), as well as smooth muscle cell/pericyte (smooth muscle actin) and leukocyte (CD45) markers, followed by Alexa-conjugated secondary antibodies (Molecular Probes, Invitrogen, Life Technologies, Carlsbad, CA). Fluorescent images were obtained in an Axioplan microscope (Carl Zeiss AG, Oberkochen, Germany); the objectives were as follows: 10× numerical aperture = 0.3 and working distance = 5.6 mm and 20× numerical aperture = 0.5 and working distance = 2.0 mm; the camera was a Zeiss AxioCamHRm 14-bit greyscale charge-coupled device; the acquisition software was Zeiss AxioVision 4.6. Quantification of the areas stained for Lyve-1, platelet endothelial cell adhesion molecule-1, and smooth muscle actin was done as described previously.[17] Shortly, we used the ImageJ software measuring the percentage of pixels that showed values above background in the appropriate color channel using the "measure" function. Prox-1 positive nuclei were counted manually. The detection of luciferase activity in EGFP/Luc *Vegfr3*$^{EGFP/Luc}$ mice was performed as described previously.[19] The National Board for Animal Experiments of the Provincial State Office of Southern Finland approved all of the animal experiments carried out in this study.

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Statistical Analysis

Significance of the differences was determined using 1-way ANOVA. When equal variances were assumed, the Tukey test was used as a post hoc test; when variances were assumed unequal, the Games-Howell test was used. For the Ba/F3 assays, separate ANOVAs were used for each concentration. The EC_{50}s of the Ba/F3 assays were calculated using the 4-parameter logistic nonlinear regression model and the ReaderFit software (Hitachi Solutions America, Ltd, South San Francisco, CA). Four-parameter logistic nonlinear regression model calculations with and without weighting gave essentially similar results. Error bars in the figures indicate the SDs.

Results

CCBE1 Enhances VEGF-C Processing and Release, Resulting in Increased VEGFR-3 Activation

CCBE1 was detected as a protein of 40 to 55 kDa molecular weight in both cell lysates and conditioned media of 293T cells transfected with a CCBE1 expression vector (Figure 1A).[20] However, most of the secreted CCBE1 migrated as a diffuse band of ≈100 kDa. Transfected VEGF-C was expressed as the uncleaved 58 kDa precursor, C-terminally processed 29/31 kDa the pro–VEGF-C form, and fully processed 21 kDa the mature form (Figure 1B, lane 1).[4,21] However, when VEGF-C and CCBE1 were cotransfected, the amounts of the unprocessed VEGF-C and pro–VEGF-C were reduced, and the mature, fully activated VEGF-C became the major species (Figure 1B, lane 2).

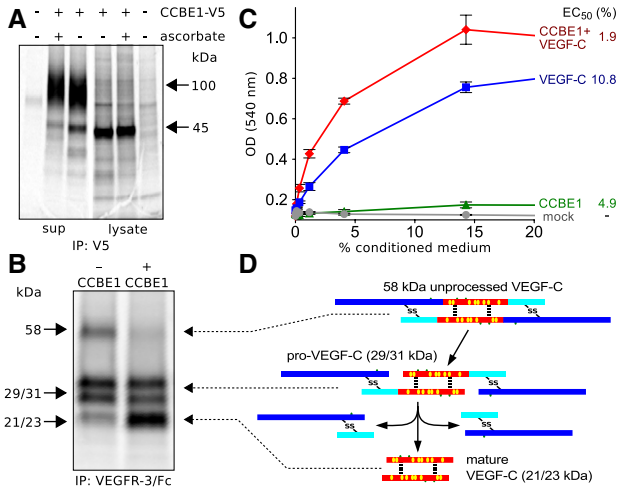


Figure 1. Collagen- and calcium-binding epidermal growth factor domains 1 (CCBE1) coexpression increases vascular endothelial growth factor (VEGF)-C proteolytic processing to the mature, fully active form. 293T cells were transfected with CCBE1 alone (**A**) or with VEGF-C+/–CCBE1 (**B**), incubated with radioactive amino acids and the medium supernatants (sup), and cell lysates were analyzed by immunoprecipitation and autoradiography. Note that both intracellular and extracellular CCBE1 is detected, and CCBE1, unlike collagen production,[20] is not dependent on ascorbate supplementation to the cell culture medium. **B**, VEGF-C precipitation with a soluble form of its receptor (VEGF receptor [R]-3/Fc) shows the expression of unprocessed, pro–VEGF-C and mature VEGF-C. **C**, Supernatants from cultures expressing both CCBE1 and VEGF-C promote the growth of Ba/F3–VEGFR-3/EpoR cells more than supernatants from cultures expressing only VEGF-C. **D**, Schematic view of the biosynthesis and processing of the VEGF-C precursor and the most prominent processed forms.[21] Arrows point to the corresponding bands in **B**. The N-terminal propeptide is shown in cyan, the VEGF homology domain in red, and the C-terminal propeptide in blue.

Cotransfection with CCBE1 also facilitated the release of VEGF-C, because the cell-layer associated amount was reduced by 80% in lysates of the cotransfected cells in short-term labeling experiments (Figure IA in the online-only Data Supplement, compare lanes 5 through 8). A similar accelerated release of VEGF-C was achieved, when the C-terminal domain of VEGF-C was removed (Figure IB in the online-only Data Supplement).

Conditioned medium from the CCBE1/VEGF-C cotransfected cultures stimulated the growth and survival of Ba/F3–VEGFR-3/EpoR and Ba/F3–VEGFR-2/EpoR cells better than the medium of cells transfected with VEGF-C alone, whereas medium from CCBE1 transfected cells alone showed very little activity, indicating that the enhanced release and cleavage resulted in increased levels of active VEGF-C (Figure 1C and Figure IC in the online-only Data Supplement). Notably, CCBE1 also slightly promoted the survival of Ba/F3–VEGFR-3/EpoR cells, presumably because of increased processing and activation of endogenous VEGF-C made by the cells.

CCBE1 Enhances VEGF-C Processing In Trans

In the developing mouse and zebrafish embryo, CCBE1 is expressed in cells adjacent to developing lymphatic vessels.[3,12] We thus determined whether CCBE1 production in trans by other cells also enhances VEGF-C processing. We transfected separate cultures of 293T cells with VEGF-C or CCBE1 and mixed the cell populations 24 hours after transfection. Alternatively, we mixed CCBE1-transfected cells with cells stably expressing VEGF-C. In these experiments, CCBE1 increased the efficiency of the extracellular processing of VEGF-C but not its release (Figure ID in the online-only Data Supplement).

CCBE1 Enhances the Lymphangiogenic Activity of VEGF-C In Vivo

Proteolytic processing of VEGF-C has been shown to increase its receptor affinity and biological activity.[4,17] To investigate whether CCBE1 enhances VEGF-C–induced lymphangiogenesis in vivo, we transduced mouse tibialis anterior muscles with AAV9 expressing CCBE1 (AAV9–CCBE1) alone or together with AAV9–VEGF-C in a 1:1 ratio or AAV9–HSA as a negative control. AAV9 encoding the mature, activated form of VEGF-C (ΔNΔC–VEGF-C) was used as a positive control.

Three weeks after the AAV transduction, the muscles were analyzed by immunohistochemistry using markers for endothelial cells (platelet endothelial cell adhesion molecule-1), lymphatic endothelial cells (lymphatic vessel endothelial hyaluronan receptor (Lyve)-1, Prox1), and leukocytes (CD45). In this assay, both VEGF-C and ΔNΔC–VEGF-C stimulated lymphangiogenesis. ΔNΔC–VEGF-C gave a considerably stronger response at the same viral dose and stimulated additionally angiogenesis (Figure 2 and Figure II in the online-only Data Supplement, bottom row). This suggested that the proteolytic processing of VEGF-C was inefficient in the AAV9-transduced muscle.

However, when VEGF-C was cotransduced with CCBE1, lymphangiogenesis was significantly enhanced, as shown by the Lyve-1 and Prox-1 staining (Figure 2). Similar to

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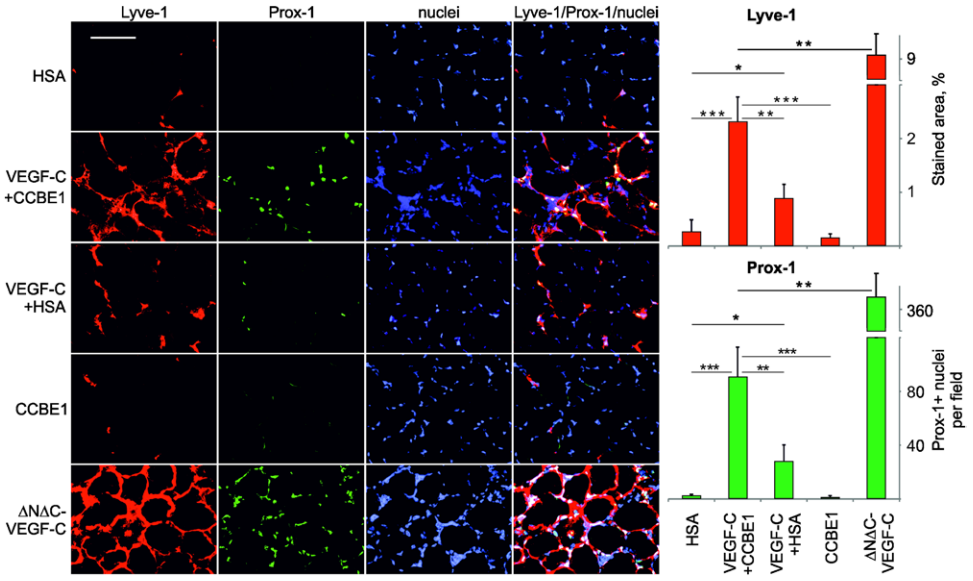


Figure 2. Collagen- and calcium-binding epidermal growth factor domains 1 (CCBE1) enhances lymphangiogenesis in vivo. Immunostaining of mouse tibialis anterior muscles transduced by adeno-associated viral vector (AAV) 9 encoding the indicated factors and stained for the indicated antigens. Note that vascular endothelial growth factor (VEGF)-C alone induces only a mild lymphangiogenic response, but its cotransduction with CCBE1 results in a strong response as detected by lymphatic vessel endothelial hyaluronan receptor (Lyve)-1 and Prox-1 staining of lymphatic vessels. As a positive control, AAV9 encoding ΔNΔC–VEGF-C (an equivalent of the fully processed, mature VEGF-C) was used. *P<0.05; **P<0.01; ***P<0.001; n ≥ 5.

the ΔNΔC–VEGF-C transduced muscle, significantly more angiogenesis and leukocyte recruitment were observed (Figures II and III in the online-only Data Supplement). These results indicated that CCBE1 enhances VEGF-C processing also in vivo.

To corroborate these findings, we used the AAV9s encoding the various VEGF-C forms and CCBE1 in mice heterozygous for a *Vegfr3*$^{EGFP/Luc}$ allele to monitor lymphangiogenesis by optical bioluminescent imaging in vivo.[19] We detected strong luciferase signals in mice cotransduced with the AAVs encoding VEGF-C and CCBE1, weaker signals in mice transduced with VEGF-C or CCBE1 alone, and no bioluminescent signals in mice transduced with HSA (Figure 3).

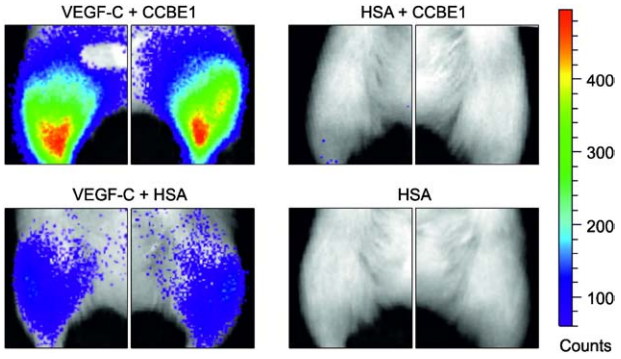


Figure 3. Vascular endothelial growth factor (VEGF) receptor (R)–3-luciferase reporter signals in mouse skeletal muscles injected with adeno-associated viral (AAV) 9 vectors encoding the indicated proteins. Note that the cotransduction with VEGF-C and collagen- and calcium-binding epidermal growth factor domains 1 (CCBE1) results in a strong luciferase signal, indicating a major lymphangiogenesis response, whereas VEGF-C or CCBE1 alone induces only little luciferase activity. The luminometry color scale is shown on the **right**.

VEGF-C and CCBE1 Are Processed by the ADAMTS3 Procollagenase

Our attempts to demonstrate a physical interaction of VEGF-C and CCBE1 were unsuccessful (Figure IVA in the online-only Data Supplement). We thus assumed that the CCBE1–VEGF-C interaction is short lived and/or indirect, perhaps mediated by the protease that removes the N-terminal propeptide of VEGF-C. We stably expressed CCBE1 in 293T cells, purified the protein, and subjected it to tryptic digestion followed by mass spectrometry. The most abundant copurified protease was ADAMTS3. Efficient N-terminal processing of pro–VEGF-C was obtained when ADAMTS3 was expressed together with VEGF-C in 293T cells (Figure 4A). To analyze whether CCBE1 enhances the ADAMTS3-mediated VEGF-C cleavage, the amounts of ADAMTS3 used for VEGF-C cleavage were titrated. When CCBE1-, VEGF-C–, and ADAMTS3-conditioned media were mixed in a ratio of 60:30:1, the ADAMTS3-mediated cleavage of VEGF-C was more efficient in the presence of CCBE1 than without (Figure 4B), and a corresponding medium had growth-promoting activity in the VEGFR-3/EpoR-expressing Ba/F3 cells (Figure 4C). When the culture media of the ADAMTS3 cotransfected samples were precipitated with ADAMTS3 antibodies or streptactin and analyzed in Western blotting with antibodies recognizing the C-terminus of CCBE1, the specific CCBE1 band migrated at 25 kDa, which corresponds to the collagen-like domain of CCBE1 (Figure 4D), indicating that ADAMTS3 may cleave CCBE1 between the epidermal growth factor and collagen homology domain. Interestingly, the DU-4475 cells produced only uncleaved CCBE1 (Figure IVB in the online-only Data Supplement), which did not promote VEGF-C activation (Figure IVC in the online-only Data Supplement).

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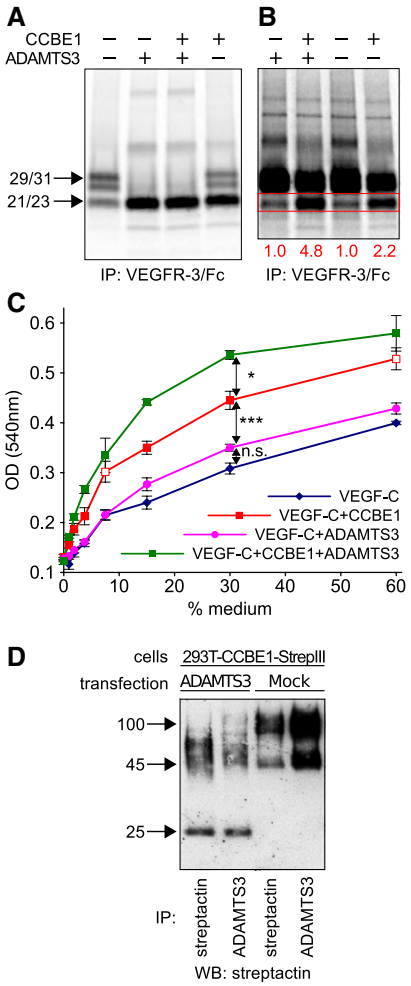


Figure 4. A disintegrin and metalloprotease with thrombospondin motifs-3 (ADAMTS3) cleaves vascular endothelial growth factor (VEGF)-C and collagen- and calcium-binding epidermal growth factor domains 1 (CCBE1). **A**, Stable VEGF-C–expressing 293T cells were transfected with the indicated expression vectors, and their culture media were immunoprecipitated with VEGF receptor (R)-3/Fc. Note that CCBE1 transfection alone results in a partial conversion of pro–VEGF-C into mature VEGF-C, whereas ADAMTS3 transfection results in the complete conversion of pro–VEGF-C to mature VEGF-C. **B**, Conditioned medium of VEGF-C–expressing cells was mixed with conditioned medium from CCBE1-, ADAMTS3-, or mock-transfected cells. Note the lack of effect of ADAMTS3 alone at 1% concentration. CCBE1 alone results in appreciable activation of VEGF-C, presumably because of the endogenous ADAMTS3 produced by 293T cells. The highest level of VEGF-C conversion to the active form occurs when both ADAMTS3 and CCBE1 are present. Fold increase of mature VEGF-C (marked by the red frame) is indicated. **C**, Supernatants of VEGF-C–expressing Chinese hamster ovary (CHO) cells were mixed with supernatants of 293T cells expressing CCBE1 and ADAMTS3 and supernatants of untransfected 293T cells at ratio of 15:16:2:47, incubated for 24 hours and assayed for their ability to promote the growth of Ba/F3–hVEGFR-3/EpoR cells. The curves were statistically different from each other at all of the data points except for those indicated without fill and the comparison VEGF-C vs VEGF-C+ADAMTS3 (n = 3). **D**, ADAMTS3 transfection results in CCBE1 cleavage, separating the C-terminal collagen-like domain from the N-terminal domain. The C-terminal domain is detected in the precipitates by the antibody against ADAMTS3. Note that full-length CCBE1 binds to protein G Sepharose nonspecifically and that the detection of any coprecipitating full-length CCBE1 is therefore not possible.

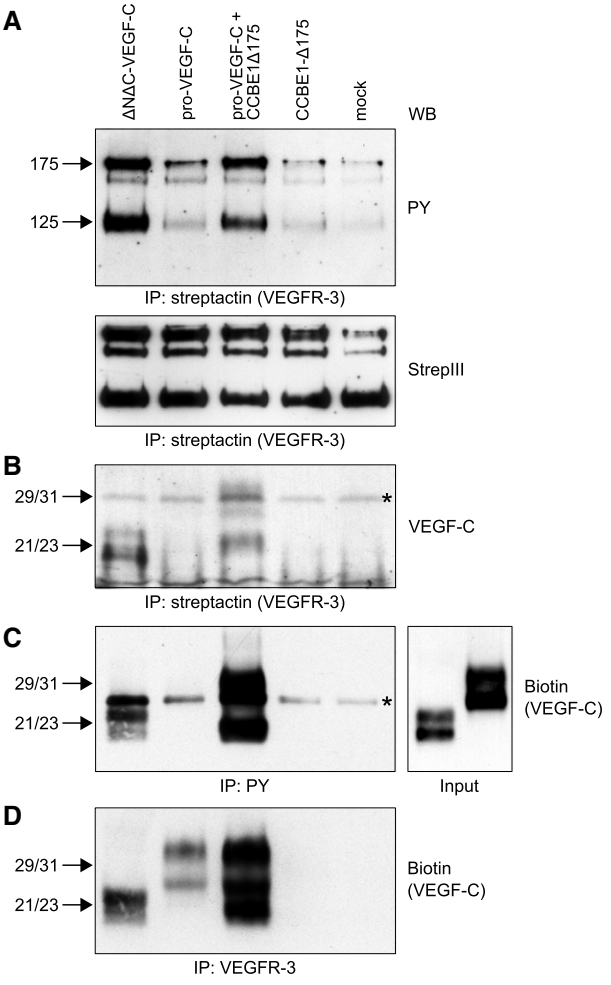


Figure 5. The N-terminal domain of collagen- and calcium-binding epidermal growth factor domains 1 (CCBE1) activates vascular endothelial growth factor (VEGF) receptor (R)-3 phosphorylation by pro–VEGF-C. VEGFR-3–expressing porcine aortic endothelial (PAE) cells were stimulated with pro–VEGF-C together with the N-terminal domain of CCBE1 (CCBE1Δ175) or with mature VEGF-C (ΔNΔC–VEGF-C). **A**, Note that pro–VEGF-C induces only a marginal activation of VEGFR-3 without CCBE1Δ175. **B**, VEGFR-3–bound mature VEGF-C was detected when CCBE1Δ175 was present during the stimulation with pro–VEGF-C, indicating that the CCBE1-enhanced processing occurs during the stimulation period. **C**, Cross-linking of VEGF-C during VEGFR-3 stimulation shows that mature VEGF-C is bound to the phosphorylated VEGFR-3. The apparent molecular weight of the mature VEGF-C produced by CCBE1-enhanced cleavage of pro–VEGF-C and recombinant ΔNΔC–VEGF-C differ because of differential glycosylation. The **right panel** shows the biotinylated VEGF-C forms used for the experiment. **D**, An experiment similar to that of **C** done with VEGFR-3 plus neuropilin-2–expressing cells. The asterisks in **B** and **C** mark nonspecific signals with the same electrophoretic mobility as the 29-kDa fragment of pro–VEGF-C.

VEGF-C Cleavage by Plasmin Is Not Influenced by CCBE1

As published previously,[22] VEGF-C was efficiently cleaved by plasmin (Figure VA in the online-only Data Supplement). The fragments obtained with low amounts of plasmin activated VEGFR-3, but this activity was lost at high plasmin concentrations (Figure VB in the online-only Data Supplement). Edman degradation of the final products revealed the N-terminal

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sequence KTQC and a complete lack of the N-terminal helix, which is incompatible with VEGFR-3 activation.[23] CCBE1 did not affect the efficiency of plasmin cleavage (Figure VC in the online-only Data Supplement).

ADAMTS3 Produced by 293T Cells Processes VEGF-C to the Mature Form

The N-terminus of the mature VEGF-C generated by incubation with recombinant, purified ADAMTS3 was identical to that reported for mature VEGF-C produced by 293 cells[4] (Figure VIA in the online-only Data Supplement). VEGF-D was not cleaved by ADAMTS3 under the same conditions (Figure VIB in the online-only Data Supplement), despite featuring a similar cleavage motif (Figure VIC in the online-only Data Supplement).

Apart from ADAMTS3, 2 other proteases, ADAMTS2 and ADAMTS14, belong to the procollagenase subfamily of ADAMTS proteases.[24] Interestingly, the ADAMTS1 gene deletion in mice results in deficient ovarian lymphangiogenesis.[25] However, unlike ADAMTS3, ADAMTS1, 2, or 14 did not cleave VEGF-C (Figure VID in the online-only Data Supplement).

We found that the cell lines that produce active, mature VEGF-C (293T, 293T-CCBE1, and PC-3 cells) express ADAMTS3, whereas the cell lines that were unable or extremely inefficient in producing active VEGF-C (CHO and NIH-3T3), expressed very little or no ADAMTS3 (Figure VII in the online-only Data Supplement). Furthermore, when ADAMTS3 was silenced in 293T cells by using lentiviral short-hairpin RNA, the VEGF-C cleavage was inhibited (Figure VIIIA in the online-only Data Supplement).

VEGF-C/VEGF-D chimeras generated by propeptide swapping were not subject to ADAMTS3 cleavage (Figure VIIIB and VIIIC in the online-only Data Supplement). Interestingly, however, 79% of VEGF-C processing was inhibited by the purified C-terminal propeptide and 43% by the N-terminal propeptide, whereas the VEGF homology domain or HSA gave no inhibition (Figure VIIID), suggesting that the VEGF-C propeptides are necessary but not sufficient for VEGF-C recognition by ADAMTS3.

The N-Terminal Domain of CCBE1 Enhances Pro–VEGF-C Cleavage to the Mature Form

Because of the difficulty in expressing sufficient amounts of full-length CCBE1, we investigated whether the isolated N-terminal domain of CCBE1 (CCBE1Δ175) can increase VEGF-C activity. We stimulated VEGFR-3–transfected porcine aortic endothelial cells with pro–VEGF-C, which resulted in very little VEGFR-3 phosphorylation when compared with mature VEGF-C (Figure 5A, lanes 1 and 2). When the recombinant CCBE1Δ175 was added to pro–VEGF-C, VEGFR-3 phosphorylation was strongly increased (Figure 5A, lane 3). Analysis of VEGFR-3 coprecipitated proteins from the pro–VEGF-C stimulated cells indicated that both pro–VEGF-C and mature VEGF-C are bound to the receptor in the presence of CCBE1Δ175 (Figure 5B, compare lanes 2 and 3). To identify which form of VEGF-C was bound to the phosphorylated VEGFR-3 receptor, we applied purified CCBE1Δ175 and biotinylated, purified pro–VEGF-C to cultures of porcine aortic endothelial–VEGFR-3 cells in PBS for 210 seconds and cross-linked VEGFR-3–associated proteins for 390 seconds. Precipitation and analysis of tyrosyl phosphorylated proteins indicated that mature VEGF-C is bound to activated VEGFR-3 when both CCBE1Δ175 and pro–VEGF-C are used for the stimulation (Figure 5C). Pro–VEGF-C alone did not coprecipitate with VEGFR-3, unless VEGFR-3 was coexpressed with the VEGF-C coreceptor neuropilin-2 (Figure 5D). However, even then, pro–VEGF-C induced very little phosphorylation of VEGFR-3 (data not shown).

Pro–VEGF-C Can Act as a Competitive Inhibitor of Mature VEGF-C

We next analyzed the ability of pro–VEGF-C to inhibit VEGFR-3 activation by mature VEGF-C. Indeed, preincubation of lymphatic endothelial cells with high amounts of pro–VEGF-C inhibited their ability to respond to mature VEGF-C (Figure 6A). Unlike mature VEGF-C, pro–VEGF-C did not stimulate the endocytosis of VEGFR-3 or the phosphorylation of the Erk, Akt, or endothelial NO synthase downstream signaling proteins in blood vascular endothelial cells or lymphatic endothelial cells (Figure 6B and 6C).

Discussion

CCBE1 is essential for embryonic lymphangiogenesis.[10–12] However, it has been unclear how it controls the lymphangiogenic response. Here we show that CCBE1 acts by regulating the cleavage of pro–VEGF-C into its active form. During its biosynthesis, unprocessed VEGF-C first undergoes a cleavage in the C-terminal part, resulting in pro–VEGF-C, and subsequently in the N-terminal part, yielding the mature form of VEGF-C.[4] Proprotein convertases such as furin mediate the C-terminal cleavage of VEGF-C,[26] but the protease that cleaves the N-terminal propeptide has not been clearly defined. We show that, whereas CCBE1 does not cleave VEGF-C, it greatly enhances the ADAMTS3-mediated N-terminal cleavage and activation of pro–VEGF-C. The N-terminal cleavage process seems inefficient in the majority of cultured cell lines, thus little of the pro–VEGF-C gets activated. Because of the remarkable difference in the lymphangiogenic potential between pro–VEGF-C and mature VEGF-C,[17] it has been assumed that, analogous to VEGF-A,[27,28] the proteolytic environment would be a critical determinant controlling VEGF-C bioavailability and activity in vivo.[4,29] Our data indicate that CCBE1 expression in tissues could regulate VEGF-C activation in the lymphatic endothelial microenvironment in a spatially controlled manner.

We were unable to demonstrate a direct interaction between CCBE1 and VEGF-C, but CCBE1 interacted with the metalloproteinase ADAMTS3, as shown by mass spectrometry and a functional assay. ADAMTS3 cleavage of pro–VEGF-C was enhanced by CCBE1, whereas plasmin cleavage was not. The expression pattern of ADAMTS3 makes it a more likely candidate for VEGF-C activation during embryonic lymphangiogenesis than plasmin,[30,31] but in wound healing and other invasive processes, where plasminogen becomes activated, VEGF-C activation (and deactivation) may occur via plasmin.

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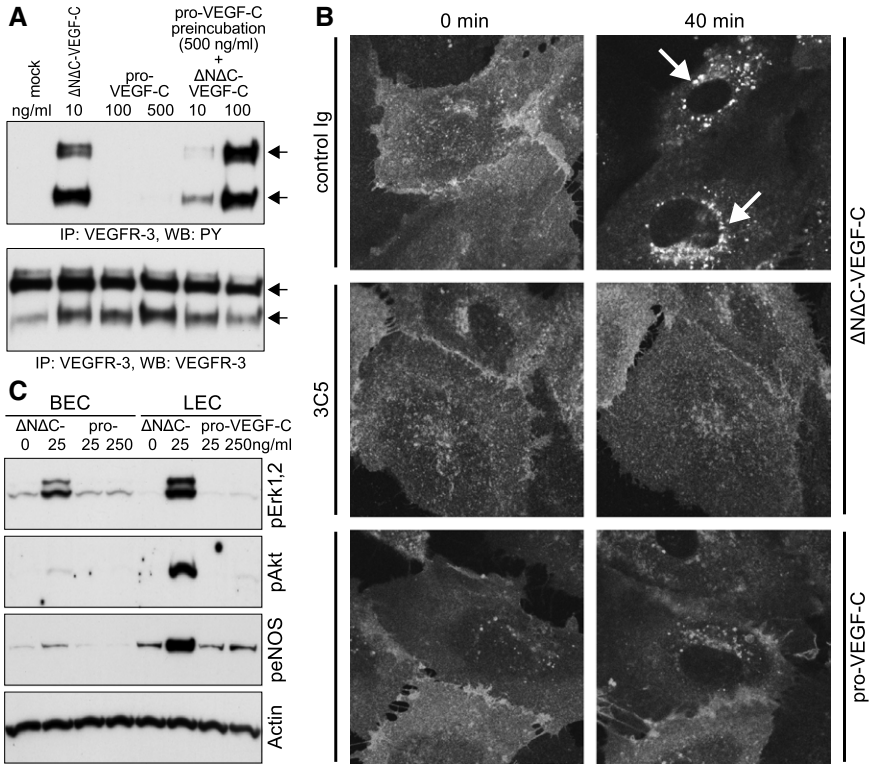


Figure 6. Pro–vascular endothelial growth factor (VEGF)-C can inhibit VEGF receptor (R)-3 activation by mature VEGF-C. **A**, Ten minutes of preincubation of lymphatic endothelial cells with pro–VEGF-C reduces their ability to respond to mature VEGF-C. Arrows denote the 125- and 175-kDa fragments of VEGFR-3. The Western blots represent separate gels. **B**, Human umbilical vein endothelial cells (HUVECs) stably expressing a VEGFR-3–green fluorescent protein (GFP) fusion protein were used for live cell fluorescence imaging after the addition of pro–VEGF-C or ΔNΔC–VEGF-C. ΔNΔC–VEGF-C resulted in a rapid internalization of VEGFR-3 (first row), which could be blocked to a large extent by the anti–VEGFR-3 antibody 3C5 (second row). Pro–VEGF-C did not significantly change the VEGFR-3 cell surface localization (third row). Arrows emphasize the concentration of VEGFR-3 in endosomes after 40 minutes of stimulation. **C**, Mature VEGF-C, but not pro–VEGF-C, induces the phosphorylation of Erk, Akt, and endothelial NO synthase (eNOS). eNOS detection was performed on the stripped pErk membrane, and actin detection was performed on the stripped pAkt membrane.

Although pro–VEGF-C is known to bind to VEGFR-3,[4,32,33] it did not bind to or activate VEGFR-3 on its own in the porcine aortic endothelial–VEGFR-3 cells. However, when we introduced neuropilin-2, we could establish binding, yet we detected very little VEGFR-3 phosphorylation. This explains the competitive inhibition of mature VEGF-C activity by pro–VEGF-C in lymphatic endothelial cells, which express neuropilin-2.[34,35]

When we applied pro–VEGF-C with CCBE1 and cross-linked proteins that were bound to activated VEGFR-3, we detected mature VEGF-C. Thus, a rapid CCBE1-assisted cleavage of receptor-bound pro–VEGF-C by a cell-surface–associated protease appears responsible for the CCBE1 enhancement of pro–VEGF-C signaling activity. This is consistent with the fact that only little of the cleavage activity is released into the medium. The demonstration of CCBE1 enhancement in conditioned cell culture supernatants required carefully titrated amounts of ADAMTS3, pro–VEGF-C, and CCBE1, whereas the CCBE1 enhancement during the short VEGFR-3 phosphorylation period of 10 minutes was robust. Endothelial cells express ADAMTS3,[36] most of which likely remains cell surface–associated because of its thrombospondin motif, which contains the high-affinity SVTCG binding site for CD36.[37]

We propose the model of VEGFR-3 activation shown in Figure 7. First, CCBE1 enables pro–VEGF-C binding to VEGFR-3. After binding, pro–VEGF-C becomes a substrate for proteases such as ADAMTS3, and the resulting in situ–generated mature VEGF-C initiates signaling. Such in situ activation of pro–VEGF-C could contribute to the lack of blood vascular effects of VEGF-C in some in vivo models,[38] despite the ability of mature VEGF-C to activate VEGFR-2. The generation of mature VEGF-C also occurred in the culture medium, albeit much less efficiently. This could explain the modest angiogenesis that accompanied the prominent lymphangiogenic effect in vivo.[39,40] Alternatively,

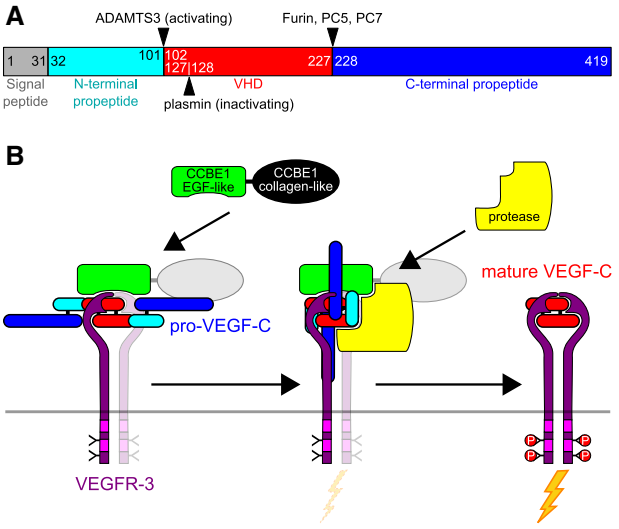


Figure 7. Schematic view of vascular endothelial growth factor (VEGF)-C and VEGF receptor (R)-3 activation by VEGF-C. **A**, Domain organization of VEGF-C and the defined cleavage sites. **B**, Pro–VEGF-C binding to VEGFR-3 is assisted by the N-terminal domain of collagen- and calcium-binding epidermal growth factor domains 1 (CCBE1). Pro–VEGF-C is then proteolytically processed in situ, and the mature VEGF-C activates VEGFR-3. Note that the transparently illustrated elements are hypothetical: VEGFR-3 could be either monomeric or dimeric during the initial binding of VEGF-C, and it is not known whether the removal of the C-terminal domain of CCBE1 is required for the CCBE1 function.

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in some instances, the angiogenic effect may be mediated via VEGFR-3.[41]

CCBE1 expression is spatially and temporally correlated with the migration routes of endothelial cells that bud from the cardinal veins.[3,12] We could detect low amounts of CCBE1 in most cultured cell lines tested. Perhaps matrix association of CCBE1 via vitronectin[11] could lead to high local CCBE1 concentrations, focusing ADAMTS3 activity to areas where VEGF-C activity is needed, for example, at sites where nascent lymphatic endothelial cells emigrate from the venous compartment. This resembles the concentration of plasminogen activator activity by vitronectin to cell surfaces and the extracellular matrix by binding to the urokinase-type plasminogen activator/soluble urokinase-type plasminogen activator receptor complex.[42]

VEGF-D, which is the closest homolog of VEGF-C,[43,44] was not cleaved by ADAMTS3, and CCBE1 did not have any effect on its activation. Alignment of VEGF-C and VEGF-D orthologs reveals that both contain multiple potential plasmin cleavage sites in the linker connecting the N-terminal propeptide with the VEGF homology domain (Figure VIC in the online-only Data Supplement). Preferential cleavage at one site over the other might explain why limited exposure to plasmin activates VEGF-C, whereas longer exposure results in VEGF-C inactivation. The net charge of the polypeptide segment between the first potential plasmin cleavage site and the ADAMTS3 cleavage site in VEGF-C is very different from that in VEGF-D. This could explain the differential action of both plasmin and ADAMTS3 on these substrates despite similar cleavage motifs.

The cleavage motif of ADAMTS3 in VEGF-C is the same as the ADAMTS2 motif in procollagens (FA[AP]↓),[45] which have been until now the only known substrates of ADAMTS3.[46] This motif is also found in bone morphogenetic protein-2 and pleiotrophin. ADAMTS3 cleavage likely requires additional interactions, because exogenously added C-terminal VEGF-C propeptide and, to a lesser extent, also the N-terminal propeptide were able to compete with the cleavage. Surprisingly, a CCBE1 cleavage product appeared in the supernatants of ADAMTS3-transfected cells, although CCBE1 lacks the FA[AP]↓ motif. The cleavage of CCBE1 into 2 separate domains may be a prerequisite for its activity, because the inability of the conditioned medium of the DU-4475 cell line to activate pro–VEGF-C was associated with a lack of CCBE1 cleavage products.

In addition to the cleavage by ADAMTS3, CCBE1 also accelerated the release of VEGF-C. Although the C-terminal cleavage is not a prerequisite for secretion,[26] the presence of the C-terminal propeptide slowed VEGF-C release compared with truncated VEGF-C forms lacking this propeptide in metabolic labeling pulse-chase experiments, indicating that facilitation of the C-terminal processing may be responsible for the enhanced release. However, the relevance of this finding is not yet clear, because it is uncertain whether cells that express both CCBE1 and VEGF-C exist in vivo.

On the basis of our findings, the lymphatic vessel defects seen in animal models lacking CCBE1 can be explained, because CCBE1 appears essential for VEGF-C activation. Furthermore, a decrease of CCBE1 expression together with other lymphangiogenic genes in the postnatal period in some tissues (Jeltsch et al, unpublished data, 2014) and potential additional substrates of ADAMTS3 suggest that CCBE1 also has lymphangiogenesis-independent roles, which may explain some of the other features of Hennekam lymphangiectasia–lymphedema syndrome.[8,47] Finally, as shown by the in vivo data, CCBE1 may offer a useful target for the modulation of VEGF-C activity, which could be used for therapeutic stimulation and inhibition of lymphangiogenesis and perhaps also angiogenesis.[48]

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Disclosures

Dr Alitalo has been a consultant for Laurantis Pharma Oy. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

There has been substantial progress in the understanding of the hereditary lymphedemas. However, the molecular mechanism is not always obvious, even when the genetic lesion has been identified. In 2009, Alders et al established the link between mutations in the *CCBE1* gene and Hennekam lymphangiectasia–lymphedema syndrome, a human hereditary condition with lymphedema as a characteristic feature. Later, genetic experiments in zebrafish and mice indicated that the *CCBE1* gene interacts with the lymphangiogenic vascular endothelial growth factor (VEGF)-C receptor-3 pathway, but the nature of the interaction remained elusive. In this article, Jeltsch et al show that the activity of VEGF-C is regulated by collagen- and calcium-binding epidermal growth factor domains 1 (CCBE1), which facilitates the proteolytic activation of a latent "pro" form of VEGF-C by the A disintegrin and metalloprotease with thrombospondin motifs-3 (ADAMTS3) metalloproteinase via a novel mode of growth factor activation. The in vivo data in the article show that CCBE1 is a potential therapeutic tool for the modulation of lymphangiogenesis and angiogenesis in a variety of diseases that involve the lymphatic system, such as lymphedema or lymphatic metastasis. In particular, application of VEGF-C has been scheduled for clinical trials to improve the incorporation of lymph node transplants into the lymphatic vascular system after mastectomy and axillary lymph node surgery. CCBE1 is a powerful activator of VEGF-C that can facilitate therapeutic lymphangiogenesis. CCBE1 and ADAMTS3 could also provide new targets for inhibition of tumor angiogenesis, lymphangiogenesis, and metastasis.

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CCBE1 Enhances Lymphangiogenesis via A Disintegrin and Metalloprotease With Thrombospondin Motifs-3–Mediated Vascular Endothelial Growth Factor-C Activation

Michael Jeltsch, Sawan Kumar Jha, Denis Tvorogov, Andrey Anisimov, Veli-Matti Leppänen, Tanja Holopainen, Riikka Kivelä, Sagrario Ortega, Terhi Kärpanen and Kari Alitalo

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Supplemental Material

Supplemental Methods

Cloning. Genes expressed via the recombinant adeno-associated virus (AAV9) vector were cloned into the psubCAG-WPRE plasmid[1], which is a derivative of psubCMV-WPRE, where the CMV promoter has been replaced with the composite CAG promoter (consisting of the chicken β-actin promoter, cytomegalovirus enhancer and β-actin intron[2]). Cloning of full-length mVEGF-C, ΔNΔC-mVEGF-C and HSA into AAV-vector (psubCAG-WPRE) was described earlier[3]. mCCBE1, fused to a V5 tag (mCCBE1-V5), was cloned as follows: A partial CCBE1 coding DNA sequence (CDS; Genebank #BC152322, Image clone ID 40140631) was inserted as a SacI/XbaI fragment into pVK1 (a pUC19-derived vector[4]). The missing nucleotides were amplified from brown adipose tissue mRNA with primers 5'-GCCGCTAGCGCCACCATGGTGCCGCCGCCT-3' and 5'-GGAGCTTGGGCACAAATGTC-3' and the CDS was completed by inserting the NheI/SacI fragment resulting in vector pVK1-CCBE1. A PCR-amplified V5-tag (obtained with primers 5'-ACCAGGAGCACCAGGAAGAC-3' and 5'-GCCTCTAGAACGCGTCTAGGTGCTGTCCAGGCCCAGCAGAGGGTTAGGGATAGG-CTTGCCTGGATAAAAATTTCTTGGGG-3') was added to the CDS as an Eco81I/XbaI fragment. From the resulting vector, the complete CDS was excised as an MluI fragment and cloned into psubCAG-WPRE.

For the *in vitro* studies, a similar vector was constructed, in which the mouse CCBE1 CDS was replaced by the human CCBE1 CDS (Genebank #NM_133459). For the co-immunoprecipitation study and the protein purification, the sequence coding for a StrepIII-tag[5], an internal ribosomal entry site and the CDS for enhanced green fluorescent protein were inserted immediately behind the CCBE1 CDS and the complete CDS was transferred

into the pMX vector[6] resulting in pMX-hCCBE1-StrepIII-IRES-EGFP. The mammalian expression constructs for VEGF-C and ΔC-VEGF-C have been described[7]. The chimeric VEGF-C/VEGF-D (CDC) expression constructs were assembled by overlapping PCR mutagenesis into the pMosaic vector[8]. The insert comprised sequences coding for amino acid residues Phe32-Ala111 and Ser228-Ser419 from human VEGF-C and intervening residues Thr92-Arg206 from human VEGF-D (version 1), and amino acid residues Phe32-Leu119 and Ser228-Ser419 from human VEGF-C and intervening residues Lys100-Arg206 from human VEGF-D (version 2).

cDNA clones for the ADAMTS constructs were obtained from the Mammalian Gene Collection. ADAMTS1 (GenBank BC040382) and ADAMTS2 (GenBank BC046456) were expression-ready (pCMV-Sport6); the ADAMTS3 cDNA clone (pCR-XL-TOPO, GenBank BC130287) was subcloned as an EcoRI fragment into the expression vector pAc5.1/V5His (Invitrogen) and pCI-neo (Promega), thus adding sequences encoding for a C-terminal V5 and hexahistidine tag. The ADAMTS14 cDNA clone (pENTR223.1, GenBank BC140263) was transferred into the expression vector pEF-DEST51 using the Gateway recombination system.

The constructs for recombinant VEGF-C expression in insect cells cells employed the pMT-Ex vector (a modified version of pMT-BiP-V5His-C[9]) and comprised sequences coding for the signal peptide of the Drosophila BiP, for amino acid residues Phe32-Ala111 for the N-terminal propeptide, Ser228-Ser419 for the C-terminal propeptide and Thr112-Arg227 for ΔNΔC-VEGF-C, followed by sequences coding for a hexahistidine tag. The construct for the expression of full-length VEGF-D comprised sequences coding for amino acid residues 22-354 in the same context. The construct for the expression of histidine-tagged human serum

albumin comprised sequences coding for the signal peptide of the Drosophila BiP and sequences coding for amino acid residues 25-609 from human serum albumin flanked both N- and C-terminally by a hexahistidine tag.

The construct for the expression of VEGF-C from its full-length cDNA was based on pFastBac1 (Invitrogen). In this construct the CDS of VEGF-C was modified by swapping the sequences coding for its endogenous signal peptide against sequences coding for the melittin signal peptide and by adding sequences coding for a C-terminal hexahistidine tag.

For the expression of the N-terminal domain of CCBE1 (CCBE1Δ175), residues 1-175 of human CCBE1 were cloned into the pFastBac (Invitrogen) baculovirus expression vector and equipped with a C-terminal Factor Xa cleavage site (amino acid residues IEGR) and a hexahistidine tag.

Antibodies. Anti-VEGF-C antiserum[10], anti-VEGF-C antibodies (R&D Systems, Minneapolis, MN, AF752), anti-V5 antibody (Invitrogen, Carlsbad, CA, #46-0705), anti-phosphotyrosine antibody 4G10 (Merck Millipore, Billerica, MA) and PY20 (BD Transduction Laboratories, Franklin Lakes, NJ), and anti-CCBE1 antibodies (Atlas Antibodies AB, Stockholm, Sweden, #HPA041374) were used for both immunoprecipitation and Western blotting. Anti-VEGF-D antibody VD1[11], anti-VEGFR-3 antibodies (Santa Cruz, Dallas, TX, sc-321), chimeric VEGFR-3/IgGFc[12] or streptactin sepharose (IBA, Göttingen, Germany) were used for immunoprecipitation. Anti-VEGF-D antibody (R&D Systems, AF286), anti-phospho-Erk1/2 antibody (Cell Signaling Technology, Inc., Danvers, MA, #9101), anti-phospho-Akt antibody (Cell Signaling Technology, Inc., #9271), anti-phospho-eNOS (BD Transduction Laboratories, #612392), streptactin-HRP conjugate (IBA and R&D

systems, #890803) were used for Western blots. The hF4-3C5 antibody used to block VEGFR-3 activation was generously provided by ImClone Systems/Eli Lilly[13].

Cell culture and generation of stable cell lines. 293T, 293F, 293GPG, 293S GnTI$^-$, PAE-VEGFR-3[14], PAE-VEGFR-3/neuropilin-2[9] and NIH-3T3 cells were grown in D-MEM 10% FCS. PC-3 cells were grown in Ham's F-12 10% FCS, DU-4475 cells in RPMI 1640 20% FCS, CHO cells in α-MEM 10% FCS or EX-CELL ACF CHO Medium and S2 cells in HyClone SFX-Insect (Thermo Scientific, Waltham, MA) or Insect-Xpress (Lonza Group, Basel, Switzerland). 293T cells were transfected with retrovirus vector, that had been produced using standard methods in 293GPG cells using the pMX-hCCBE1-StrepIII-IRES-EGFP construct. The cells were cultured for one week and sorted once with FACS for EGFP. 293F cells were transfected with the expression plasmid pCI-neo-hADAMTS3-V5-H6, selected with G418 and clonal lines were established by the ring cloning technique. Stable expression of CCBE1 and ADAMTS3 was confirmed by Western blotting. LECs, BECs and HUVECs were purchased from Promocell (Heidelberg, Germany) and maintained according to the instructions of the supplier. The Ba/F3-hVEGFR-2/EpoR cell line was generated similarly to the Ba/F3-mVEGFR-2/EpoR[15]; however, pCI-neo was used instead of pEF-BOS. The junctional amino acid sequences of the chimera were ...FFIIEGAQEKTNLEGS (end of VEGFR-2 part) – (start of mEpoR part) LILTLSLILVLISLLLTVLALLSHRRTLQQKIWPGIPSPESEFE... This chimeric construct was electroporated with one 30 ms 1400V pulse (Neon transfection device, Invitrogen) into the Ba/F3 cells. Cells were grown in medium containing 2 ng/ml mIL-3 for 36 hours, after which they were split and selection was started for three weeks with 1.2 mg/ml G418. Cells were maintained with sub-optimal mIL-3 concentration (0.4 ng/ml) and optimal VEGF-A and

VEGF-C concentrations (200 and 300 ng/ml, respectively).

Protein expression and purification. S2 cells were transfected using Effectene (Qiagen, Venlo, The Netherlands). Stable cell pools were selected for 3 weeks with 400 µg/ml hygromycin starting 2 days after transfection. For protein production, the cells were adapted to suspension culture and induced for 4-5 days with 1 mM $CuSO_4$. After batch-binding to Ni^{2+}NTA sepharose from the pH-adjusted conditioned supernatant, the Ni^{2+}NTA sepharose was loaded onto a column, washed with 20 mM imidazole, and eluted with a step-gradient of 250 mM imidazole. The protein was further size-separated on a Superdex 200 column with PBS as a running buffer.

Recombinant baculovirus was produced using the FactBac system (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Sf9 cells were infected and conditioned medium harvested 4 days post infection. Protein purification was performed as above. CCBE1Δ175 was purified in Hepes-buffered saline supplemented with 2 mM $CaCl_2$.

CCBE1 was enriched from 200 ml conditioned medium of 293T cells expressing StrepIII-tagged CCBE1 using streptactin sepharose (IBA) according to the instructions of the manufacturer, and the eluted peak fraction was dialyzed against TBS. Histidine-tagged ADAMTS3 was purified from 1.5 litres of conditioned medium of stably transfected 293F cells (D-MEM supplemented with 2%FCS, 30µM $ZnCl_2$ and 0.1U heparin/ml). The collected medium was dialyzed against phosphate buffered saline, pH-adjusted to 8.0 and batch-bound protein was eluted with a step-gradient of 250 mM imidazole from the Ni^{2+}NTA sepharose (IBA) after washing with 20 mM imidazole and dialyzed against 1xTBS. Biotinylated ΔNΔC-VEGF-C and pro-VEGF-C were generated from purified proteins using sulfosuccinimidyl-6-

[biotin-amido]hexanoate (Thermo Scientific, #21335), according to the instructions of the supplier.

Cleavage of pro-VEGF-C and pro-VEGF-D. The indicated amounts of plasmin (Sigma-Aldrich, St. Louis, MO, P1867) were incubated for 18 hours with 2.5 µg of pro-VEGF-C. 1.15 µg of pro-VEGF-C was incubated in TBS, as indicated, with 4.15 µg 293T-cell-derived ADAMTS3 or 15 µg of S2-cell-derived ADAMTS3. 1.7 µg of pro-VEGF-D was incubated with 7.5 µg recombinant 293T-cell-derived ADAMTS3 in TBS for 30 hours with or without 10 µl recombinant CCBE1. Analysis of the cleavage was performed by SDS-PAGE/Western. All incubations were at 37°C and recombinant CCBE1 was included, as indicated, at a concentration of approximately 2.5 µg/ml.

VEGF-C/CCBE1 co-immunoprecipitation. 293T cells were transfected with either CCBE1-StrepIII or VEGF-C constructs. Conditioned media were used in a streptactin pull-down analysis either separately or as a mix of CCBE1 and VEGF-C. The mixed media were incubated for 10 min at RT before being applied to the pull-down. The precipitates were analyzed with anti-CCBE1 antibody and anti-VEGF-C antiserum after SDS-PAGE and Western blotting. Input represents 25 µl of VEGF-C conditioned medium, which was loaded as a positive control.

ADAMTS3/CCEB1 co-immunoprecipitation. 293T-CCBE1-StrepIII cells were transfected with ADAMTS3 or mock expression constructs. Conditioned media were immunoprecipitated with ADAMTS3 antibodies (Santa Cruz, sc-21486) and protein G sepharose or used in a streptactin pull-down. Precipitates were detected with streptactin-HRP conjugate after SDS-PAGE and Western blotting.

Mass spectroscopy. Enriched CCBE1 protein was subjected to liquid chromatography-mass spectrometry as previously described[16]. In short, mass spectrometry analysis was performed on an Orbitrap Elite ETD mass spectrometer (Thermo Scientific) using the Xcalibur version 2.7.1 coupled to a nLCII nanoflow system (Thermo Scientific) via a nanoelectrospray ion source. Peak extraction and subsequent protein identification was achieved using Proteome Discoverer software (Thermo Scientific). Calibrated peak files were searched against human protein databases by the SEQUEST search engine. Database searches were limited to tryptic peptides with a maximum of 1 missed cleavage; carbamidomethyl cysteine and methionine oxidation were set as fixed and variable modifications, respectively.

Pulse chase. 293T cells were transfected and grown for 36 hours on 6-cm dishes to near confluency. Cells were starved for 30 min in met-/cys-deficient D-MEM, 5% dialyzed FCS, after which cells were metabolically labeled for 2 hours with [^{35}S]-cysteine/[^{35}S]-methionine. Thereafter, cells were washed with warm PBS and 5 ml of chase medium was added (D-MEM, 10% FCS + 2 mM cold L-methionine + 2mM cold L-Cysteine). At the indicated time points, the dishes were placed on ice and the medium was removed for analysis.

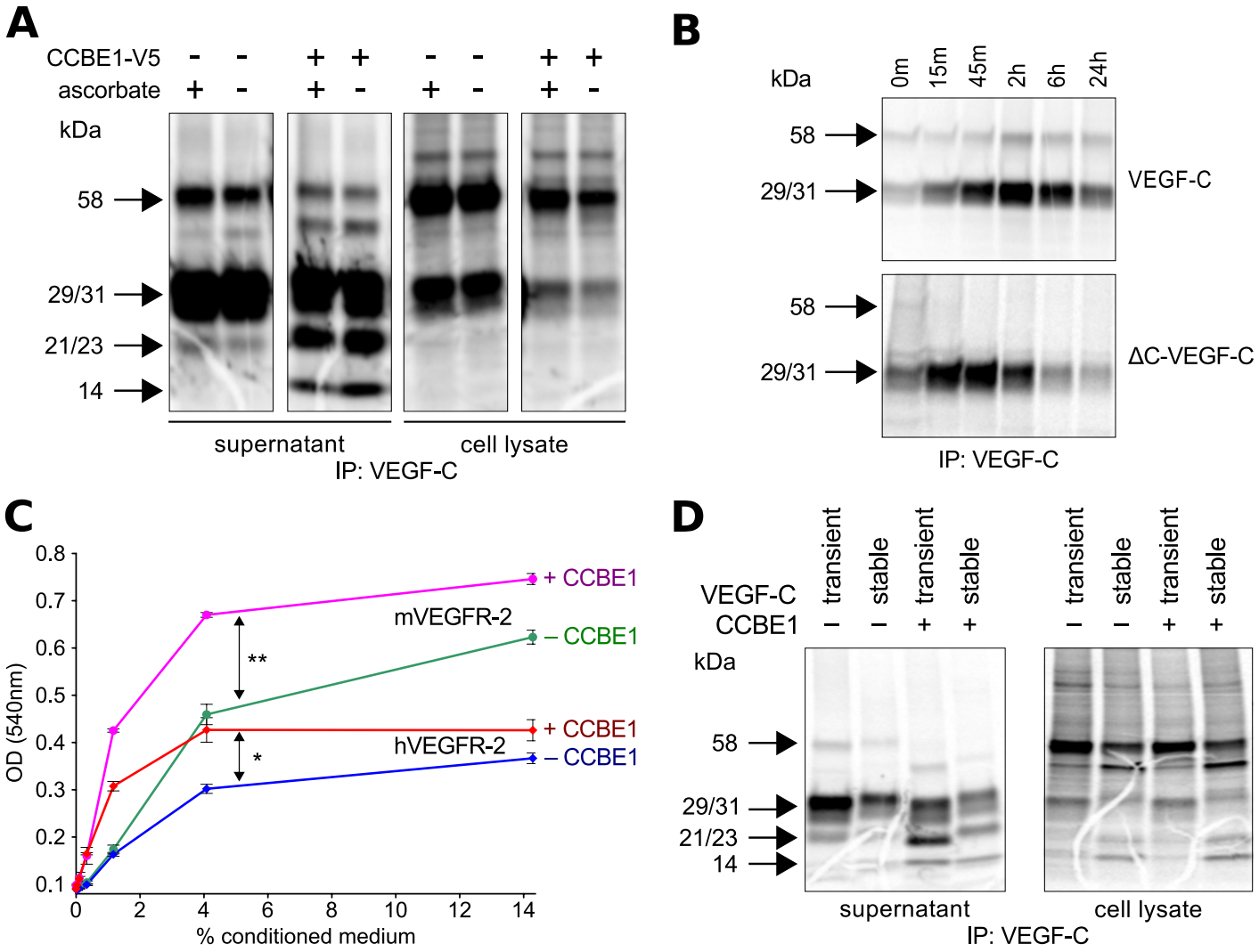
Competition of VEGF-C cleavage by VEGF-C propeptides. Purified histidine-tagged proteins were included in the labeling medium of the VEGF-C/CCBE1-cotransfected 293T cells at a concentration of 25 µg/ml. The labeling medium was conditioned for 30-72 hours after transfection, depleted from histidine-tagged proteins with Ni^{2+}NTA sepharose, and VEGF-C was immunoprecipitated, separated by PAGE and visualized by exposure to X-ray film. Inhibition of the N-terminal cleavage of VEGF-C was measured by quantitating the 14 kDa N-terminal cleavage product from the laser-scanner read-out.

Quantitative PCR for ADAMTS2, -3 and -14. Total RNA was isolated from the cells with NucleoSpin RNA II kit according to the protocol (Macherey-Nagel, Düren, Germany) and cDNA synthesis was performed using iScript cDNA synthase kit (Bio-Rad; Hercules, CA). QPCR was carried out using SYBR green chemistry with Bio-Rad CFX96 Real-Time System. All data were normalized to GAPDH and quantification was performed using the 2-DDCT method. The following primers were used: hADAMTS2 (fwd 5'-AAATCTACCATGACGAGTCC -3', rev 5'- TCATGGACTTTCCATAGCTC -3'), hADAMTS3 (fwd 5'- CCATTCCTATGACTGTCTCC -3', rev 5'-CCAACACCAAAATCAAAACG -3'), hADAMTS14 (fwd 5'-CAACTACTCAATGGATGAGC -3', rev 5'- AAGGTCCTGAATGCCAAG -3') hGAPDH (fwd 5'- CCACTAGGCGCTCACTGTTC -3', rev 5'- CCCCATACGACTGCAAAGAC -3'), mADAMTS2 (fwd 5'- ACTACAACATTGAGGTCCTG -3', rev 5'-TCATGGTAGATTTCGTTGAC -3'), mADAMTS3 (fwd 5'-CGATACATCCATTCCTATGAC -3', rev 5'- GTACACATCTTGTAGCCAAC -3') , mADAMTS14 (fwd 5'- TAGCTTTCAGGACCTTTGAG -3', rev 5'-CTTGGTCTTGCAGAAGTATG -3') m/chGAPDH (fwd 5'-ACAACTTTGGCATTGTGGAA -3', rev 5'- GATGCAGGGATGATGTTCTG -3'), chADAMTS2 (fwd 5'- GGAGCTTGGCCGATACCTAC -3', rev 5'-TGTTTGCAGGGGTCAAAGGT -3'), chADAMTS3 (fwd 5'-GGTCTCCATACCACCCTCCT -3', rev 5'- GGCTGACCTGACTGGATGTC -3') , chADAMTS14(fwd 5'- GGCTGACAGAGGGAAGTGTC -3', rev 5'-GGATTCCTGCAAAGGTTGCG -3').

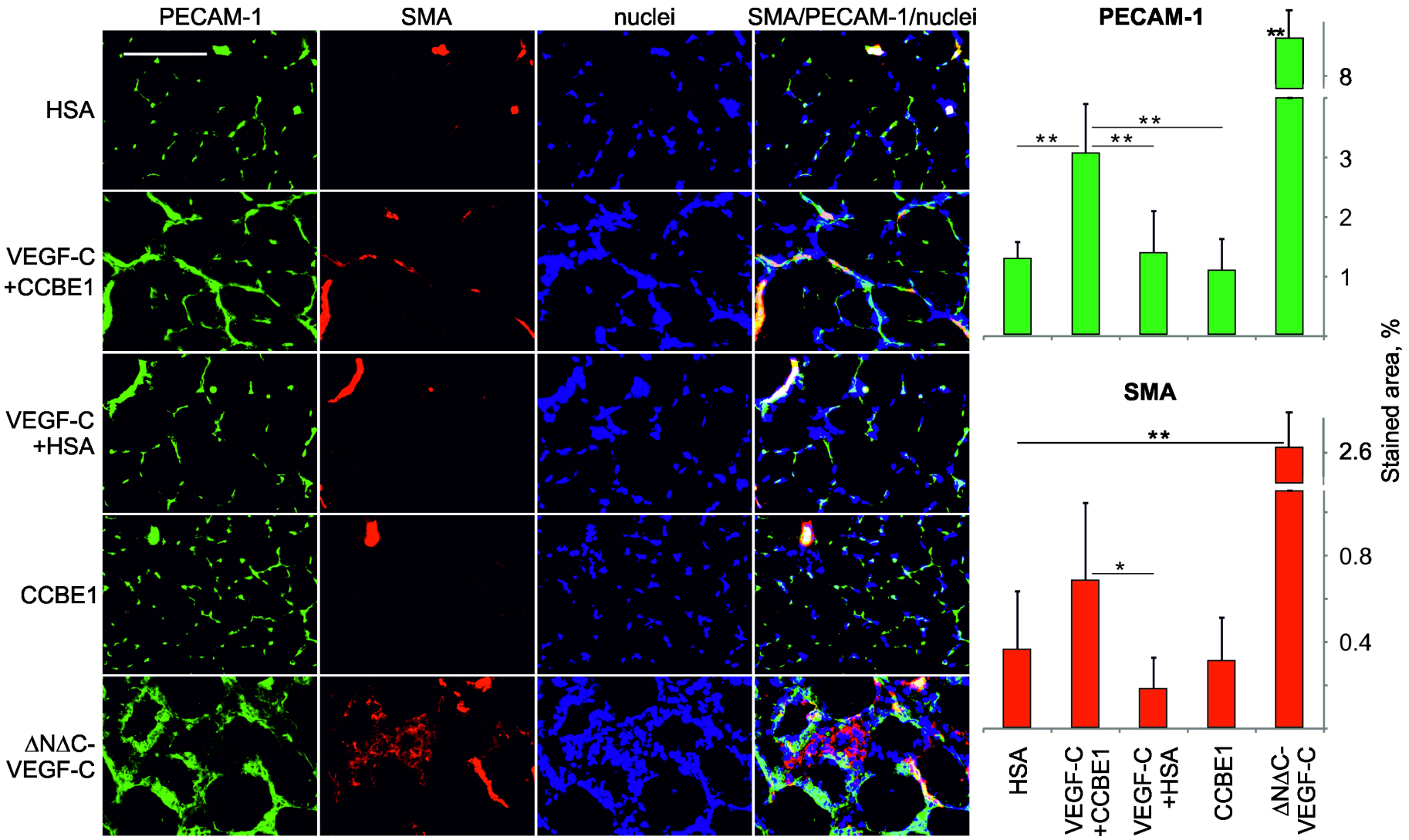
Downregulation of ADAMTS3 by lentiviral shRNA. Constructs expressing ADAMTS3

shRNA (TRCN0000050571) and non-target control shRNA were from the RNAi Consortium shRNA library[17]. Lentivirus, generated by using the standard methods according to the RNAi Consortium, was used to transduce 293T cells. After selection of stable pools, the efficiency of VEGF-C cleavage was assayed as described in the *Materials and methods* section under *Transfection, metabolic labeling and protein analysis*.

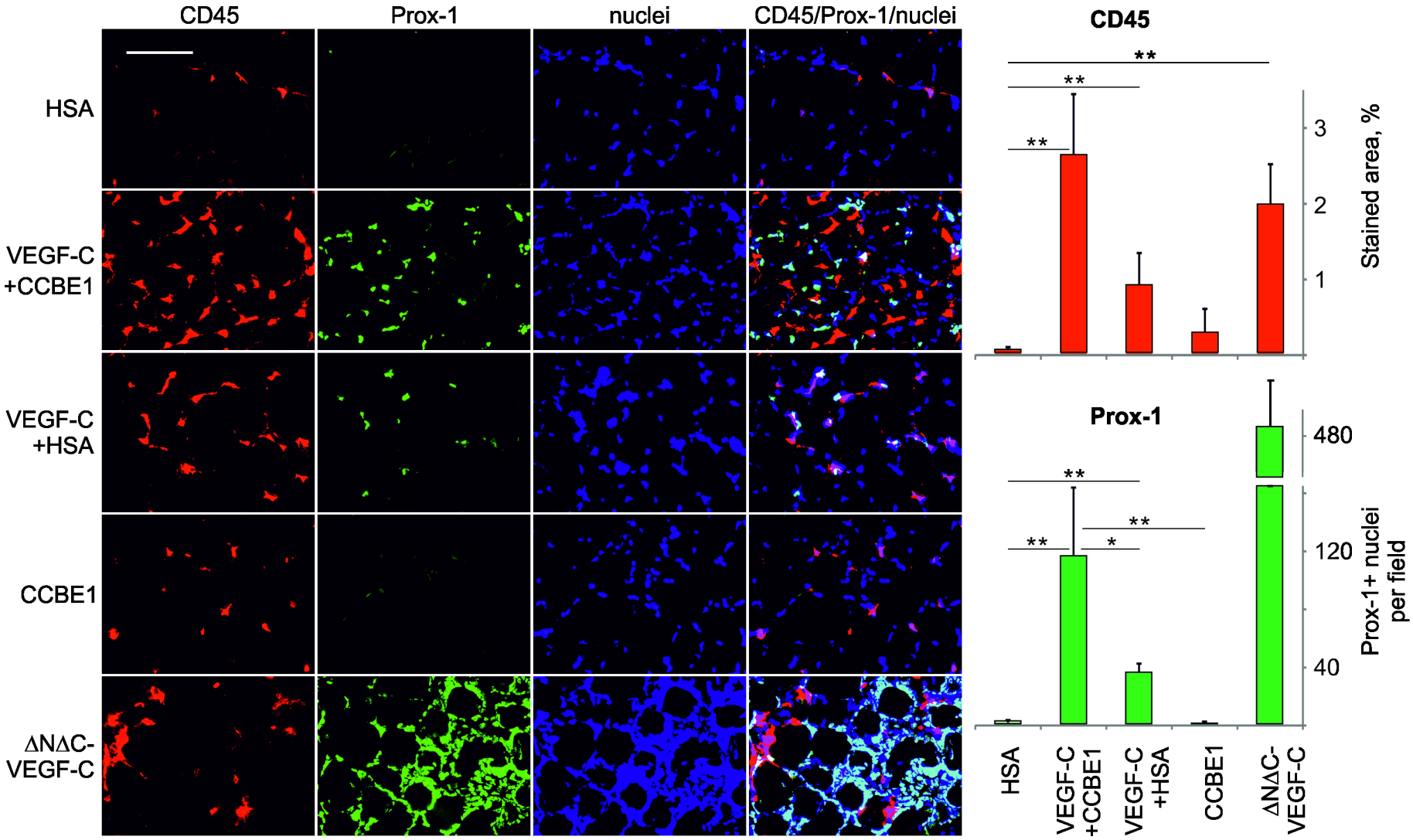
Supplemental Figures



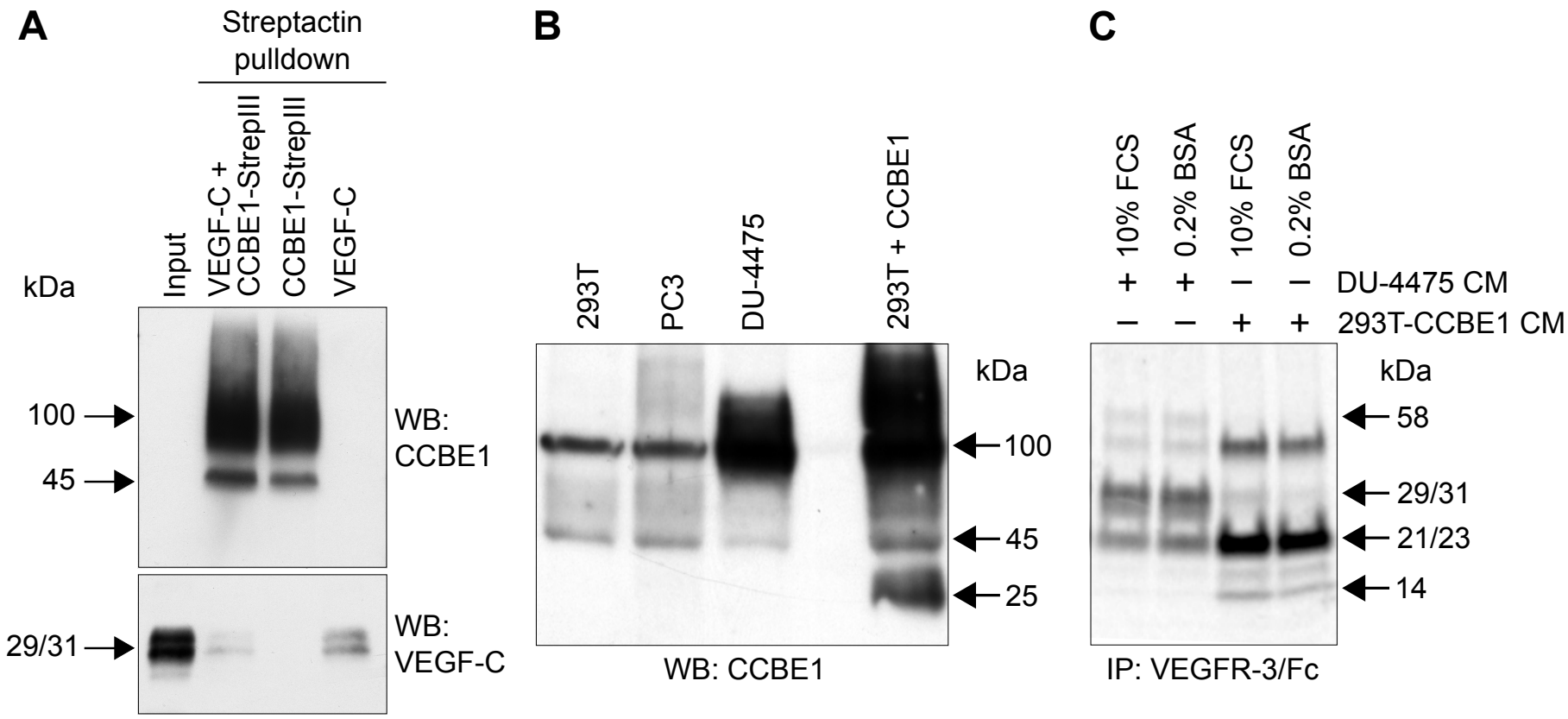
Jeltsch et al., Fig. S1



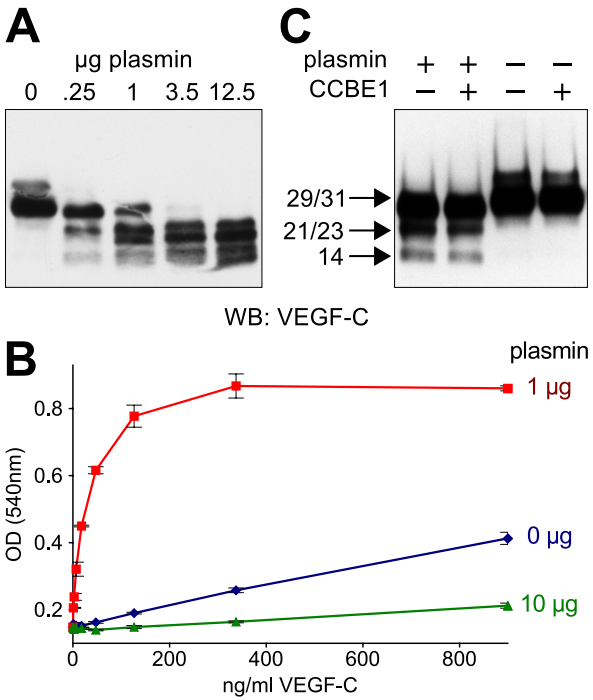
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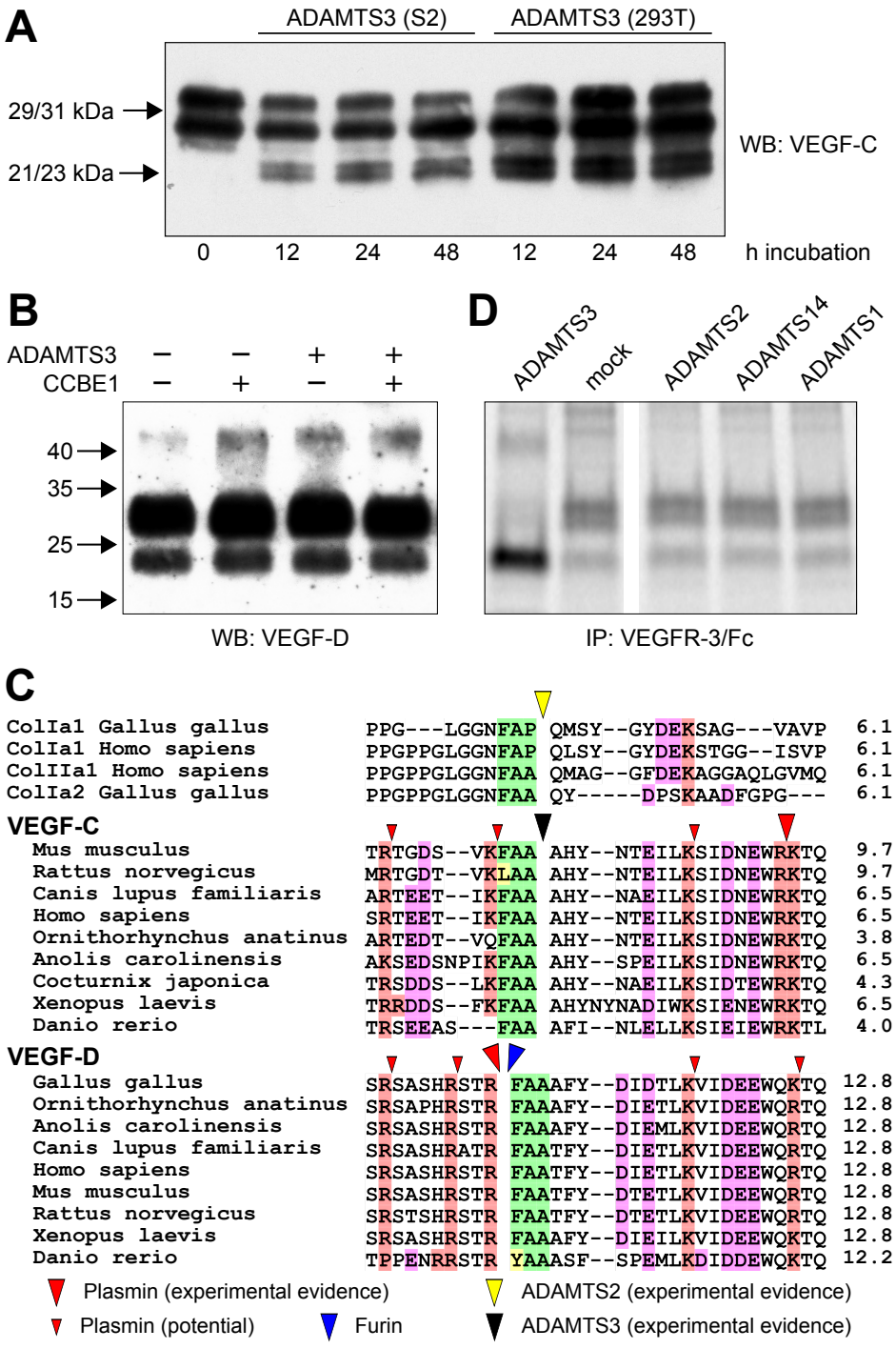
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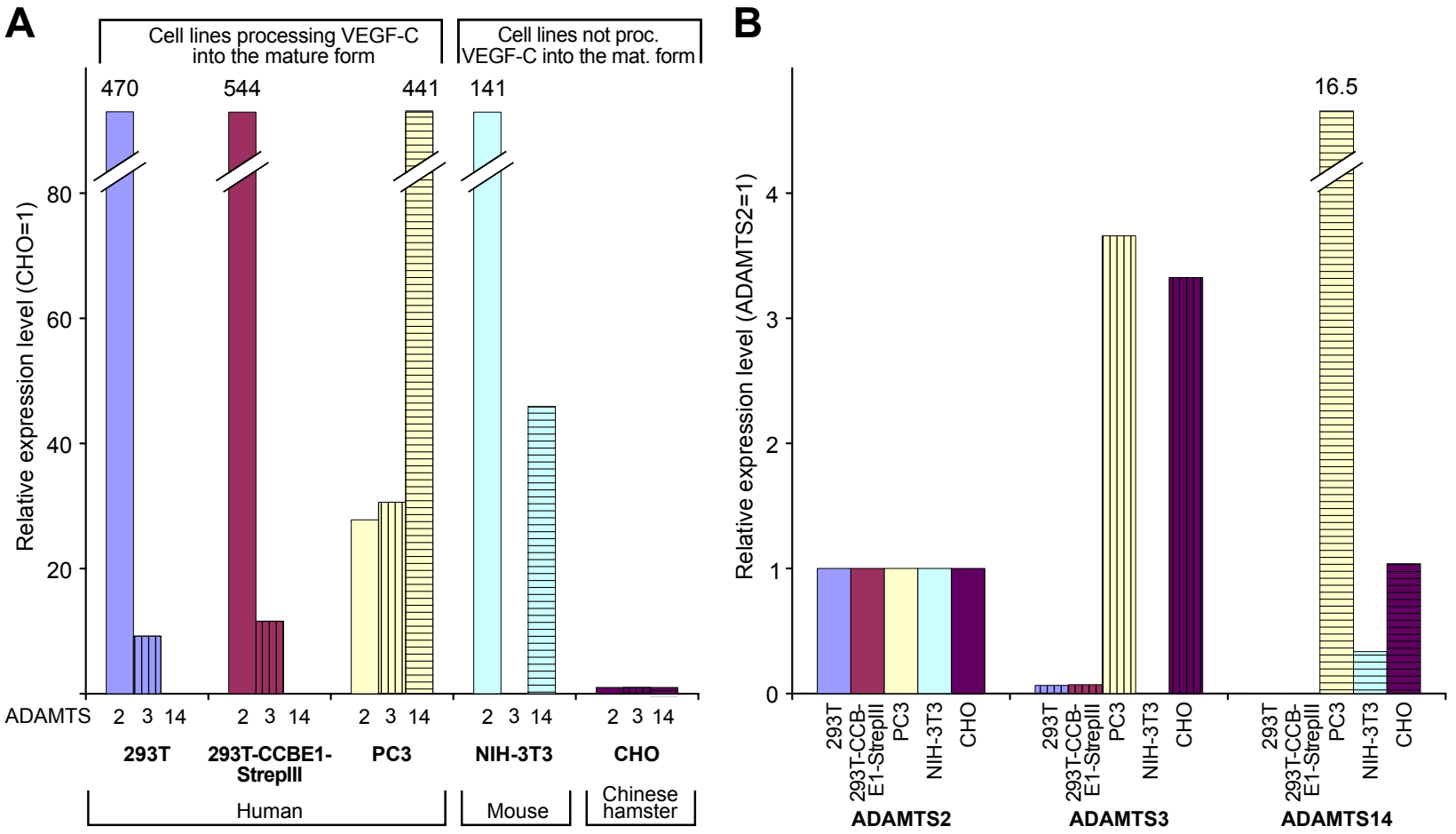
Jeltsch et al., Fig. S4



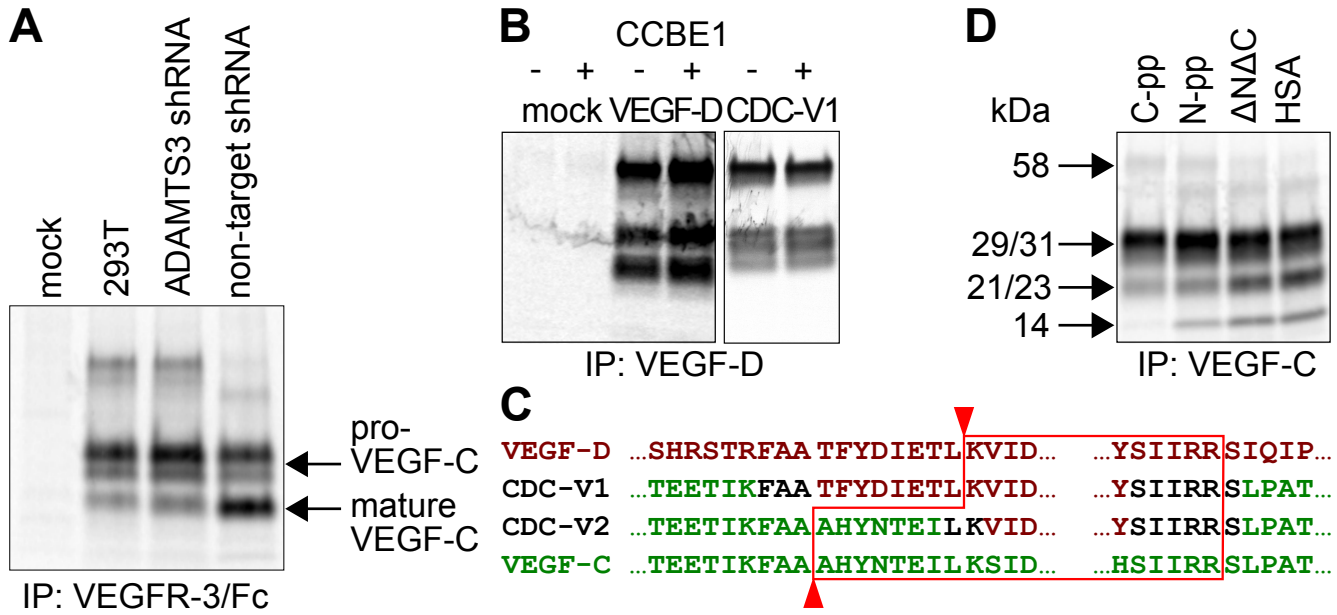
Jeltsch et al., Fig. S5



Jeltsch et al., Fig. S6



Jeltsch et al., Fig. S7



Jeltsch et al., Fig. S8

Supplemental Figure Legends

Supplemental Figure 1. CCBE1 enhances VEGF-C release and cleavage. (A) The figure shows VEGF-C immunoprecipitation from supernatants and lysates of cells transfected with VEGF-C with and without CCBE1, as shown. The molecular weights on the left show the mobility of the major VEGF-C forms. Note that the amount of the mature 21 kDa form of VEGF-C increases (8-fold) in the supernatant and the amounts of the uncleaved VEGF-C and pro-VEGF-C are reduced (by 74% and 51%, correspondingly) upon CCBE1 cotransfection (compare lanes 1, 2 to 3, 4). The 14 kDa fragment resulting from the N-terminal cleavage of VEGF-C appears only in the supernatants of the cotransfected cells. Cotransfection with CCBE1 seems to facilitate the release of VEGF-C as the cell layer associated VEGF-C polypeptides are reduced by 80% in the cell lysates of the CCBE1-cotransfected cells (compare lanes 5, 6 to 7, 8). (B) In a metabolic labeling-pulse-chase analysis, wild-type VEGF-C secretion peaks at 2h, whereas a VEGF-C mutant without the C-terminal propeptide (ΔC-VEGF-C) peaks already between 15 and 45 minutes. (C) Conditioned medium from cultures expressing both CCBE1 and VEGF-C has increased activity in promoting the growth of Ba/F3 cells expressing mouse (m) or human (h) VEGFR-2/EpoR chimeras compared to medium from cultures expressing only VEGF-C. The curves were statistically different from each other at all shown data points except for x=0 ($P<0.05$ marked with * and at $P<0.01$ marked with **; n = 3). (D) Separate cell populations were transfected with VEGF-C or CCBE1 and then mixed for the metabolic labeling period, as indicated. Note also a small difference in the migration of VEGF-C between the stably and transiently transfected cells, resulting from the different glycosylation pattern of VEGF-C produced by the stably transfected 293S GnTI$^-$ cells[18].

Supplemental Figure 2. CCBE1 co-transduction with VEGF-C stimulates angiogenesis. Immunohistochemistry for endothelial (PECAM-1) and smooth muscle cell (SMA) markers in *tibialis anterior* muscles. Quantification of the stained areas is shown on the right. Statistically significant differences of $P<0.05$ are marked with *, of $P<0.01$ with ** and of $P<0.001$ with ***; $n \geq 5$.

Supplemental Figure 3. Recruitment of CD45+ leukocytes and lymphatic vessels by CCBE1/VEGF-C co-transduction. Prox1 transcription factor was used as the marker for lymphatic endothelial cells. Analysis was done as in the experiments described in Supplemental Figure 2.

Supplemental Figure 4. CCBE1 and VEGF-C do not interact in a stable manner. CCBE1 is produced by 293T, PC3 and DU-4475 cell lines, but DU-4475-produced CCBE1 does not promote VEGF-C cleavage. (A) Media conditioned with StrepIII-tagged CCBE1 and VEGF-C were mixed, but no retention of VEGF-C could be detected on the streptactin column (second lane). The faint signal results from non-specific binding of VEGF-C to the column (compare to the fourth lane, for which only media conditioned with VEGF-C was applied to the column). (B) The ConA-bound fraction of conditioned cell culture medium was analyzed by SDS-PAGE/Western blotting. Note that the 293T and PC3 cell lines express only small amounts of endogenous CCBE1, whereas the DU-4475 cells express large amounts. Two species of CCBE1 can be detected in the supernatant, migrating at about 45 and 100 kDa, respectively. In the transfected 293T cells, a short CCBE1 form of 25 kDa is observed. (C) Addition of conditioned medium (CM) from CCBE1-producing 293T cells to VEGF-C-producing cells promotes cleavage of VEGF-C, while CM from DU-4475 cells does not. This effect was observed whether serum-containing (10% FCS) or

serum-free (0.2% BSA) medium was used for the conditioning.

Supplemental Figure 5. Plasmin cleavage of VEGF-C and the activity of the cleavage products in the Ba/F3-VEGFR-3/EpoR assay. (A) Pro-VEGF-C is cleaved by plasmin. (B) While low amounts of plasmin resulted in a strong activation of VEGF-C, high amounts led to inactivation of VEGF-C. (C) Cleavage of VEGF-C by plasmin is not enhanced by CCBE1.

Supplemental Figure 6. Potential cleavage of VEGF-D by ADAMTS3, and VEGF-C by other ADAMTS family members. (A) VEGF-C is cleaved by recombinant ADAMTS3. (B) VEGF-D was incubated with or without ADAMTS3, but no difference in the ratio between mature VEGF-D and the pro-VEGF-D form was observed. The presence of CCBE1 had no effect. (C) Alignment of the cleavage context for plasmin and ADAMTS2/3 substrates. The cleavage contexts of the four known procollagen targets of ADAMTS2 were aligned with VEGF-C and VEGF-D sequences. The ADAMTS2 cleavage sites are indicated by a yellow triangle, the ADAMTS3 cleavage site by a black triangle, plasmin cleavage sites are indicated by red triangles and the furin cleavage site by a blue triangle. The numbers denote the theoretical isoelectric point of the stretch comprising the 10 amino acid residues N-terminal to the ADAMTS3 cleavage site. Background color-coding for the amino residues: magenta, acidic; red, basic; green, ADAMTS2/3 cleavage motif; yellow, exceptions to the cleavage motif. (D) Transfection of ADAMTS1 and the procollagenase-type ADAMTS2 and ADAMTS14 into VEGF-C expressing 293F cells does not result in increased generation of mature VEGF-C.

Supplemental Figure 7. Comparison of expression levels of ADAMTS2, ADAMTS3 and ADAMTS14 by quantitative PCR. (A) Comparison of the expression levels between

different cell types relative to CHO cells =1. (B) Comparison within one cell type relative to ADAMTS2 = 1. Note that the cell lines that do process VEGF-C into its mature form express ADAMTS3, while those cells that are unable or extremely inefficient in processing VEGF-C into its mature form do express very low amounts or no ADAMTS3.

Supplemental Figure 8. Suppression of VEGF-C processing by ADAMTS3 shRNA and effect of the N- and C-terminal propeptides of VEGF-C on processing. (A) The processing of pro-VEGF-C into mature VEGF-C was reduced in 293T cells by lentiviral ADAMTS3 shRNA compared to the non-target shRNA. Note that the baseline processing of VEGF-C is increased in the cells transduced with a non-targeting control shRNA compared to the non-transduced cells. (B) The proteolytic processing of VEGF-D and the VEGF-D/VEGF-C chimera version 1 (CDC-V1) in the presence or absence of CCBE1 cotransfection. Processing of CDC-V2 was also not affected by CCBE1 (data not shown). (C) Alignment of the amino acid sequence of the chimeras and their parent proteins. VEGF-C-derived sequences are shown in green, VEGF-D-derived sequences in red. The boxed region marks where the proteolytic cleavages occur in VEGF-C and VEGF-D. (D) Competitive inhibition of CCBE1 action by the VEGF-C propeptides. Note that the amounts of both the mature 21/23 kDa form of VEGF-C and the 14 kDa N-terminal propeptide are reduced by competition with the C- and N-terminal propeptides. C-pp, C-terminal propeptide; N-pp, N-terminal propeptide; HSA, human serum albumin; ΔNΔC, C-and N-terminally truncated form of VEGF-C similar to mature VEGF-C[9].

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