CCBE1 enhances lymphangiogenesis via ADAMTS3-mediated VEGF-C activation

Michael Jeltsch, PhD[1,2,3], Sawan Kumar Jha, BSc[2], Denis Tvorogov, PhD[2]; Andrey Anisimov, PhD[1,2]; Veli-Matti Leppänen, PhD[1]; Tanja Holopainen, MD[2]; Riikka Kivelä, PhD[2]; Sagrario Ortega, PhD[4]; Terhi Kärpanen, PhD[5]; Kari Alitalo, MD, PhD[1,2,6,*]

[1]Wihuri Research Institute, [2]Translational Cancer Biology Program and [3]Department of Biomedicine, Biomedicum Helsinki, University of Helsinki, Finland

[4]Biotechnology Programme, Spanish National Cancer Research Centre, Madrid, Spain

[5]Hubrecht Institute, Utrecht, The Netherlands

[6]Helsinki University Central Hospital, Helsinki, Finland

*Author for correspondence: Dr. Kari Alitalo, Wihuri Research Institute, Biomedicum Helsinki, P.O.B. 63 (Haartmaninkatu 8), 00014 University of Helsinki, Finland; Phone: +358-9-19125511; Fax: +358-9-19125510; E-mail: kari.alitalo@helsinki.fi

Word count: 6852

Jeltsch: CCBE1 enhances VEGF-C processing via ADAMTS3

Journal Subject Code: 129 (Basic Science Research/Angiogenesis)

Abstract

Background. Hennekam lymphangiectasia–lymphedema syndrome (OMIM 235510) is a rare autosomal recessive disease, which is associated with mutations in the collagen- and calcium-binding EGF domains 1 *(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 CCBE1 interactions with the 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 while 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 (ADAMTS3) protease, resulting in the mature 21/23-kDa form of VEGF-C, which induces increased VEGF-C receptor signaling. Adeno-associated viral vector (AAV) mediated transduction of CCBE1 into mouse skeletal muscle enhanced lymphangiogenesis and angiogenesis induced by AAV-VEGF-C.

Conclusions. These results identify ADAMTS3 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 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.

Key Words: angiogenesis, metalloproteinases, vasculature, growth substances, endothelium

Introduction

VEGF-C is the main driver of lymphangiogenesis in embryonic development and in various lymphangiogenic processes in adults[1]. It acts via 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 due to 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 (VHD) in order for VEGF-C to reach its full signaling potential[4]. VEGF-C can activate also the main angiogenic receptor VEGFR-2 when both propeptides are cleaved off[4]. Hence, the mature VEGF-C can stimulate also angiogenesis.

Mutations in VEGF-C and VEGFR-3 have been shown to result in hereditary lymphedema[5, 6, 7]. Another hereditary condition with lymphedema as a cardinal symptom is Hennekam lymphangiectasia–lymphedema syndrome (HS)[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 CCBE1 causes the lymphatic phenotype. CCBE1 is a two-domain protein with an N-terminal potential Ca-binding domain with EGF-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 two of all identified mutations affect the collagen-like domain. All 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 based on 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 which 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 the migration signal provided by VEGF-C, while the later block in *Ccbe1*-deficient embryos was attributed to a defect in endothelial cell migration, perhaps due to lack of migratory cues from the extracellular matrix, of which CCBE1 is thought to be a component[12].

Both *CCBE1+/-* and *VEGF-C+/-* heterozygous embryos show a reduction of Prox1-positive endothelial cells emigrating from the cardinal veins[3]. The double heterozygous *CCBE1+/-;VEGF-C+/-* embryos have an aggravated version of this phenotype, suggesting that CCBE1 and VEGF-C participate synergistically to 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 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. 24 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. In order to produce unlabeled protein, the culture media were exchanged and supernatants and lysates harvested 48 hours later. After immunoprecipitation, the samples were electrophorated in 4-20% 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 read-outs or scanned X-ray film using the ImageJ software (NIH, 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 (PAE) cells expressing VEGFR-3 or VEGFR-3 plus neuropilin-2 were washed with PBS and starved over night in D-MEM 0.2%BSA. ΔNΔC-VEGF-C, pro-VEGF-C and CCBE1Δ175 were diluted to 0.02, 0.4 and 5 µg/ml in 1 ml D-MEM/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 mM, and crosslinking 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 was analyzed by SDS-PAGE/Western.

VEGFR-3 trafficking in human umbilical vein endothelial cells (HUVECs). HUVECs stably transfected with the pMXs-VEGFR-3-GFP vector[16] were grown on glass-bottom microwells (MatTek Co., Ashland, MA) for 24 h. The FCS concentration was reduced to 0.5%, and 12 h 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 GFP signal was recorded at the 488 nm wavelength. The human VEGFR-3 blocking antibody hF4-3C5 was used at 5 μg/ml.

Recombinant AAV production. AAV9 vectors were made by a three-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 $6x10^{10}$. Three weeks after transduction, the mice were sacrificed 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 (PECAM-1) as well as smooth muscle cell/pericyte (smooth muscle actin, SMA) and leukocyte (CD45) markers, followed by Alexa-conjugated secondary antibodies (Molecular Probes, Invitrogen). Fluorescent images were obtained in an Axioplan 2 microscope (Carl Zeiss AG, Oberkochen, Germany); the objectives were: 10x NA=0.3 WD 5.6 and 20x NA=0.5 WD 2.0; the camera was a Zeiss AxioCamHRm 14-bit greyscale CCD; the acquisition software was Zeiss AxioVision 4.6. Quantification of the areas stained for Lyve-1, PECAM-1 and SMA was done as previously described[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 previously described[19]. The National Board for Animal Experiments of the Provincial State Office of Southern Finland approved all animal experiments carried out in this study.

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

Results

CCBE1 enhances VEGF-C processing and release, resulting in increased VEGFR-3 activation. CCBE1 was detected as a protein of 40-55 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 about 100 kDa. Transfected VEGF-C was expressed as the uncleaved 58 kDa precursor, C-terminally processed 29/31-kDa "pro-VEGF-C" form and fully processed 21 kDa 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).

Cotransfection with CCBE1 facilitated also the release of VEGF-C as the cell-layer associated amount was reduced by 80% in lysates of the cotransfected cells in short-term labeling experiments (Supplemental Figure 1A, compare lanes 5, 6 to 7, 8). A similar accelerated release of VEGF-C was achieved, when the C-terminal domain of VEGF-C was removed (Supplemental Figure 1B).

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, while 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 Supplemental Figure 1C). Notably, also CCBE1 promoted slightly 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 if 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 (Supplemental Figure 1D).

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]. In order to investigate if CCBE1 enhances VEGF-C induced lymphangiogenesis *in vivo*, we transduced mouse *tibialis anterior* muscle with adeno-associated virus serotype 9 vector (AAV9) expressing CCBE1 (AAV9-CCBE1) alone or together with AAV9-VEGF-C in a 1:1 ratio, or AAV9-human serum albumin (HSA) as a negative control. AAV9 encoding the mature, activated form of VEGF-C (ΔNΔC-VEGF-C) was used as a positive control.

Two weeks after the AAV transduction, the muscles were analyzed by immunohistochemistry using markers for endothelial cells (PECAM-1), lymphatic endothelial cells (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 Supplemental Figure 2, bottom row). This suggested that the proteolytic processing of VEGF-C was inefficient in the AAV9 transduced muscle.

However, when VEGF-C was co-transduced with CCBE1, lymphangiogenesis was significantly enhanced, as shown by the LYVE-1 and Prox-1 staining (Figure 2). Similar to the ΔNΔC-VEGF-C transduced muscle, significantly more angiogenesis and leukocyte recruitment were observed (Supplemental Figures 2 and 3). 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 co-transduced 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).

VEGF-C and CCBE1 are processed by the ADAMTS3 procollagenase. Our attempts to demonstrate a physical interaction of VEGF-C and CCBE1 were unsuccessful (Supplemental Figure 4A). 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 co-purified 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). In order to analyze if 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 co-transfected 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 EGF and collagen homology domains. Interestingly, the DU-4475 cells produced only uncleaved CCBE1 (Supplemental Figure 4B), which did not promote VEGF-C activation (Supplemental Figure 4C).

VEGF-C cleavage by plasmin is not influenced by CCBE1. As previously published[22], VEGF-C was efficiently cleaved by plasmin (Supplemental Figure 5A). The fragments obtained with low amounts of plasmin activated VEGFR-3, but this activity was lost at high plasmin concentrations (Supplemental Figure 5B). Edman degradation of the final products revealed the N-terminal 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 (Supplemental Figure 5C).

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] (Supplemental Figure 6A). VEGF-D was not cleaved by ADAMTS3 under the same conditions (Supplemental Figure 6B), despite featuring a similar cleavage motif (Supplemental Figure 6C).

Apart from ADAMTS3, two 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 (Supplemental Figure 6D).

We found that the cell lines that produce active, mature VEGF-C (293T, 293T-CCBE1 and PC-3 cells) express ADAMTS3, while the cell lines that were unable or extremely inefficient in producing active VEGF-C (CHO, NIH-3T3), expressed very little or no ADAMTS3 (Supplemental Figure 7). Furthermore, when ADAMTS3 was silenced in 293T cells by using lentiviral shRNA, the VEGF-C cleavage was inhibited (Supplemental Figure 8A).

VEGF-C/VEGF-D chimeras generated by propeptide swapping were not subject to ADAMTS3 cleavage (Supplemental Figure 8B and C). Interestingly however, 79 % of VEGF-C processing was inhibited by the purified C-terminal propeptide and 43% by the N-terminal propeptide, whereas the VHD or HSA gave no inhibition (Supplemental Figure 8D), 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 to express sufficient amounts of full-length CCBE1, we investigated if the isolated N-terminal domain of CCBE1 (CCBE1Δ175) can increase VEGF-C activity. We stimulated VEGFR-3 transfected PAE cells with pro-VEGF-C, which resulted in very little VEGFR-3 phosphorylation when compared to mature VEGF-C (Figure 5A, lanes 1 and 2). When the recombinant CCBE1Δ175 was added with pro-VEGF-C, VEGFR-3 phosphorylation was strongly increased (Figure 5A, third lane). 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 what 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 PAE-VEGFR-3 cells in PBS for 210 s and cross-linked VEGFR-3 associated proteins for 390 s. 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 co-precipitate with VEGFR-3, unless VEGFR-3 was co-expressed 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, pre-incubation 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 eNOS downstream signaling proteins in blood vascular endothelial cells or lymphatic endothelial cells (Figure 6B and C).

Discussion

CCBE1 is essential for embryonic lymphangiogenesis[10, 11, 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 while 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 indicates 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.

While 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 PAE-VEGFR-3 cells. However, when we introduced neuropilin-2, we could establish binding, yet 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, while 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 due to 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 occurred also 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, 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 which bud from the CV[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 (uPA)/soluble uPA 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 (Supplemental Figure 6C). Preferential cleavage at one site over the other might explain, why limited exposure to plasmin activates VEGF-C, while 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, since 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 two separate domains may be a prerequisite for its activity as 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. Even though the C-terminal cleavage is not a prerequisite for secretion[26], the presence of the C-terminal propeptide slowed VEGF-C release compared to 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 as it is uncertain whether cells that express both CCBE1 and VEGF-C exist *in vivo*.

Based on our findings, the lymphatic vessel defects seen in animal models lacking CCBE1 can be explained, as 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 (unpublished data of the authors) and potential additional substrates of ADAMTS3 suggests that CCBE1 has also 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 modulation of VEGF-C activity, which could be used for therapeutic stimulation and inhibition of lymphangiogenesis and perhaps also angiogenesis[48].

Acknowledgments

We thank Marcel Lackner for assistance in the VEGFR-3 phosphorylation studies, Tanja Laakkonen for the generation of the AAVs, Tapio Tainola for DNA sequencing and laboratory management and Markku Varjosalo and Sini Miettinen from the Proteomics Unit of the Institute of Biotechnology for mass spec analysis and protein sequencing. Dr. Bronek Pytowski (ImClone LLC/Eli Lilly & Co.) generously provided the hF4-3C5 antibody and Dr. Steven Stacker and Dr. Mark Achen the VD1 antibody.

Funding Sources

The Academy of Finland (grant numbers 265982, 272683, 273612 and 273817), European Research Council (ERC-2010-AdG-268804, Marie Curie Actions FP7/2007-2013REA 317250), the Leducq Foundation (11CVD03) and the Finnish Foundation for Cardiovascular Research are acknowledged for funding. Tanja Holopainen has been supported by the Jalmari and Rauha Ahokas Foundation and the K. Albin Johansson Foundation.

Disclosures

Kari Alitalo has been involved in consultance for Laurantis Pharma Oy.

References

1. Alitalo K. The lymphatic vasculature in disease. *Nat Med*. 2011;17:1371-1380.

2. Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, Jeltsch M, Jackson DG, Talikka M, Rauvala H, Betsholtz C, Alitalo K. Vascular endothelial growth factor c is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol*. 2004;5:74-80.

3. Hagerling R, Pollmann C, Andreas M, Schmidt C, Nurmi H, Adams RH, Alitalo K, Andresen V, Schulte-Merker S, Kiefer F. A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy. *EMBO J*. 2013;32:629-644.

4. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkkinen N, Alitalo K. Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J*. 1997;16:3898-3911.

5. Gordon K, Schulte D, Brice G, Simpson MA, Roukens MG, van Impel A, Connell F, Kalidas K, Jeffery S, Mortimer PS, Mansour S, Schulte-Merker S, Ostergaard P. Mutation in vascular endothelial growth factor-c, a ligand for vascular endothelial growth factor receptor-3, is associated with autosomal dominant milroy-like primary lymphedema. *Circ Res*. 2013;112:956-960

6. Irrthum A, Karkkainen MJ, Devriendt K, Alitalo K, Vikkula M. Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. *Am J Hum Genet*. 2000;67:295-301.

7. Karkkainen MJ, Ferrell RE, Lawrence EC, Kimak MA, Levinson KL, McTigue MA, Alitalo K, Finegold DN. Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. *Nat Genet*. 2000;25:153-159.

8. Van Balkom ID, Alders M, Allanson J, Bellini C, Frank U, De Jong G, Kolbe I, Lacombe D, Rockson S, Rowe P, Wijburg F, Hennekam RC. Lymphedema-lymphangiectasia-mental retardation (Hennekam) syndrome: A review. *Am J Med Genet*. 2002;112:412-421.

9. Alders M, Mendola A, Ades L, Al Gazali L, Bellini C, Dallapiccola B, Edery P, Frank U, Hornshuh F, Huisman SA, Jagadeesh S, Kayserili H, Keng WT, Lev D, Prada CE, Sampson JR, Schmidtke J, Shashi V, van Bever Y, Van der Aa N, Verhagen JM, Verheij JB, Vikkula M, Hennekam RC. Evaluation of clinical manifestations in patients with severe lymphedema with and without CCBE1 mutations. *Mol Syndromol*. 2013;4:107-113.

10. Alders M, Hogan BM, Gjini E, Salehi F, Al-Gazali L, Hennekam EA, Holmberg EE, Mannens MM, Mulder MF, Offerhaus GJ, Prescott TE, Schroor EJ, Verheij JB, Witte

M, Zwijnenburg PJ, Vikkula M, Schulte-Merker S, Hennekam RC. Mutations in CCBE1 cause generalized lymph vessel dysplasia in humans. *Nat Genet*. 2009;41:1272-1274.

11. Bos FL, Caunt M, Peterson-Maduro J, Planas-Paz L, Kowalski J, Karpanen T, van Impel A, Tong R, Ernst JA, Korving J, van Es JH, Lammert E, Duckers HJ, Schulte-Merker S. CCBE1 is essential for mammalian lymphatic vascular development and enhances the lymphangiogenic effect of vascular endothelial growth factor-c in vivo. *Circ Res*. 2011;109:486-491.

12. Hogan BM, Bos FL, Bussmann J, Witte M, Chi NC, Duckers HJ, Schulte-Merker S. Ccbe1 is required for embryonic lymphangiogenesis and venous sprouting. *Nat Genet*. 2009;41:396-398.

13. Achen MG, Roufail S, Domagala T, Catimel B, Nice EC, Geleick DM, Murphy R, Scott AM, Caesar C, Makinen T, Alitalo K, Stacker SA. Monoclonal antibodies to Vascular Endothelial Growth Factor-D block its interactions with both VEGF receptor-2 and VEGF receptor-3. *Eur J Biochem*. 2000;267:2505-2515.

14. Stacker SA, Vitali A, Caesar C, Domagala T, Groenen LC, Nice E, Achen MG, Wilks AF. A mutant form of Vascular Endothelial Growth Factor (VEGF) that lacks VEGF receptor-2 activation retains the ability to induce vascular permeability. *J Biol Chem*. 1999;274:34884-34892.

15. Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, Kauppinen R, Jackson DG, Kubo H, Nishikawa S, Yla-Herttuala S, Alitalo K. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med*. 2001;7:199-205.

16. Ghalamkarpour A, Holnthoner W, Saharinen P, Boon LM, Mulliken JB, Alitalo K, Vikkula M. Recessive primary congenital lymphoedema caused by a VEGFR3 mutation. *J Med Genet*. 2009;46:399-404.

17. Anisimov A, Alitalo A, Korpisalo P, Soronen J, Kaijalainen S, Leppänen V-M, Jeltsch M, Ylä-Herttuala S, Alitalo K. Activated forms of VEGF-C and VEGF-D provide improved vascular function in skeletal muscle. *Circ Res*. 2009;104:1302-1312.

18. Michelfelder S, Varadi K, Raupp C, Hunger A, Korbelin J, Pahrmann C, Schrepfer S, Muller OJ, Kleinschmidt JA, Trepel M. Peptide ligands incorporated into the threefold spike capsid domain to re-direct gene transduction of AAV8 and AAV9 in vivo. *PLoS ONE*. 2011;6:e23101.

19. Martinez-Corral I, Olmeda D, Dieguez-Hurtado R, Tammela T, Alitalo K, Ortega S. In vivo imaging of lymphatic vessels in development, wound healing, inflammation, and tumor metastasis. *Proc Natl Acad Sci USA*. 2012;109:6223-6228.

20. Murad S, Grove D, Lindberg KA, Reynolds G, Sivarajah A, Pinnell SR. Regulation of collagen synthesis by ascorbic acid. *Proc Natl Acad Sci USA*. 1981;78:2879-2882.

21. Karpanen T, Alitalo K. Molecular biology and pathology of lymphangiogenesis. *Annu Rev Pathol*. 2008;3:367-397.

22. McColl BK, Baldwin ME, Roufail S, Freeman C, Moritz RL, Simpson RJ, Alitalo K, Stacker SA, Achen MG. Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. *J Exp Med*. 2003;198:863-868.

23. Leppanen VM, Tvorogov D, Kisko K, Prota AE, Jeltsch M, Anisimov A, Markovic-Mueller S, Stuttfeld E, Goldie KN, Ballmer-Hofer K, Alitalo K. Structural and mechanistic insights into VEGF receptor 3 ligand binding and activation. *Proc Natl Acad Sci USA*. 2013;110:12960-12965.

24. Porter S, Clark IM, Kevorkian L, Edwards DR. The ADAMTS metalloproteinases. *Biochem J*. 2005;386:15-27.

25. Brown HM, Dunning KR, Robker RL, Pritchard M, Russell DL. Requirement for ADAMTS-1 in extracellular matrix remodeling during ovarian folliculogenesis and lymphangiogenesis. *Dev Biol*. 2006;300:699-709.

26. Siegfried G, Basak A, Cromlish JA, Benjannet S, Marcinkiewicz J, Chretien M, Seidah NG, Khatib AM. The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. *J Clin Invest*. 2003;111:1723-1732.

27. Park JE, Keller GA, Ferrara N. The vascular endothelial growth factor (VEGF) isoforms: Differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell*. 1993;4:1317-1326.

28. Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J Cell Biol*. 2005;169:681-691.

29. Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, Detmar M. Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by Vascular Endothelial Growth Factor-C in melanoma. *Am J Pathol*. 2001;159:893-903.

30. Le Goff C, Somerville RP, Kesteloot F, Powell K, Birk DE, Colige AC, Apte SS. Regulation of procollagen amino-propeptide processing during mouse embryogenesis by specialization of homologous ADAMTS proteases: Insights on collagen biosynthesis and dermatosparaxis. *Development*. 2006;133:1587-1596.

31. Castellino FJ, Ploplis VA. Structure and function of the plasminogen/plasmin system. *Thromb Haemost*. 2005;93:647-654.

32. Lee J, Gray A, Yuan J, Luoh SM, Avraham H, Wood WI. Vascular endothelial growth factor-related protein: A ligand and specific activator of the tyrosine kinase receptor Flt4. *Proc Natl Acad Sci USA*. 1996;93:1988-1992.

33. Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V, Alitalo K. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development*. 1996;122:3829-3837.

34. Kärpänen T, Heckman CA, Keskitalo S, Jeltsch M, Ollila H, Neufeld G, Tamagnone L, Alitalo K. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. *FASEB J*. 2006;20:1462-1472.

35. Xu Y, Yuan L, Mak J, Pardanaud L, Caunt M, Kasman I, Larrivee B, Del Toro R, Suchting S, Medvinsky A, Silva J, Yang J, Thomas JL, Koch AW, Alitalo K, Eichmann A, Bagri A. Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3. *J Cell Biol*. 2010;188:115-130.

36. Aung HH, Lame MW, Gohil K, He G, Denison MS, Rutledge JC, Wilson DW. Comparative gene responses to collected ambient particles in vitro: Endothelial responses. *Physiol Genomics*. 2011;43:917-929.

37. Li WX, Howard RJ, Leung LL. Identification of SVTCG in thrombospondin as the conformation-dependent, high affinity binding site for its receptor, CD36. *J Biol Chem*. 1993;268:16179-16184.

38. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukumura D, Jain RK, Alitalo K. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science*. 1997;276:1423-1425.

39. Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI, Kholová I, Kauppinen RA, Achen MG, Stacker SA, Alitalo K, Ylä-Herttuala S. VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. *Circ Res*. 2003;92:1098-1106.

40. Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Ylä-Herttuala S, Jäättelä M, Alitalo K. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res*. 2001;61:1786-1790.

41. Tammela T, Zarkada G, Wallgard E, Murtomaki A, Suchting S, Wirzenius M, Waltari M, Hellstrom M, Schomber T, Peltonen R, Freitas C, Duarte A, Isoniemi H, Laakkonen P, Christofori G, Yla-Herttuala S, Shibuya M, Pytowski B, Eichmann A, Betsholtz C, Alitalo K. Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature*. 2008;454:656-660.

42. Chavakis T, Kanse SM, Yutzy B, Lijnen HR, Preissner KT. Vitronectin concentrates proteolytic activity on the cell surface and extracellular matrix by trapping soluble urokinase receptor-urokinase complexes. *Blood*. 1998;91:2305-2312.

43. Leppänen V-M, Jeltsch M, Anisimov A, Tvorogov D, Aho K, Kalkkinen N, Toivanen P, Ylä-Herttuala S, Ballmer-Hofer K, Alitalo K. Structural determinants of Vascular Endothelial Growth Factor-D receptor binding and specificity. *Blood*. 2011;117:1507-1515.

44. Stacker SA, Stenvers K, Caesar C, Vitali A, Domagala T, Nice E, Roufail S, Simpson RJ, Moritz R, Karpanen T, Alitalo K, Achen MG. Biosynthesis of Vascular Endothelial Growth Factor-D involves proteolytic processing which generates non-covalent homodimers. *J Biol Chem*. 1999;274:32127-32136.

45. Dombrowski KE, Prockop DJ. Cleavage of type I and type II procollagens by type I/II procollagen N-proteinase. Correlation of kinetic constants with the predicted conformations of procollagen substrates. *J Biol Chem*. 1988;263:16545-16552.

46. Fernandes RJ, Hirohata S, Engle JM, Colige A, Cohn DH, Eyre DR, Apte SS. Procollagen II amino propeptide processing by ADAMTS-3. Insights on dermatosparaxis. *J Biol Chem*. 2001;276:31502-31509.

47. Al-Gazali LI, Hertecant J, Ahmed R, Khan NA, Padmanabhan R. Further delineation of Hennekam syndrome. *Clin Dysmorphol*. 2003;12:227-232.

48. Norrmén C, Tammela T, Petrova TV, Alitalo K. Biological basis of therapeutic lymphangiogenesis. *Circulation*. 2011;123:1335-1351.

Figure legends

Figure 1. CCBE1 coexpression increases 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 intra- 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 (VEGFR-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. *: $P<0.05$, ***: $P<0.001$, n.s.: no statistically significant difference; $n = 4$. (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 panel B. The N-terminal propeptide is shown in cyan, the VEGF homology domain in red and the C-terminal propeptide in blue.

Figure 2. CCBE1 enhances lymphangiogenesis *in vivo*. Immunostaining of mouse *tibialis anterior* muscles transduced by AAV9 encoding the indicated factors and stained for the indicated antigens. Note that VEGF-C alone induces only a mild lymphangiogenic response, but its co-transduction with CCBE1 results in a strong response as detected by 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. *, ***, n.s.: as in Figure 1; **: $P<0.01$; $n \geq 5$.

Figure 3. VEGFR-3-luciferase reporter signals in mouse skeleteal muscles injected with AAV9 vectors encoding the indicated proteins. Note that the co-transduction with VEGF-C and CCBE1 results in a strong luciferase signal, indicating a major lymphangiogenesis response, while VEGF-C or CCBE1 alone induces only little luciferase activity. The luminometry color scale is shown on the right.

Figure 4. ADAMTS3 cleaves VEGF-C and CCBE1. (A) Stable VEGF-C-expressing 293T cells were transfected with the indicated expression vectors and their culture media were immunoprecipitated with VEGFR-3/Fc. Note that CCBE1 transfection alone results in a partial conversion of pro-VEGF-C into mature VEGF-C, while 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 due to the endogenous ADAMTS3 produced by 293T cells. The highest levels 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 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 24h 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 data points except for those indicated without fill and the comparison VEGF-C versus 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 non-specifically and that the detection of any co-precipitating full-length CCBE1 is therefore not possible.

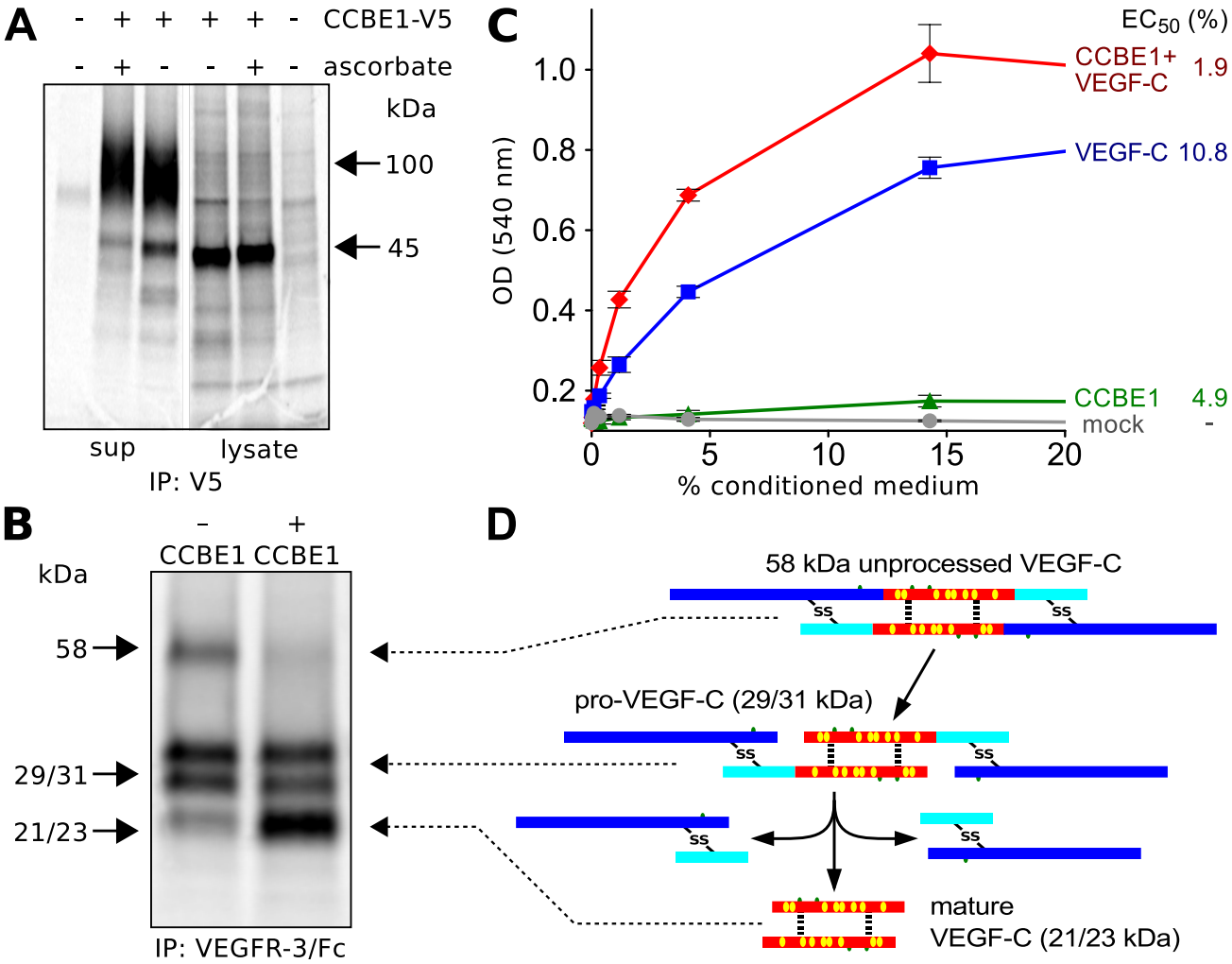
Figure 5. The N-terminal domain of CCBE1 activates VEGFR-3 phosphorylation by pro-VEGF-C. VEGFR-3 expressing PAE cells were stimulated with pro-VEGF-C together with the N-terminal domain of CCBE1 (CCBE1Δ175), or with mature VEGF-C (ΔNΔC-VEGF-C). (A) Note that pro-VEGF-C induces only a marginal activation 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 non-specific signals with the same electrophoretic mobility as the 29 kDa fragment of pro-VEGF-C.

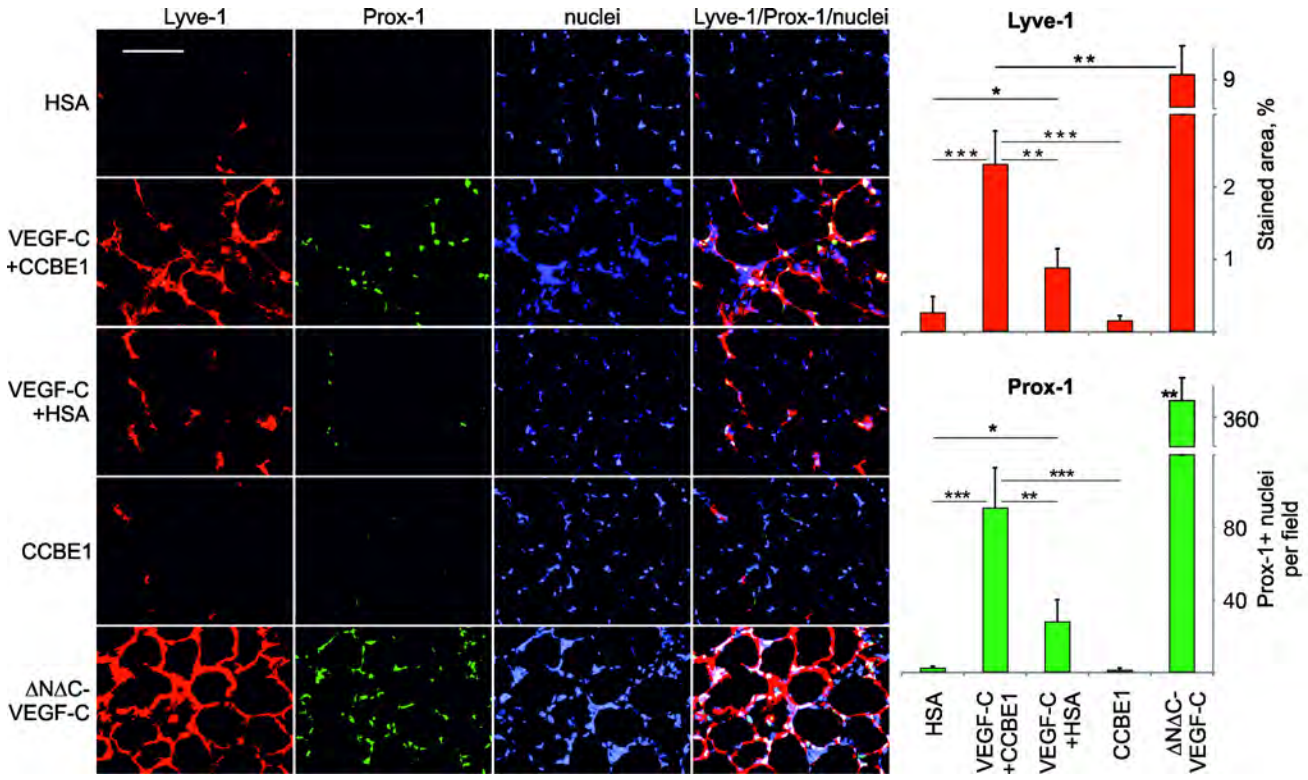
Figure 6. Pro-VEGF-C can inhibit VEGFR-3 activation by mature VEGF-C. (A) 10 min pre-incubation of lymphatic endothelial cells with pro-VEGF-C reduces their ability to respond to mature VEGF-C. Arrows denote the 125 and 175 kDa fragments of VEGFR-3. The Western blots represent separate gels. (B) HUVECs stably expressing a VEGFR-3-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 change significantly 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 eNOS. eNOS detection was performed on the stripped pErk membrane, and actin detection was performed on the stripped pAkt membrane.

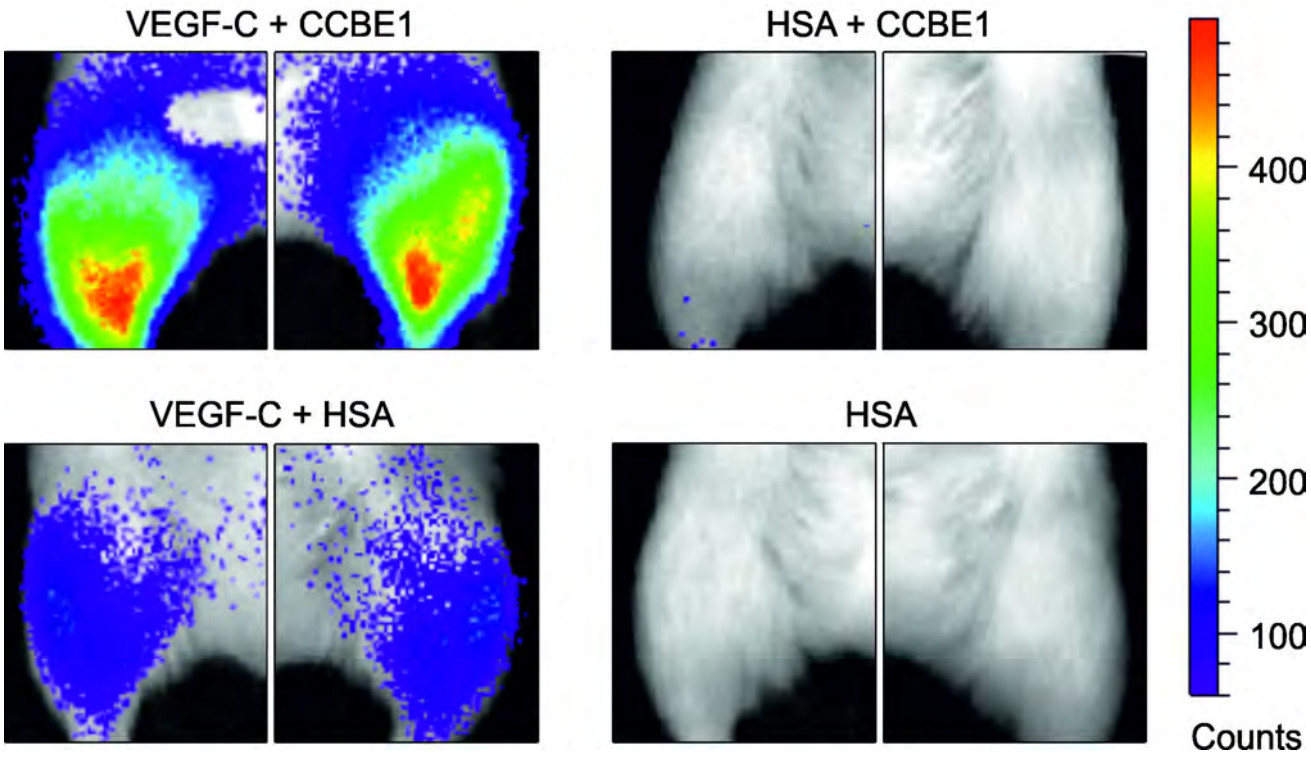
Figure 7. Schematic view of VEGF-C and VEGFR-3 activation by VEGF-C. (A) Domain organization of VEGF-C and the defined cleavage sites. (B) Pro-VEGF-C binding to VEGFR-3 is assisted by the N-terminal domain of CCBE1. Pro-VEGF-C is then proteolytically processed *in situ* and the mature VEGF-C activates 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 if the removal of the C-terminal domain of CCBE1 is required for the CCBE1 function.



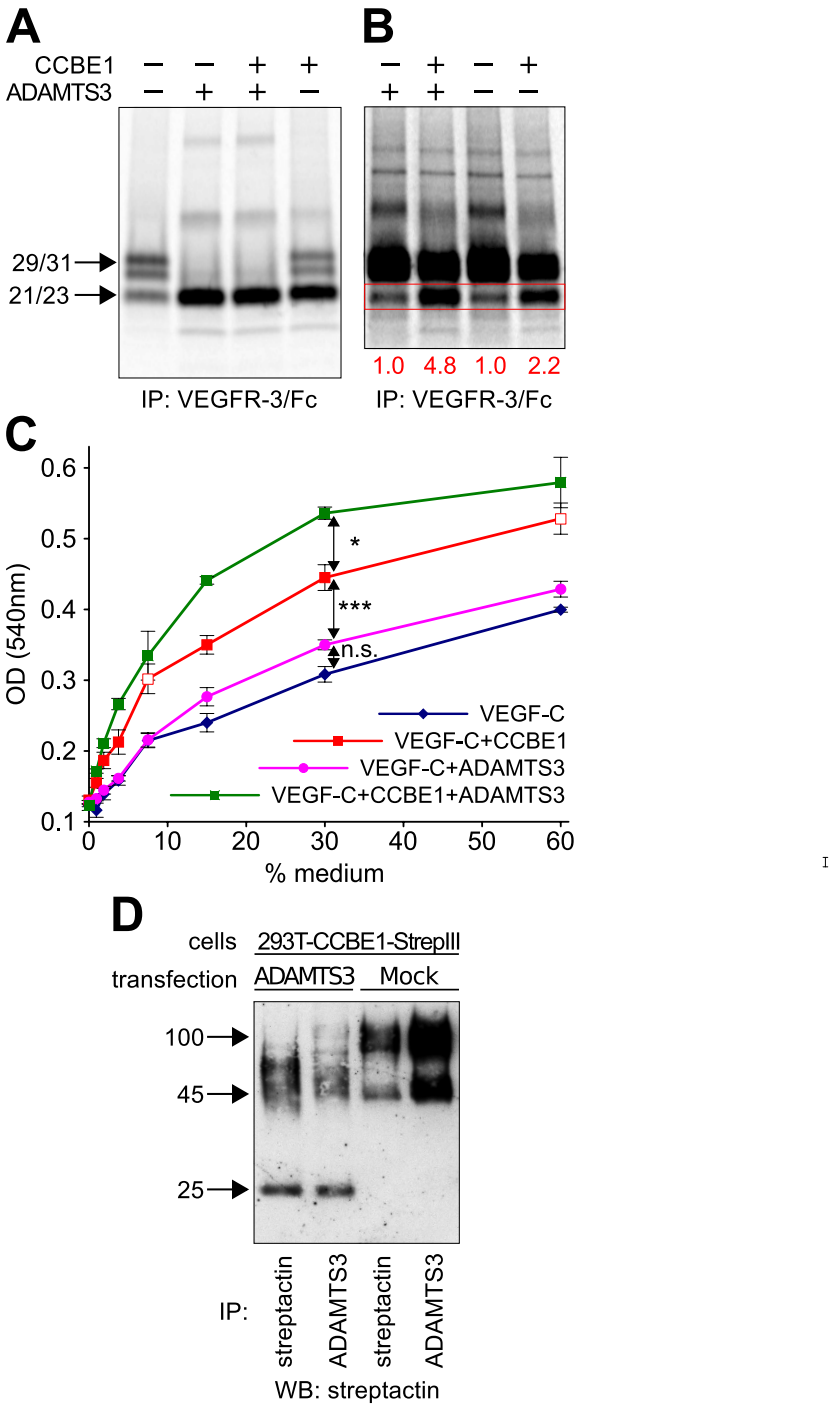
Jeltsch et al., Fig. 1



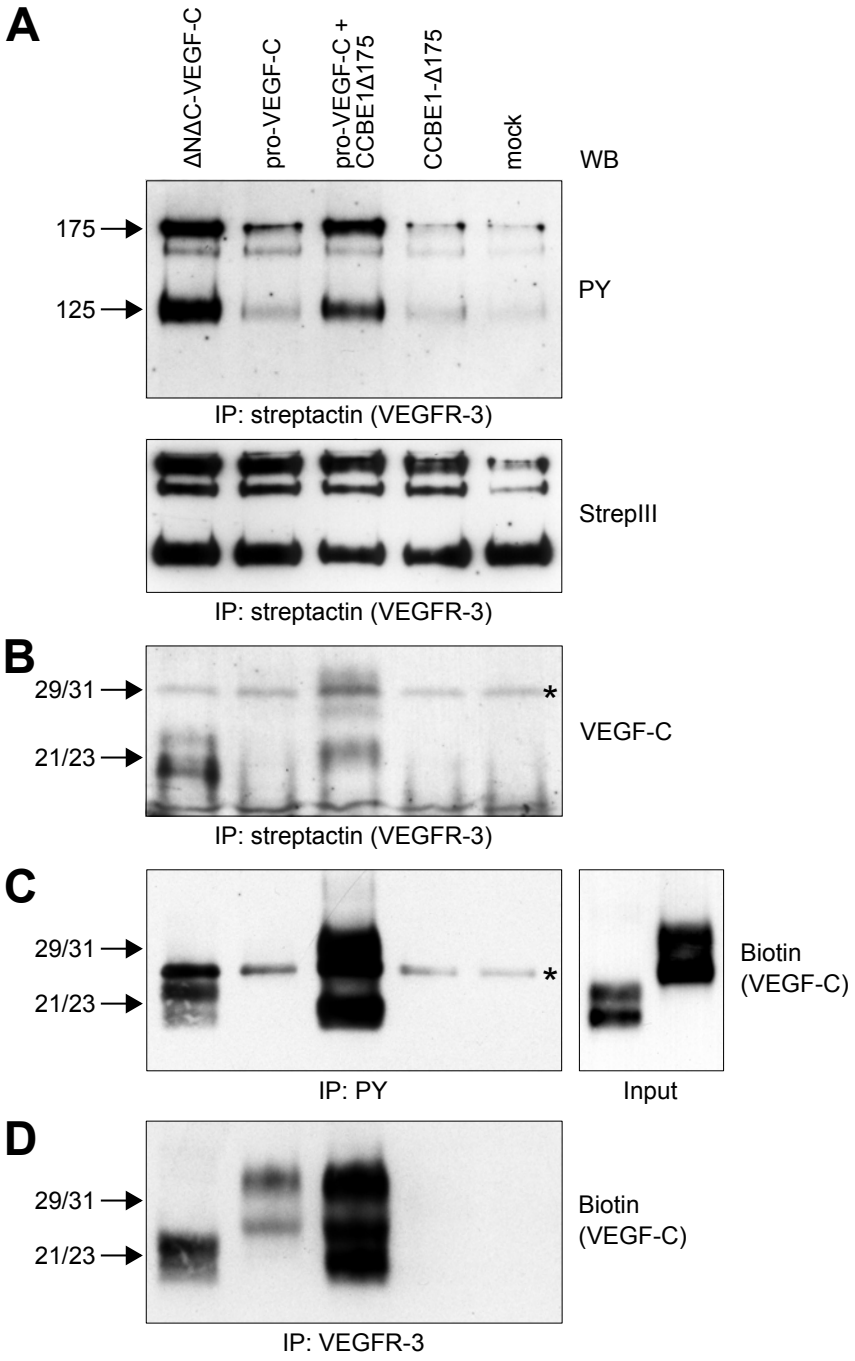
Jeltsch et al., Fig. 2



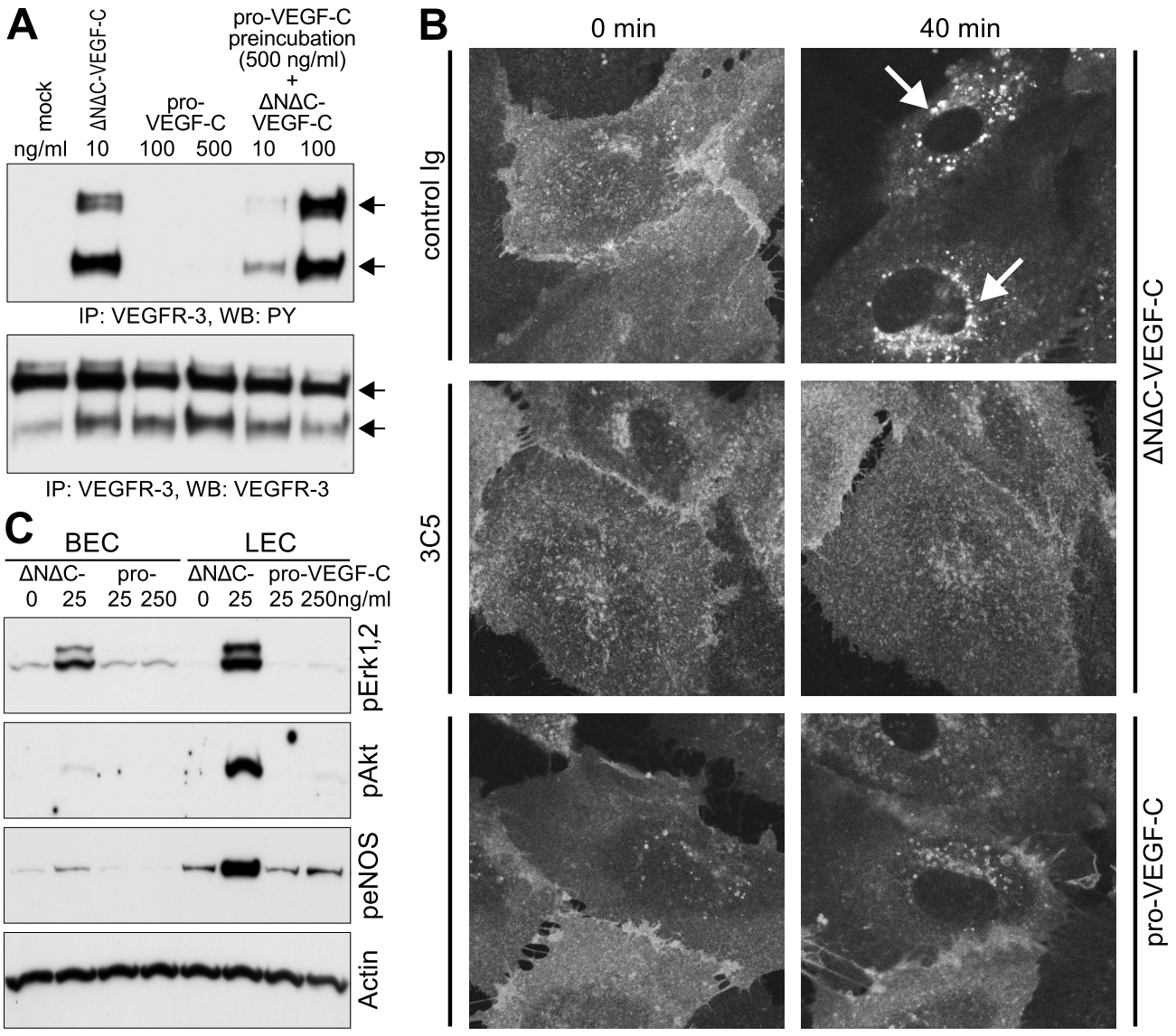
Jeltsch et al., Fig. 3



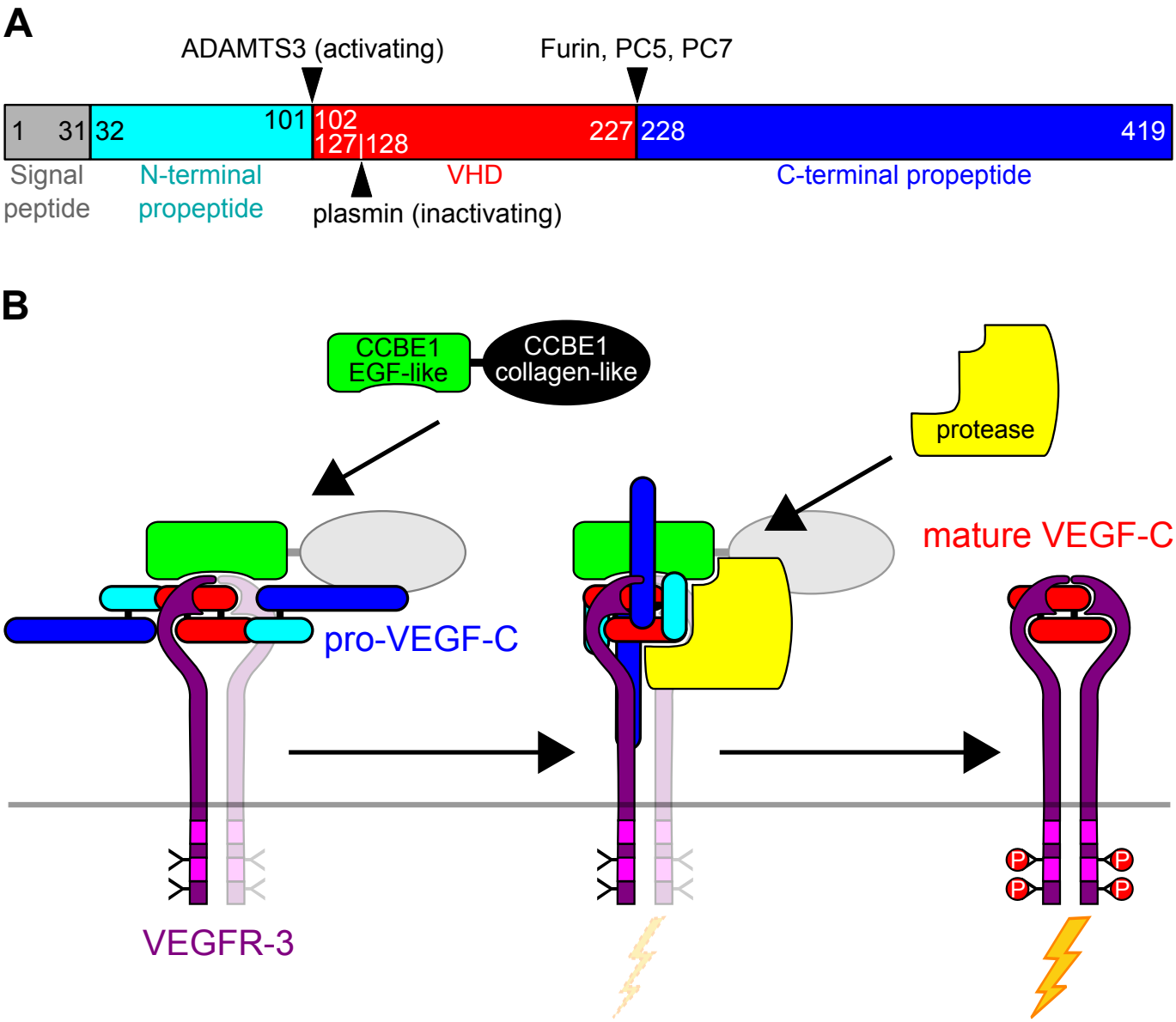
Jeltsch et al., Fig. 4



Jeltsch et al., Fig. 5



Jeltsch et al., Fig. 6



Jeltsch et al., Fig. 7

Clinical Perspective Summary

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 CCBE1 gene interacts with the lymphangiogenic VEGF-C growth factor-receptor (VEGFR-3) pathway, but the nature of the interaction remained elusive. In this manuscript, Jeltsch et al. show that the activity VEGF-C is regulated by CCBE1, which facilitates the proteolytic activation of a latent "pro" form of VEGF-C by the ADAMTS3 metalloproteinase via a novel mode of growth factor activation mechanism. The *in vivo* data in the manuscript 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.