Molecular Medicine

Functional Dissection of the CCBE1 Protein
A Crucial Requirement for the Collagen Repeat Domain

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Rationale: Collagen- and calcium-binding EGF domain–containing protein 1 (CCBE1) is essential for lymphangiogenesis in vertebrates and has been associated with Hennekam syndrome. Recently, CCBE1 has emerged as a crucial regulator of vascular endothelial growth factor-C (VEGFC) signaling.

Objective: CCBE1 is a secreted protein characterized by 2 EGF domains and 2 collagen repeats. The functional role of the different CCBE1 protein domains is completely unknown. Here, we analyzed the functional role of the different CCBE1 domains in vivo and in vitro.

Methods and Results: We analyzed the functionality of several CCBE1 deletion mutants by generating knock-in mice expressing these mutants, by analyzing their ability to enhance Vegfc signaling in vivo in zebrafish, and by testing their ability to induce VEGFC processing in vitro. We found that deleting the collagen domains of CCBE1 has a much stronger effect on CCBE1 activity than deleting the EGF domains. First, although CCBE1ΔCollagen mice fully phenocopy CCBE1 knock-out mice, CCBE1ΔEGF knock-in embryos still form rudimentary lymphatics. Second, Ccbe1ΔEGF, but not Ccbe1ΔCollagen, could partially substitute for Ccbe1 to enhance Vegfc signaling in zebrafish. Third, CCBE1ΔEGF, similarly to CCBE1, but not CCBE1ΔCollagen could activate VEGFC processing in vitro. Furthermore, a Hennekam syndrome mutation within the collagen domain has a stronger effect than a Hennekam syndrome mutation within the EGF domain.

Conclusions: We propose that the collagen domains of CCBE1 are crucial for the activation of VEGFC in vitro and in vivo. The EGF domains of CCBE1 are dispensable for regulation of VEGFC processing in vitro, however, they are necessary for full lymphangiogenic activity of CCBE1 in vivo. (Circ Res. 2015;116:1660-1669. DOI: 10.1161/CIRCRESAHA.116.304949.)

Key Words: CCBE1 protein ■ endothelium, vascular ■ Hennekam lymphangiectasia-lymphedema syndrome ■ lymphangiogenesis ■ vascular endothelial growth factor

The lymphatic vasculature plays a key role in tissue fluid homeostasis, fat absorption, and trafficking of immune cells.1 Dysfunction of lymphatic vessels leads to edema formation, and lymphatic vessels have also been implicated in other pathological conditions, such as inflammation and tumor metastasis.2

In vertebrates, lymphatic vessels originate from blood endothelial cells in the cardinal vein. In mice, lymphatic endothelial cells (LECs) are first specified on the dorsal side of the cardinal vein by expression of the lymphatic endothelial marker Prox1.3 Subsequently, LECs migrate away as strings of loosely connected cells and reorganize into 2 large, lumened lymphatic vessels, the dorsolaterally situated peripheral longitudinal lymphatic vessel (PLLV) and the more ventrally localized primordial thoracic duct (pTD).4,5 From these structures, a lymphatic network emerges through sprouting lymphangiogenesis.

In zebrafish, lymphatic precursor cells arise when venous sprouts migrate dorsally from the cardinal vein at 32 hours post fertilization. Roughly half of these sprouts connect to an artery to form intersegmental veins, while the other half migrate dorsally to the horizontal myoseptum region and form a pool of lymphatic precursors cells, the parachordal lymphangioblasts.6,7 These parachordal lymphangioblasts subsequently migrate dorsally and ventrally along arteries to reorganize into the main lymphatic vessels, the TD, and the dorsal longitudinal lymphatic vessels.8

The main lymphangiogenic signaling pathway involves vascular endothelial growth factor-c/vascular endothelial growth
factor receptor-3 (VEGFC/VEGFR3) signaling. Both in mouse and in fish, VEGFC/VEGFR3 signaling is crucial for the sprouting of venous cells from the cardinal vein.5,9,10 In VEGFC knock-out mice, LECs are specified, but fail to migrate away from the cardinal vein.11 Thus, the current model postulates that VEGFC acts as a morphogen to activate VEGFR3 signaling in LECs, which drives LECs out of the cardinal vein. In Vegfc mutant zebrafish, as well as in Vegfr3/Flt4 kinase dead mutants, venous sprouts fail to migrate out of the cardinal vein and subsequently, the formation of parachordial lymphangio-blast and the major lymphatic vessels (TD and dorsal longitudinal lymphatic vessels) is impaired.9,12 In humans, mutations in VEGFR3 or VEGFC have been linked to Milroy and Milroy-like disease, a form of hereditary primary lymphedema.13,14 Recently, collagen- and calcium-binding EGF domains 1 (CCBE1) was identified to be essential for lymphatic development in zebrafish and mice.12,15 CCBE1 encodes a secreted protein that contains an EGF domain and a calcium-binding EGF domain (Ca-EGF) at the N-terminal and 2 collagen domains at the C-terminal. In zebrafish, Ccbe1 is required for lymphovenous sprouting from the cardinal vein.6,9,10 In VEGFC knock-out mice, LECs are specified, but fail to migrate away from the cardinal vein (CV), hence resembling the VEGFC phenotype. However, in contrast to VEGFC knock-out mice, where no lymphatic sprouts are formed, CCBE1 knock-out embryos display a distinct migratory effect and exhibit abnormal, short Prox1-positive sprouts which fail to segregate from the cardinal vein.5 In both zebrafish and mice, CCBE1 interacts genetically with Vegfc.5,10 Recently, it has been shown that CCBE1 is a crucial regulator of VEGFC processing and regulates Vegfc-mediated induction of Vegfr3 signaling during embryonic lymphangiogenesis.10,17 In order for VEGFC to reach its full signaling capacity, the VEGFC protein needs to be activated by cleavage of the N- and C-terminal propeptides from the VEGF homology domain.16 In fact, CCBE1 regulates activation of VEGFC by enhancing proteolytic processing of VEGFC via the metalloprotease ADAMTS3.17 Importantly, CCBE1 is linked to Hennekam syndrome (HS), a human recessive disease characterized by lymphedema, lymphangiectasia, and mental retardation.18 To date, 9 different mutations in CCBE1 were identified to be causative19 for HS, with most mutations encoding missense mutations that affect the N-terminal part of the protein, namely the EGF domain, the Ca-EGF domain, or the cysteine-rich sequence upstream of the EGF domain. Only 2 of the known mutations affect the collagen repeat domains, probably affecting the stability of a triple helix conformation of the collagen domain by altering conserved glycines in a Gly-X-Y motif.19 One mutation introducing a frame-shift and encoding a predicted truncated protein lacking the collagen domain was found as a compound heterozygous mutation together with one of the other mutations.

Until now, it is unknown which parts of the CCBE1 protein are needed for its function in vivo. Thus, we performed a functional analysis of the different domains of CCBE1 using 3 different approaches. First, we generated knock-in mice expressing deletion mutants of CCBE1. Second, we tested whether mutant Ccbe1 molecules were able to increase the activity of Vegfc in an in vivo zebrafish assay. Third, we analyzed whether mutant CCBE1 molecules can activate VEGFC processing in vitro. We found that deletion of the collagen domain inhibited CCBE1 function in vivo phenocopying CCBE1 knock-out mice. Furthermore, a CCBE1ΔColCollagen mutant was not able to activate VEGFC signaling in vivo and in vitro. Surprisingly, however, we found that deletion of the EG domains had no effect on VEGFC activation by CCBE1 in vitro and a CCBE1ΔEGF mutant is still partially active in vivo as CCBE1ΔEGF mice develop rudimentary lymphatics. Consistent with this, Ccbe1ΔEGF is able to increase Vegfc signaling in zebrafish.

Collectively, our data suggests that the collagen domains of CCBE1 are crucial for its ability to increase VEGFC activity in vivo and in vitro. The EG domains are necessary for its full lymphangiogenic activity in vivo; however, they are dispensable for enhancement of VEGFC processing in vitro.

Our results provide important insights into the functionality of CCBE1 protein domains, with possible implications for therapeutic lymphedema treatment.

Methods

Mouse Lines

Mouse lines were maintained at the Hubrecht Institute using standard husbandry conditions. Experiments were approved by the local Animal Experimentation Committee (DEC). Generation of knock-in mice is described in the Supplement.

Zebrafish Lines

The Tg(fli1a:EGFP) and Tg(shh:vegfc) were described previously.10,20

VEGFC Processing In Vitro

Analyzing VEGFC processing in vitro is described in detail in the Supplement.

Results

CCBE1 Variants Lacking Either the EGF or the Collagen Repeat Domains Cannot Substitute for WT CCBE1 In Vivo

To investigate the relevance of the different domains of CCBE1 in vivo, we generated knock-in mice expressing different deletion mutants of CCBE1, lacking either the EGF and Ca-EGF domains (CCBE1ΔEGF mice) or lacking both collagen repeat domains (CCBE1ΔCol mice; Figure 1A). The
EGF domains were removed by generating mice with floxed exons 4 and 5 (Figure 1B). Cre-mediated recombination led to an in-frame deletion of exon 4 and 5 removing both EGF domains. Correct splicing from exon 3 to 6 was verified by sequencing of cDNA from homozygous CCBE1ΔEGF embryos (Online Figure 1A). For CCBE1ΔCol mice, removal of the 2 collagen repeat domains was achieved by replacement of exon 7 of CCBE1 with the cDNA of exon 7 to 11 lacking amino acids 248 to 337 followed by a transcriptional stop (Figure 1C). Analogous to this knock-in, a CCBE1 full length (CCBE1FL) knock-in mouse was generated as a control in which exon 7 is replaced with the cDNA of exon 7 to 11 including the collagen domains followed by a transcriptional stop. Expression levels of CCBE1ΔCol and CCBE1FL in knock-in mice were confirmed by Western blotting (Online Figure 1B). Homozygous CCBE1FL control knock-in mice were viable and fertile and did not show any overt morphological defects (Figure 1D). Homozygous fetuses expressing CCBE1ΔEGF or CCBE1ΔCol presented with severe edema at E14.5 and die in utero, thus phenocopying the CCBE1 full knock-out in which a LacZ cassette was placed into the CCBE1 locus (CCBE1LacZ; Figure 1D).15 Heterozygous animals for both CCBE1ΔEGF and CCBE1ΔCol mutants were viable and fertile and did not show any overt edemic phenotype, but fetuses compound heterozygous for CCBE1ΔEGF and CCBE1ΔCol show the same phenotype as mice homozygous for the 2 alleles (Online Figure 1C).

Thus, mutant CCBE1 proteins lacking either the EGF domains or the collagen repeat domains cannot substitute for wild-type (WT) CCBE1 in vivo.

**CCBE1ΔCollagen Mice Lack All Lymphatic Structures, But Lumenized Lymphatic Structures Develop in CCBE1ΔEGF Fetuses**

Previously, we have shown that CCBE1 knock-out mice lack all lymphatic vessels, whereas the patterning of blood vessels is not affected.15 To analyze lymphatic and blood vessel development in mice expressing CCBE1 lacking either the EGF domains or the collagen repeat domains, we performed skin stainings of mutant fetuses at E14.5 to E16.5 (Figure 2A, Online Figure II) using Lyve-1 and Prox1 as lymphatic markers and the pan-endothelial marker CD31 to visualize blood vessels. We observed no abnormalities in blood vessels in any of the mice. Moreover, CCBE1FL knock-in mice do not show obvious differences in lymphatic vessel development in the skin compared with WT animals. Homozygous CCBE1ΔCol fetuses phenocopy CCBE1LacZ mice lacking all lymphatic vessels. Interestingly, however, CCBE1ΔEGF mice do exhibit some lymphatic structures in the skin. Like lymphatic vessels in WT control fetuses, these structures sprouted into the direction of the dorsal midline at E14.5, but they failed to develop into a contiguous, branched plexus at both time points analyzed. At E16.5, patches of LECs had aggregated and formed large spherical structures.
Next, we performed Lyve-1 stainings of paraffin sections of E14.5 fetuses (Figure 2B) to investigate lymphatic development close to the CV. Although the pTD and large lymphatic vessels in other areas, such as the skin, could be readily identified in WT control and in CCBE1FL knock-in fetuses, lymphatic vessels were undetectable in CCBE1ΔCol or CCBE1LacZ mice. Analogous to the whole mount stainings, lumenized lymphatic structures were found in the skin of E14.5 CCBE1ΔEGF fetuses. Moreover, lymphatic structures were also identified at the position of the pTD; however, these structures were smaller than in WT control animals.

Collectively, these results reveal that although both the collagen domains and the EGF domains are necessary for lymphatic development, a CCBE1ΔEGF mutant does retain partial functionality in vivo.

**Specified LECs Migrate From the Cardinal Vein in CCBE1ΔEGF Embryos, But Not in CCBE1ΔCollagen or CCBE1LacZ Embryos**

To analyze lymphatic development in more detail and at an earlier stage, we visualized the formation of the first lymphatic structures by whole mount staining and optical clearing of E12.5 embryos. Recently, it had been shown that specified LECs leave the CV as streaks of cells to form the first 2 large, lumenized lymphatic vessels, the PLLV, and the pTD. In CCBE1LacZ knock-out embryos, as well as in CCBE1ΔCol embryos, Prox1+ or Lyve-1+ LECs were unable to leave the CV and were not detected outside of the CV. In CCBE1ΔEGF mice, however, some Prox1+ cells were able to leave the cardinal vein but only formed discontinuous lymphatic structures at the positions where the pTD and PLLV are normally located (Figure 3).

Thus, the CCBE1ΔCol mutant has no lymphangiogenic activity in vivo. The CCBE1ΔEGF mutant is able to induce migration of lymphatic progenitor cells out of the CV at early time points of lymphatic development, although it cannot induce the formation of the main lymphatic structures, the PLLV, and pTD.

**Second Collagen Domain of Ccbe1 is Essential for Enhancing Vegfc Signaling in Zebrafish**

To confirm and extend our findings in an independent system, we used an assay that assesses Vegfc signaling in zebrafish. A gene of choice is expressed specifically in the ventral-most region of the neural tube, the floor plate, from the shh promoter. The effect of the transgene on adjacent intersegmental vessels can then be monitored in vivo and over time. Previously, we have shown that Tg(shh:vegfc;shh:ccbe1) zebrafish, expressing both vegfc and ccbe1 as independent transgenes in the floor plate, exhibit aberrant ectopic turning of arteries at 32 hours post fertilization, whereas transgenic zebrafish carrying single transgenes do not. This phenotype can be attributed to high...
Vegf/Vegfr3 signaling in arteries. Injection of ccbe1 mRNA in Tg(shh:vegfc) embryos that only express vegfc from the shh promoter also efficiently induced strong arterial sprouting (Figure 4B and 4H), whereas injection of ccbe1 mRNA into WT embryos had no effect (Online Figure III). First, we tested the activity of a ccbe1 mutant, fofhu3613, in this assay. fofhu3613 harbors...
a mutation in the EGF domain (D162E), leading to a loss of lymphatic development in zebrafish mutants. Unexpectedly, fofhu3613 mRNA has levels of activation that are indiscernible from wt ccbe1 (Figure 4B, 4C, and 4H). Interestingly and in accordance with the mouse data, ccbe1ΔEGF was still able to induce misturning of arteries, albeit to a lower degree than WT ccbe1 (Figure 4D and 4H). In contrast, ccbe1ΔCol mRNA injection did not induce ectopic arterial turning (Figure 4E and 4H). To further narrow down which part of the collagen domains was required for its function, we tested mutant forms of ccbe1 in which either the first or the second collagen domain was removed. Interestingly, we found that deletion of the first collagen domain, ccbe1ΔColA, only led to a partial loss of function in the ability to induce ectopic turning, whereas loss of the second collagen domain, ccbe1ΔColB, led to a complete loss of function (Figure 4F–4H).

Thus, the EGF and Ca-EGF domain, as well as the first collagen domain, are required for full function of the Ccbe1 protein, however, only removal of the second collagen domain fully renders the Ccbe1 protein dysfunctional in regulating Vegfc signaling in zebrafish. These data are in alignment with the above-described mouse mutant analysis.

### Second Collagen Domain of CCBE1 Is Required for Enhancement of VEGFC Processing by CCBE1 In Vitro

CCBE1 has recently been shown to enhance proteolytic processing of VEGFC, which leads to an increase in bioavailability of the mature 21 kDa form of VEGFC. To assess how the various functional domains affect the ability of CCBE1 to increase mature VEGFC levels, we coexpressed human VEGFC, together with WT or various mutant forms of CCBE1 in HEK-293T cells. Conditioned media containing secreted VEGFC and CCBE1 were analyzed by Western blotting. As reported before, CCBE1 clearly stimulated the proteolytic processing of VEGFC as is apparent from an increased amount of the 21 kDa form of VEGFC. Surprisingly, we found that the EGF domains are not required for CCBE1 function in VEGFC processing in vitro as coexpression of CCBE1ΔEGF led to an increase in mature VEGFC comparable with that of WT CCBE1 (Figure 5B). Moreover, we tested whether CCBE1ΔEGF had different kinetics than WT CCBE1 by combining conditioned media from CCBE1 and VEGFC expressing cells for various periods of times. Interestingly, we could find no difference between CCBE1 and CCBE1ΔEGF (Online Figure IV). Also by lowering the amount of transfected CCBE1,
we could not detect any difference in the activity of CCBE1 and CCBE1ΔEGF (Online Figure V). Moreover, CCBE1Δ170E, which harbors a point mutation that is equivalent to the fofhu3613 mutation in zebrafish, was also able to enhance processing of VEGFC (Online Figure V), further corroborating that perturbation of the EGF domain does not impair CCBE1-mediated processing of VEGFC in vitro.

In contrast, the CCBE1 mutant that lacks the collagen domains (CCBE1ΔCol) lost the ability to increase VEGFC processing (Figure 5B). Both deletion of solely the collagen domains or truncation of the C-terminal half of CCBE1 (CCBE1 1–175) abolished its activity (Online Figure VI). Furthermore, we metabolically labeled cells that were transfected with VEGFC and CCBE1 variants and precipitated VEGFC using VEGFR3-Fc, which further confirmed these results (Online Figure VII). In conclusion, the collagen domains are of crucial importance for the effect of CCBE1 on proteolytic processing of VEGFC.

To test how the different CCBE1 variants regulate VEGFC-mediated processes, such as proliferation and downstream signaling on LECs, we incubated LECs with conditioned media from HEK-293T cells. Conditioned medium containing VEGFC and CCBE1 or CCBE1ΔEGF were able to increase proliferation even further compared with medium containing only VEGFC or VEGFC and CCBE1ΔCol (Online Figure VIII). To investigate downstream signaling, we also analyzed phosphorylation of ERK. In agreement with the proliferation assay, combinations of VEGFC with either CCBE1 or CCBE1ΔEGF induced a stronger phosphorylation of ERK than VEGFC on its own or VEGFC in the presence of CCBE1ΔCol (Figure 5C). These findings show that the collagen domains are crucial for the VEGFC-mediated activation of LECs.

Because the zebrafish experiments indicated that the 2 collagen domains are not equally important for CCBE1 function in mediating VEGFC signaling in vivo, we tested whether these domains would also have differential effects on VEGFC processing in vitro. Strikingly, deletion of the first collagen domain (CCBE1ΔColA) was dispensable for activation of VEGFC processing in this assay, however, deletion of the second collagen domain (CCBE1ΔColB) inhibited activation of VEGFC processing by CCBE1 (Figure 5D, compare lanes 12 and 13). Moreover, even deletion of amino acids 92 to 294, which deletes all parts of the protein from the EGF domains to the first collagen domain (CCBE1ΔEGFΔColA), did not have an overt effect on CCBE1 function in this assay (Figure 5D).

Because CCBE1 has been suggested to mediate processing of VEGFC via the ADAMTS3 protease,17 we also tested if ADAMTS3 cooperates with mutant forms of CCBE1. We generated a stable cell line expressing VEGFC and subsequently coexpressed ADAMTS3 and CCBE1. We found that overexpression of ADAMTS3 on its own induced a mild increase of proteolytic processing which was further increased by coexpression of CCBE1 or CCBE1ΔEGF (Figure 5D). In agreement with our other findings, lack of the collagen domains led to an almost complete abrogation of the synergism between CCBE1 and ADAMTS3 (Figure 5D). We found that under these experimental conditions CCBE1ΔCol gave a minor increase in VEGFC processing. The increase in processing was more pronounced, when we used higher amounts of CCBE1 (variant) medium (Online Figure IX). These data suggest that
CCBE1ΔCol retains a weak capacity to mediate VEGFC processing in the presence of ADAMTS3, but only to a degree that is much lower than WT CCBE1 or CCBE1AE GF.

We further confirmed that the synergism of CCBE1 with ADAMTS3 depended more on the second collagen domain, as CCBE1ΔColA enhanced processing as effectively as WT CCBE1, whereas CCBE1ΔColB showed much less activity. However, CCBE1ΔColB expression did cause the appearance of low levels of mature VEGFC, which was not seen to that extent in response to CCBE1ΔCol (Figure 5D, compare lane 11–13). This probably reflects partial functional identity of ColA, which is only apparent in the presence of high levels of ADAMTS3. Next, we tested the binding capacities of the different CCBE1 variants to ADAMTS3. We consistently observed a lower binding propensity of CCBE1ΔCol to bind ADAMTS3 than WT CCBE1 (Online Figure X).

Taken together, these data suggest that the collagen domains, especially the second collagen domain, are of major importance for processing of VEGFC by CCBE1.

Hennekam Mutation in the Collagen Domain, But Not in the Ca-EGF Domain Fully Impairs CCBE1 Function

CCBE1 mutations have been shown to be causative for HS, a congenital autosomal recessive disease, which is characterized by lymphangiectasia, generalized lymphedema and mental retardation. Most of the mutations inducing HS have been identified affecting the N-terminal part of the protein, containing a cysteine-rich sequence N terminal of the EGF domains and the EGF and Ca-EGF domains (Figure 6A). Only 2 mutations have been found within the collagen domains, with 1 patient harboring a mutation in the second collagen domain, G327R. To interrogate the effect of the mutations responsible for the HS phenotype on the functionality of CCBE1, we tested 2 constructs that bear mutations found in patients with HS. One mutation, R158C, is located within the Ca-EGF domain and 1 mutation, G327R, within the second collagen repeat. First, we tested the functionality of these 2 mutants in vivo by injecting zebrafish ccbe1 mRNAs that bear the corresponding mutations into Tg(shh:vegfc) embryos. Whereas a zebrafish R150C mutation, equivalent to human R158C and located in the Ca-EGF domain, only marginally lowered activity compared with WT ccbe1 in this assay, the mutation in the collagen domain, G313R in zebrafish, completely abolished the ectopic Ccbe1 effect (Figure 6B and 6C). Moreover, in an in vitro processing assay using human CCBE1, the R158C mutation in the Ca-EGF domain did not affect functionality of CCBE1 compared with the WT molecule, whereas the G327R mutation in the second collagen domain led to a strong loss of VEGFC maturation (Figure 6D).

Discussion

CCBE1 is one of the few genes that are indispensable for embryonic lymphatic development. It has been shown that CCBE1 is a crucial regulator of VEGFC activation via the protease ADAMTS3. However, which CCBE1 domains are necessary to exert its function is not known. Here, we have conducted a functional domain analysis, based on different in vitro and in vivo systems, to gain comprehensive insight into the requirement of the different conserved protein domains for CCBE1 activity.

Several lines of evidence have stressed the importance of the EGF domains in the past. First, the majority of mutations in CCBE1 in patients with HS have been found within or in close proximity to the EGF domains. Second, in the original and subsequent screens that identified zebrafish ccbe1 as an essential gene for lymphatic development, several alleles were identified in which the causative mutation was found to be located in the EGF or Ca-EGF domain. To gain more insight into the function of the EGF and Ca-EGF domains, we generated knock-in mice expressing a CCBE1 variant lacking these domains. Surprisingly, unlike the full CCBE1 knock-out fetuses which lack all lymphatic structures, CCBE1AE GF fetuses do develop rudimentary, lumenized lymphatic structures in the skin, and at the position of the pTD and PLLV. In the skin, these lumenized larger structures sprouted into the same direction to the dorsal midline as lymphatic vessels in WT fetuses. They did, however, fail to form a properly branched lymphatic network. In CCBE1AE GF embryos, remnant, fragmented lymphatic structures were located at the position of the pTD and PLLV indicating that specified LECs do migrate out of the cardinal vein roof to the same location as LECs in WT control embryos. In accordance with this, a Ccbe1AE GF mutant was partially functional in zebrafish, where the injection of ccbe1AE GF can still exert aberrant arterial sprouting in the Tg(shh:vegfc) zebrafish. Although fophu3613 could increase Vegfc signaling in arteries to the same extent as wt ccbe1, it cannot substitute for wt ccbe1 in zebrafish because in contrast to wt ccbe1 mRNA, it is not able to rescue a ccbe1 mutant. Unexpectedly, loss of the EGF domains did not have any effect on the ability of CCBE1 to enhance processing of VEGFC in cell culture into the active form detected by the antibodies used. Thus, in vitro, the EGF and Ca-EGF domains are dispensable for the function of CCBE1 in VEGFC activation. However, as a CCBE1AE GF molecule cannot substitute for WT CCBE1 in mice and has reduced activity in increasing Vegfc signaling in zebrafish, the EGF domains do have a crucial function in vivo. The precise role of the EGF and Ca-EGF domains in vivo is not yet clear and needs further investigation in the future. It might be that this domain is necessary in other processes, such as binding to extracellular matrix components (ECM). Consistent with this notion, a CCBE1ΔCollagen-Fc protein has been shown to bind to vitronectin, Collagen I, Collagen IV, and Collagen V in an in vitro binding assay. Alternatively, the EGF domains could be involved in other aspects of VEGFC/VEGFR3 signaling, such as receptor binding, possibly also mediated through prior or simultaneous interaction of CCBE1 with ECM. Indeed, in a different assay, a cornea pocket assay, the N-terminal part of CCBE1 was able to enhance the lymphangiogenic effect of purified, fully processed VEGFC.

In all 3 different model systems tested, we show that loss of the collagen domains has a profound effect on CCBE1 function. CCBE1ΔCol knock-in mice phenocopy the CCBE1LacZ full knock-out mice characterized by a failure of specified LECs to leave the cardinal vein. This was further confirmed by a total lack of enhancement of VEGFC signaling by ccbe1Δcol in Tg(shh:vegfc) zebrafish and the inability of CCBE1ΔCol to induce proteolytic processing of VEGFC. In particular, the second collagen domain was found to be of higher importance, as both our zebrafish and our in vitro experiments show that loss of the second collagen domain alone strongly reduce the function of the
CCBE1 protein. The crucial role of the collagen domains may be because of their involvement in direct activation of ADAMTS3. Indeed, coexpression of ADAMTS3 and CCBE1ΔCol did not synergize in VEGFC processing and CCBE1ΔCol has a reduced capacity to bind ADAMTS3. Previously, it has been shown that a recombinant protein containing only amino acids 1 to 175 of CCBE1 was able to induce processing of recombinant pro-VEGFC. However, a comparative analysis between the N- and C-terminal parts of CCBE1 was not performed in that assay. Also in our system, we detected a slight increase in the amount of fully processed VEGFC when ADAMTS3 was overexpressed and high amounts of CCBE1ΔCol were used. Considering that CCBE1ΔCol also binds ADAMTS3, albeit with reduced affinity, ADAMTS3 may have binding epitopes both in the N- and C-terminal parts of CCBE1. Nevertheless, in vitro, even in excess, the activity of the N-terminal part of CCBE1 was markedly reduced compared with WT CCBE1 or the C-terminal part of CCBE1. Thus, these data suggest that, whereas the N-terminal part of CCBE1 can have some activity in proteolytic processing, this activity is clearly reduced compared with WT CCBE1 or the C-terminal part of CCBE1.

Our findings shed new light on the molecular defects that underlie HS and may provide a rationale that explains why so few HS mutations were found in the collagen domains. To date, most patients exhibit missense mutations affecting the N-terminal domain clustered in the EGF domain, the Ca-EGF or the cysteine-rich domain upstream of the EGF domain. We could show that a mutation within the Ca-EGF domain inducing HS, C158R, and a mutation that leads to lymphatic defects in zebrafish, fophu3613, does not affect the ability of CCBE1 to activate VEGFC processing in vitro and can still enhance Vegfc signaling in vivo, indicating that these mutations are hypomorphic alleles. In fact, the phenotype of fophu3613 mutant zebrafish can partially be rescued by expressing high levels of full length Vegfc in the floorplate of zebrafish. These results and the fact that a CCBE1ΔEGF molecule is partially active in vivo suggest that the HS mutations found within the EGF and EGF-Ca domains of CCBE1 are most likely hypomorphic alleles that retain some function.

In contrast, only 1 of 13 patients identified to date harbors a mutation within the second collagen domain. This mutation, G327R, probably affects the secondary structure of the collagen repeat. In our analyses, we found that the G327R mutation strongly reduced CCBE1 function in vitro and in vivo, almost completely impairing its ability to activate VEGFC processing. This result is surprising as such a strong loss of function would most likely result in a complete absence of lymphatics, and it is unlikely that many patients could survive who have mutations in the collagen-B domain. Therefore, we consider it more likely that predominantly weaker alleles of CCBE1 are viable and can be found in patients, for example, alleles that affect the EGF domain. Full loss-of-function situations are unlikely to be viable in humans. Of note, the only known patient bearing the G327R mutation is the only surviving child of 4 siblings. Furthermore, this patient is the only patient known to exhibit vascular defects indicating that this mutation has a stronger effect than the other HS mutations found.

Taken together our findings show that the collagen domains of CCBE1 are indispensable for its function in VEGFC activation in vitro and in vivo. The EGF domains are dispensable for this function in vitro, but are necessary for full activity of Ccbe1 in enhancing Vegfc/VegfR3 signaling in vivo and for proper lymphangiogenesis in the mouse embryo. Contrary to the intuitive belief that the collagen domains bind to the ECM and that the EGF domain is more functionally relevant, we show here that it is the collagen repeat domain that is crucial for enhancement of proteolytic processing of VEGFC by CCBE1, presumably by activating ADAMTS3. The role of the EGF domain should be subject of future investigations but previous work has indicated their capacity to bind ECM proteins. In zebrafish, ccbe1 is expressed along the migration route of lymphatic precursor cells; thus, it is possible that binding of the EGF domains to ECM serves to direct specific locations of CCBE1 activity. At these dedicated areas, the collagen repeat domains of CCBE1 mediate enhanced processing of VEGFC by activation of ADAMTS3. Thus, CCBE1 provides positional information for VEGFC signaling, which orchestrates the migration of LECs (Figure 7). These molecular insights provide new entry points for therapeutical approaches to either stimulate or inhibit lymphangiogenesis by modulating CCBE1 function.

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is Known?**

- Collagen- and calcium-binding EGF domains 1 (CCBE1) protein is required for lymphatic vessel development in fish and in mice.
- CCBE1 is associated with Hennekam syndrome, characterized by lymphedema, lymphangiectasia, and developmental delay.
- CCBE1 regulates vascular endothelial growth factor-C (VEGF-C) signaling via activation of VEGF-C processing by ADAMTS3.

**What New Information Does This Article Contribute?**

- The second collagen domain of CCBE1 is crucial for the activation of VEGF-C processing via ADAMTS3 in vitro and for its lymphangiogenic activity in vivo.
- The EGF domains of CCBE1 are negligible for the activation of VEGF-C processing via ADAMTS3 in vitro, but are needed for its full lymphangiogenic activity in vivo.
- A Hennekam syndrome mutation within the collagen domain affects the function of CCBE1 stronger than a Hennekam syndrome mutation within the EGF domains.

CCBE1 is a secreted protein, necessary for lymphangiogenesis in fish and in mice. In humans, CCBE1 is associated with the Hennekam syndrome, characterized by lymphatic abnormalities. The causative mutations in CCBE1 are mostly located in the N-terminal part of the protein. CCBE1 exerts its function via activation of proteolytic processing of the main lymphatic growth factor VEGFC via the protease ADAMTS3. To date, it is not known which parts of the protein are necessary for its function. We show here that the collagen domains of CCBE1 are crucial for its lymphangiogenic activity in vivo as well as for the activation of VEGFC via ADAMTS3. The EGF domains are necessary for its full lymphangiogenic activity in vivo, but are dispensable for its ability to induce VEGFC processing in vitro. The crucial role of the collagen domains possibly explains why few Hennekam mutations are found in the collagen domains. This study furthers our understanding of how CCBE1 orchestrates lymphangiogenesis and has implications for new pro- or anti lymphangiogenic therapeutic approaches.
Functional Dissection of the CCBE1 Protein: A Crucial Requirement for the Collagen Repeat Domain

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Supplemental Material

Supplemental Methods

Antibodies and reagents:

The following antibodies were used: Anti-Lyve-1 (#11-034, Angiobio), Anti-Lyve-1 (AF2125, R&D Systems), Anti-CD31 Mec13.3 (#550274, Pharmingen), anti-Prox1 (102-PA32, ReliaTech), anti-Prox1 (AF2727, R&D Systems), anti-VEGFC (isoform 103 antibody, antibodies-online), anti-CCBE1 (HPA041374 Sigma), anti-ERK1/2 (#4696, Cell Signaling), anti Phospho-ERK (#9101, Cell Signaling), anti-HA tag antibody (ab9110, abcam), anti-FlagM2 antibody (#3165, Sigma, and ANTI-FLAG® M2 Affinity Gel (A2220, Sigma). All Alexa-coupled antibodies were purchased from Invitrogen, HRP coupled antibodies were used from Dako.

Constructs:

cce1 (wt), ccce1fof, ccce1-myc, ccce1-mycR150 and ccce1myc-G313R cDNA clones have been described previously1, 2. Zebrafish ccce1ΔEGF was generated by amplification of ccce1 pCS2 followed by religation. Zebrafish ccce1Δcol, ccce1ΔcolA and ccce ΔcolB were generated by site-directed mutagenesis using wt ccce1 in PCS2 as template. Human CCBE1 was subcloned into the pBabe vector in Xho1-EcoR1 restriction sites. Mutant variants of CCBE1 for expression in human cells were generated by site directed mutagenesis. Human CCBE1 Flag and human CCBE1 1-175 Flag were subcloned bluntly using Stu1 restriction enzyme in PCS2. ADAMTS3 was subcloned from an ADAMTS3 clone (RC211602, Origene) into PCS2 and pLV using EcoR1 sites. Lentiviral constructs for CCBE1 variants were generated by PCR from CCBE1 pBABE constructs and cloned in pLV vector, pLV VEGFC was generated by PCR from VEGFC PCS2 using Spe1/Xho1 sites. Primer sequences used for generation of constructs are given in Online Table I.

Online Table I

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Cell culture

Human LECs were a kind gift from Dr. Tatiana Petrova. LECs were cultured in EGM-2-MV medium (Lonza). HEK-293Ts were cultured in DMEM supplemented with 10% FBS and pen/strep.

Generation of stable 293T cell lines

Recombinant lentiviruses were produced by the transfection of 293T cells with pLV constructs described above and helper plasmids psPax2 and plPVSVG. Transduction of 293Ts was performed at a multiplicity of infection of 1, with the addition of Polybrene to a final concentration of 8 g ml$^{-1}$. One day post infection, the medium was replaced with fresh medium (supplemented with puromycin).

WST assay

LECs were cultured in 96 well plates at a concentration of 5000 cells per well and grown in the indicated conditioned medium for 24 hours. Subsequently proliferation was assessed by adding WST reagent (Roche) according to the manufacturer’s protocol. After 3 hours of incubation absorbance was measure at 450 nm using a microplate reader.

Targeting strategy for the generation of CCBE1ΔEGF and CCBE1ΔCol mice:

CCBE1ΔEGF mice were generated by homologous recombination of mouse SV129 ES cells using a targeting vector that contains a 4.2 kb long arm of homology 5’ of exon 4, an FLPe
flanked neomycin resistance cassette, a LoxP flanked exon 4 to 5 and a 2 kb short arm of homology 3’ of exon 5. Neomycin-resistant clones were screened by Southern blot analysis. The Neomycin resistance cassettes were removed by crossing to FLPe mice, and exon 4 and 5 of CCBE1 were deleted by crossing floxed mice to PGK-Cre mice. For the generation of CCBE1ΔCol mice, mouse SV129 ES cells were transfected using a targeting vector comprising a 4.5 kb arm of homology 5’ of exon 7, the cDNA of exon 7 to 11 lacking bp encoding aa 248 to 337 followed by a Flag-HA tag, a transcriptional stop, an FLPe flanked neomycin resistance cassette followed by a single LoxP site and a 4.2 kb arm of homology 3’ of exon 7. CCBE1FL mice were generated analogous to CCBE1ΔCol mice encoding the complete cDNA of exon 7 to 11. The Neomycin resistance cassette was removed by crossing to FLPe mice. All mouse lines were crossed at least 5 times to C57Bl6 mice before analysis.

Immunoprecipitation of Flag-HA tagged CCBE1ΔCol and CCBE1FL proteins from CCBE1ΔCol and CCBE1FL embryos

E14.5 embryos were removed from the uterus, homogenized with an Ultra Turrax in RIPA buffer plus 1 mM NaVO₄ and 2x complete EDTA free protease inhibitors. Lysates were incubated over night at 4°C and centrifuged at 4°C for 30 min. For immunoprecipitation, lysates were incubated for 2 hrs at 4°C with anti-Flag beads, washed three times with lysis buffer and analyzed by SDS-page. Flag-HA tagged CCBE1 proteins were detected by Western blotting using anti-HA antibodies.

mRNA injection into zebrafish eggs

ccbe1 mRNA was transcribed from a NotI-linearised template using SP6 RNA polymerase and the mMessage mMachine Kit (Ambion), and injected at 400 pg/embryo.

VEGFC processing in vitro

Proteolytic processing of VEGFC was performed as described previously³. Briefly, HEK293T cells were transfected with VEGFC and/or CCBE1 cDNA using XtremeGene (Roche) according to manufacturer’s instructions. Conditioned media were collected and mixed with Laemmli sample buffer. When indicated, conditioned media were collected, mixed at the indicated ratio and incubated at 37°C before mixing with sample buffer. Western blotting analysis was performed using VEGFC (VEGFC isoform 103 antibody, antibodies-online) and CCBE1 (HPA041374, Sigma) antibodies. Note that antibodies used to detect VEGFC are able to recognize both the intermediate 31 kD form and the fully processed mature 21 kD form of VEGFC. CCBE1 antibodies recognize an epitope in the C-terminus of the protein, allowing detection of all variants used in this study unless stated otherwise. When indicated, VEGFC was coexpressed with VEGFR-3-Fc⁴, which was immunoprecipitated using protein A-beads (GE-Healthcare).

Whole mount staining of murine fetal skin

Whole mount staining of dorsal skin preparations of fetuses from staged matings was performed as previously described⁵ using anti-PECAM1, anti-Lyve-1 and anti-Prox1 as primary antibodies. Images were acquired on a Leica SP8 confocal microscope.
Immunohistochemistry

Embryos were fixed, processed into paraffin blocks and immunostaining of sections was performed according to standard procedures. Envision+ kit (DAKO) was used as a secondary reagent and stainings were developed using DAB. Slides were counterstained with hematoxylin.

Whole mount staining and optical clearing of mouse embryos

Whole mouse embryos were stained and cleared as described previously using anti-Prox1, anti-PECAM-1 and anti-Lyve-1 as primary antibodies. Cleared embryos were imaged on a Leica SP8 confocal microscope. 3D stacks were generated using Fiji (Image J).

Supplementary References:


Online Figure Legends

Online Figure I: CCBE1ΔEGF and CCBE1ΔCol cannot substitute for wildtype CCBE1 in vivo.

(A) Sequencing of cDNA isolated from homozygous CCBE1ΔEGF embryos showing correct splicing from exon 3 to 6. (B) Immunoprecipitation of Flag-HA tagged CCBE1ΔCol and CCBE1FL proteins. Tagged CCBE1 proteins were immunoprecipitated from whole embryos at E14.5 using anti-Flag beads and were detected by Western blotting using anti-HA antibodies. Equal loading of the IP is shown by an anti-tubulin Western blot of the whole embryo lysate. (C) Edema formation in CCBE1ΔEGF/CCBE1ΔCol fetuses at E14.5.
Online Figure II: Rudimentary lymphatics develop in the skin of CCBE1ΔEGF, but not of CCBE1ΔCollagen fetuses.

Skin stainings of blood and lymphatic vessels visualized by PECAM-1, Lyve-1 and Prox1 staining in E14.5 embryos. CCBE1Col fetuses phenocopy CCBE1LacZ mice with a complete lack of lymphatic vessels in the skin. CCBE1ΔEGF skins feature remnant lymphatic structures which are unable to form a branched lymphatic network.

Online Figure III: CCBE1 lacking the second collagen domain cannot activate VEGFC in vivo, whereas deletion of the EGF domains or the first collagen domain leads to a modest decrease of activation.

Percentage of embryos showing wt (no arterial sprouting), mild (aberrant ISAs spanning up to two somites) or severe (aberrant ISAs spanning more than two somites) arterial sprouting in Tg(shh:vegfc) and wt embryos that are uninjected or were injected with the indicated ccbe1 variant mRNAs. Tg(shh:vegfc) embryos: n=101, 427, 149, 182, 93, 149, 188 for injection of wt ccbe1, ccbe1fof, ccbe1ΔEGF, ccbe1Δcol, ccbe1ΔcolA and ccbe1ΔcolB mRNA, respectively. Wt embryos: n= 55, 117, 42, 53, 52, 69, 56 for injection of wt ccbe1, ccbe1fof, ccbe1ΔEGF, ccbe1Δcol, ccbe1ΔcolA and ccbe1ΔcolB mRNA, respectively.

Online Figure IV: Kinetics of activation of VEGFC processing via CCBE1 are unaltered by loss of EGF domains

Stable cell lines were generated that express either VEGFC, CCBE1, CCBE1ΔEGF or CCBE1ΔCol (293T-VEGFC, 293T-CCBE1, 293T-CCBE1ΔEGF, 293T-CCBE1ΔCol). Conditioned media were combined in a ratio of 4:1 (VEGFC:CCBE1 variant) and incubated for 3 or 6 hrs at 37 degrees, after which the media were combined with Laemmli buffer. VEGFC isoforms were detected by Western blotting. At 3 hrs of incubation no processing of VEGFC could be detected under these conditions, but after 6 hrs CCBE1 and CCBE1ΔEGF could clearly enhance processing of VEGFC, while CCBE1ΔCol could not.

Online Figure V: Titration of CCBE1 variants shows that loss of the EGF domains or a D170E mutation do not affect the activity of CCBE1

Various amounts of indicated CCBE1 constructs were cotransfected in 293T cells with a constant amount of VEGFC (1 ug) and a VEGFC processing assay was performed. Note that even 4 ug of CCBE1ΔCol shows no activity compared to 0,5 ug of wt CCBE1. In contrast CCBE1ΔEGF and CCBE1D170E show activities comparable to wt CCBE1.

Online Figure VI: CCBE1 1-175 Flag is unable to mediate processing of VEGFC

HEK-293T cells were transfected with indicated constructs and a processing assay for VEGFC was performed. Note that CCBE1 1-175 could not be detected with the CCBE1 antibody used for the other CCBE1 constructs and therefore we used a Flag-tag to allow detection of this protein. CCBE1 1-175 is unable to effectively induce processing of VEGFC.

Online Figure VII: Proteolytic processing of VEGFC by various CCBE1 variants analyzed by radiolabeling and VEGFR-3-FC immunoprecipitation
293T cells were transfected with VEGFC with and without CCBE1 constructs and incubated with radioactive amino acids. Supernatants were analyzed by VEGFR-3-Fc immunoprecipitation and autoradiography. The partially processed VEGFC 31 kD, the mature VEGFC 21 kD and the fold change of the mature VEGFC are indicated. Fold changes were calculated by densitometric analysis of 21 kD bands using ImageJ.

Online Figure VIII: Proliferation of LECs in response to VEGFC and CCBE1 variants

Human LECs were serum starved for 6 hours and then incubated with conditioned media from the stable cell lines 293T-VEGFC and 293T-CCBE1, 293T-CCBE1ΔEGF or 293T-CCBE1ΔCol in a ratio of 1:1 (VEGFC:CCBE1 variant). Proliferation of LECs in the presence of indicated proteins was assessed by WST-1 assay after 24 hrs of incubation. Values are reported as relative WST signal (absorbance at 450nm) compared to LECs incubated in medium from control 293T cells. Values represent averages of 5 independent experiments done in triplicate. Two asterisks indicate a statistically significant difference from the control value (p<0,01; paired t-test). N.S. indicates a non-significant difference from control value (p>0,05; paired t-test).

Online Figure IX: Proteolytic processing of VEGFC by high levels of CCBE1 variants

Cells stably expressing VEGFC (293T-VEGFC cells) were transfected with VEGFR-3-Fc and medium was harvested after 3 days. This conditioned VEGFC and VEGFR-3-Fc containing medium was combined with medium derived from 293T cells stably transfected with CCBE1 variants (see Online Figure IV) and conditioned medium from 293T-ADAMTS3 cells, which stably express ADAMTS3. The media were combined in the following ratio: 293T-VEGFC cotransfected with VEGFR-3-Fc (8,3%), 293T-ADAMTS3 (8,3%), 293T-CCBE1variant (83,3%) yielding high levels of CCBE1 that cannot be generated by transient transfection. The combined media were incubated at 37 °C for 24 hours. In this experimental setup VEGFC levels were too low to detect in unconcentrated medium. Therefore VEGFC was concentrated by immunoprecipitating VEGFR-3-Fc and VEGFC processing was assessed via Western blot. Under these conditions CCBE1ΔCol stimulates proteolytic processing of VEGFC, albeit to a much lower degree compared to wt CCBE1 or CCBE1ΔEGF. This increase was only reproducible under these conditions.

Online Figure X: CCBE1ΔCollagen has a reduced affinity for ADAMTS3

Stable cell lines expressing CCBE1, CCBE1ΔEGF, or CCBE1ΔCol were transiently transfected with ADAMTS3. CCBE1 variants were immunoprecipitated and associated ADAMTS3 was analyzed by Western blot. Both proteins are extremely sticky which makes these experiments demanding and leads to background bands even in the negative control. However, we consistently observed a reduced binding of CCBE1ΔCol to ADAMTS3 compared to binding of wt CCBE1 and CCBE1ΔEGF to ADAMTS3.
Online Figure I

A

CCBE1ΔEGF sequenced cDNA

exon 3

exon 6

B

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<th>kDa</th>
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Flag IP/ HA western

Total lysate/ Tubulin western

C

WT

CCBE1ΔEGF/CCBE1ΔCol

Online Figure II

WT | CCBE1ΔEGF | CCBE1ΔCollagen | LacZ

Lyve-1

CD31

Prox-1
Online Figure III
Online Figure IV

![Diagram of VEGFC expression levels at 3 hr and 6 hr with corresponding protein bands for Control, CCBE1, CCBE1 ΔEGF, CCBE1 ΔCol, CCBE1 ΔEGF, and CCBE1 ΔCol.]

Online Figure V

![Diagram of VEGFC and CCBE1 expression levels with varying amounts of CCBE1 variant transfected (0.5 ug, 2 ug, 4 ug).]

VEGFC

Control  CCBE1  CCBE1 ΔEGF  CCBE1 ΔCol  CCBE1 ΔEGF  CCBE1 ΔCol

31 kD
21 kD

3 hr  6 hr

CCBE1

55 kD
40 kD

Amount of CCBE1 variant transfected

0.5 ug  2 ug  4 ug
Online Figure VIII

Proliferation of LECs

N.S.

**

**

N.S.

Relative W3G signal

- VEGFC  VEGFC + CCB1  VEGFC + CCB1 deltaFGF  VEGFC + CCB1 deltaCollagen
Online Figure IX

Medium ratio: 293T-VEGFC cotransfected with VEGFR-3-Fc (8,3%); 293T-ADAMTS3 (8,3%); 293T-CCBE1 variant (83,3%)

VEGFC/VEGFR-3-Fc & ADAMTS3

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Short exposure
Long exposure

Online Figure X

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