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Original Article

ORIGINAL ARTICLE

Loss of ADAMTS3 activity causes Hennekam lymphangiectasia–lymphedema syndrome 3

Pascal Brouillard[1,‡], Laura Dupont[2,‡], Raphael Helaers[1], Richard Coulie[1], George E. Tiller[3,†], Joseph Peeden[4], Alain Colige[2,‡] and Miikka Vikkula [1,5,*,‡]

[1]Human Molecular Genetics, de Duve Institute, University of Louvain, 1200 Brussels, Belgium, [2]Laboratory of Connective Tissues Biology, University of Liège, 4000 Liège, Belgium, [3]Pediatric Medical Genetics, Vanderbilt University Medical Center, Nashville 37232, TN, USA, [4]East Tennessee Children's Hospital, University of Tennessee Medical Center, Knoxville, TN 37916, USA and [5]Walloon Excellence in Life Sciences and Biotechnology (WELBIO), de Duve Institute, University of Louvain, 1200 Brussels, Belgium

*To whom correspondence should be addressed at: Human Molecular Genetics, de Duve Institute, University of Louvain, Avenue Hippocrate 74, Box B1.74.06, B-1200 Brussels, Belgium. Tel: +32 27647496; Fax: +32 27647460; Email: miikka.vikkula@uclouvain.be

Abstract

Primary lymphedema is due to developmental and/or functional defects in the lymphatic system. It may affect any part of the body, with predominance for the lower extremities. Twenty-seven genes have already been linked to primary lymphedema, either isolated, or as part of a syndrome. The proteins that they encode are involved in VEGFR3 receptor signaling. They account for about one third of all primary lymphedema cases, underscoring the existence of additional genetic factors. We used whole-exome sequencing to investigate the underlying cause in a non-consanguineous family with two children affected by lymphedema, lymphangiectasia and distinct facial features. We discovered bi-allelic missense mutations in *ADAMTS3*. Both were predicted to be highly damaging. These amino acid substitutions affect well-conserved residues in the prodomain and in the peptidase domain of ADAMTS3. *In vitro*, the mutant proteins were abnormally processed and sequestered within cells, which abolished proteolytic activation of pro-VEGFC. VEGFC processing is also affected by CCBE1 mutations that cause the Hennekam lymphangiectasia–lymphedema syndrome syndrome type1. Our data identifies *ADAMTS3* as a novel gene that can be mutated in individuals affected by the Hennekam syndrome. These patients have distinctive facial features similar to those with mutations in CCBE1. Our results corroborate the recent *in vitro* and murine data that suggest a close functional interaction between ADAMTS3 and CCBE1 in triggering VEGFR3 signaling, a cornerstone for the differentiation and function of lymphatic endothelial cells.

Introduction

The lymphatic system is essential for tissue and body fluid homeostasis. During development, lymphatic endothelial cells differentiate from veins upon combined action of the PROX1 and SOX18 transcription factors. Several other proteins are subsequently involved in the development of the lymphatic system. Defective function of these proteins may result in primary (congenital) lymphedema. To date, mutations in twenty-seven genes have been associated with either isolated or syndromic primary lymphedema. These include *CCBE1*, *FLT4*, *FOXC2*, *GATA2*, *GJA1*, *GJC2*, *HGF*, *HRAS*, *IKBKG*, *ITGA9*, *KIF11*,

[†]Present address: Department of Genetics, Kaiser Permanente, 4900 W. Sunset Blvd, Los Angeles, CA 90027, USA.
[†]The authors wish it to be known that, in their opinion, the two first authors should be regarded as joint First Authors, and the two last authors should be regarded as joint Last Authors.

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KRAS, *PTPN11*, *PTPN14*, *RAF1*, *RASA1*, *SOS1*, *SOX18* and *VEGFC* (reviewed by Brouillard et al (1)), as well as *CELSR1* (2), *EPHB4* (3), *FAT4* (4), *PIEZO1* (5,6), *RELN* (7), *RIT1* (8), (9), *TSC1* and *TSC2* (10). These genes account for about one third of patients with primary lymphedema.

The central axis controlling lymphangiogenesis involves the VEGFR3 receptor (encoded by *FLT4*) and its ligand, VEGFC. More than 100 patients (families) have been reported with dominant, recessive, or *de novo* inactivating mutations in VEGFR3 (1), and two with a loss-of-function mutation in VEGFC (11,12). One recessive homozygous mutation in PTPN14, a VEGFR3-specific phosphatase, was discovered in a large consanguineous family (13). Moreover, the extracellular protein CCBE1, which is involved in the activation of VEGFC (14), is mutated in individuals with the Hennekam syndrome (OMIM 235510) (15,16). Patients with this recessive syndrome have generalized lymphatic anomalies, a dysmorphic face and cognitive impairment. Mice deficient in *Ccbe1* die due to lymphatic abnormalities (17). Recent biochemical data indicate that ADAMTS3 is a direct partner of CCBE1 required for VEGFC activation (14).

Here, we report the identification of a novel gene mutated in primary lymphedema. We discovered compound heterozygous mutations in *ADAMTS3* in two siblings with a phenotype reminiscent of the Hennekam lymphangiectasia–lymphedema syndrome. The unaffected parents were each a carrier for one of the mutations. We show that each of the ADAMTS3 mutations results in sequestration of the enzyme within the cell, and thus, in the compound heterozygous state of the affected siblings, a complete loss of ADAMTS3 function in regard to VEGFC activation. This underscores the crucial role of ADAMTS3 in the regulation of VEGFR3 signaling pathway.

Results

A family with mutations in *ADAMTS3*

The parents in family LE-69 are of Western European origin and non-consanguineous, with no familial history of primary lymphedema or other lymphatic problems. They were clinically examined and appeared healthy. The mother gave birth to two children, a girl (LE-69-10) and a boy (LE-69-11), who both presented with polyhydramnios and congenital lymphedema of the lower extremities (as well as hydroceles for the boy) (Fig. 1A).

The girl had a prolonged intensive care unit stay, primarily related to her lymphedema and feeding problems. She also has protein-losing enteropathy, which at one time required elemental formula feeding via a gastrostomy tube. Her lymphedema is rather widespread, but worse in the left lower extremity. Increasing severity occurs in a dependent course, but is present in her face, abdomen, genitalia and legs. The facial lymphedema is minimal. Her physical features of note include: flat facies, hypertelorism (intercanthal distance 4.25 cm), synophrys, pseudo-strabismus, upward-slanting palpebral fissures, and anteverted peaked nostrils. She has an unusual stellate shaped umbilicus. She has a normal chest and cardiovascular exam. She has no hepatosplenomegaly. She has growth hormone deficiency, which is responding well to therapy, and complex migraines. Otherwise, she is a healthy adolescent.

Her brother has had a more benign course. He had less edema at birth. He had a spontaneous pneumothorax and required a chest tube. He also had protein-losing enteropathy, but to a lesser degree than his sister, and never required gastrostomy feeding. He has no hepatic involvement and no evidence of growth hormone deficiency. He has facial features similar to those of his sister, including hypertelorism (intercanthal distance 4 cm) and strabismus. He has minimal facial lymphedema. Lymphedema in the extremities is more pronounced on the left side. Secondary changes of his feet are milder than those of his sister.

A rather unusual finding noticed in the girl, and to a lesser degree in her brother, is the nearly complete resolution of the lymphedema during febrile illness. She may even do without her compression garments during these episodes. This is not due to dehydration, nor to use of non-steroidal anti-inflammatory agents. Incidentally, the girl has a chromosome 14p deletion inherited from the unaffected father; no karyotype was performed for the boy.

In order to identify the genetic cause of primary lymphedema in this family, we explored the whole-exome sequencing data from the index patient (LE-69-10, Fig. 1A) using Highlander (Helaers et al, under revision). We unraveled only one heterozygous polymorphism in CCBE1 (rs200772179, present 61 times in gnomAD (http://gnomad.broadinstitute.org)) and no change in FAT4. However, we discovered two concomitant nucleotide changes in *ADAMTS3*. The corresponding amino acid substitutions were predicted to be damaging or deleterious by Polyphen 2, Mutation Taster, SIFT, LRT, and with medium or high impact by Mutation Assessor. Variant *c.503 T > C* (nucleotide 503 from the ATG of *ADAMTS3*) results from an A-to-G substitution at position 73, 414, 196 on chromosome 4 (based on reference genome assembly hg19), which predicts a leucine 168 to a proline substitution (p.Leu168Pro). This change was found in the affected siblings (LE-69-10 and -11) and the unaffected father (Fig. 1A). The second variant found in the girl (LE-69-10), was an A-to-G change at position 73, 188, 804, corresponding to a *c.872 T > C* (p.Ile291Thr) substitution. It was also detected in the brother and the unaffected mother. Neither variant was present in the 60, 706 WES of ExAC, the 250 trios of GoNL (Genome of the Netherlands), nor our internal cohort of 645 sequenced samples. The *c.503 T > C* (p.Leu168Pro) is present once within the 245, 264 alleles in gnomAD, whereas the *c.872 T > C* (p.Ile291Thr) is not. Thus, the changes are extremely rare, which corroborates the note that the two children are compound heterozygotes for two recessive mutations in *ADAMTS3*. No other homozygous or compound heterozygous mutation in *ADAMTS3* was found within 78 additional primary lymphedema index patients sequenced.

ADAMTS3-L168 and I291 are conserved residues

The *c.503 T > C*; p.Leu168Pro (L168P) and *c.872 T > C*; p.Ile291Thr (I291T) substitutions were predicted to be damaging by several software programs. However, they were not localized in domains known to be crucial for ADAMTS3 activity, such as the furin cleavage site or the amino acids of the peptidase domain involved in zinc binding and catalytic activity (Fig. 1B). Therefore, we aligned the amino acid sequences of ADAMTS3 from different species (from human to fish and reptile), focusing on the regions encompassing the mutations (Fig. 2A). The prodomain (ending at the furin cleavage site, Fig. 1B) is only moderately conserved. However, the L168P mutation is in the center of a fully conserved 9-amino acid stretch (N-C-D-G-L-A-G-M-I), suggesting an important role in enzymatic function. The second mutation is located in the first half of the metalloproteinase domain, between the furin and the zinc-binding catalytic site (Fig. 1B). This domain is well conserved among different species, and remarkably, the I291T mutation is located within one of the most conserved stretches of the sequence (Fig. 2A, right panel).

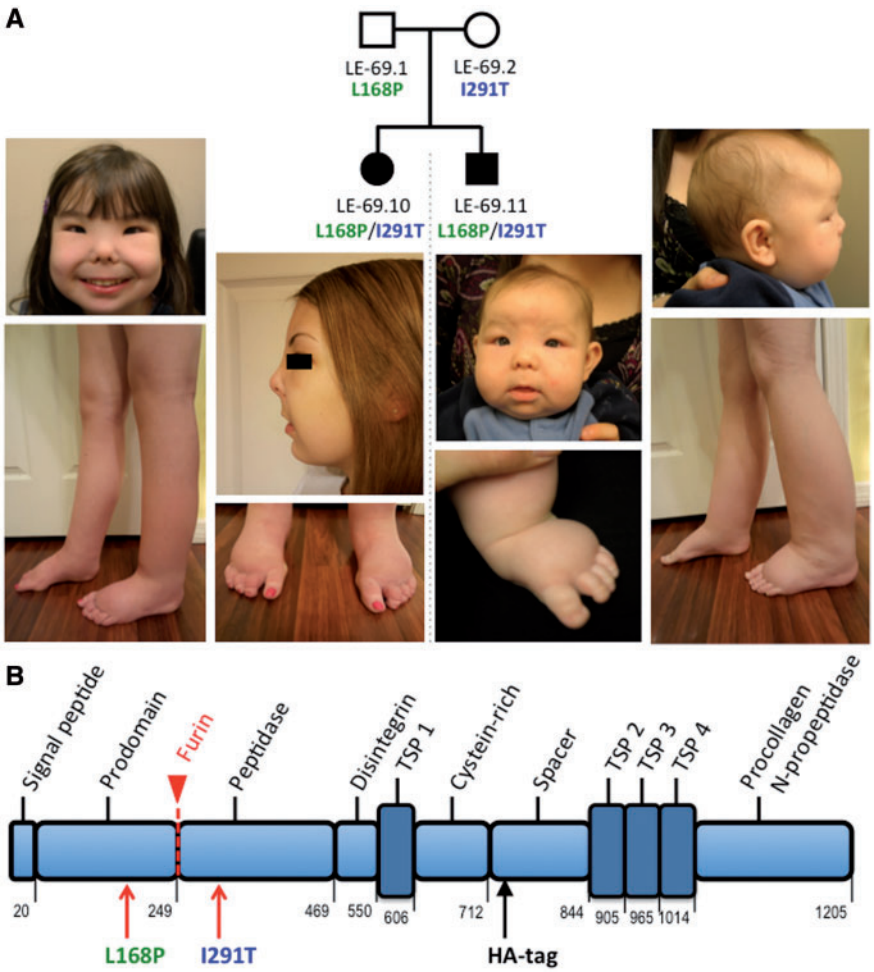

Figure 1. ADAMTS3 mutations in a recessive lymphedema family. (**A**) Pedigree of family LE-69 with ADAMTS3 mutations and pictures of the faces, legs and feet of the two affected children, at different ages. (**B**) Structure and domains of ADAMTS3 protein, showing the furin cleavage site (red arrowhead), the position of the HA-tag and the mutations c.503T>C; p.Leu168Pro (L168P) and c.872T>C; p.Ile291Thr (I291T). TSP, thrombospondin domain.

We also verified the nature of the amino acids at these positions in the 18 other human ADAMTS proteins (Fig. 2B). The L168 residue is embedded in a specific signature (S-X-C-X-G-L-X-G) in the prodomain of all ADAMTSs (except ADAMTS13, which contains a truncated prodomain). Proline is never present at this position. Alignment at the level of the second mutation also revealed a conserved sequence and showed that either I or L are the preferred amino acids (with M and A being present in ADAMTS17 and ADAMTS4, respectively). T is never observed at this position. Alignments were finally performed with sequences of human ADAM family members (a family distinct but related to ADAMTS) (Fig. 2C). This revealed the same S-X-C-X-G-L-X-G signature at the level of the L168P mutation and showed that an aliphatic amino acid (I, V, L) is found at the I291T position, as in the ADAMTSs. Altogether these data strongly favor a damaging effect of the two mutations identified in our patients.

Mutant ADAMTS3 proteins are abnormally cleaved and not secreted in the conditioned medium

The extent of the damaging effect of the identified mutations could not be predicted. Therefore, we developed a model allowing regulation of the level of production of the recombinant proteins in order to create conditions suitable for the identification of small activity differences. Cell layers and culture media obtained from control and induced cells were analyzed by Western blotting. With an antibody targeting the C-terminal end of ADAMTS3, a 166 kDa product, corresponding to the propeptide, was identified in the cell layer of the wild-type (WT) and the two mutants (Fig. 3A). No immunoreactive material was detected in the conditioned medium.

A second antibody, raised against an ADAMTS3 fragment encompassing the prodomain, confirmed the presence of a 166 kDa product in the cell layer of all the clones (Fig. 3B, upper band). In cells expressing WT-ADAMTS3, additional bands were observed in the cell layer (at 139 and 36 kDa) and in the conditioned culture medium (36 and 25 kDa) (see Supplementary Material, Fig. S1 for details of the fragments), demonstrating secretion and illustrating the complex process of maturation and cleavage of WT-ADAMTS3. The active form of ADAMTS3, which has lost its prodomain and should migrate at 100–110 kDa, cannot be detected by this antibody. The distribution pattern for the L168P mutant indicated that the mutant enzyme accumulated in the cell layer as the 166 kDa full-length polypeptide, while it was undetectable in the culture medium (Fig. 3B). For

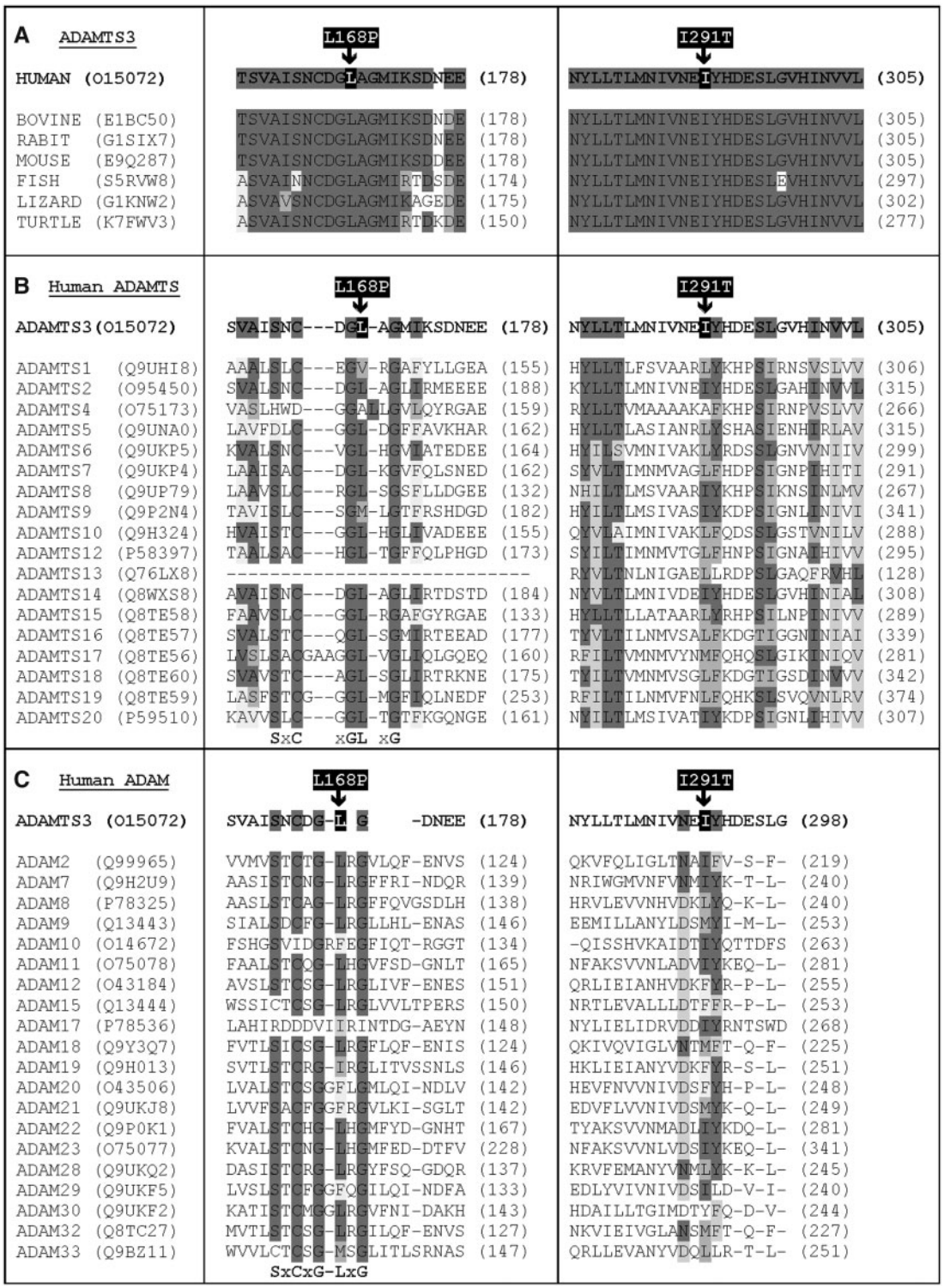

Figure 2. Amino acid conservation around ADAMTS3-L168P and I291T mutations. Alignments of human ADAMTS3 protein sequence with (A) ADAMTS3 in other species; (B) the 18 other human ADAMTSs; and (C) the 20 human ADAMs. UniProt identification codes are provided between parentheses after the names. Positions of the last amino acid in aligned sequences are also reported. Conserved residues as compared to human ADAMTS3 are highlighted in dark grey. Middle and light grey boxes illustrate substitutions by amino acids with similar or close physicochemical properties, respectively.

the I291T mutant, the 166 kDa pro-ADAMTS3 was also observed in the cell layer, together with other products (of 125, 97 and 47 kDa) absent from cells expressing the WT enzyme. No signal was detected in the culture medium.

The maturation process of ADAMTS3 involves the cleavage of the prodomain by proprotein convertases, such as furin, as well as the removal of most of the procollagen N-propeptidase C-terminal domain by cleavage at a site that remains to be defined

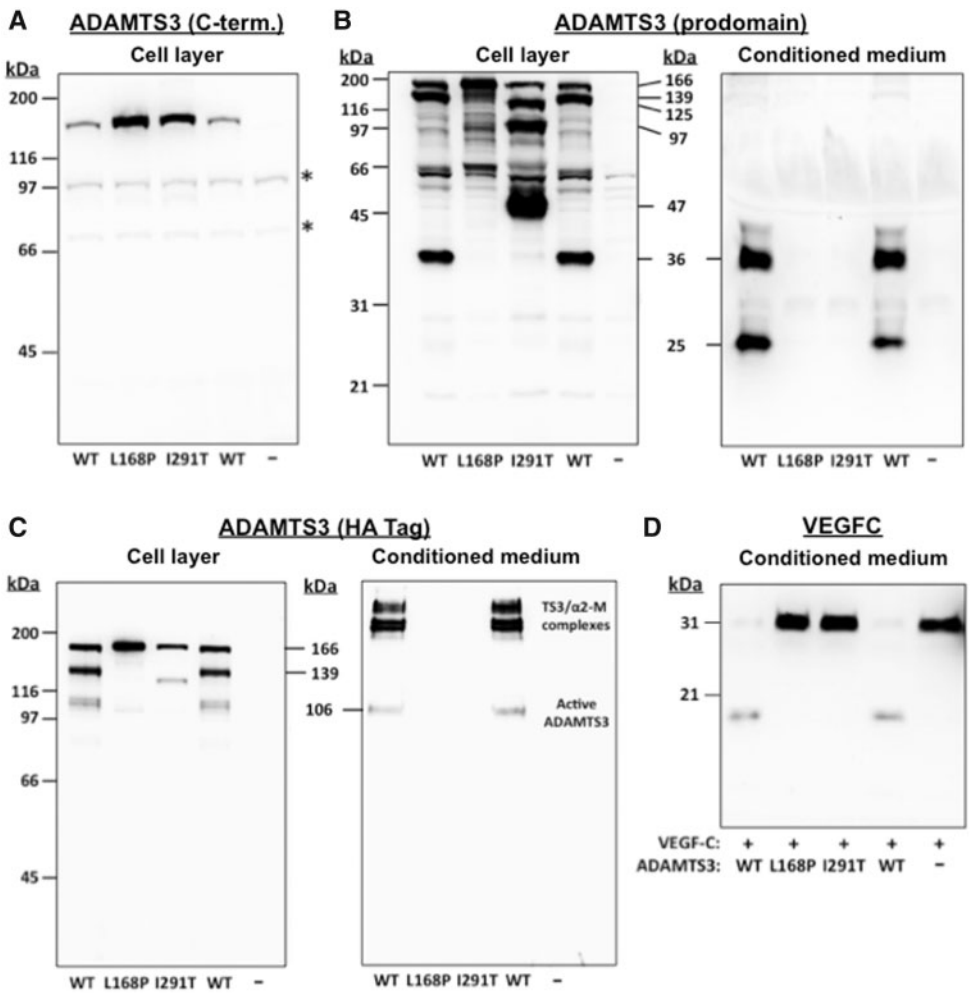

Figure 3. Impact of ADAMTS3 mutations on its activation and on VEGFC processing. HEK293 cells transfected with empty vector (–) and clones conditionally expressing (A, B) WT, L168P or I291T-ADAMTS3 or (C) HA-tagged forms, were cultured in presence of doxycyclin. Cell layers and conditioned culture media were collected after 48 h and analyzed using antibodies specific for (A) the C-terminal domain of ADAMTS3, (B) the prodomain or (C) the HA epitope. Details of the different fragments generated are given in Supplementary Material, Figure S1. (**A**) C-terminus-specific antibody sc-21488 detected the 166 kDa ADAMTS3 propeptide in the cell layer. Two background bands (*) were also present in the negative control. (**B**) N-terminal prodomain-specific antibody AF5465 showed bands at 166, 139 and 36 kDa in the cell layer for WT-ADAMTS3, and at 36 and 25 kDa in the conditioned medium. The processing was strongly altered for the mutants, most notably by absence of products detected in the culture medium. (**C**) HA-tagged proteins confirmed the complex pattern of proteolytic maturation. The active 106 kDa product was predominantly secreted in the conditioned culture medium, where it is mostly covalently associated with alpha2-macroglobulin. (**D**) VEGFC processing in control HEK293 cells (–) and clones expressing the different forms of ADAMTS3 were co-cultured with HEK293 cells expressing VEGFC at a 1:1 ratio. The antibody AF2179 strongly recognized pro-VEGFC but less efficiently its active mature form. Almost complete conversion of the precursor was observed in presence of WT-ADAMTS3, but no significant cleavage could be detected for the mutants.

(Supplementary Material, Fig. S1) (18). Since the commercially available ADAMTS3 antibodies are specific for the N- or the C-terminal, they did not allow visualization of the active central domain. To overcome this, we inserted an HA-epitope in the spacer region of ADAMTS3 (Fig. 1B, Supplementary Material, Fig. S1), according to a strategy successfully used previously for ADAMTS2 (18). The use of an anti-HA antibody confirmed the presence of the 166 and 139 kDa products, as well as products migrating at 100–110 kDa in cells expressing WT-ADAMTS3 (Fig. 3C). In the conditioned medium, a 106 kDa band was identified, corresponding to the released active form of ADAMTS3. Moreover, very high molecular weight products were observed, consisting of cleaved ADAMTS3 in complex with alpha2-macroglobulin (Fig. 3C). The latter is an almost universal inhibitor of endoproteinases, including matrix metalloproteinases and ADAMs. It presents a cleavable 'bait' that, once proteolytically cleaved, causes conformational changes that entrap the proteinase. It becomes then covalently anchored by transacylation. Formation of a covalent complex with alpha2-macroglobulin is therefore a sign of proteolytic activity of ADAMTS3 (19). For both mutants, the patterns of proteolytic maturation of ADAMTS3 were clearly altered, and no ADAMTS3 was detected in the conditioned medium (Fig. 3C).

ADAMTS3 mutants are unable to cleave VEGFC

Absence of immunoreactive material in the conditioned medium of cells expressing the mutant ADAMTS3 did not completely rule out the possibility that an active ADAMTS3 fragment, which would encompass the metalloproteinase domain but not the epitopes recognized by the antibodies, could be secreted and active. Therefore, the functional consequences of the ADAMTS3

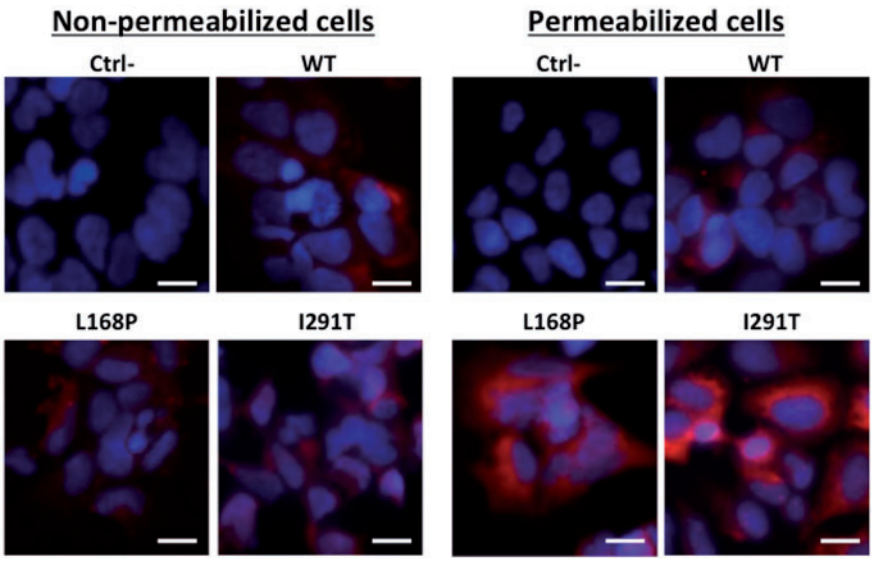

Figure 4. Effects of ADAMTS3 mutations on its secretion. Visualization of ADAMTS3 on non-permeabilized (left) and permeabilized (right) cells stained with SC-21486 antibody and a secondary antibody coupled to Alexa Fluor 546 (red). Nuclei were stained with DAPI (blue). Control cultures (Ctrl-) consisted of HEK293 cells containing the expression vector for WT-ADAMTS3 but in the absence of doxycycline, to prevent ADAMTS3 expression. WT, L168P and I291T refer to HEK293 clones cultured for 48 h in presence of doxycycline to induce expression of WT-ADAMTS3, or L168P or I291T-ADAMTS3 mutants, respectively. Scale bars = 10 μm.

mutations on processing of pro-VEGFC were evaluated. HEK293 cells express endogenously CCBE1, which has been shown to increase the maturation of pro-VEGFC by ADAMTS3. Control HEK293 cells or HEK293 cells expressing the different forms of ADAMTS3 (WT, L168 or I291) were mixed at a 1:1 ratio with HEK293 cells expressing pro-VEGFC (Fig. 3D). A complete conversion was observed in presence of WT-ADAMTS3, as illustrated by the almost total disappearance of the 31 kDa band, which is cleaved into a smaller product (less efficiently recognized by the antibody). In contrast, absence of cleavage was observed for the L168P and I291T-ADAMTS3 mutants.

ADAMTS3 mutants are sequestered within cells

To determine if the mutant ADAMTS3 enzymes accumulated in cells or were secreted but remained associated with the cell layer (Fig. 3B), we performed immunofluorescence studies on non-permeabilized and permeabilized cells (Fig. 4). Cells expressing WT-ADAMTS3 were faintly labeled, with or without permeabilization, with the N-terminal antibody (not shown) and the C-terminal antibody (Fig. 4). Thus, taking into account all our experiments, processing and secretion of WT-ADAMTS3 was efficient, with normal cleavage of the C-terminal propeptidase domain and release into the conditioned medium. Similar amounts of ADAMTS3 were seen around non-permeabilized cells for the mutant proteins, but a significant accumulation of both L168P and I291T mutants was noted inside the cells after permeabilization, demonstrating impaired secretion (Fig. 4). Altogether, these data demonstrate altered maturation and absence of secretion of ADAMTS3 mutants, explaining the absence of processing of pro-VEGFC into active VEGFC (Fig. 3D).

Discussion

The Hennekam syndrome, characterized by lymphangiectasia, lymphedema, and typical facial features and cognitive impairment, was described in 1989 (20). In 2009, *CCBE1* recessive mutations were shown to cause this syndrome (HKLLS1; OMIM 235510) (15). CCBE1 is involved in processing of VEGFC, the main VEGFR3 ligand involved in lymphangiogenesis. CCBE1 deficiency in mice and zebrafish results in drastically impaired lymphatic vasculature (15,17,21). A second gene, *FAT4*, encoding a protocadherin, has been reported to cause a Hennekam-like syndrome (HKLLS2; OMIM 616006) by homozygous or combined heterozygous mutations (4). The function of FAT4 in lymphatic vasculature is unknown.

According to current *in vitro* and animal model data, CCBE1 requires ADAMTS3 for efficient processing and activation of VEGFC (Fig. 5A). Pro-VEGFC binding to VEGFR3 is assisted by the N-terminal domain of CCBE1, but CCBE1 itself does not process VEGFC (14); rather, the cleavage is performed by ADAMTS3.

The CCBE1 N-terminal domain also binds to the extracellular matrix, thereby restricting the localization of VEGFC processing, whereas the C-terminal domain of CCBE1, containing two collagen repeats, is required for VEGFC activation (22). Indeed, lack of the C-terminal domain mimics the *Ccbe1* knockout (22). The ADAMTS3-CCBE1 complex can also form independently of VEGFR3 and cleave VEGFC, but proper localization is required for adequate lymphangiogenesis (23). ADAMTS3 seems a requisite for this. Indeed, we reported elsewhere that an ADAMTS3 change ($c.1694\,G>A$; p.Arg595Gln), which does not change VEGFC processing but reduces ADAMTS3 interaction with CCBE1, renders ADAMTS3 unable to retain CCBE1 at the cell surface, resulting in disruption of VEGFR3 signaling (24).

Here, we identified two compound heterozygous recessive mutations in *ADAMTS3* in two siblings with primary lymphedema, lymphangiectasia and distinct facial features. The symptoms are reminiscent of those of Hennekam syndrome 1, caused by CCBE1 mutations (OMIM 235510), underscoring the likely cooperative function of the two proteins in the same cellular process. We propose to name this clinical entity caused by ADAMTS3 mutations Hennekam lymphangiectasia–lymphedema syndrome 3 (HKLLS3), given that HKLLS2 (OMIM 616006) is caused by FAT4 mutations (4).

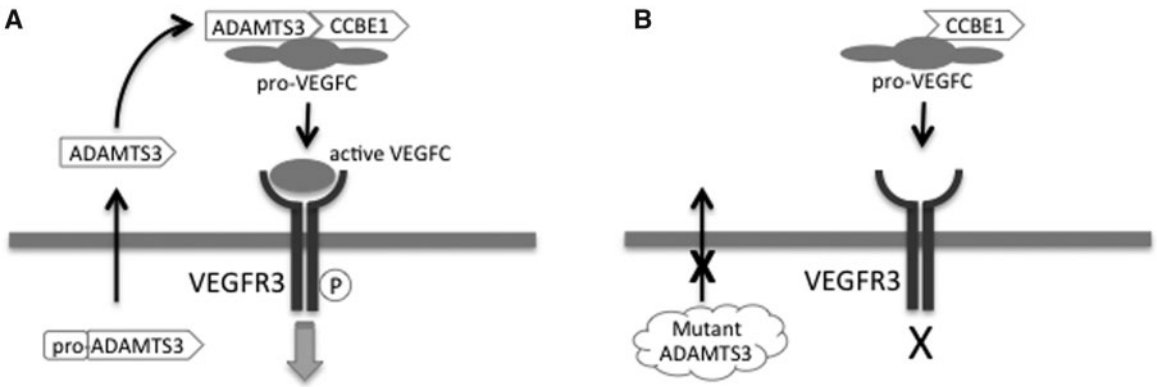

Figure 5. Mechanism of VEGFC/VEGFR3 activation is altered by ADAMTS3 mutations. (**A**) Pro-ADAMTS3 is activated and secreted in the extracellular space where it cleaves pro-VEGFC into active VEGFC, in conjunction with CCBE1. Binding of activated VEGFC to VEGFR3 induces phosphorylation of the receptor, which triggers downstream signaling. (**B**) Mutants of ADAMTS3 (*c.503T>C*; p.Leu168Pro and *c.872T>C*; p.Ile291Thr) are sequestered in the cell and cannot induce VEGFC cleavage and activation.

The two identified mutations (*c.503T>C*; p.Leu168Pro and *c.872T>C*; p.Ile291Thr) result in loss of function of ADAMTS3 and defective VEGFC activation (Fig. 5B). Each of the mutations was present in one of the parents, who were unaffected. Thus, haploinsufficiency of ADAMTS3 is not pathogenic; 50% of enzyme is sufficient to cleave and activate enough VEGFC. This is in agreement with our previous observations that heterozygous *Adamts3*$^{+/-}$ mice were healthy and reproduced well (25).

Our *in vitro* experiments demonstrated that the two mutants of ADAMTS3 displayed an abnormal electrophoretic profile (Fig. 3). Similar to ADAMTS2 (18), the activity of ADAMTS3 is largely regulated by several proteolytic processes (Supplementary Material, Fig. S1). Two main cleavages are required to confer full activity. First, the prodomain must be excised by proprotein convertases, such as furin, at a specific basic site (MRRRRH in ADAMTS3), between the prodomain and the peptidase domain. The second crucial cleavage occurs at the beginning of the C-terminal procollagen N-propeptidase (PNP) domain, at a site not yet precisely identified. This cleavage might be performed through an autocatalytic process. Persistence of the PNP domain in the homologous ADAMTS2 strongly reduced its activity and leads to formation of aggregates at the cell surface and in the extracellular matrix (Colige *et al.*, unpublished). Although the exact mechanism of inhibition is unknown, the PNP domain could reduce interactions with the substrates (18).

The discovery of reduced mutant ADAMTS3 activity could reflect very low bio-availability of the enzyme. In our experiments, the maturation process of the WT-ADAMTS3 occurred as expected. In contrast, the two mutations have dramatic consequences on ADAMTS3 secretion and maturation. None of the mutants was detected in the conditioned medium (Fig. 3), whereas strong intracellular accumulation was evident by immunofluorescence (Fig. 4). The L168P mutant accumulates in the cell layer in its full-size, possibly because its cleavage by proprotein convertases is hampered. For the I291T mutant, products of abnormal sizes were also observed (Fig. 3B), clearly illustrating maturation defects. Altogether, absence of secretion and abnormal processing explain why the mutants of ADAMTS3 were unable to cleave and activate pro-VEGFC in the extracellular compartment.

Amelioration of the lymphedema with fever in our patients is an intriguing clinical observation. It raises the hypothesis that the mutations are temperature sensitive and that elevated temperatures may result in improved protein secretion. To address this question, we cultured WT and mutants ADAMTS3 proteins at different temperatures (37, 39 and 41 °C), in presence of VEGFC (Supplementary Material, Fig. S2). However, no secretion of the mutants was seen in any condition, and even the secretion and activation of the WT ADAMTS3 was impaired at 41 °C. Thus, this clinical observation remains unexplained.

The bi-allelic loss of function of ADAMTS3 reported here is not lethal in human beings. In contrast, the *Adamts3*$^{-/-}$ mice died around E15.0 with severe edema due to complete lack of lymphatic vessels (25). Several hypotheses can be made to explain these differential effects. Species-specific compensation by other enzymes during embryonic development could occur, although ADAMTS2 and ADAMTS14 (the two closest homologs of ADAMTS3) are unable to cleave pro-VEGFC *in vitro* (Colige *et al.*, unpublished). Activation and secretion of the mutant ADAMTS3 might also be less affected *in vivo* during embryogenesis than in HEK293 cells, providing sufficient residual activity for embryonic survival, but insufficient activity for normal development and/or homeostasis of the lymphatic network.

In addition to endothelial cells, ADAMTS3 is significantly expressed in other tissues, such as in cartilage where it is considered to be the enzyme responsible for cleavage of the aminopropeptide of type II procollagen. Since our patients do not have developmental delay or major skeletal defects, it probably indicates that lack of ADAMTS3 secretion is compensated, at least partially, by another collagen aminopeptidase, likely ADAMTS2 (25). However, involvement of ADAMTS3 in type II procollagen maturation could explain the characteristic facial features of the subjects. It also suggests that periodic evaluation of cartilage and joint integrity of the patients may be indicated. ADAMTS3 is also strongly expressed in the brain, but no obvious neurological disorder was identified in the patients. Finally, the *Adamts3* $^{-/-}$ mice present with reduced liver development (25). No obvious signs of hepatic dysfunction were evidenced in our patients, albeit no extensive liver evaluation was performed.

In conclusion, our discovery of *ADAMTS3* mutations in human subjects with lymphangiectasia, lymphedema and distinct facial features (Hennekam lymphangiectasia–lymphedema syndrome 3) reveals another key component in the development of primary lymphedema. Therefore, individuals with phenotypic signs reminiscent of the Hennekam syndrome need to be screened not only for *CCBE1* and *FAT4* mutations, but also for alterations in *ADAMTS3*. These data give unequivocal evidence for the important physiological role that ADAMTS3 plays in VEGFC activation in man.

Materials and Methods

Patients and sequencing

DNA samples and phenotypic data from family LE-69 (Fig. 1A) were collected (G.T. and J.P.). Informed consent was obtained from the patients prior to their participation in the study, as approved by the Ethical Committee of the Medical Faculty at University of Louvain (Brussels, Belgium) and the Committee for the Protection of Human Subjects of Vanderbilt University (Nashville, TN, USA). DNA was extracted using the Wizard Genomic kit (Promega) and concentration measured on a Nanodrop 8000 (Thermo Scientific). Exome sequencing of the index case (LE-69.10) was performed on a SOLiD 5500xl with a Wildfire module (Life Technologies), after exon capture with SureSelect v5 kit (Agilent). The reads were aligned with Lifescope (Life Technologies) and bam files were imported into Highlander for analysis (http://sites.uclouvain.be/highlander/), a software developed in our laboratory (Helaers et al, under revision). Variants were called with the Genome Analysis Toolkit (GATK, Broad Institute) and annotated with a number of software programs, including dbNSFP v2 (26).

Filtering in Highlander was performed using following criteria: variants within a list of ~600 candidate genes; not detected in >3 distinct pathologies including lymphedema; read depth of at least 10 at this position; absent or present at <0, 15% (corresponding to the estimated prevalence of primary lymphedema in the population) in the Exome Aggregation Consortium database (ExAC, http://exac.broadinstitute.org); predicted damaging by at least four prediction software programs; and visually evaluated in the Integrative Genomics Viewer (IGV, http://software.broadinstitute.org/software/igv/). The underlying hypothesis was recessive inheritance, and thus only genes with a homozygous mutation or two independent mutations were kept.

We used PCR and Sanger sequencing with genomic primers 5′-CTTTAACATCACGGCATTTGG-3′ and 5′-TCGTCTTCGCATTATTCCAAG-3′ to study co-segregation of the mutation *c.503 T > C*; p.Leu168Pro (L168P), and 5′-TGACTACCTTTCAGAGTTCAG-3′ and 5′-TGGAAGTCTCCACTCATGTG-3′ for mutation *c.872 T > C*; p.Ile291Thr (I291T) in the family.

Expression vectors

The pcDNA4/TO vector containing the human *ADAMTS3* coding sequence (26) was used for site-directed mutagenesis. Oligonucleotides were designed using QuikChange Primer Design (Agilent). For *c.503 T > C* (p.Leu168Pro), we used primers 5′-TCAGCAACTGTGATGGTC**C**GGCTGGAATGATAAAAAG-3′ and 5′-CTTTTTATCATTCCAGCC**G**GACCATCACAGTTGCTGA-3′. For *c.872 T > C* (p.Ile291Thr), we used 5′-CCTGACCCTAATGAACATTGTGAATGAAA**C**TTACCATGATGAGTCC-3′ and 5′-GGACTCATCATGGTAA**G**TTTCATTCACAATGTTCATTAGGGTCAGG-3′. In brief, 10 ng of plasmid DNA was used as template for PCR amplification with *Pfu Turbo* DNA polymerase. The amplification products were verified on 1% agarose gels, *Dpn*I digested to remove template DNA, and transformed in JM109 bacteria for amplification. The entire coding sequence was verified using Sanger sequencing.

To create different hemagglutinin (HA)-flagged ADAMTS3 proteins, the wild-type vector was linearized with *Xho*I (New England Biolabs) and used as a template for PCR-amplification with Phusion High Fidelity DNA polymerase (New England Biolabs). Primer A (5′-CACCACG**GGTACC**TTTACCCATACGATGTTCCAGATTACGCTAAGATGTTTGATATACCCCCTGGGGCT-3′) contained the only endogenous *Kpn*I site in ADAMTS3 (bold), followed by a sequence of 27 nt encoding the HA epitope (underlined) inserted between nucleotides *c.2187_2188*. Primer B (5′-TTGTTCGGGCCCAAGCTTGGTAC-3′) was downstream of the *Kpn*I site in the multiple cloning site of the vector. The HA-coding sequence was therefore inserted at the beginning of the spacer domain (Fig. 1B). This *Kpn*I cassette was subsequently replaced in the wild-type (WT) and mutant expression vectors. For VEGFC expression, the full-length human coding sequence (22) was inserted between the *Bam*HI and *Xba*I sites of pcDNA4/TO (TetOn System; Invitrogen). Plasmids were purified with the Plasmid Maxi kit (Qiagen) and sequence-verified.

Stable cell lines

Human embryonic kidney HEK293 cells expressing a tetracyclin-sensitive repressor (27) were transfected with wild-type or mutant ADAMTS3 or VEGFC expression vectors, using GeneJuice Transfection Reagent (Novagen). After selection (Zeocin, 300 μg/ml; Thermo Fisher Scientific), subcloning was performed to identify clones producing the recombinant proteins only when induced with 1 μg/ml doxycycline (Sigma-Aldrich). Selected clones were subsequently cultured in DMEM supplemented with Zeocin (300 μg/ml), blasticidin (4 μg/ml) and 10% fetal bovine serum.

ADAMTS3 profiling

Transfected HEK293 cells conditionally expressing wild-type or mutant ADAMTS3 (HA-tagged or not) were cultured until confluent. The culture medium was then replaced by fresh medium containing doxycycline (1 μg/ml) to induce ADAMTS3 expression. After 48 h, the conditioned media were collected and the cell layers were scraped in Laemmli denaturation buffer (with 100 mM DTT). Samples were separated by electrophoresis (7.5 or 12.5% SDS-PAGE) and transferred to PVDF membrane (NEN Life Sciences Products). Membranes were blocked (60 min, 3% BSA in PBS-Tween) and incubated with the anti-ADAMTS3 antibody (sc-21486, Santa Cruz, recognizing the C-terminal part, or AF5465, R&D Systems, raised against the N-terminal part) or with the anti-HA antibody (11867423001, Roche) for 18 h at 4 °C. The secondary peroxidase-conjugated antibodies (1 h at RT; 1/2000) were from Dako. Peroxidase activity was revealed with an enhanced chemiluminescence assay (ECL™ Prime Western Blotting System, Sigma Aldrich) in an ImageQuant™ LAS 4000 (GE Healthcare).

VEGFC processing

HEK293 cells transfected to conditionally express ADAMTS3 or VEGFC were co-cultured at a 1:1 ratio. At confluence, doxycycline was added for 48 h. The conditioned media were collected and the cell layers were scraped in Laemmli denaturation buffer (with 100 mM DTT). Samples were resolved on 12.5% SDS-PAGE) and transferred to PVDF. Membranes were blocked (60 min in 3% BSA in PBS-Tween) and incubated 18 h at 4 °C with the anti-VEGFC antibody (AF2179, R&D Systems), followed by an anti-sheep peroxidase-conjugated secondary antibody (1/2000, Dako). Peroxidase activity was revealed as described above.

ADAMTS3 cytochemistry

HEK293 cells conditionally expressing wild-type or mutant ADAMTS3 were cultured until 50% confluent, then treated with

doxycycline for 48 h. After fixation (4% formaldehyde in PBS for 10 min), cells were either left untreated, or permeabilized (3 min in PBS containing 0.1% Triton X-100). ADAMTS3 staining was performed using two different antibodies (sc-21486 from Santa Cruz and AF5465 from R&D Systems, at a 1/50 dilution) for 2 h at 37 °C, followed by Alexa Fluor-546 donkey anti-goat IgG (A11056, Life Technologies) or Fluor-488 rabbit anti-sheep IgG (ab150181, Abcam) for 1 h. Nuclei were counter-stained with DAPI (1/1000, Life Technologies, D1306) in PBS. After mounting in Dako fluorescent mounting medium (s3023, Dako), cells were observed with an Eclipse TiS microscope (Nikon).

Genbank accession number for *ADAMTS3* mRNA: NM_014243. The variants were submitted to LOVD (adamts3.lovd.nl).

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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References

1. Brouillard, P., Boon, L. and Vikkula, M. (2014) Genetics of lymphatic anomalies. *J. Clin. Invest.*, **124**, 898–904.
2. Gonzalez-Garay, M.L., Aldrich, M.B., Rasmussen, J.C., Guilliod, R., Lapinski, P.E., King, P.D. and Sevick-Muraca, E.M. (2016) A novel mutation in CELSR1 is associated with hereditary lymphedema. *Vasc. Cell*, **8**,
3. Martin-Almedina, S., Martinez-Corral, I., Holdhus, R., Vicente, A., Fotiou, E., Lin, S., Petersen, K., Simpson, M.A., Hoischen, A., Gilissen, C. *et al.* (2016) EPHB4 kinase-inactivating mutations cause autosomal dominant lymphatic-related hydrops fetalis. *J. Clin. Invest.*, **126**, 3080–3088.
4. Alders, M., Al-Gazali, L., Cordeiro, I., Dallapiccola, B., Garavelli, L., Tuysuz, B., Salehi, F., Haagmans, M.A., Mook, O.R., Majoie, C.B. *et al.* (2014) Hennekam syndrome can be caused by FAT4 mutations and be allelic to Van Maldergem syndrome. *Hum. Genet.*, **133**, 1161–1167.
5. Fotiou, E., Martin-Almedina, S., Simpson, M.A., Lin, S., Gordon, K., Brice, G., Atton, G., Jeffery, I., Rees, D.C., Mignot, C. *et al.* (2015) Novel mutations in PIEZO1 cause an autosomal recessive generalized lymphatic dysplasia with non-immune hydrops fetalis. *Nat. Commun.*, **6**, 8085.
6. Lukacs, V., Mathur, J., Mao, R., Bayrak-Toydemir, P., Procter, M., Cahalan, S.M., Kim, H.J., Bandell, M., Longo, N., Day, R.W. *et al.* (2015) Impaired PIEZO1 function in patients with a novel autosomal recessive congenital lymphatic dysplasia. *Nat. Commun.*, **6**, 8329.
7. Hong, S.E., Shugart, Y.Y., Huang, D.T., Shahwan, S.A., Grant, P.E., Hourihane, J.O., Martin, N.D. and Walsh, C.A. (2000) Autosomal recessive lissencephaly with cerebellar hypoplasia is associated with human RELN mutations. *Nat. Genet.*, **26**, 93–96.
8. Koenighofer, M., Hung, C.Y., McCauley, J.L., Dallman, J., Back, E.J., Mihalek, I., Gripp, K.W., Sol-Church, K., Rusconi, P., Zhang, Z. *et al.* (2016) Mutations in RIT1 cause Noonan syndrome: additional functional evidence and expanding the clinical phenotype. *Clin. Genet.*, **89**, 359–366.
9. Milosavljevic, D., Overwater, E., Tamminga, S., de Boer, K., Elting, M.W., van Hoorn, M.E., Rinne, T. and Houweling, A.C. (2016) Two cases of RIT1 associated Noonan syndrome: Further delineation of the clinical phenotype and review of the literature. *Am. J. Med. Genet. A*, **170**, 1874–1880.
10. Geffrey, A.L., Shinnick, J.E., Staley, B.A., Boronat, S. and Thiele, E.A. (2014) Lymphedema in tuberous sclerosis complex. *Am. J. Med. Genet. A*, **164A**, 1438–1442.
11. Gordon, K., Schulte, D., Brice, G., Simpson, M.A., Roukens, M.G., van Impel, A., Connell, F., Kalidas, K., Jeffery, S., Mortimer, P.S. *et al.* (2013) 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.*, **112**, 956–960.
12. Balboa-Beltran, E., Fernandez-Seara, M.J., Perez-Munuzuri, A., Lago, R., Garcia-Magan, C., Couce, M.L., Sobrino, B., Amigo, J., Carracedo, A. and Barros, F. (2014) A novel stop mutation in the vascular endothelial growth factor-C gene (VEGFC) results in Milroy-like disease. *J. Med. Genet.*, **51**, 475–478.
13. Au, A.C., Hernandez, P.A., Lieber, E., Nadroo, A.M., Shen, Y.M., Kelley, K.A., Gelb, B.D. and Diaz, G.A. (2010) Protein tyrosine phosphatase PTPN14 is a regulator of lymphatic function and choanal development in humans. *Am. J. Hum. Genet.*, **87**, 436–444.
14. Jeltsch, M., Jha, S.K., Tvorogov, D., Anisimov, A., Leppanen, V.M., Holopainen, T., Kivela, R., Ortega, S., Karpanen, T. and Alitalo, K. (2014) CCBE1 enhances lymphangiogenesis via A disintegrin and metalloprotease with thrombospondin motifs-3-mediated vascular endothelial growth factor-C activation. *Circulation*, **129**, 1962–1971.
15. Alders, M., Hogan, B.M., Gjini, E., Salehi, F., Al-Gazali, L., Hennekam, E.A., Holmberg, E.E., Mannens, M.M., Mulder, M.F., Offerhaus, G.J. *et al.* (2009) Mutations in CCBE1 cause generalized lymph vessel dysplasia in humans. *Nat. Genet.*, **41**, 1272–1274.
16. Connell, F., Kalidas, K., Ostergaard, P., Brice, G., Homfray, T., Roberts, L., Bunyan, D.J., Mitton, S., Mansour, S., Mortimer, P. *et al.* (2010) Linkage and sequence analysis indicate that CCBE1 is mutated in recessively inherited generalised lymphatic dysplasia. *Hum. Genet.*, **127**, 231–241.
17. Bos, F.L., Caunt, M., Peterson-Maduro, J., Planas-Paz, L., Kowalski, J., Karpanen, T., van Impel, A., Tong, R., Ernst, J.A., Korving, J. *et al.* (2011) CCBE1 is essential for mammalian

lymphatic vascular development and enhances the lymphangiogenic effect of vascular endothelial growth factor-C *in vivo*. *Circ. Res.*, **109**, 486–491.
18. Colige, A., Ruggiero, F., Vandenberghe, I., Dubail, J., Kesteloot, F., Van Beeumen, J., Beschin, A., Brys, L., Lapiere, C.M. and Nusgens, B. (2005) Domains and maturation processes that regulate the activity of ADAMTS-2, a metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types I-III and V. *J. Biol. Chem.*, **280**, 34397–34408.
19. Baker, A.H., Edwards, D.R. and Murphy, G. (2002) Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J. Cell Sci.*, **115**, 3719–3727.
20. Hennekam, R.C., Geerdink, R.A., Hamel, B.C., Hennekam, F.A., Kraus, P., Rammeloo, J.A. and Tillemans, A.A. (1989) Autosomal recessive intestinal lymphangiectasia and lymphedema, with facial anomalies and mental retardation. *Am. J. Med. Genet.*, **34**, 593–600.
21. Hogan, B.M., Bos, F.L., Bussmann, J., Witte, M., Chi, N.C., Duckers, H.J. and Schulte-Merker, S. (2009) Ccbe1 is required for embryonic lymphangiogenesis and venous sprouting. *Nat. Genet.*, **41**, 396–398.
22. Roukens, M.G., Peterson-Maduro, J., Padberg, Y., Jeltsch, M., Leppanen, V.M., Bos, F.L., Alitalo, K., Schulte-Merker, S. and Schulte, D. (2015) Functional dissection of the CCBE1 protein: a crucial requirement for the collagen repeat domain. *Circ. Res.*, **116**, 1660–1669.
23. Bui, H.M., Enis, D., Robciuc, M.R., Nurmi, H.J., Cohen, J., Chen, M., Yang, Y., Dhillon, V., Johnson, K., Zhang, H. *et al.* (2016) Proteolytic activation defines distinct lymphangiogenic mechanisms for VEGFC and VEGFD. *J. Clin. Invest.*, **126**, 2167–2180.
24. Jha, K.S., Rauniyar, K., Karpanen, T., Leppanen, V.M., Brouillard, P., Vikkula, M., Alitalo, K. and Jeltsch, M. (2017) Efficient activation of the lymphangiogenic growth factor VEGF-C requires the C-terminal domain of VEGF-C and the N-terminal domain of CCBE1. *Sci. Rep.*, **7**, 4916.
25. Janssen, L., Dupont, L., Bekhouche, M., Noel, A., Leduc, C., Voz, M., Peers, B., Cataldo, D., Apte, S.S., Dubail, J. *et al.* (2016) ADAMTS3 activity is mandatory for embryonic lymphangiogenesis and regulates placental angiogenesis. *Angiogenesis*, **19**, 53–65.
26. Liu, X., Jian, X. and Boerwinkle, E. (2013) dbNSFP v2.0: a database of human non-synonymous SNVs and their functional predictions and annotations. *Hum. Mutat.*, **34**, E2393–E2402.
27. Bekhouche, M., Leduc, C., Dupont, L., Janssen, L., Delolme, F., Vadon-Le Goff, S., Smargiasso, N., Baiwir, D., Mazzucchelli, G., Zanella-Cleon, I. *et al.* (2016) Determination of the substrate repertoire of ADAMTS2, 3, and 14 significantly broadens their functions and identifies extracellular matrix organization and TGF-beta signaling as primary targets. *FASEB J.*, **30**, 1741–1756.