An Evolutionarily Conserved Role for Polydom/Svep1 During Lymphatic Vessel Formation

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ABSTRACT

Rationale: Lymphatic vessel formation and function constitutes a physiologically and pathophysiologically important process, but its genetic control is not well understood.

Objective: Here, we identify the secreted Polydom/Svep1 protein as essential for formation of the lymphatic vasculature. We analyzed mutants in mice and zebrafish to gain insight into the role of Polydom/Svep1 in the lymphangiogenic process.

Methods and Results: Phenotypic analysis of zebrafish polydom/svep1 mutants showed a decrease in venous and lympho-venous sprouting, which leads to an increased number of intersegmental arteries. A reduced number of primordial lymphatic cells populated the horizontal myoseptum region, but failed to migrate dorsally or ventrally, resulting in severe reduction of the lymphatic trunk vasculature. Corresponding mutants in the mouse Polydom/Svep1 gene showed normal egression of Prox-1+ cells from the cardinal vein at E10.5, but at E12.5 the tight association between the cardinal vein and lymphatic endothelial cells at the first lymph-venous contact site was abnormal. Furthermore, mesenteric lymphatic structures at E18.5 failed to undergo remodeling events in mutants, and lacked lymphatic valves. In both fish and mouse embryos, the expression of the gene suggests a non-endothelial and non-cell autonomous mechanism.

Conclusions: Our data identify zebrafish and mouse Polydom/SVEP1 as essential extracellular factors for lymphangiogenesis. Expression of the respective genes by mesenchymal cells in intimate proximity with venous and lymphatic endothelial cells is required for sprouting and migratory events in zebrafish, and for remodeling events of the lymphatic intra-luminal valves in mouse embryos.

Keywords: Polydom/Svep1, lymphangiogenesis, zebrafish, mouse model, lymphatic capillary, mouse mutant, valve

Nonstandard Abbreviations and Acronyms:

CV     cardinal vein
DA     dorsal aorta
dpf    days post-fertilization
ECM    extracellular matrix
EGF    epidermal growth factor
ENU    ethyl-nitroso-urea
hpf    hours post-fertilization
ISLV   intersegmental lymphatic vessel
LEC    lymphatic endothelial cell
HMS    horizontal myoseptum
ISVs   intersegmental vessels
PL     parachordal lymphangioblast
PLCG1  phospholipase c gamma 1
PCV    posterior cardinal vein
pLLV   peripheral longitudinal lymphatic vessel
pTD    primordial thoracic duct
TD     thoracic duct
VEGF-C vascular endothelial growth factor-C
VEGFR-3 vascular endothelial growth factor receptor-3
INTRODUCTION

The lymphatic vasculature serves key physiological roles during embryonic and adult life\(^1,2\). It controls fluid homeostasis and retrieves water and macromolecules from the interstitium, it takes part in immune surveillance, and it is responsible for the uptake and transport of dietary lipids from the small intestine.

Formation and maturation of the lymphatic vasculature has been studied in a number of vertebrate systems, including mice\(^3\), Xenopus\(^4\) and zebrafish\(^5,6\). In mice, future lymphatic endothelial cells (LECs) egress from the cardinal vein (CV) as a network of loosely connected cells that express Prox-1, and then go on to form two initial lymphatic vessels, the primordial thoracic duct (pTD) close to the CV, and the more peripheral longitudinal lymphatic vessel (pLLV)\(^3\). The migration of LECs depends on a number of secreted factors such as VEGF-C\(^7\) and CCBE1\(^8\), which act in concert with ADAMTS3\(^9,10\) to generate mature and biologically active VEGF-C protein\(^11,12\). VEGF-C in turn binds to VEGFR3 on venous and lymphatic endothelium. This system is evolutionarily conserved, and mutants in the respective genes lead to severely impaired lymphatic vasculature formation in both murine\(^7-9\) and zebrafish\(^11,13\) embryos. Later, once the primary lymphatic vascular network has been established, further morphological changes occur, resulting in the formation of capillaries that take up lymph from the interstitium, and collecting lymphatic vessels, which transport the contents of the lymphatic system back to the venous system. Mature collecting vessels are typically covered by smooth muscles cells, show basement membrane deposition, and contain intraluminal valves to prevent back-flow of lymph\(^2\). Much remains to be learned about the maturation steps towards fully functional lymphatic capillaries and collecting vessels, but the transcription factors Foxc2 and NFATc1 are known to be required for this process\(^14,15\). Foxc2 mutant mouse embryos fail to form lymphatic valves, and human patients for lymphedema-distachiasis (Online Mendelian Inheritance in Man reference no. 153400) have been described to lack FOXC2 function\(^15,16\). A somewhat milder phenotype is found in embryos with an endothelial-specific Integrin-alpha9 deficiency, where valve leaflets are rudimentary, with disorganized fibronectin matrix\(^17\).

In zebrafish, the sprouting of venous and future lymphatic ECs occurs simultaneously, between 30 and 36 hours post-fertilization (hpf). While venous sprouts connect to arterial intersegmental vessels (ISVs) and remodel them into veins, those sprouts that will form the trunk lymphatic vasculature migrate to the embryonic midline (the horizontal myoseptum (HMS)) and form a population of single cells, termed parachordal lymphangioblasts (PLs)\(^13\). PL cells populate the HMS region transiently, before they initiate a distinct migratory phase, causing them to move either dorsally to form the dorsal longitudinal lymphatic vessel (DLLV), or ventrally to form the thoracic duct (TD). Migration in this phase always occurs along arteries\(^18-20\). Later aspects of lymphatic vascular formation in zebrafish are less well defined, and the formation of valves, e.g., has not been described.

Here, we report a novel gene function associated with lymphangiogenesis during early embryonic stages of both zebrafish and mouse. The extracellular protein Polydom/Svep1, which is not expressed by endothelial cells, has not previously been connected to lymphatic vessel formation, but is here shown to result in migratory defects and remodelling abnormalities in zebrafish and mouse mutant embryos, respectively.
METHODS

Zebrafish ethyl-nitroso-urea (ENU) mutagenesis\textsuperscript{13}, the screening procedure\textsuperscript{13}, analysis of zebrafish mutants\textsuperscript{13}, BAC recombinering\textsuperscript{21} were carried out as previously described. All remaining animal procedures, in situ hybridizations\textsuperscript{3} and immunohistochemistry\textsuperscript{3} were carried out as previously described, or as detailed in the online information.

RESULTS

Ly02-512 zebrafish mutants display defects of the lymphatic vasculature.

In a forward genetic screen designed to uncover novel gene functions within the zebrafish lymphatic system\textsuperscript{13}, we identified a mutant, initially termed Ly02-512, which lacked all, or parts of, the TD and displayed edema around the eye, heart and intestine at 5 days post fertilization (dpf). Mutants developed a swim bladder and showed no signs of delayed development at 5dpf (Figure 1A,B), but developed other defects such as brain and jaw abnormalities as well as heart defects (Figure 1A,B, and data not shown). A closer examination at 5dpf demonstrated that in Ly02-512 mutant embryos the blood vasculature appeared normal and functional, with the exception of an increase in the number of arterial ISVs (highlighted in red), at the expense of venous ISVs (Figure 1B). This was clearly observed at 5dpf in double transgenic embryos which express RFP under the control of the \textit{flt1enh} promoter\textsuperscript{18} in arterial ISVs, and GFP under the control of the pan-endothelial \textit{fli1a} promoter\textsuperscript{22}. The TD, visualized using the same combination of transgenes (Figure 1C) or by highlighting the perfused blood vessels with rhodamine dextran angiography (Figure 1D), was absent in mutant embryos, while it could easily be identified in all sibling embryos as a thin vessel immediately ventral to the dorsal aorta (DA) (Figure 1C,D, arrows). However, the venous sprouting and TD defects in Ly02-512 mutants were not as severe as compared to \textit{ccbe1}, \textit{vegfc} or \textit{flt4/vegfr3} mutants, in which hardly any venous sprouts or TD are formed\textsuperscript{11,13,23,24}. Venous sprouts that did form in Ly02-512 mutants appeared to sprout correctly and formed normal intersegmental veins, or morphologically normal PLs. Also, when quantifying the fate of venous sprouts, we could not find a difference in fate decisions for lympho-venous sprouts: there was no preference for venous sprouts to form definitive veins or PLs (see below).

Ly02-512 represents a premature stop allele of the polydom/svep1 gene.

In order to identify the genetic lesion causing the phenotype, we employed a positional mapping strategy using CA-repeat markers. Initial bulk segregant analysis placed the gene on linkage group 7 between markers z4.29 and z7.4 (Figure 2A), comprising a region of roughly 350kb containing 12 genes (Zv9). Analysis of over 3500 mutant embryos and over 7000 meiosis revealed no further recombinants, which is why we applied a BAC rescue approach. We identified three independent BACs that were predicted to span the whole genomic region between the markers in question (Figure 2B), and generated independent transgenic lines with the rationale that one of the transgenic BAC alleles should be able to rescue the mutant phenotype. While BACs CH73-220H7 and DKEY-108N6 failed to rescue, the BAC DKEY-8E16 reduced the number of phenotypically mutant embryos from the expected 25% to 4.1% (2/49) (Figure 2C). Since two recombinants (2/7096) for z4.29 placed the region of interest to the right side of the marker, thereby excluding five of the six genes on BAC DKEY-8E16, the only remaining candidate gene on the BAC was \textit{polydom/svep1}. Sequencing of all exons of the \textit{polydom/svep1} gene in mutant embryos revealed a premature stop codon in exon 14 of the gene (K836X; numbering based on a predicted protein starting 49 amino acids upstream of the annotation in Zv9, coded by 5’ flanking region and including a start methionine and a signal sequence).
Subsequently, two additional mutant alleles from the screen with similar phenotypes were found to fail to complement the svep1<sup>K836X</sup> allele, with a fully penetrant TD phenotype (i.e., all mutant embryos lacked at least part of the TD) in all three cases (Figure 2D). Sequencing cDNA from mutant embryos demonstrated nonsense codons in the respective mutants, leading to a C355X conversion in Ly04-093 and an Y1918X conversion in Ly05-265 (Figure 2E). In order to exclude differential splice products that would produce transcripts without the affected exons, we carried out RT-PCR analysis of sibling and mutant embryos from the Ly04-093 and the Ly02-512 alleles. RT-PCR and subsequent sequencing revealed no alternative splice products (Online Figure I). Injection of murine SVEP1 mRNA was able to rescue the zebrafish mutant phenotype (Online figure II). Based on (1) the genetic mapping results, (2) the successful rescue with BAC DKEY-8E16 and murine SVEP1 mRNA, and (3) the identification of 3 independent mutant alleles, we concluded that the phenotype was caused by mutations in the zebrafish polydom/svep1 gene. Furthermore, a morpholino directed against the gene and an independently generated TALEN allele (11 base-pair deletion in the first exon; Morooka et al., submitted) also result in an identical phenotype, strongly suggesting that all 4 mutant alleles represent loss-of-function situations. The protein product prediction of the gene comprised 3555 amino acids, contained a signal peptide but no predicted transmembrane domain, and harbored different domains (hence the name Polydom<sup>25</sup>) such as the ones that are abbreviated in the acronym SVEP<sup>26</sup>: Sushi, von Willebrand, epidermal growth factor (EGF), and pentraxin domain(s). Figure 2F provides a schematic representation of the domain structure of the predicted protein, with the position of the nonsense codons indicated.

The migration of lymphatic endothelial cells is impaired in polydom/svep1 zebrafish mutants.

Polydom/svep1 mutants were initially uncovered because of their aberrant lymphatic development, and we wanted to analyze the origin of the phenotype in more detail. Initially, we analyzed the number of secondary (venous) sprouts from the posterior cardinal vein (PCV). We counted secondary sprouts in phospholipase c gamma 1 (plcg1) morpholino (MO) injected egg lays from polydom/svep1<sup>+/−</sup> parents. The interference with Plcg1 activity suppresses arterial development<sup>27</sup>, allowing for easier appreciation of venous sprouting events in a fli1a:GFP transgenic background (Figure 3A). Polydom/ svep1<sup>+/−</sup> embryos showed a small but statistically significant reduction of secondary sprouts compared to polydom /svep1<sup>+/+</sup> siblings, while polydom /svep1<sup>−/−</sup> embryos only showed about 40% of the venous sprouts found in wild type embryos (Figure 3F). This aspect of the phenotype was variable, and the effect on venous sprouting differed both between different clutches and within a single clutch of mutant embryos. Still, all mutant embryos show an increase in arteries over veins (Figure 3E,H).

We then examined the formation of PL cells, which in wildtype embryos form from those venous sprouts that do not stably connect to arterial ISVs. The formation of PL cells can be seen in Figure 3B and C, demonstrating robust formation of PLs at the HMS region between 32.5hpf and 46.5hpf in wild type sibling embryos. By contrast, in polydom/svep1 mutants, the number of PL cells forming was reduced (Figure 3B,C,G). The population of PL cells in mutant embryos behaved normally regarding migration patterns along the horizontal midline region, but at 2.5dpf, when in wildtype embryos PL cells started to migrate dorsally or ventrally along arterial ISVs (Figure 3D, a-c; online movie), in mutant embryos PL cells did not show the typical migratory behavior away from the HMS (Figure 3D, d-f), but rather remained in the midline region.

To substantiate whether PL cells at the level of the HMS are specified correctly, we carried out Prox-1 antibody staining in situ at a point in time when PL cells populate the HMS region at 2dpf. Fli1aeGFP positive PL cells were identified, and the proportion of cells simultaneously expressing Prox-1 was quantified (Figure 4A,B). We found no difference in the proportion of Prox-1+ PL cells, and conclude that the specification of PL cells is not deficient in svep1 mutant embryos.
Next, we analyzed whether Vegfc/Vegfr3 signaling is impaired in svep1 mutants. To this end, we measured the downstream signaling pathway of Vegfr3 by immunofluorescence staining of phospho-ERK in the PCV at the time of venous sprouting (Le Guen et al., 2014). At 32hpf, pERK staining was found in a subset of nuclei within the PCV endothelium. Whereas significant differences in pERK staining could be observed with ccebl morphants (Figure 4C) and vegf-C mutants (Figure 4D), we could not detect differences of pERK staining in svep1 mutant versus sibling embryos (Figure 4E,F). This suggests that VEGFR3/pERK signaling is not affected in svep1 mutants, or affected at considerably lower levels than in the absence of Ccebl and Vegf-C.

Next, we assessed the response of endothelial cells of svep1 mutant embryos to ectopically provided human VEGFC expressed in the floorplate of zebrafish embryos. We could not observe a difference in Vegfc signaling in svep1 mutants and siblings as at 2dpf exogenous VEGFC led to comparable levels of lymphovenous hyper-sprouting in sibling control and svep1 mutant embryos (Online Figure III A-B). In line with this finding, double svep1/ccbe1 heterozygous zebrafish embryos did not show TD defects, indicating that Svep1 is not a component of the vegfc/vegfr3 pathway (Online Figure III C – D).

**Polydom/svep1 is expressed by cells in close connection with vessels, but not by endothelial cells.**

In order to understand the polydom/svep1 expression pattern, we performed in situ hybridizations which demonstrated expression in multiple locations, including expression in the region of the PCV and the intersegmental vessels (Online Figure IV). To achieve cellular resolution in living embryos, we generated a svep1:Gal4FF transgenic line, introducing a Gal4FF cassette in the location of the predicted first ATG of svep1 in the DKEY-8E16 BAC. Analyzing the expression in svep1:Gal4FF; UAS:eGFP transgenic embryos confirmed expression at multiple sites, including the epidermis, the cleithrum and operculum, and the trunk region (data not shown). We here restrict ourselves to the analysis of polydom/svep1 expression in regions with relevance to the vascular system. At 34hpf, we found individual cells expressing svep1/polydom in positions directly abutting the DA and the PCV, as depicted in Figure 5A. Of note, in a transgenic kdrl:mCherry background, the population of svep1/polydom-positive cells (green) was not overlapping with endothelial cells (red), indicating expression by non-endothelial cells. The number of cells expressing the gene increased by 72hpf, when a large number of svep1/polydom-positive cells surrounded the PCV (Figure 5B,F, and 5G, blue arrow-head). Given that PL cells migrate dorsally or ventrally almost exclusively along arterial ISVs, we examined whether svep1/polydom-expression is preferentially found around intersegmental arteries. We found no preference for arterial over venous ISVs, and in a fltl enh:RFP line which highlights arteries (red, Figure 5B,C), svep1/polydom-positive cells were found in an equal distribution around both venous and arterial ISVs. Again, as previously observed for endothelial cells of the PCV, svep1/polydom-positive cells were found in tight association with ISVs (Figure 5C), but there was no overlap between the two cell populations, further supporting the notion that endothelial cells do not express svep1/polydom. This was confirmed by RT-PCR of cultured human umbilical vein endothelial cells (HUVEC) and LECs, in which POLYDOM/SVEP1 mRNA could not be detected, whereas POLYDOM/SVEP1 mRNA was abundant in the fibroblastic cell line VH32 (Online Figure VB).

Around 48hpf, the first PL cells are identifiable in the HMS region, and we noticed that also svep1/polydom-positive cells were present in the region, usually closely abutting PL cells (Figure 5D). The close association of PL cells and polydom/svep1 expressing cells became even more evident at 72-78hpf (Figure 5E,F,G). In order to determine whether PL cells populate the HMS early enough to possibly induce svep1 expression, we followed time lapse sequences and observed that the expression of polydom/svep1 appeared to be independent of the presence of PL cells: at 39hpf, expression of one or two polydom/svep1-expressing cells became apparent, and only a few hours later PL cells also started to populate the region (Figure 5H). Strikingly, there are always cells that express polydom/svep1 in the HMS region close to arteries at those points in time when PL cells need to make the critical transition to migrate dorsally or...
ventrally, suggesting that the presence of Polydom/Svep1 protein might be instrumental in governing this migratory step.

At later stages when the TD formed in zebrafish embryos, polydom /svep1 expression was abundantly detected between the DA and the PCV, i.e. in the region of TD formation (Figure 5I). Neither at this point in time nor at earlier stages was it possible to unambiguously identify the nature of the cells that expressed polydom /svep1. We excluded muscle pioneer cells and neurons at 48hpf (data not shown) and hence consider it most likely that mesenchymal cells are the cells producing Polydom/Svep1. This is consistent with in vitro expression data using mammalian cell lines (Online Figure VB), and in situ antibody staining in mouse embryos (Morooka et al., submitted).

**Polydom/Svep1 is required at later stages of lymphatic vascular development and valve formation in mice.**

To examine a possible evolutionarily conserved role of the Polydom/Svep1 protein, we generated knock out mice for the murine orthologue of the gene, based on the EUCOMM ‘knockout first’ strategy (Figure 6A, and see online information for details). Crossing of heterozygous carriers yielded homozygous mutant embryos (Figure 6B, C, and Online Figures VI and VII), which were obtained at a normal Mendelian ratio (n=130 total, with 31 Polydom/Svep1 +/-, 67 Polydom/Svep1 +/-, 32 Polydom/Svep1 -/- from 17 liters at E14.5). Homozygous wildtype and heterozygous embryos were indistinguishable, but mutant embryos at E14.5 or older could often be recognized due to edema formation, which was somewhat variable in phenotypic strength (Online Figure VII). Mutant embryos at E18.5 always showed edema (Figure 6C). In order to determine the exact onset and cause of the phenotype, we analyzed sibling and mutant E11.0, E12.5 and E13.5 embryos using whole mount antibody staining. Using VEGFR3 antibodies to stain venous and lymphatic cells, and PROX-1 antibodies to highlight LECs, we did not observe any differences in formation of the first lymphatic structures between sibling and mutant E11.0 and E12.5 embryos. Egression of future lymphatic ECs from the CV occurred normally in mutant embryos (Figure 7A-F), and formation of the pTD and the pLLV progressed in mutant embryos in a manner indistinguishable from wildtype siblings. At E12.5, however, we noticed that the formation of the first lympho-venous contact site did not develop normally in mutant embryos. While in sibling embryos the endothelial layer of the CV and the cells of the pTD were observed in close juxtaposition, in mutant embryos these layers were spatially separated and clearly distinguishable (Figure 7G,H). In wild type embryos we observed an accumulation of PROX-1/VEGFR3 positive cells at the lympho-venous connection site (Figure 7E, large arrow), while this typical clustering of cells did not occur in mutant embryos (7F, large arrow). Also in optical cross-sections (Figure 7G-J), the tight association of lymphatic and venous endothelium, a characteristic of the site of prospective valve formation in sibling embryos was not observed in mutant embryos.

Next, we analysed lymphatic remodelling in E18.5 embryos by staining mesenteries for VEGFR-3, CD31 and PROX-1. In wildtype sibling embryos remodelling of lymphatic vessels was apparent, including the formation of lymphatic valve structures that retained high levels of all three markers used, while downregulated elsewhere in collecting lymphatic vessels (Figure 8A-E). We noted that valve structures were absent in the mesentery of homozygous mutant embryos (Figure 7F-J). We also observed that the lymphatic vessels in mutants were not lumenized, were considerably smaller than in siblings, and retained higher levels of VEGFR3, CD31 and PROX-1 expression (Figure 8A,B,F,G).
DISCUSSION

Lymphangiogenesis and maturation of lymphatic vessels are essential to form a functional lymphatic vasculature\(^1,\,2\). Here, we introduce the Polydom/Svep1 protein as a key factor for embryonic lymphangiogenesis, and demonstrate that in both zebrafish and mouse mutants lack of the protein function results in defective lymphatic vasculature and lymphedema formation.

The formation of a functional lymphatic vascular plexus is a complex process that requires a series of sequential events. First, lymphatic endothelial sprouting occurs within the main embryonic vein. Second, cells that separate from the CV aggregate into lymphatic vascular structures, and these structures form a lumen. Third, in higher vertebrates remodelling steps result in the formation of a hierarchical lymphatic vasculature, where collecting lymphatic vessels develop intra-luminal valves and obtain pericyte coverage\(^14,\,15\). The latter events have not been reported in teleosts, but earlier events such as sprouting from the embryonic cardinal vein appear to be genetically highly conserved: Prox-1 expression in future LECs is a marker for lymphatic fate\(^28-30\), Vegfc is the chemoattractant that is required for sprouting from the vein\(^5,\,7\), and Vegfr3 constitutes the main receptor for Vegfc within the venous and lymphatic endothelium\(^24,\,31,\,32\). Given the significant evolutionary distances between the different phyla it is not surprising that there are some differences: e.g., zebrafish Prox-1 function is required maternally\(^28\) rather than zygotically\(^29\), and lympho-venous sprouting in zebrafish is a simultaneous process rather than a sequential one as in mice. Nevertheless, the genetic analysis of key players in the process demonstrates a high degree of genetic conservation in respect to Prox-1, Vegfc, Cebe1 and Vegfr-3 function\(^7,\,8,\,11,\,13,\,24,\,28,\,29,\,33\).

We identified a zebrafish mutant that shows reduced venous sprouting. The degree of phenotypic severity varies, but there is a consistent and significant reduction of venous sprouting. This results in a decrease in venous ISV formation, in a consequential increase of arteries, and in a reduction of the number of PL cells in the HMS region. The PL cells that do succeed in populating the HMS region exhibit an additional phenotype: the PL cells fail to initiate migration along arteries in a dorsal or ventral direction and remain in the HMS region. Consequently, mutant embryos lack most, or all of the TD. This later aspect of the phenotype is reminiscent of PL migratory defects along arteries in embryos deficient of chemokine signaling\(^20\), but in the absence of Cxcl12a/Cxcr4a/b signaling the initial sprouting from the PCV and the subsequent migration of PL cells to the HMS region occurs normally. We therefore consider it unlikely that Svep1 is involved in chemokine signaling.

Three mutant alleles of the polydom/svep-1 gene were identified, and all three alleles encode nonsense mutations that are predicted to result in truncated proteins. The polydom/svep1 gene is predicted to encode a large secreted protein comprising 3555 amino acids, which is likely to be part of the extracellular matrix (ECM). The protein contains many different domains (hence the name Polydom)\(^25,\,26\), among which are Sushi repeats, a von Willebrand factor type A domain, EGF and EGF-calcium binding repeats, and a pentraxin domain (hence the alternative name Svep1). None of these domains provide an intuitive explanation for the involvement of the protein in lymphangiogenesis. Polydom/Svep1 has been reported to bind strongly to Integrin \(\alpha 9\beta 1\)\(^34\), which in turn has been shown to play a critical role in valve morphogenesis in mice\(^17,\,34\). We have confirmed that Integrin \(\alpha 9\beta 1\) and SVEP1 protein are co-localized (Online Figure VIII), and turned to the zebrafish to test a possible genetic interaction. We generated a zebrafish integrin \(\alpha 9\) mutant allele using the TALEN technology, but homozygous mutant embryos are viable and do not show a lymphatic phenotype (Online Figure IX). Also integrin \(\alpha 9\) mutants that were heterozygous for polydom/svep1 show no phenotype. Furthermore, the EDDMMEVPY motif within the 21\(^{st}\) CCP module, which constitutes the sequence binding most strongly to Integrin \(\alpha 9\beta 1\) protein\(^34\) is lacking in the zebrafish Polydom/Svep1 protein. This, and the absence of a lymphatic phenotype in zebrafish integrin \(\alpha 9\) mutants makes it unlikely that Polydom/Svep1 acts exclusively through Integrin \(\alpha 9\). Of course, other integrins might constitute binding partners for Polydom/Svep1, and we have analyzed integrin \(\alpha 4\) and \(\alpha 5\) mRNA
distribution. While the expression patterns of integrin $\alpha 4$ and $\alpha 5$ do not overlap with polydom/svep1 mRNA distribution in all aspects (Online Figure X), we are nevertheless currently generating mutant alleles for these genes as well. We have also considered the presence of RGD motifs in the Polydom/Svep1 protein, and indeed the zebrafish protein is predicted to contain two RGD domains. However, other vertebrate species do only contain one such motif (rat, human) or none (mouse, cat, guinea pig, sheep). Furthermore, the sites of RGD motifs within the respective proteins are not conserved between different vertebrate species. We thus consider it unlikely that RGD motifs are functionally relevant.

In order to describe polydom/svep1 expression with cellular resolution and to make use of the unique imaging properties of early zebrafish embryos, we generated a transgenic line with a Gal4FF cassette inserted into the predicted start ATG position of the polydom/svep1 locus. Gene expression was predominantly found around the PCV, around ISVs, and in the HMS region. While expression around the PCV is consistent with a venous sprouting defect in mutant embryos, the temporal and spatial expression pattern of very few cells per somite at the HMS is particularly intriguing: expression commences just at the time before PL cells populate the region (Figure 5H), and in a position which coincides later with the area where PL cells migrate dorsally or ventrally. The defect in PL cell migration is a unique feature of the polydom/svep1 mutant, and the temporally and spatially restricted expression of the gene is likely to suggest that providing Polydom/Svep1 protein by a few cells is required to either instruct, or to allow PL cells to move ventrally or dorsally along arteries.

The nature of the cells expressing polydom/svep1 could not be unambiguously verified. Co-expression of the svep1:Gal4FF;UAS:GFP signal with any endothelial-specific reporter was never observed, suggesting non-endothelial expression. We have furthermore excluded muscle pioneer or neuronal cells as the source of Polydom/Svep1 protein, and consider it therefore most likely that mesenchymal cells or pericytes produce Svep1. This is consistent with in vitro data of human cells and in situ lacZ expression data in mice, which also support a non-endothelial expression domain (Online Figure V). Furthermore, functional tests by Morooka et al. (submitted) did not yield a lymphatic phenotype in Tie2:Cre-mediated, endothelial-specific knock-outs of Polydom/Svep1. Therefore, it is most likely that mesenchymal cells in both mouse and fish express Polydom/Svep1, and it will be interesting to see what regulates the highly specific expression pattern in the zebrafish HMS.

The mouse Polydom/Svep1 mutants present with edema formation. The phenotypic strength varies to some extent and is, in this respect, reminiscent of the variable venous sprouting phenotype in zebrafish mutants. At E18.5, the edema formation could be mild or quite severe (Online Figure VII). Nevertheless, all mutant embryos develop edema at E18.5. Based on the early venous sprouting defects in zebrafish mutants, a similar defect might have been predicted to occur in mouse mutants. However, whole mount embryo analysis at E11.0 and E12.5 failed to provide evidence for early migratory defects in PROX-1 positive, future LECs. Rather, we noticed that the first defect in mutants appears at the first lympho-venous contact site at E12.5 and E13.5 (Figure 7G-J). While in wildtype siblings a significant accumulation of Prox-1+ cells is observed in the region of lympho-venous juxtaposition, there are only few cells in mutants that aggregate in the correct location, and their association is not very tight (Figure 7H,J). Of note, a similar phenotype has not been reported in integrin $\alpha 9$ mutants, rendering support to the notion that integrin $\alpha 9$ is unlikely to fully explain the Polydom/Svep1 phenotype.

During later stages of development, we focused on lymphatic maturation and remodeling events in the intestinal mesentery, as valve morphogenesis has been studied in this tissue in detail. Mutant Polydom/Svep1 embryos show only thin lymphatic structures associated with apparently normally developed arteries and veins, without an apparent lumen and without any valves. Hence, while in zebrafish embryos the lack of Polydom/Svep1 results in a reduced number of LECs to sprout from the CV and a failure to execute the correct migratory pattern at the level of the HMS, in mouse mutants early events occur normally. Rather, Polydom/Svep1 controls remodeling steps both early and late. This raises questions about
the evolutionary implications of these different phenotypes, which manifest themselves in the same vascular bed. A better understanding of the molecular pathway which Polydom/Svep1 is a part of will be required to fully understand this phenomenon.

In summary, we here show the ECM protein Polydom/Svep1 to be required for key events during lymphatic vascular formation in fish and mice. While there are differences in how the respective phenotypes emerge, intriguingly in both species the protein is provided by non-endothelial cells close to the lymphatic vascular bed, and in both organisms the gene is essential for lymphatic vascular development.

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DISCLOSURES

None.

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FIGURE LEGENDS

Figure 1: Characterization of a zebrafish mutant that affects the formation of the lymphatic vascular system. A, Gross morphology of the Ly02-512 mutant. The mutant embryos appear without major macroscopic defects or developmental delay, and form a swim bladder at 5dpf. However, closer examination shows abnormalities in the jaw (arrowhead), and the embryo displays oedema around the heart, eye, and intestine (arrows). B, Analysis of the vasculature in a fli1a:GFP; flt1erm2:RFP transgenic background, highlighting arteries (red). Note the normal overall patterning of the blood vasculature despite the higher proportion of intersegmental arteries in the mutant embryo. C, Mutant embryos lack all or most aspects of the TD, which in wildtype siblings is positioned just ventral to the DA (white arrows in C and D), but is absent in mutants (asterisks). D, Rhodamine dextran injection into the cardinal vein shows normal blood circulation in Ly02-512 mutants.

Figure 2: Ly02-512 encodes an allele of polydom/svep1. A, Positional cloning approach to identify the molecular lesion in Ly02-512 mutant embryos. Using polymorphic markers, Ly02-512 was mapped to a region between markers z4.29 and z7.4 comprising approximately 350kb on linkage group 7. B, Three different BAC constructs that were independently inserted as transgenes into the Ly02-512 line containing 12 candidate genes within the linkage group. C, In Ly02-512 mutant embryos which contained the DKEY-8E16 BAC the TD phenotype was rescued (p<0.001), suggesting DKEY-8E16 to contain the gene of interest. D, Two other mutant lines identified in the forward genetic screen, Ly04-093 and Ly05-265, failed to complement Ly02-512 and each other, suggesting they represent alleles of the same gene. E, Sequencing of the three independent mutant alleles revealed three different non-sense substitutions, predicted to result in truncated versions of the Polydom/Svep1 protein. F, Schematic presentation of the Polydom/Svep1 protein domain structure and the positions of the truncations in the three mutant lines. Red rectangle: signal peptide; blue pentagon: von Willebrand factor type A domain; yellow ovals: SUSHI repeat; green pentagons: EGF-like and calcium-binding EGF-like domains; pink hexagon: pentaxin domain.

Figure 3. Polydom/svep1 mutants show reduced venous and lympho-venous sprouting. A and F, Quantification of sprouts from the PCV in wildtype (wt) siblings and polydom/svep1 mutants, in pleg1 morphant embryos. Knock-down of pleg1 suppresses arterial formation, hence only venous structures can be observed in a fli1a:GFP transgenic background. Heterozygous embryos show a significant reduction in venous sprouting events from the PCV, and this is further exacerbated in mutant embryos at 54 hpf (wt siblings: n=29, heterozygous embryos: n=53, polydom/svep1 mutants: n=26). B, Still frames from confocal time-lapse imaging of a wildtype sibling and polydom/svep1 mutant embryo in a fli1a:GFP; flt1erm2:RFP double transgenic background are shown over the course of 32.5 to 46.5hpf. Both the number of secondary sprouts from the PCV (yellow arrowheads) and PL cells (white arrowheads) was reduced in mutant embryos. C and G, polydom/svep1 mutant embryos form a reduced number of PLs at the HMS region. Confocal images of wild type sibling and polydom/svep1 mutant embryos at 48hpf in fli1a:GFP; flt1erm2:RFP background and quantification of PLs at 54hpf (WT siblings: n=8, heterozygous embryos; n=11, polydom/svep1 mutants: n=6). D, PL cells at the level of the HMS fail to migrate along intersegmental arteries in the polydom/svep1 mutants. Still frames from confocal time-lapse imaging of a wildtype sibling and a polydom/svep1 mutant embryo in a fli1a:GFP transgenic background are shown over the course of 2.5dpf to 3.5dpf. E and H, An increased number of arterial ISVs at the expense of venous ISVs in polydom/svep1 mutants is highlighted by flt1erm2:RFP expression in fli1a:GFP background at 5dpf (siblings: n=20, polydom/svep1 mutants: n=10). Values are presented as means ± standard deviation. ** = P<0.01; *** = P<0.001 in all panels.

Figure 4. PCV cells express pERK and PL cells express Prox-1 in svep1 mutant embryos. A, Partial maximal projection of antibody staining against PROX-1(red) and fli1a:GFP (green) in embryos from an svep1+/--; fli1a:GFP incross at 48hpf. Prox-1 positive PL cells are indicated by an arrowhead. B, Quantification of Prox-1 positive PL cells across 9 somites at the HMS in siblings (96 out of 106 counted
PLs are Prox-1 positive in 18 embryos) and svep1 mutants (28 out of 30 PLs are Prox-1 positive in 8 mutant embryos). C-E, pErk-positive cells were quantified in the cardinal vein by scoring RFP and GFP co-expression (indicated by arrows) laterally across 6 somites in the trunk. (C), in ccbe1 MO injected embryos (total number of 6 pERK positive cells in 5 ccbe1 morphants; total number of 36 pERK positive cells in 6 non-injected siblings) and (D) vegfc<sup>hut6410</sup> mutants (total number of 17 pERK positive cells in 7 mutants and 67 pERK positive cells in 8 siblings) the amount of pErk positive cells is significantly reduced whereas in svep1 mutants (E,F) no difference in pErk can be detected in the PCV (total number of 151 pERK positive cells in 31 siblings compared to 48 pERK positive cells in 9 svep1 mutants). F, Partial maximal projections of antibody staining against pERK (red) and flila:EGFP (green) in svep1+/-; flila:GFP incrosses show no difference in the amount of pERK positive cells in the PCV at 32hpf. Bar graphs show mean ± standard deviation. For statistical analysis, the Mann-Whitney test was applied in all panels.

**Figure 5.** Zebrafish polydom/svep1 is expressed dynamically at regions of venous and lymphatic endothelial cell migratory activity. A, The first polydom/svep1 expression appears around 34hpf in non-endothelial cells along the DA and PCV, as depicted by svep1:Gal4FF;UAS:GFP expression in a kdrl: mCherry transgenic background. B, By 48hpf, the number of polydom/svep1 positive cells along the PCV has increased, and polydom/svep1 positive cells can abundantly be found in the immediate vicinity of both arterial (highlighted in red in fli1<sup>end</sup>:RFP background) and venous (marked with asterisks) ISVs. C, Higher magnification of an independent region at 48hpf, demonstrating the tight connection between endothelial cells (red) and polydom/svep1 positive cells (green). D-F, Between 48hpf and 72hpf, when PL cells populate the HMS region and start to migrate dorsally and ventrally along arterial ISVs, individual cells in the midline of the embryo start to express polydom/svep1. The polydom/svep1 expressing cells are in a very immediate contact with migrating PL cells. G, Cross section of a 72hpf svep1:Gal4FF;UAS:GFP embryo in a kdrl:mCherry background. Note the close association of PL cells (red) and polydom/svep1 positive cells (green) in the HMS region (white arrow heads), and of PCV cells and polydom/svep1 positive cells (blue arrow head). The DA (red arrow head) is not covered by polydom/svep1 positive cells at this time point any more. H, Still frames of a confocal time lapse imaging of a svep1:Gal4FF;UAS:GFP;kdrl:mCherry embryo from 36hpf to 51hpf. Polydom/svep1 positive cells (green) and PL cells (red) start appearing at the horizontal midline region around 39hpf. Note the almost simultaneous appearance of both cell types. The full movie can be seen online. I, At 3dpf and 5dpf, polydom/svep1 expression can be seen in between the DA and the PCV, exactly in the region, which the lymphatic cells of the future TD will populate.

**Figure 6: Characterization of Polydom/Svep1 function in mice.** A, Targeting construct to create a Polydom/Svep1 knock-in mouse. The LacZ construct disrupts the locus and is predicted to result in a protein truncated after amino acid 559. The primers used for genotyping are indicated (fw, forward primer; mt-rv, mutant reverse primer; wt-rv, wild type reverse primer). B, Genotyping using the PCR primers clearly distinguishes the wildtype from the mutant allele. C, E18.5 litters from heterozygous parents contained mutant and sibling embryos in normal Mendelian ratios. Heterozygous siblings are indistinguishable from wildtype embryos, but mutant embryos show a clear nuchal edema (arrow).

**Figure 7: The first detectable abnormalities in Polydom/Svep1 mutant embryos occur at the level of lympho-venous valve formation.** In order to determine the first phenotypic abnormalities in mutant embryos, we used whole mount imaging on the ultramicroscope at E11.0, E12.5 and E13.5 embryos. Combined antibody-staining highlighting CD31, PROX-1 and VEGFR-3 expression at E11.0 (A and B), and Prox-1 and VEGFR-3 at E12.5 (C-H), did not reveal any alterations between wildtype sibling and mutant embryos. In all cases examined, we could not detect differences in future LECs egressing from the cardinal vein, and the formation of the primitive pTD and the pLLV appeared unchanged. However, we did notice at E12.5 a difference at the level of the first lymphovenous connection: here, Prox-1 positive cells of the CV and Prox-1 positive LECs come together in tight association (G), but this association was not found.
in mutant embryos (H). I,J, Closer view of the lympho-venous contact site in volume reconstructions at E13.5. While in wildtype embryos the dual contact sites are massive structures composed of multiple cells, in Polydom/Svep1 deficient embryos only very few individual cells are actually in contact to the high Prox-1 positive expression domain inside the CV. Scale bars correspond to 100 μm in all panels.

**Figure 8:** Polydom/Svep1 mutant embryos fail to remodel mesenteric lymphatic vessels at E18.5, and do not form valves. Lymphatic structures in mutant mesenteries are closely associated with blood vessels as they are in siblings, but they are significantly smaller in size, appear non-luminized and do not contain valve structures. A-E, Whole mount immunofluorescent staining of mesenteric vessels for VEGFR-3, CD31 and PROX-1 at E18.5 of a Svep1+/- and -/- embryo. At this time of the development the maturation of lymphatic vessels is well underway, including the formation of lymphatic valve structures that retain high levels of PROX-1 protein. CD31 and VEGFR-3 levels are down-regulated in the lymphatic vessels, but remain high in the lymphatic valve regions. A, Overlay picture of VEGFR-3, CD31 and PROX-1 staining with the boxed region shown in higher magnification in B-E; F-J, Immunofluorescent staining of vessels in a E18.5 Svep1-/- embryo indicates that the size of the lymphatic vessel is dramatically decreased. PROX-1 expression remains high in the LECs and lymphatic valves fail to form. F, Overlay picture of VEGFR-3, CD31 and PROX-1 staining with the boxed area shown in higher magnification in G-J. Scale bars correspond to 50μm in all panels.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Lymphangiogenesis involves a complex array of embryonic cellular movements and, at later stages, an extensive set of maturation events to allow lymphatic vessels to exert their function.

- In vertebrates, the development of the lymphatic system is evolutionarily conserved.

What New Information Does This Article Contribute?

- The extracellular protein Polydom/SVEP1 is essential for lymphatic vessel formation in fish and mice.

- Zebrafish embryos mutant in svep1 show defective venous sprouting and aberrant migration behaviour of future lymphatic endothelial cells.

- Murine Polydom/SVEP1 mutant embryos develop severe edema in utero, caused by defective lympho-venous connections, missing valve structures and remodelling defects.

Lymphatic vessels play key roles in many physiological processes, but the genetic control of lymphatic vessel formation and lymphatic endothelial cell function is poorly understood. We show here that the large secreted Polydom/SVEP1 protein, which has previously not been connected to lymphatic vessel formation or function, controls key steps of lymphatic development in zebrafish and mice. The combined analysis of zebrafish and mouse mutants demonstrates that the Polydom/SVEP1 protein influences different aspects of lymphatic development in the respective species: zebrafish mutant embryos exhibit defects in early lymphatic endothelial cell migration, while mouse mutant embryos show defects at later stages of development. This study introduces a new key gene function to the field of lymphatic endothelial cell biology, and demonstrates that Polydom/SVEP1 is indispensable for different steps during lymphatic development.
Figure 1

A

Sibling

Mutant

B

Sibling

Mutant

fli1a:GFP;flt1^{enh}.RFP

C

Sibling

Mutant

DA1

PCV

PCV

D

fli1a:GFP;angiography
Figure 2

A

z4999
23.02 Mb
16/2276

z4.29
23.98 Mb
2/16

z7.4
24.33 Mb
2/14

z13.24
24.82 Mb
14/2043

Chr 7

svep1

ptgr1l

haus3

ptgr1

poln

C7H4orf48

whsc2

btr09

tesk1

faah2b

B

DKEY-8E16

zgc:153151
CR318603.2
gmpr2

nedd8

slc7a8a

DKEY-10BN6

CH73-220H7

C

100%

Mut

Het

WT

Mut

Het

WT

Siblings without DKEY-8E16 BAC

Embryos with DKEY-8E16 BAC

no TD
TD 4-20 somites
TD < 4 somites
TD > 20 somites

Sibling

Mutant

Rescued mutant

fli1a:GFP

D

100%

WT

Mut

Ly02-512

Ly02-512

Ly04-093

x

x

Ly04-093

Ly05-265

Ly05-265

E

Ly02-512

Ly04-093

Ly05-265

WT Sibling

Het Sibling

Mutant

Stop

Stop

Stop

F

Ly04-093
C355X
Ex4

Ly02-512
K836X
Ex14

Ly05-265
Y1918X
Ex35
Figure 3

A  wt sibling    svep1-/-

plgl1 MO injected     fli1a:GFP

B

wt sibling  svep1-/-

DAI  PCV

32.5hpf  36hpf  39.5hpf  43hpf  46.5hpf

C  wt sibling    svep1-/-

fli1a:GFP; flt1<sup> thugs</sup>:RFP

D  2.5dpf  3dpf  3.5dpf

E

wt sibling  svep1-/-

pl  alsv  da  pcv

2.5dpf  3dpf  3.5dpf

F

secondary sprouts / embryo

WT  Het  Mut

***

G

Plts / embryo

WT  Het  Mut

**

H 60%  30%

Sib  Mut

***
Figure 6

A

B

C

wildtype allele
mutant allele

Svep1 +/+  Svep1 +/-  Svep1 -/-
Figure 8
An Evolutionarily Conserved Role for Polydom/Svep1 During Lymphatic Vessel Formation
Terhi Kärpanen, Yvonne Padberg, Serge A van de Pavert, Cathrin Dierkes, Nanami Morooka, Josi Peterson-Maduro, Glenn van de Hoek, Max Adrian, Naoki Mochizuki, Kiyotoshi Sekiguchi, Friedemann Kiefer, Dörte Schulte and Stefan Schulte-Merker

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SUPPLEMENTAL MATERIAL

Online Methods:

Antibodies and reagents:
The following antibodies and reagents were used: anti-Prox-1 (102-PA32, Reliatech), Prox1 rabbit mAb [AngioBio Co, # 11-002], phospho-ERK1/2 antibody XP rabbit mAb [Cell signaling#4370], anti-Lyve-1 (103-PA50, Reliatech), anti-VEGFR-3(AF743, R&D), CD31 (550274, BD), rat monoclonal anti-mouse PECAM-1 (clone 5D2.6 and clone 1G5.1, provided by Dr S Butz), goat anti-mouse integrin α9 (AF3827, R&D), anti-GFP chicken polyclonal [ab13970], goat α-rabbit IgG-HRP (Life Technologies). A rabbit antibody directed against the N-terminal region of mouse Polydom was generated by the Sekiguchi laboratory, BetaBlue™ Staining Kit (71074-3FRZ, Novagen), Hoechst 33342, Tyramide-FITC/Cy3/Cy5 (NEL744001KT, Perkin Elmer).

Generation of the Svep1 knock-out first allele.
We obtained ES-cells with a modified version of the gene (Figure 6A) (EUCOMM, project 93128): the modified allele of the murine Svep1/Polydom locus contains a cassette with a lacZ element, allowing both expression analysis of the gene trough ß Galactosidase activity (Online Figure V) as well as interrupting the coding frame after exon 7. ES cells were injected into blastocysts, and mosaic pups resulted from this approach after transferring blastocysts to foster mothers. Putative carrier mice were crossed to wildtype mice, and genotyping yielded individuals that were identified as heterozygous in the F1 generation (see Figure 6B)

Primers for genotyping knock-out first mice

| Primer         | Primer sequence                |
|----------------|-------------------------------|
| Wt primer fw   | AGGCAATTCAGGTACAACCATCTGG     |
| Wt primer rv   | TCCTGCTGCTCCCCAGTCTTTGCC      |
| Mut primer rv  | CAACGGGTTCTTCTTTAGTCC         |

Primers used in this study for genotyping fish:

| Primer     | Primer sequence     |
|------------|--------------------|
| Itga9 Fw   | agcacaagagagacgacgc |
| Itga9 Rv   | Ccactgagcagtaaagg  |
| Svep1_WT   | GAAGGTGACCAAGTATGCTGGGAGATGATGTCTCTCTGc  |
| Svep1_MUT  | GAAGGTCCAGGTCAACGGATGGGAGATGATGTCTCTCTGCt |
| Svep1_Common| CAGGCACTGTGCAAGGAAAGTCACC |

Primers used for in situ hybridization

| Primer               | Primer sequence               |
|----------------------|------------------------------|
| Svep1 in situ fw     | CACACAACACACATGCACAC         |
| Svep1 insitu Rv T3   | cattaacctcaataagggaATCCCATGAGGCTCAACAT |
| Itga9 insitu fw      | CCAACACCTCCGACTACACT         |
| Itga9 insiturv T3    | cattaacctcaataagggaATCCGGCACCCTGTGTAACACGC |
| Itga4 insitu fw      | TGTTATCTGCTGTTGCTGCTGCTGCT |
| Itga4 insiturv T3    | cattaacctcaataagggaATCCGGCACCCTGTGTAACACGC |
| Itga5 insitu fw      | TCCTCAAAACCTCCGACTACACT     |
| Itga5 insiturv T3    | cattaacctcaataagggaATCCGGCACCCTGTGTAACACGC |
Whole-mount stainings
Whole-mount staining of dorsal skin preparations of fetuses from staged matings was performed as previously described using anti-PECAM1, anti-Lyve-1 and anti-Prox-1 as primary antibodies. Images were acquired on a Leica SP8 confocal microscope.

Immunohistochemistry for mouse embryos
Embryos were fixed, stained with BetaBlue™ Staining Kit overnight and processed into paraffin blocks. Immunostaining of sections was performed according to standard procedures. Envision+ kit (DAKO) was used as a secondary reagent. Slides were counterstained with hematoxylin.

Analysis of zebrafish pERK by immunohistochemistry
*Ccei* morpholino injected embryos, and embryos derived from *vegfc* and a *svep1* incrosses were fixed overnight at 32hpf and stained with α-pErk and α-GFP according to the previously described protocol. The embryos were imaged and p-Erk positive cells were quantified in the cardinal vein by scoring RFP and GFP co-expression laterally across 6 somites in the trunk.

Analysis of zebrafish Prox-1 by immunohistochemistry
Embryos from a *svep1* incross were fixed and stained at 48hpf as described previously with the following modifications. After acetone treatment, embryos were treated with Proteinase K for 30 minutes at room temperature. Antibodies used were chicken α-GFP (1:400), α-Prox-1 (1:500), and goat α-rabbit IgG-HRP (1:1000). Prox-1 positive PL cells were counted manually across 8 somites, scoring expression of Tg(fli1a:nEGFP) detected by α-GFP in green and α-Prox-1 in red.

mRNA injection into zebrafish
Murine Svep1 plasmid was obtained from the Sekiguchi lab and was transcribed from a AvrII-lineralized template using RiboMax Large Scale RNA production System T7 and injected at 1 ng/embryo.

In situ hybridizations
Anti-sense RNA probes were generated by PCR from cDNA, were transcribed with T3 RNA-polymerase and carried out on TL fish as described in Schulte-Merker (2002).

Ultramicroscopy
After whole-mount immunostaining with anti-Prox1, anti-PECAM-1 and anti-VEGFR-3, embryos were optically cleared with Benzylbenzoate/Benzylalkohol (BABB) and were imaged with an ultramicroscope (La Vision Biotech, Bielefeld). Stacks were captured with a step size of 1 µm and at different magnifications. 3D reconstruction, morphometric analysis and analysis of ultramicroscopy stacks were performed by using Voreen Software.

Generating a mutant allele of zebrafish integrin α9
TALEN-mediated genome editing for the generation of mutants was performed as described before. The TALEN binding sites in itgu9 exon 1 are: TAL1, 5’-TGATCTACAATATCCAGTGGT-3’; TAL2, 5’-GCGACGTTTTTCGGATA-3’ which generated a 7bp deletion from position +125 until +131 downstream of the ATG (according to transcript ENSDART0000004575.6).

VEGFC over-expression assay
Ectopic over-expression of human VEGFC in the floorplate was driven by a sonic hedgehog promoter and a floorplate specific activator region, and an estimate of the expression of hVEGFC was obtained and monitored by simultaneous expression of tagRFP. Plasmids encoding hVEGFC cDNA and the floorplate specific promoter and enhancer regions flanked by MiniTol2sites were co-injected at 25 ng/µl together with tol2 transposase mRNA (25 ng/µl) into zebrafish eggs of crosses of *svep1* heterozygous carriers at the 1-2 cell stage. Embryos were selected and sorted at 2 dpf based on comparable expression of tagRFP and imaged on a Leica SPE confocal microscope. For quantification of vessel sprouting, both
the sum of the GFP+ of all z-planes and the number of vessel branch points per area (200 x 300 microns) were analyzed using ImageJ (NIH, Bethesda, Maryland, USA).

**Statistical analysis**
Data sets were tested for normality (Shapiro-Wilk) and equal variance. P-values were determined by Student's t-test. When normality test failed, Mann-Whitney test was performed.
Online Figure I: Alternative splicing does not occur in svep1<sup>093</sup> and svep1<sup>512</sup> mutants: A: Part of the svep1 locus (exon1-16), depicted with primer sequences used for RT-PCR. Mutation of svep1<sup>093</sup> (mutation in exon 4) and the svep1<sup>512</sup> (mutation in exon 14) mutants are indicated by an asterisk; B: RT-PCR of svep1<sup>093</sup> and svep1<sup>512</sup> with two different primer pairs indicate that no alternative splicing occurs in both mutants.
Online Figure II: Rescue of svep1 mutants with murine Svep1 mRNA: A: Lateral view of polydom/svep1 sibling (top) and mutant (bottom) embryos at 5dpf. Embryos had been injected at the 1-2 cell stage with 1ng of murine Svep1 mRNA generated by in vitro transcription. At 5dpf the extent of the TD (arrows) across ten body segments was scored (arrows), and embryos were subsequently genotyped. Note the complete thoracic duct structure in the mutant embryo. B: Quantification of a separate experiment, where control embryos were left un-injected, while experimental embryos were injected with 1ng of murine Svep1 mRNA. Embryos were scored as described above, and subsequently genotyped. All un-injected mutant embryos developed thoracic duct fragments in maximally 40% of their trunk segments, while in injected embryos 30% showed a thoracic duct in 50-70% of their trunk segments, and 20% of injected mutant embryos even showed complete rescue.
Online Figure III: VEGFC over-expression and Svep1 and Ccbe1 interaction: (A – B) svep1 mutant endothelial cells respond to VEGFC. (A) Confocal projections of siblings and Svep1 mutants expressing VEGFC IRES RFP in the floorplate versus non injected control at 2 dpf, transgene: fli1a:GFP. Forced expression of human VEGFC in the floorplate led to excessive vessel sprouting both in siblings and in svep1 mutants (B) Quantification of endothelial vessel area as measured by GFP+ area surrounding a position of comparable RFP expression. Data sets were tested for normality (Shapiro-Wilk) and equal variance. P-values were determined by Student's t-test. Values are presented as means ± standard error of mean values (SEM). ns = not significant; * = P<0.05; ** = P<0.01; *** = P<0.001. (C - D) svep1 and ccbe1 do not genetically interact (C) Confocal projections of wt and svep1/ccbe1 double heterozygous animals do not show any defect in TD generation as compared to wildtype controls at 5 dpf, transgene: fli1a:GFP. Arrows indicate the position of the TD (D). Quantification of the extent of TD formation across ten body segments in the trunks of wildtype, single heterozygote and double heterozygote embryos do not indicate genetic interaction between svep1 and ccbe1. One out of 3 independent experiments is shown.
Online Figure IV: Comparison of *svep1* expression domains in the *svep1:GalFF; UAS:GFP* transgenic line with whole mount *in situ* hybridization of non-transgenic embryos. *Polydom/svep1* expression at 48hpf (F,G,H,I,J) and 72hpf (A,B,C,D,E) of TL fish. A,B: Higher magnification of the ceratohyal underneath the eye of a transgenic embryo (A) and upon *in situ* hybridization at 72hpf; C: negative control embryo at 72hpf, lacking RNA antisense probe; D,E: *svep1* is expressed in the branchial arch region at 72hpf, which can be seen in both the transgene (D) and by *in situ* hybridization (E); (C) serves as a negative control for (D,E) as well. F,G: *svep1* expression in the fin bud at 48hpf; H,I: *svep1* expression abutting the middle cerebral vessel at 48hpf; J: Lateral view of *polydom/svep1* expression in the region of the PCV at 48hpf; K: *svep1* expression (indicated by the arrows) covering ISVs (compare to the transgenic expression in Figure 5B, C, main text). Notochord staining in (J,K) represents background staining and was also observed in all negative controls. Lateral views in all panels, anterior to the right.
Online Figure V: *Polydom/Svep1* is expressed by non-endothelial cells in the region of the CV and of the pTD. A: Left: Transverse paraffin section of an E12.5 *Svep1*<sup>+/LacZ</sup> embryo stained with anti-LYVE-1 to highlight the lymphatic structure. LacZ expression, which is visualized by β-galactosidase, is detected in close proximity to the CV and the pTD. LacZ expression is also found in close proximity to the dorsal aorta. B: Based on RT-PCR data, *Polydom/Svep1* is expressed by VH32 fibroblasts but not by HUVEC cells and LECs.
Online Figure VI: The lymphatic structures are dramatically decreased in size in Polydom/Svep1 deficient embryos at E14.5: Left: Polydom/Svep1 deficient embryos developed edema at the back. Middle: Hematoxylin staining on transverse paraffin sections of heterozygous and mutant Polydom/Svep1 embryos. Polydom/Svep1 deficient embryos show a decreased size of the lymphatic structure compared to the heterozygous sibling. LS, lymphatic structure; CV, cardinal vein; DA, dorsal aorta. Right: Fluorescent staining on paraffin section using antibodies against podoplanin (green), LYVE-1 (red) and endumucin (blue) shows that the lymphatic structure (white arrows) is very small in Polydom/Svep1 deficient embryos as compared to the large jugular lymphatic structure (JLS) in the wildtype sibling embryo.
Online Figure VII: Variation of the phenotype in Polydom/Svep1 deficient embryos at E18.5. Despite of the fact that the severity of the edema varies considerably in Polydom/Svep1 deficient embryos, mutants were distinguishable from the heterozygous and wildtype siblings in all cases. While some *Polydom/Svep1* mutants only display edema at the dorsal side, others also have edema at the ventral side.
Online Figure VIII: Integrin α9β1 and SVEP1 protein are co-localized: Whole-mount immunofluorescence staining for Polydom/Svep-1(red) and Integrin a9 (green) in the wild-type mesentery at E18.5. L, lymphatic vessel; N, nerve. Nuclei are counter-stained with DAPI. Scale bar indicates 100 μm.
Online Figure IX: Genetic interaction of Svep1 and Itga9: A-C: Lateral view of representative examples of fli1a:GFP transgenic embryos that are either wildtype for both svep1 and integrin α9 (A), mutant for integrin α9 (B), or mutant for integrin α9 and heterozygous for svep1 (C). Neither of the genetic combinations shows a thoracic duct (arrows) phenotype. Hence, we have found no evidence for a genetic interaction of integrin α9 and svep1. D: Quantification of the embryos presented in (A-C).
Online Figure X: Whole mount in situ hybridization of integrin α9, α4 and α5 show vessel-specific expression: The expression of itgα4 (A) itgα9 (B) and itgα5 (C) at 32 hpf as detected by in situ hybridization (lateral views). A: itgα4 is expressed in the mid cerebral vessel (MCev) and in the notochord; B,C: itg α9 and itgα5 is expressed in the PCV and the intersegmental vessels.

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