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 a9 (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	CAACGGGTTCTTCTGTTAGTCC

Primers used in this study for genotyping fish:

Primer	Primer sequence
Itga9_Fw	agcacaagagatgagaccgc
Itga9_Rv	Ccactcgagcgattaacgga
Svep1_WT	GAAGGTGACCAAGTTCATGCTGGGGAGATGATGTCTCTTGCa
Svep1_MUT	GAAGGTCGGAGTCAACGGATTGGGGAGATGATGTCTCTTGCt
Svep1_Common	CAGGCACTGTGCAGGTAAAGTCATT

Primers used for in situ hybridization

Primer	Primer sequence
Svep1 in situfw	CACACAACAACACATGCCAAG
Svep1 insitu Rv_T3	cattaaccctcactaaagggaaGAATCCATGGAGGCTCAACAT
Itga9_insitufw	CCCAAACCTCCGACTTACACT
Itga9_insiturv_T3	cattaaccctcactaaagggaaATCCGGCACTTGTAAACAGC
Itga4_insitufw	TGTTATCTGCGTGTGGTGCT
Itga4_insiturv_T3	cattaaccctcactaaagggaaGCTGCTCACACTGGCTGTT
Itga5_insitufw	TCTTCAAGCGAACTCCATACG
Itga5_insiturv_T3	cattaaccctcactaaagggaaCGACTACCTCATTCTCGCTTG

Whole-mount skin stainings

Whole mount staining of dorsal skin preparations of fetuses from staged matings was performed as previously described [1] 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

Ccbe1 morpholino injected embryos, and embryos derived from *vegfc* ^{hu6410} and a *svep1*^{512} incrosses were fixed overnight at 32hpf and stained with α-pErk and α-GFP according to the previously described protocol [2]. 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*^{512} incross were fixed and stained at 48hpf as described previously [2] with the following modifications. After acetone treatment, embryos were treated with Proteinase K for 30 minutes at room temperature. Antibodies used were chicken a-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[6] 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).[3]

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 [4]. The TALEN binding sites in Itgα9 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 ENSDART00000045475.6).

VEGFC over-expression assay

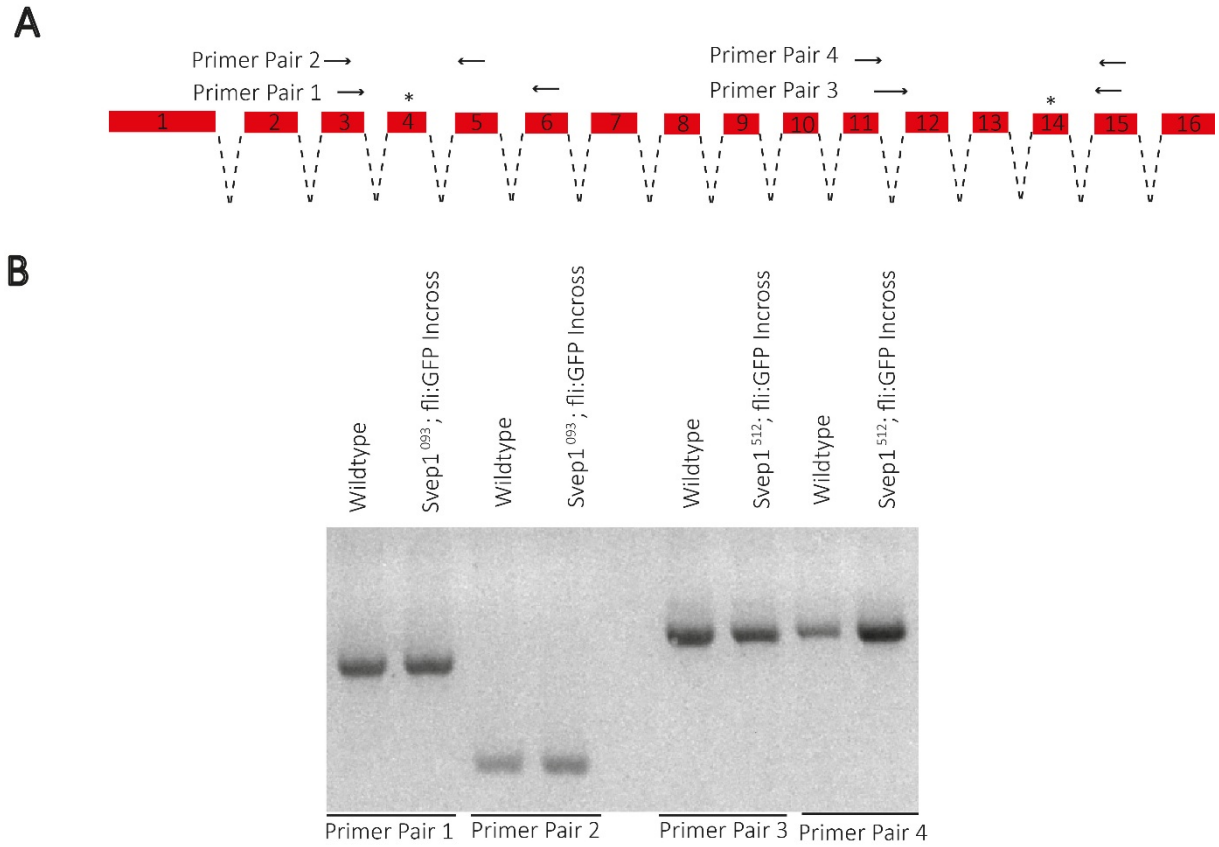
Ectopic over-expression of human VEGFC in the floorplate was driven by a sonic hedgehog promoter and a floorplate specific activator region, and an estimate of the expression of hVEGFC was obtained and monitored by simultaneous expression of tagRFP [5]. Plasmids encoding hVEGFC cDNA and the floorplate 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).

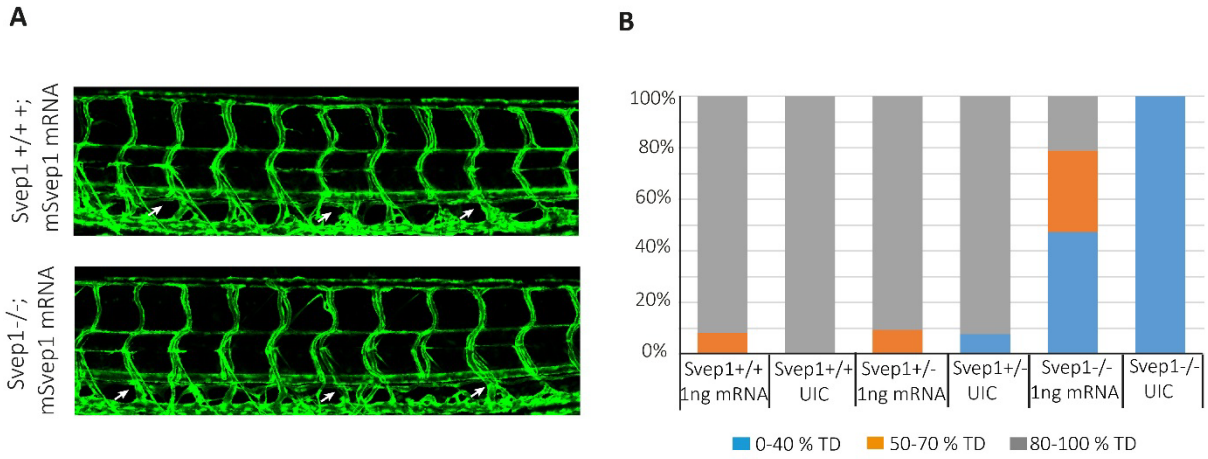
Statistical analysis

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.

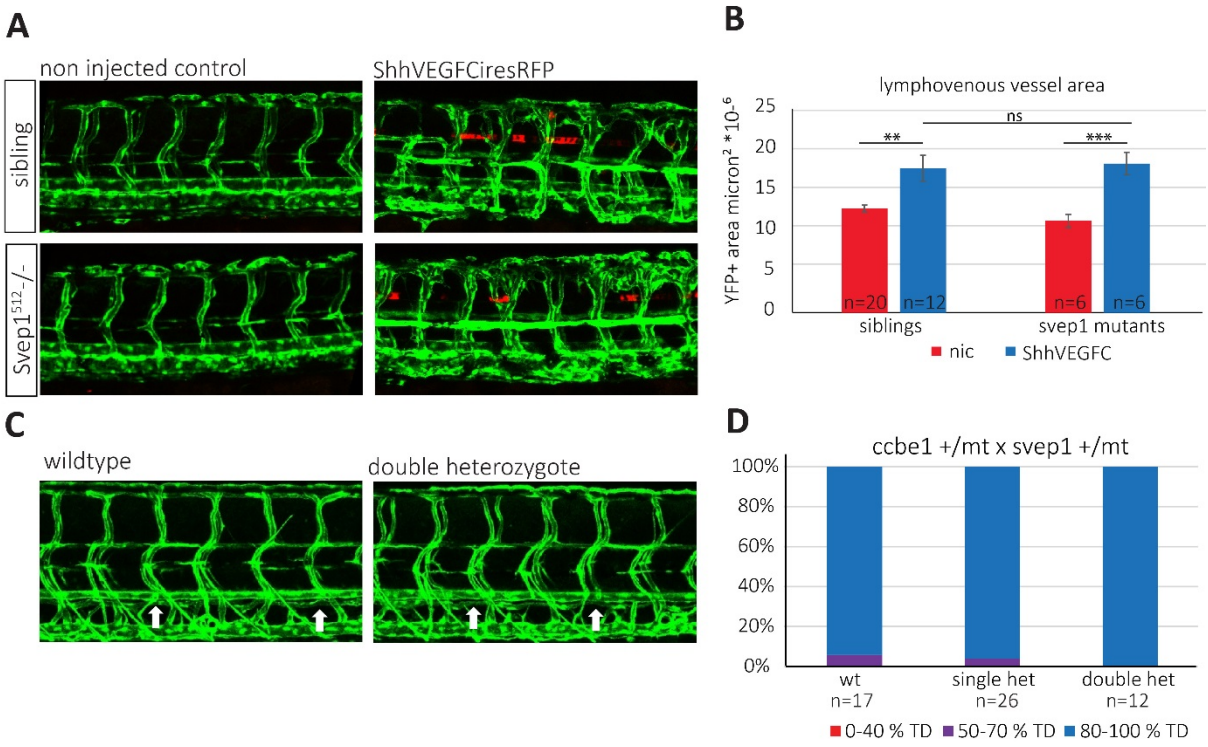
Online Figures



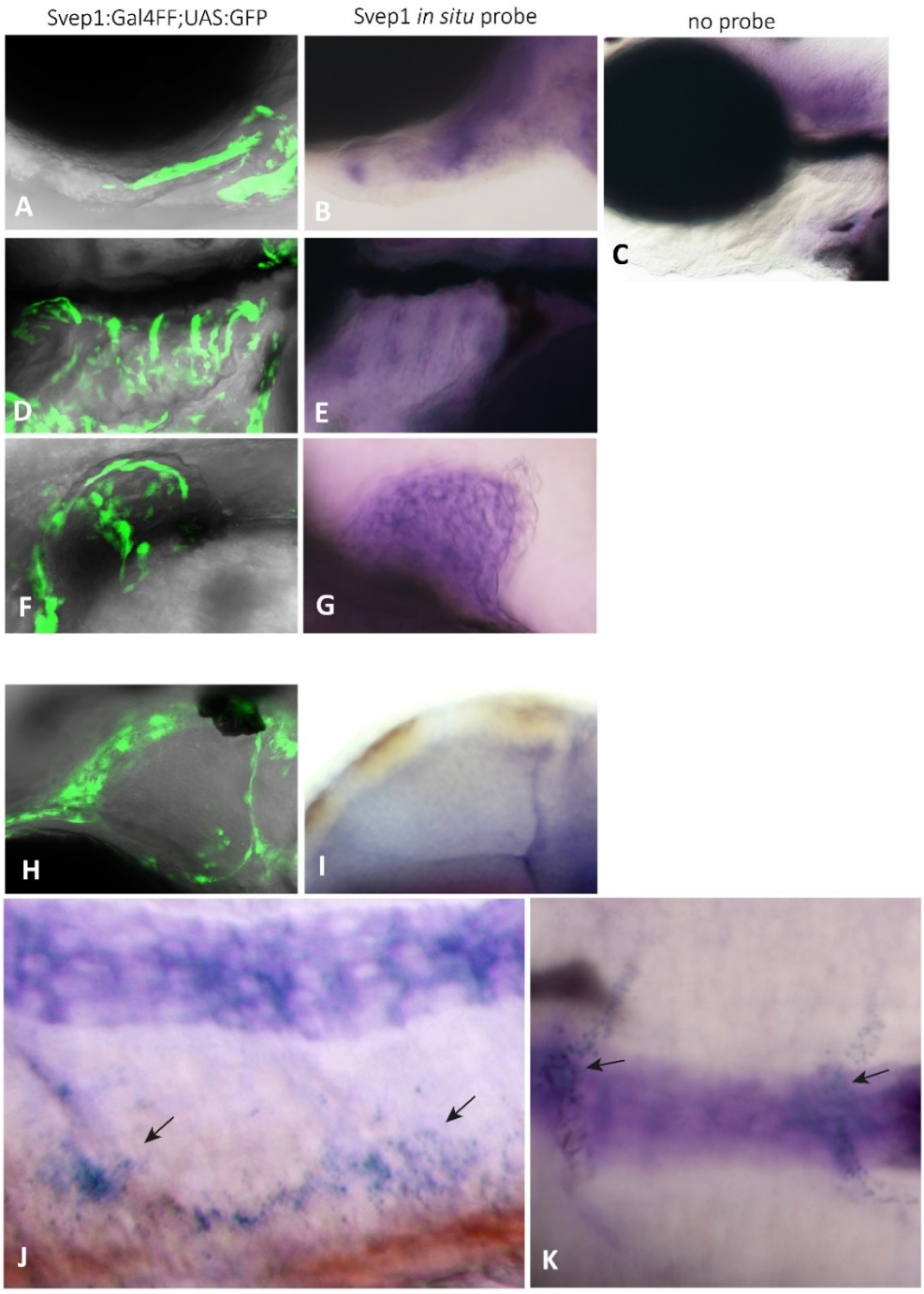
Online Figure I: Alternative splicing does not occur in *svep1* ^{093} and *svep1* ^{512} mutants: A: Part of the *svep1* locus (exon1-16), depicted with primer sequences used for RT-PCR. Mutation of *svep1* ^{093} (mutation in exon 4) and the *svep1* ^{512} (mutation in exon 14) mutants are indicated by an asterisk; B: RT-PCR of *svep1*^{093} and *svep1*^{512} with two different primer pairs indicate that no alternative splicing occurs in both mutants.



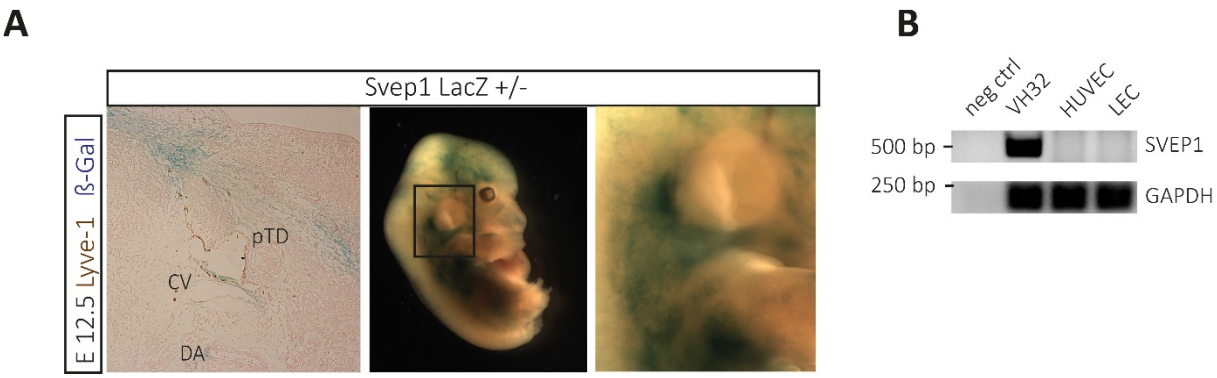
Online Figure II: Rescue of *svep1* mutants with murine *Svep1* mRNA: A: Lateral view of *polydom/svep1* sibling (top) and mutant (bottom) embryos at 5dpf. Embryos had been injected at the 1-2 cell stage with 1ng of murine *Svep1* mRNA generated by *in vitro* transcription. At 5dpf the extent of the 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.



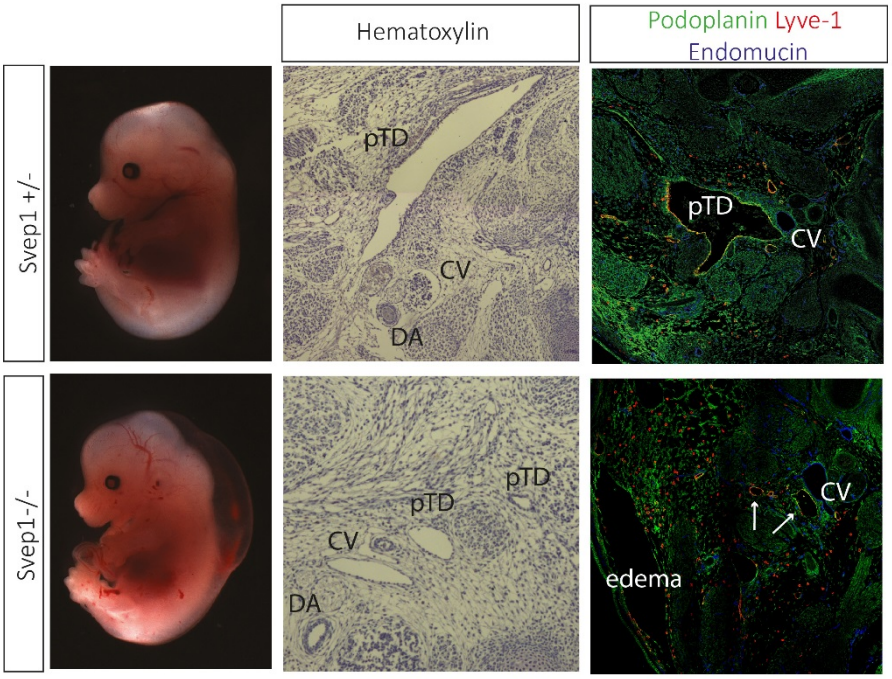
Online Figure III: VEGFC over-expression and Svep1 and Ccbe1 interaction: (A – B) *svep1* mutant endothelial cells respond to VEGFC. (A) Confocal projections of siblings and *Svep1* mutants expressing *VEGFC IRES RFP* in the floorplate versus non injected control at 2 dpf, transgene: *fli1a*:GFP . Forced 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.



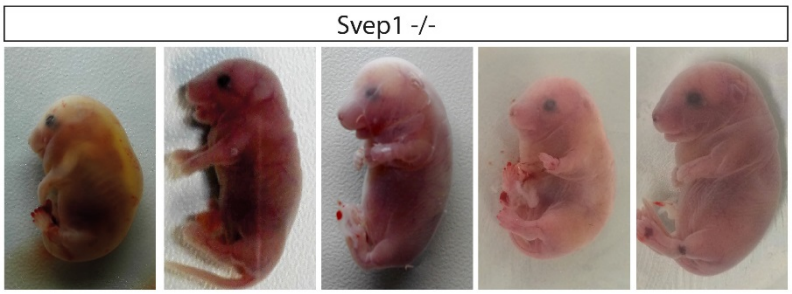
Online Figure IV: Comparison of *svep1* expression domains in the *svep1:GalFF; UAS:GFP* transgenic line with whole mount *in situ* hybridization of non-transgenic embryos. *Polydom/svep1* expression at 48hpf (F,G,H,I,J) and 72hpf (A,B,C,D,E) of TL fish. A,B: Higher magnification of the ceratohyal underneath 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.



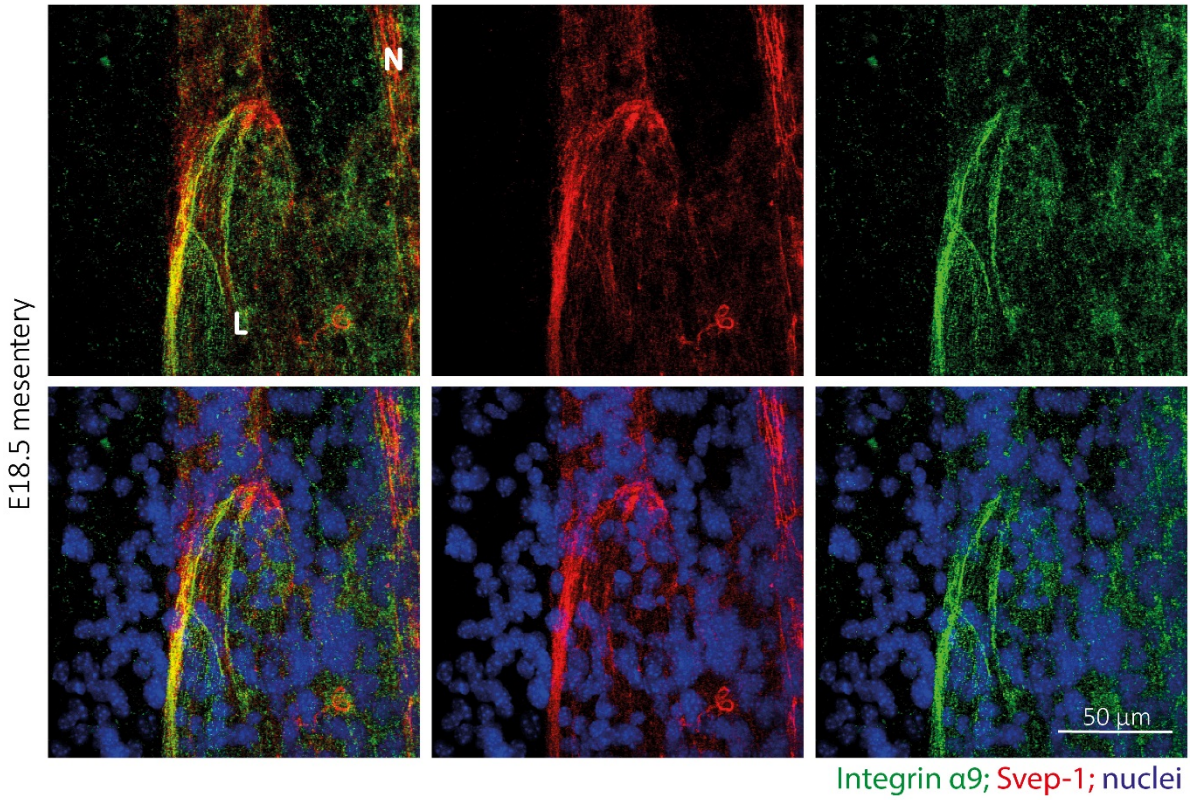
Online Figure V: *Polydom/Svep1* is expressed by non-endothelial cells in the region of the CV and of the pTD. A: Left: Transverse paraffin section of an E12.5 *Svep1*$^{+/LacZ}$ embryo stained with anti-LYVE-1 to highlight the lymphatic structure. LacZ expression, which is visualized by β-galactosidase, 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.



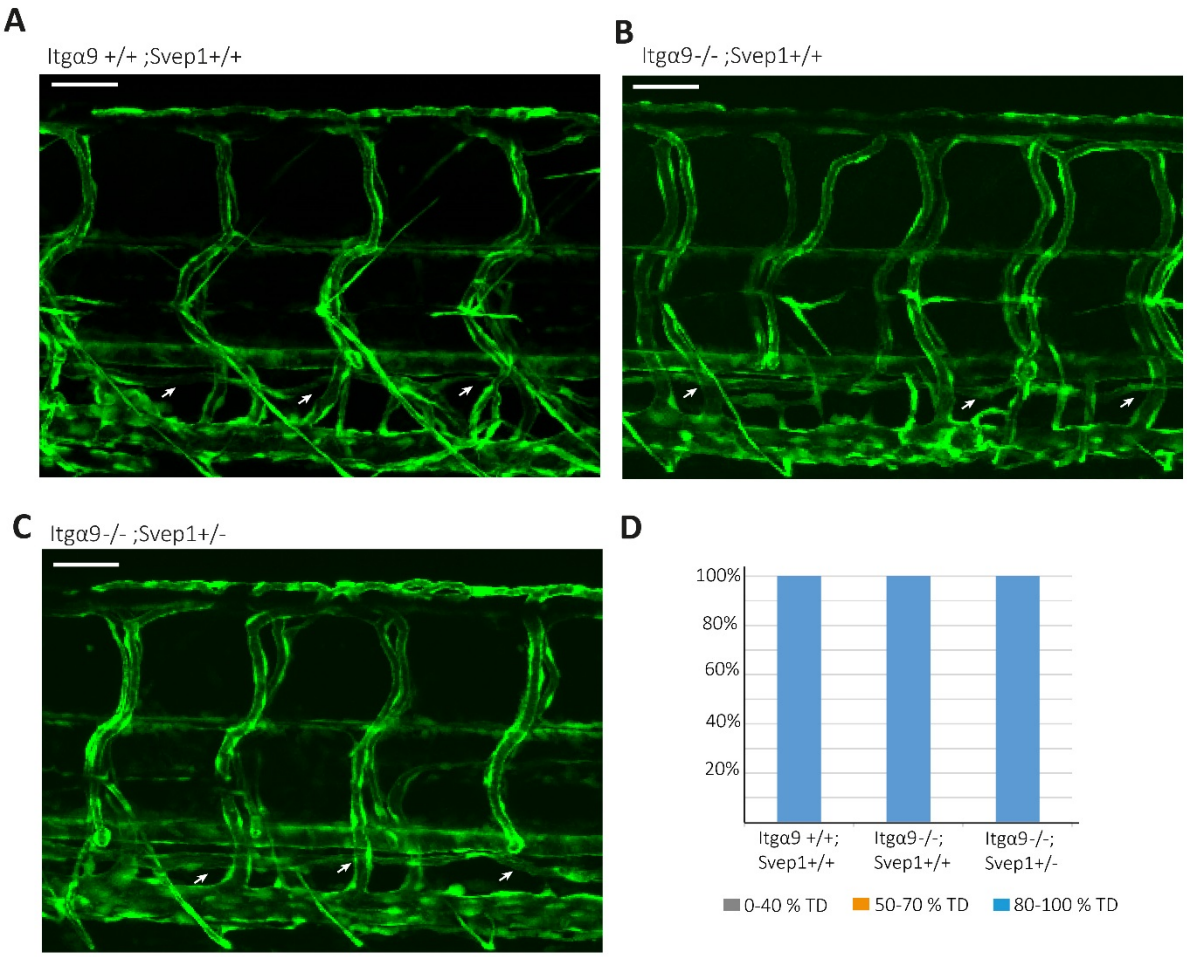
Online Figure VI: The lymphatic structures are dramatically decreased in size in Polydom/Svep1 deficient embryos at E14.5: Left: Polydom/Svep1 deficient embryos developed edema at the back. Middle: Hematoxylin staining on transverse paraffin sections of heterozygous and mutant Polydom/Svep1 embryos. 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.



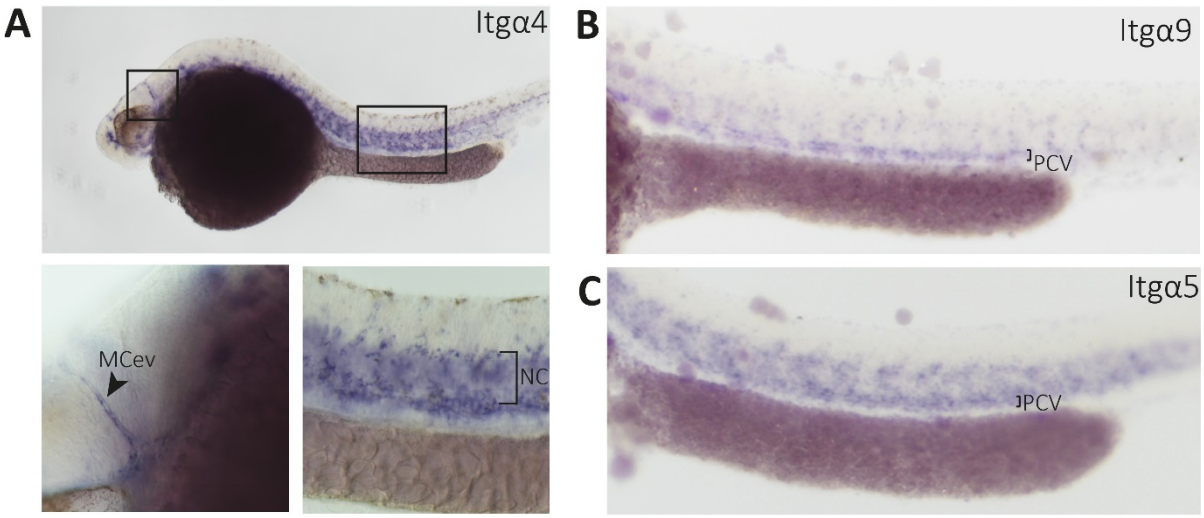
Online Figure VII: Variation of the phenotype in Polydom/Svep1 deficient embryos at E18.5. Despite of the fact that the severity of the edema varies considerably in Polydom/Svep1 deficient embryos, mutants were distinguishable from the heterozygous and wildtype siblings in all cases. While some *Polydom/Svep1* 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 Itgα9: 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.

Online References

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