A truncation allele in vascular endothelial growth factor c reveals distinct modes of signaling during lymphatic and vascular development

Jacques A. Villerfanc1, Stefania Nicoli1-*, Katie Bentley2‡, Michael Jeltsch3, Georgia Zarkada3, John C. Moore1, Holger Gerhardt2, Kari Alitalo3 and Nathan D. Lawson1§

SUMMARY
Vascular endothelial growth factor C (Vegfc) is a secreted protein that guides lymphatic development in vertebrate embryos. However, its role during developmental angiogenesis is not well characterized. Here, we identify a mutation in zebrafish vegfc that severely affects lymphatic development and leads to angiogenesis defects on sensitized genetic backgrounds. The um18 mutation prematurely truncated Vegfc, blocking its secretion and paracrine activity but not its ability to activate its receptor Flt4. When expressed in endothelial cells, vegfcum18 could not rescue lymphatic defects in mutant embryos, but induced ectopic blood vessel branching. Furthermore, vegfc-deficient endothelial cells did not efficiently contribute to tip cell positions in developing sprouts. Computational modeling together with assessment of endothelial cell dynamics by time-lapse analysis suggested that an autocrine Vegfc/Flt4 loop plays an important role in migratory persistence and filopodia stability during sprouting. Our results suggest that Vegfc acts in two distinct modes during development: as a paracrine factor secreted from arteries to guide closely associated lymphatic vasculature and as an autocrine factor to drive migratory persistence during angiogenesis.

KEY WORDS: Vegfc, Angiogenesis, Lymphatic, Zebrafish

INTRODUCTION
Evolutionarily conserved transport systems comprise blood and lymphatic vessels that are lined by distinct endothelial cell types. Blood vessels allow circulation of blood cells, gas exchange and delivery of metabolic products to and from tissues. The lymphatic system consists of blind-ended vessels that return fluid and macromolecules from interstitial tissue space back into blood circulation, while serving as a conduit for the development and transport of lymphoid cells (Tammela and Alitalo, 2010). During embryogenesis, blood vessels arise from endothelial cell progenitors that coalesce and differentiate into arteries and veins (Cleaver and Krieg, 2010). Subsequently, new blood vessels sprout from pre-existing ones by angiogenesis (Poole and Coffin, 1989). Later, lymphatic progenitors are specified from endothelial cells in the embryonic cardinal vein (Srinivasan et al., 2007), which sprout to form lymphatic vessels.

Vascular endothelial growth factor C (Vegfc) is the central regulator of lymphatic development (Tammela and Alitalo, 2010). Vegfc is expressed as a pro-peptide and cleaved to yield a secreted factor that acts on lymphatic endothelial cells (Joukov et al., 1997). Ectopic vegfc expression can cause lymphatic hyperplasia (Jeltsch et al., 1997) and mice or zebrafish lacking vegfc fail to develop lymphatic vessels and exhibit a block in primary sprouting of lymphatic progenitors from the cardinal vein (Karkkainen et al., 2004; Küchler et al., 2006; Yaniv et al., 2006). Vegfc functions primarily through binding and activation of the Vegfr3 receptor tyrosine kinase (Joukov et al., 1996) (hereafter referred to as Flt4), which is specifically expressed in both blood vascular and lymphatic endothelial cells (Kaipainen et al., 1995; Kukk et al., 1996). While Vegfc can also activate Vegfr2, a Flt4-specific form of Vegfc can induce lymphatic hyperplasia (Veikkola et al., 2001). Vegfc and its receptor are also important for lymphatic homeostasis in adults. Notably, mutations in human FLT4 that reduce kinase activity cause congenital lymphedema (Karkkainen et al., 2000). Thus, Vegfc/Flt4 signaling is essential for lymphatic development and homeostasis in vertebrates.

In addition to their roles in lymphatic development, Vegfc and Flt4 have been implicated in angiogenesis. In the circulatory system, flt4 is expressed in actively sprouting endothelial cells, where it becomes restricted to tip cells (Siekmann and Lawson, 2007; Tammela et al., 2008) and loss of flt4 in mouse or zebrafish causes defects in angiogenesis (Covassin et al., 2006; Dumont et al., 1998; Tammela et al., 2011; Tammela et al., 2008). Interestingly, flt4 is a central target of repression by the Notch pathway during angiogenesis. Notch activation downregulates flt4 expression (Lawson et al., 2001), whereas Notch deficiency causes increased and ectopic flt4 expression (Siekmann and Lawson, 2007; Tammela et al., 2008). Furthermore, reduced flt4 signaling attenuates the hyperangiogenic phenotype associated with loss of Notch (Benedito et al., 2012; Siekmann and Lawson, 2007). However, the role of vegfc during angiogenesis is less clear. Although transgenic overexpression of Vegfc in mice can induce lymphatic hyperplasia (Jeltsch et al., 1997), it also induced angiogenesis, which was restricted to embryonic blood vessels that expressed Flt4 and was caused by overexpressing Vegfc in endothelial cells themselves (Lohela et al., 2008). Furthermore,
vegfc is normally expressed in endothelial cells during development (Covassin et al., 2006; Tammela et al., 2008), suggesting that a Vegfc/Flt4 autocrine loop might be important for angiogenesis.

Here, we describe a zebrafish mutant, um18, that fails to develop lymphatic vessels and displays mild angiogenesis defects on a sensitized genetic background. The um18 mutation caused premature truncation of zebrafish Vegfc, which hindered its secretion but not its ability to activate Flt4. Vegfcum18 could not rescue lymphatic formation, but caused ectopic blood vessel branching when expressed in endothelial cells. Additionally, vegfc-deficient cells displayed autonomous defects in tip cell potential, suggesting an important autocrine role in angiogenesis. Computational modeling and time-lapse analysis of angiogenesis in Vegfc-deficient embryos suggested an important role in maintaining filopodia stability and migratory persistence. Together, our results suggest that Vegfc signals via distinct autocrine and paracrine mechanisms to pattern blood and lymphatic vessels, respectively.

MATERIALS AND METHODS
Zebrafish handling and maintenance
Zebrafish were maintained in accordance with approved University of Massachusetts Medical School IACUC protocols. Tg(fli1a:egfp); kdb; Tg(kdr:rasmcherry) embryos lines have been described elsewhere (Covassin et al., 2006; Herbert et al., 2009; Lawson and Weinstein, 2002).

Antibodies
A glutathione-S-transferase (GST)-fusion protein containing the first 225 amino acids of zebrafish Flt1b was expressed in BL21 E. coli, purified on glutathione-Sepharose beads (GE Healthcare) and cleaved from the GST using PreScission Protease (GE Healthcare). Flt4 peptide (NH2-CASDELERFEHKHRGA; New England Peptide, Gardner, MA, USA) or Flt1b protein was used to raise rabbit polyclonal antisera (Capralogics, Hardwick, MA, USA).

Identification and cloning of um18
N-ethyl-N-nitrosourea (ENU) mutagenesis and haploid screening were performed as described previously (Covassin et al., 2009), except that embryos were screened for loss of primordial hindbrain channel (PHBC). F1 females were outcrossed to TL males and progeny from individual F2 embryos were screened for loss of primordial hindbrain channel (PHBC). F1 females were outcrossed to TL males and progeny from individual F2 embryos were screened for loss of primordial hindbrain channel (PHBC). F1 females were outcrossed to TL males and progeny from individual F2 embryos were screened for loss of primordial hindbrain channel (PHBC). F1 females were outcrossed to TL males and progeny from individual F2 embryos were screened for loss of primordial hindbrain channel (PHBC). F1 females were outcrossed to TL males and progeny from individual F2 embryos were screened for loss of primordial hindbrain channel (PHBC). F1 females were outcrossed to TL males and progeny from individual F2 embryos were screened for loss of primordial hindbrain channel (PHBC).

Phenotypic analysis
General morphology and circulation were assessed using transmitted light on an MZ FLIII stereomicroscope (Leica). Images were recorded on an AxioCam MRc digital camera (Zeiss). Vascular morphology in Tg(fli1a:egfp); kdb embryos was imaged using a Leica DMI2000 confocal microscope (HC PL APO 20×/0.70 CS objective). Microangiography was performed as described previously (Isogai et al., 2001). Intersomitic vessel (ISV) connections were scored as arterial or venous based on flow direction and counted from the fifth to the last somite at 72 hpf. ISV length was determined in live or fixed embryos subjected to GFP immunostaining as described (Covassin et al., 2006). To quantify endothelial nuclei, embryos were fixed in 2% paraformaldehyde overnight at 4°C and permeabilized with 0.5% Triton X-100 in PBS for 30 minutes. Samples were incubated in blocking solution (PBS containing 0.1% Triton X-100, 1% normal goat serum, 1% BSA, 0.01% sodium azide), followed by Flt1b antisera at 1:1000 in blocking solution. Embryos were washed four times for 15 minutes each, followed by an overnight wash at 4°C and staining with goat anti-rabbit Alexa-Fluor 568 antibody (Molecular Probes) at 1:1000 in blocking solution. We counted nuclei in three adjacent ISVs and dorsal longitudinal anastomotic vessels in embryos injected with 15 ng control MO or Dll4 MO (Siekmann and Lawson, 2007). Ectopic branching points were counted for the same ISVs.

Analysis of Vegfc knockout mouse embryos
Mouse studies were approved by the Committee for Animal Experiments of the District of Southern Finland. Vegfc+/lacZ mice are described elsewhere (Karkkainen et al., 2004). Embryos were staged according to Edinburgh Mouse Atlas criteria, harvested at E9.5, fixed in 4% paraformaldehyde, washed in PBS and processed for whole-mount staining as described previously (Tammela et al., 2011). Endomucin staining (sc-65495, Santa Cruz), followed by Alexa Fluor 488-conjugated anti-rat antibody (Molecular Probes), was used to visualize blood vessels in the embryos. Samples were mounted with Vectashield mounting medium (Vector Labs) containing 4,6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories) and analyzed with a confocal microscope (Zeiss LSM 510 Meta, 10× objective with NA 0.45). Three-dimensional projections were digitally reconstructed from confocal z-stacks. Branching points in cranial vessels and ISV length between somites 14 and 16 were manually counted using ImageJ software (NIH; version 1.43r). Statistical analysis was carried out with PASW Statistics 18.0 software (SPSS). For comparison of means a two-way ANOVA test followed by Tukey’s HSD post-hoc test were used. Data are presented as mean ± s.e.m. with statistical significance indicated.

Cloning and plasmid construction
Zebrafish vegfc, vegfcum18 and vegfcat middle entry clones (pMEvegfc, pMEvegfcum18, pMEvegfcat) lacking stop codons were constructed by Gateway cloning as described (Villefranc et al., 2007) (see supplementary material Table S1 for primer sequences). pMEvegfc, pMEvegfcum18, pMEvegfcat or pMEegfp3 (Villefranc et al., 2007) were used in LR reactions with pDestTo12p2A, p5Flieb and p3E-2Amcherry to yield pTo12flieb:vegfc-2Amcherry, pTo12flieb:vegfcum18-2Amcherry, pTo12flieb:vegfcat-2Amcherry and pTo12flieb:egfp-2Amcherry. To generate mammalian expression constructs, pMEvegfc or pMEvegfcum18 were used in an LR reaction with pCSDest2 (Villefranc et al., 2007) and p3E-2Amcherry to produce pCSEvegfc-2Amcherry and pCSEvegfcum18-2Amcherry, pMT-ExzVegfc-wt and pMT-ExzVegfcum18 were constructed by cloning a BamHI/Nol-cleaved PCR product of zebrafish vegfc into pMBiV-5His-C (Invitrogen), which was modified to include a hygromycin cassette and a frameshift mutation between the BiP signal peptide and the multiple cloning site such that the 5′-GAT-3′ from the BglII site codes for an aspartic acid residue. A human VEGFCum18 middle entry vector was constructed as above using the primers listed in supplementary material Table S1. pME-VEGFCum18 was LR cloned into pCSDest (Villefranc et al., 2007) to create pCS-VEGFCum18. To generate pCS-flt4-ha, we created pMEMfl4 lacking its endogenous stop codon (supplementary material Table S1). pMEMfl4 and p3E-HA were LR cloned into pCSDest2 to give pCS-flt4-ha (Villefranc et al., 2007).

Transient transgenic vegfc expression
We co-injected 25 pg pTo12 constructs into one-cell stage embryos with 25 pg tol2 transposase mRNA. To assay ISV defects, we injected wild-type Tg(fli1a:egfp); kdb embryos and counted ectopic Cherry-positive vessel branches in six hemisegments at 30 hpf. For rescue experiments, we injected embryos from an incross of Tg(fli1a:egfp); kdb; Tg(kdr:rasmcherry) carriers. Injected embryos with Cherry-positive vessels were scored for TD formation at 5 dpf and genotyped for um18.

Production of Vegfc-conditioned media
S2 cells were transfected with either pMT-ExzVegfc or pMT-ExzVegfcum18 and stable cell pools were created according to standard protocols. To produce protein, cells were induced with 1 mM CuSO4 and conditioned cell supernatant was harvested 3.5 days post-induction.
Yolk membrane angiogenesis assay

Four nanoliters of Vegfc-, Vegfcum18- or negative control-conditioned medium was injected into the perivitelline space of anesthetized 48-hpf Tg(fli1a:egfp)y1 embryos as previously (Nicoli et al., 2009). Ectopic subintestinal vessels (SIVs) were visualized by confocal microscopy and counted at 3.5 dpf. For xenografts, NIH3T3 cells were transfected with 1 μg pCSvegfc-2Amcherry or pCSvegfcum18-2Amcherry using Lipofectamine 2000 (Invitrogen), grown for 48 hours, and subjected to fluorescence-activated cell sorting (FACS) to isolate expressing cells. Approximately 6000 Cherry-positive cells were diluted in Matrigel (Sigma) and injected into the perivitelline space as above. Xenografts were imaged at 2 days post-engraftment by confocal microscopy. Imaris (Bitplane) was used to quantify the relative number of vegfc-expressing cells (red voxels) and vessels (green voxels) as described previously (Nicoli et al., 2010).

Analysis of VEGFC secretion

NIH3T3 cells were transfected with 1 μg pSport6CMV-VEGFC (Open Biosystems) or pCS-VEGFCum18 using Lipofectamine 2000. Medium was collected at 30 and 72 hours post-transfection and concentrated using centrifugal filter units (Ultragel 10K, Millipore) while cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% NP40, 0.1% SDS) containing protease inhibitors (Complete Mini, Roche). Blots were probed with a polyclonal human anti-VEGFC antibody (Joukov et al., 1996), stripped, and reprobed with a monoclonal anti-Tubulin antibody (kindly provided by Dr Michael Green, University of Massachusetts Medical School, Worcester, MA, USA).

Flt4 activation assay

NIH3T3 cells were transiently transfected with 1 μg pCS/flt4-HA. After 24 hours, cells were starved for 24 hours and stimulated for 7 minutes with zebrafish Vegfc-, Vegfcum18- or negative control-conditioned medium. Cells were lysed in RIPA buffer containing phosphatase and protease inhibitors and used for immunoprecipitation using zebrafish Flt4 polyclonal antibody. Immunoprecipitates were subjected to SDS-PAGE and western blotting. Blots were probed with a monoclonal antibody against phosphotyrosine (4G10 clone, Millipore), stripped, and reprobed with monoclonal anti-HA antibody (BABCO).

Mosaic analysis

Transplantations were performed as described (Covassin et al., 2009). Donors were injected with control (5 ng) or Vegfc MO (2.5 and 5 ng). Alternatively, cells were taken from embryos derived from an incross of Tg(fli1a:egfp)y1;vegfcum18 carriers and donors were genotyped following transplantation. At 30 hpf, the location of donor cells in host vessels was calculated and analyzed using a Fisher’s exact test as described previously (Nicoli et al., 2012).

Computational modeling

The memAgent-Spring model (MSM) (Bentley et al., 2008; Bentley et al., 2009) was used to investigate four possible autocrine effects of Vegfc-Flt4 signaling to identify the best match to experimental data. The MSM has previously been calibrated to match wild-type sprouting data; therefore, Vegfc/Flt4 signaling is encompassed in existing parameters. We modeled the loss of Vegfc by a reduction in an existing model parameter. For each mechanism simulated, a single parameter k was created to control the strength of this loss, under four different conditions as follows:

Type I – Vegfc directly affects Dll4 and migration. (a) Vegfc/Flt4 acts to improve signaling via Vegfr2 by a constant amount. For MO, k is a small fixed amount subtracted from the total local VEGF of a membrAgent. (b)
Vegfc/Flt4 signaling proportionally reduces Vegf2 signaling. For MO, $k$ is the proportion of the total local VEGF of a memAgent removed.

Type 2 – Vegfc indirectly affects Dll4 but directly affects migration. (a) Vegfc/Flt4 induces initiation of filopodia. For MO, $k$ is the probability of a filopodia extending in response to VEGF stimulation. (b) Vegfc/Flt4 promotes persistence and stability of filopodia. For MO, $k$ is the probability of a filopodium to randomly begin retracting.

In each case, $k$ was directly calibrated using a sensitivity analysis to match transplant data using the approach below (see supplementary material Table S2 for parameter values). If a matching parameter could be found, each mechanism was then tested in a simulation modeling the dorsal migration of a tip cell through the somites, as defined below. Type 1b and Type 2b matched the chimeric data, but only Type 2b was able to match the ISV extension data.

Modeling chimeric transplant data
To model transplant data, embryos were considered as a single simulation of a vessel comprising ten cells in a continuous gradient of Vegfa, linearly increasing in the $y$-axis. A simple uniform, random contribution of Vegfc-deficient cells to the vessel was capped at a maximum of 50% MO cells to control cells. Hence, some ‘embryos’ could have zero MO cells and a maximum of five in random positions. Only embryos that contained at least one MO cell were counted. The same method was used to quantify chimeric simulations as was used experimentally.

Modeling ISV tip cell extension
To simulate the ISV migration, a version of the model was implemented to focus on measuring the distance reached by a traveling tip cell. In this case, DL1–Notch signaling was switched off as the selection process was assumed to have occurred already and a tip cell selected. A linear, increasing gradient of Vegfa was created in a column of three-dimensional space, 10 μm wide in the center above the tip cell, reaching up for 100 μm. Concentration settings were as previously described (Bentley et al., 2009): 0.04 molecules of VEGF multiplied by the $y$-axis position in each grid site. This assumes that VEGF is not accessible in somites to the left or right of the column and thus the tip will not grow into somite space.

Time-lapse analysis
Tg(fli1a:egfp)y1 embryos were injected with 5 ng control or Vegfc MO and mounted in agarose at 16 hpf. Images of control and morphant embryos were captured in parallel on a LSM7 MP laser-scanning microscope (Zeiss) equipped with a Chameleon Ti:Sapphire pulsed laser (Coherent) every 5 minutes over 8 hours using ZEN 2009 software with the Multi-time macro (Zeiss). Time-lapse series were loaded into ImageJ to analyze ISV sprouting and filopodial extension/retraction. ISV length (from the base of the dorsal aorta to the leading edge of the ISV) and filopodia length were measured manually in each frame using ImageJ. We measured the lengths of at least ten filopodia from five separate embryos at 5-minute intervals for a 60 minute span. Filopodia measurements were made on ISVs of comparable length in control- and Vegfc MO-injected embryos. For ISV length, we limited analysis to ISVs that were between 3 and 6 μm within the first few movie frames. We normalized the starting time of each ISV sprout to their first detectable length and recorded length every 5 minutes for a total of 300 minutes. For ISV forward and retraction rates, we calculated the change in distance between successive time points from 0 to 30 μm in length for 16 ISVs from eight embryos (control MO) or 17 ISVs from six embryos (Vegfc MO). Negative (retraction) and positive (forward) values for each ISV were analyzed for frequency and averaged. Statistical significance was measured using a paired $t$-test (ISV length) or a Wilcoxon test (retraction/forward rates).

RESULTS
A truncated form of vegfc perturbs lymphatic development
A screen for zebrafish embryos lacking a primordial hindbrain channel (PHBC) (supplementary material Fig. S1) identified the um18 mutant, which is linked to the vegfc locus (Fig. 1A). Analysis of vegfc coding sequence in um18 mutants revealed a CAA (Gln202) to TAA (stop) transition (Fig. 1B) that truncates Vegfc 13 amino acid residues upstream of a conserved cleavage site recognized by the proprotein convertase furin (Fig. 1C) (Joukov et al., 1997; Siegfried et al., 2003). Despite the presence of a premature stop codon, we did not observe a decrease in vegfc transcript levels in mutant embryos (data not shown). At 30 hpf, wild-type and mutant siblings were generally indistinguishable (Fig. 1D,E, left). vegfcum18 mutants displayed a partially penetrant defect in PHBC formation (Fig. 1D,E, middle; supplementary material Table S3), which recovered by 40 hpf (data not shown), and fully penetrant defects in lymphatic development. Both heterozygous and homozygous vegfcum18 mutant embryos failed to form a thoracic duct (TD), which is the first lymphatic vessel in zebrafish embryos, at 5 dpf (Fig. 1D,E, right; supplementary material Table S3, Fig. S2). We detected vegfcum18 carriers at expected frequencies in adults, suggesting that they could recover from this defect (data not shown). We also noted loss of parachordal lymphangioblasts, which give rise to the TD (Küchler et al., 2006; Yaniv et al., 2006), at 2 dpf in vegfcum18 mutant and heterozygous embryos (supplementary material Fig. S3A-E). In addition, vegfcum18 mutants had fewer intersomitic vein connections than wild-type siblings (supplementary material Fig. S3F-H and Table S4), suggesting an early defect in venous sprouting of lymphatic progenitors.

Fig. 2. vegfcum18 enhances angiogenesis defects in kdrly17 mutant embryos. (A–D) Confocal micrographs of trunk blood vessels in Tg(fli1a:egfp)y1 (A) wild-type, (B) vegfcum18 mutant, (C) kdrly17 mutant and (D) kdrly17/vegfcum18 double-mutant zebrafish embryos at 30 hpf. Arrows indicate intersomitic vessels (ISVs). Anterior is to the left, dorsal is up. (E) ISV length in embryos of the indicated genotype at 30 hpf. Values are the average from two clutches of embryos. *P<0.05; N.S., not significant; error bars indicate s.e.m. DA, dorsal aorta; PCV, posterior cardinal vein. Scale bar: 50 μm.
To confirm that defects in um18 mutants were due to loss of vegfc, we performed rescue experiments. Since vegfc is normally expressed in the dorsal aorta (Covassin et al., 2006), we drove transgenic endothelial expression using an enhancer from the fli1a gene (Villefranc et al., 2007). To visualize expressing cells, vegfc coding sequences were fused to mcherry separated by a viral 2A peptide sequence in order to express both proteins (Provost et al., 2007). Mutant embryos expressing egfp-2Amcherry or vegfcum18-2Amcherry in arterial endothelial cells failed to form a TD (Fig. 1F,G,I). By contrast, vegfc-2Amcherry rescued TD formation in vegfcum18 mutants (Fig. 1H,I). Interestingly, a furin-resistant form of Vegfc (Vegfcum18), which prevents its cleavage and retains the C-terminus, can partially rescue lymphatic formation in vegfcum18 mutants (supplementary material Fig. S4A,B). Together, these observations suggest that the C-terminal domain of Vegfc is essential for lymphatic development.

The vegfcum18 mutation has a weak effect on angiogenesis

Morpholino (MO)-mediated knockdown of zebrafish Vegfc or Flt4 causes a mild, partially penetrant block in intersomitic vessel (ISV) formation (Covassin et al., 2006). Furthermore, in Vegfc-deficient mouse embryos at E9.5, we observed decreased branching of cranial vessels as well a shortening of ISVs (supplementary material Fig. S5A,B), similar to zebrafish embryos injected with vegfc MO (supplementary material Fig. SSC-F), confirming an important conserved role for vegfc in developmental angiogenesis. However, ISVs appeared normal in vegfcum18 mutants, suggesting that this truncated form of Vegfc might be sufficient for angiogenesis. To further determine the effect of this mutation on angiogenesis, we assessed ISV formation in vegfcum18 mutant embryos in which this process was mildly inhibited or enhanced.

Zebrafish embryos mutant for kdr<sup>y17</sup>, a zebrafish vegf receptor 2 ortholog, show variable ISV defects that are enhanced by reducing fli4 (Covassin et al., 2009; Covassin et al., 2006). We generated Tg(fli1a:egfp)<sup>y1</sup> zebrafish that were heterozygous for kdr<sup>y17</sup> and vegfcum18 and assessed ISV length in their progeny. In vegfc<sup>um18</sup> mutant embryos with wild-type kdr, ISV length was normal (Fig. 2A,B,E). However, we noted a slight and significant decrease in ISV length in kdr<sup>y17</sup>;vegfc<sup>um18</sup> double-heterozygous embryos that was further enhanced by loss of both copies of wild-type vegfc (Fig. 2E). ISVs were significantly shorter in kdr<sup>y17</sup<y17mutant embryos that were wild-type for the vegfc locus (Fig. 2C,E), with a progressive decrease in kdr<sup>y17</sup>;vegfc<sup>um18</sup> (Fig. 2E) and kdr<sup>y17</sup>y17;vegfc<sup>um18</sup>um18 double-mutant embryos (Fig. 2C-E). We also observed ISVs in vegfc<sup>um18</sup> embryos lacking the Notch ligand, delta-like 4 (dll4), which causes increased ISV endothelial cell number and branching (Leslie et al., 2007; Siekmann and Lawson, 2007) (Fig. 3A,B,G,H). These defects were similar in vegfc<sup>um18</sup> and heterozygous embryos lacking dll4 (Fig. 3C,D), although slightly less severe (Fig. 3G,H). Likewise, vegfc<sup>um18</sup> homozygous mutant embryos displayed milder increases in branching and no increase in cell number following dll4 knockdown when compared with wild-type siblings (Fig. 3B,E-H). Taken together, these observations suggest that the vegfc<sup>um18</sup> mutation has a mild effect on ISV formation that is evident on a sensitized genetic background.

Vegfc<sup>um18</sup> is not efficiently secreted

To investigate the effect of the um18 truncation on the Vegfc protein, we first determined whether Vegfcum18 could activate Flt4. We generated conditioned medium from Drosophila S2 cells expressing either Vegfc or Vegfcum18 fused to the BiP secretion signal to reduce possible effects of the C-terminal truncation on secretion. In cells expressing zebrafish Flt4, both Vegfc- and Vegfcum18-conditioned media induced receptor tyrosine phosphorylation to similar levels (Fig. 4A). Furthermore, injection of Vegfc- or Vegfcum18-conditioned medium into the perivitelline space of zebrafish embryos caused similar levels of ectopic subintestinal vessel (SIV) formation, whereas mock conditioned medium did not (Fig. 4B-E).

We next examined the effect of um18 on Vegfc secretion. Since an appropriate antibody against zebrafish Vegfc was not available, we used the human equivalent of Vegfc<sup>um18</sup> (VEGFCum18). In this case, constructs contained the native VEGFC secretion signal. We detected both unprocessed and processed forms of VEGFC in lysates and medium from transfected cells (Fig. 4F). By contrast, VEGFCum18 accumulated in cell lysates and was not in the medium at 30 hours post-transfection (Fig. 4F,G), although it was detectable by 72 hours (data not shown). We did not detect changes in protein stability caused by the loss of the C-terminal domain in these cases (supplementary material Fig. S6A,B). Interestingly, the furin-
resistant form of Vegfc that is capable of partially rescuing vegfcum18 lymphatic defects is secreted, despite its inability to be cleaved (supplementary material Fig. S4B,C). To further investigate the effect of the observed secretion defect in vivo, we assessed the ability of xenografted cells expressing zebrafish vegfcum18 to rescue lymphatic defects. At 2 days post-graft, cells expressing wild-type Vegfc-2Amcherry were highly vascularized whereas those expressing Vegfcum18-2Amcherry were not (Fig. 4H-J). Together, these observations suggest that the um18 mutation in Vegfc prevents its efficient secretion, but not its ability to activate Flt4.

An endothelial cell-autonomous role for Vegfc during angiogenesis

vegfc is expressed in the hypochord, which lies adjacent to the dorsal aorta, as well as in aortic endothelial cells themselves (Covassin et al., 2006). We further find that vegfc is expressed in ISV endothelial cells as they sprout from the aorta (supplementary material Fig. S6C), suggesting that vegfc might play an endothelial cell-autonomous role during angiogenesis. To investigate this possibility, we first determined the effect of Vegfc and Vegfcum18 on ISVs by overexpressing them in endothelial cells. Transient endothelial expression of either vegfc or vegfcum18 in ISVs induced ectopic ISV branching into the horizontal myoseptum, whereas egfp-2Amcherry did not (Fig. 5A-D). Although vegfcum18 elicited a more variable effect, this difference was not statistically significant (Fig. 5D). This effect is in contrast to the inability of vegfcum18 to rescue lymphatic development (see Fig. 1G,I).

We next determined the endothelial cell-autonomous effect of vegfc deficiency on ISV growth by performing mosaic analysis. We transplanted cells from donor Tg(fli1a:egfp)y1 embryos injected with 5 ng Vegfc or control MO, or derived from an incross of vegfcum18 heterozygous carriers, into Tg(kdrl:rasmcherry)s896 host embryos and assessed their positions in trunk blood vessels at 30 hpf. Control donor endothelial cells contributed to all trunk blood vessel positions with relatively equal frequency (Fig. 5E,G). By contrast, vegfc morphant cells displayed less frequent contribution to both the distal tip cell position and posterior cardinal vein than control morphant cells (Fig. 5F,G). Although we noted a similar trend in the behavior of vegfcum18 mutant cells, the differences were not statistically significant (Fig. 5H). Together, these observations demonstrate that vegfc acts in an endothelial cell-autonomous manner to promote ISV growth.

Vegfc is required for persistent forward migration during angiogenesis

To investigate cellular mechanisms that might be affected by a putative Vegfc/Flt4 autocrine loop, we incorporated these signals into a previously described agent-based computational model (Bentley et al., 2008; Bentley et al., 2009). We then simulated sprouting with Vegfc acting in two possible ways: (1) through a direct effect on Notch signaling and dll4 levels or (2) through a...
Fig. 5. Endothelial cell-autonomous effects of Vegfc on angiogenesis. (A-C) Confocal micrographs of trunk blood vessels in Tg(fli1a:egfp)y1 zebrafish embryos at 30 hpf. Anterior to the left, dorsal is up. Transgene expression (red fluorescence) is indicated in normal (arrow) or ectopically branched (arrowheads) ISVs. Embryos were co-injected with pTol2-transposase mRNA and (A) pTol2-fliebs:egfp-2ACherry, (B) pTol2-fliebs:vegfc-2ACherry or (C) pTol2-fliebs:vegfcum18-2ACherry. (D) Quantification of ectopic ISV branching in cells expressing the fliebs:egfp-2ACherry (n=12 embryos) or fliebs:vegfc-2ACherry (n=18 embryos) transgene. Values indicate the proportion of embryos with Cherry-positive ISVs displaying ectopic branching. (E,F) Confocal micrographs of trunk blood vessels at 30 hpf of Tg(kdr:rasmcherry)um1 embryos transplanted with Tg(fli1a:egfp)y1 cells (green) from embryos injected with 5 ng control (E) or Vegfc MO (F) at 5 ng. Arrow indicates a stalk cell. (G,H) The proportion of embryos displaying donor cells in the indicated trunk vessel position. (G) Donor cells from embryos injected with the indicated MO. (H) Donor cells were from embryos derived from an incross of vegfcum18 carriers. *P<0.05; N.S., not significant; error bars indicate s.e.m. Scale bars: 50 μm.

direct effect on the migratory apparatus, with indirect effects on Notch. Only the latter mechanism yielded an output similar to both the experimentally derived transplantation and ISV length data. Simulating a direct effect on Notch failed to recapitulate ISV growth defects, although it could mimic the transplantation data (Fig. 6A; supplementary material Movies 1, 2). Cells lacking vegfc also showed a moderately reduced ability to contribute to the tip cell position in competition simulations, consistent with mosaic analyses (supplementary material Table S2 and Movies 3, 4). These results suggest that a decrease in filopodia persistence might cause slower ISV growth rates in Vegfc-deficient embryos.

To test the simulation predictions, we performed two-photon time-lapse analysis of ISVs at 5-minute intervals in Tg(fli1a:egfp)y1 embryos injected with 5 ng control or Vegfc MO. We focused on the first few hours of ISV growth to eliminate secondary effects resulting from ISVs being at different positions relative to surrounding pro-angiogenic growth factors (e.g. Vegfa) in control and Vegfc-deficient embryos at later time points. This analysis revealed that ISVs grew more slowly in embryos injected with Vegfc MO, which is qualitatively consistent with growth curves generated by the simulations (Fig. 6A,B). We also noted a slight reduction in the average time of filopodia persistence in vegfc-deficient embryos (13.27 minutes in control MO-injected embryos versus 10.14 minutes in vegfc-deficient embryos; Fig. 6C) as predicted by the simulations, although this fell just under the cut-off for statistical significance (P=0.0501). We observed that early ISV growth was characterized by both forward migration and retraction back towards the dorsal aorta in control MO- and Vegfc MO-injected embryos (supplementary material Movies 5 and 6). Whereas the frequency of forward migration or retraction was unchanged (data not shown), the average magnitude of retraction, but not forward movement, was significantly greater in ISVs lacking vegfc compared with the control (Fig. 6D,E). Interestingly, we noted similar behaviors in simulations of growing tip cells during competition (compare supplementary material Movie 3 and Movie 4). Thus, vegfc appears to contribute to the persistent forward migration of ISVs and may also affect filopodial stability during angiogenesis.

DISCUSSION
Vegfc is an essential regulator of lymphatic vessel development (Hogan et al., 2009; Karkkainen et al., 2004; Tammela et al., 2008). However, less is known about its role in developmental angiogenesis or about the relative importance of distinct Vegfc domains for its function. Here, we find that vegfc is important for endothelial tip cell migration during angiogenesis, consistent with the proposed role of its receptor flt4 (Siekmann et al., 2008). Our observations further suggest that vegfc functions in an endothelial cell-autonomous manner in this regard. Indeed, vegfc is expressed in ISVs as they sprout from the dorsal aorta and mosaic analysis demonstrates that vegfc-deficient endothelial cells are less likely to contribute to the tip cell position in ISVs. These results are consistent with the ISV growth defects observed in rspo1 mutant zebrafish, which lack endothelial vegfc expression, and can be rescued with an endothelial cell-specific vegfc transgene (Gore et al., 2011). Interestingly, ISV tip cells in rspo1 mutant embryos displayed significantly fewer filopodia, consistent with our observation that Vegfc contributes to filopodia stability.

Co-expression of flt4 and vegfc in ISVs and the cell-autonomous requirement for vegfc suggests that a Vegfc/Flt4 autocrine loop is important to promote tip cell migration and angiogenesis (supplementary material Fig. S7B). Vascular endothelial growth factor A (Vegfa) can also act in an autocrine loop to promote endothelial cell survival (Lee et al., 2007). However, we did not observe overt indications of apoptosis in vegfc-deficient embryos
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model simulations is necessarily qualitative; as in most quantitative analyses, discrepancies are expected because the output from the model cannot be quantitatively validated. Although ISV growth defects following Vegfc knockdown were model-predicted effects on ISV growth by time-lapse analyses. Persistence. Accordingly, we observed a pathway in ISV growth, filopodia stability and directional persistence. This mechanism might also explain differences in ISV defects between vegfcum18 mutants and other examples of vegfc deficiency in zebrafish, which can more severely affect ISV sprouting (e.g. vegfc morphants or rspo1 mutant embryos). Although our results suggest that a Vegfc/Fli4 autocrine loop promotes angiogenesis, we cannot rule out possible paracrine roles. Indeed, macrophage-expressed Vegfc is thought to play a central role in postnatal mouse retinal angiogenesis (Tammela et al., 2011). Thus, further work using cell type-specific ablation of vegfc will be essential to better define the signaling modalities in each of these contexts.

The memAgent-Spring computational model has previously proved insightful for revealing the importance of a Vegf-Notch feedback loop in modulating endothelial tip and stalk cell behaviors (Bentley et al., 2008; Bentley et al., 2009). Simulations using this model correctly predicted that cells within a growing sprout would continuously compete for the tip cell position and that the ‘winner’ is determined by relative levels of Vegf and Notch (Jakobsson et al., 2010). However, these simulations had not previously incorporated Vegfc or its receptor. Our integration of a Vegfc/Fli4 autocrine loop into this model suggested an important role for this pathway in ISV growth, filopodia stability and directional persistence. Accordingly, we observed in vivo evidence for the model-predicted effects on ISV growth by time-lapse analyses. Although ISV growth defects following Vegfc knockdown were somewhat milder in vivo than those predicted by the model, these quantitative discrepancies are expected because the output from the model simulations is necessarily qualitative; as in most computational models, the input parameters are clearly limited to the known factors under investigation in this study. Furthermore, detailed quantitative values, such as the number of receptor molecules per cell or receptor/ligand binding affinities, are currently lacking and so could not be included in the model. Nevertheless, both the model and in vivo observations point toward an important role for Vegfc in maintaining filopodia stability and directional persistence during angiogenesis. Importantly, this mechanism might not otherwise have been identified without the use of the memAgent-Spring model and resulting predictions gained from its application.

Whereas angiogenesis is mildly affected by the um18 mutation, lymphatic development is severely blocked, with defects even in heterozygous embryos. These phenotypes are consistent with the inability of the mutant protein to be efficiently secreted, underscoring the function of Vegfc as a paracrine factor that directs lymphatic development. In mouse embryos, Vegfc from mesenchymal cells adjacent to the cardinal vein promotes the sprouting of lymphangioblasts (Karkkainen et al., 2004). In zebrafish, venous sprouts that will give rise to the lymphatic system likewise grow towards Vegfc, which is expressed in the endothelial cells of the dorsal aorta. Interestingly, the arterial vasculature in the zebrafish guides patterning of the developing lymphatic system (Bussmann et al., 2010). Given that vegfc is specifically expressed in arterial endothelial cells and that its secretion is essential for lymphatic development, our results support a model in which arteries guide the development of nascent lymph vessels, in part, through the paracrine action of Vegfc (supplementary material Fig. S7B).

The um18 mutation eliminates the Vegfc C-terminal domain, which contains tandemly repeated cysteine-rich motifs resembling those of the silk protein BR3P (Kukk et al., 1996). Domain swap studies have demonstrated that the C-terminal domain could potentiate angiogenic responses when fused to Vegfa (Keskitalo et al., 2007). Conversely, fusion of the Vegfc VEGF homology domain (VHD) to the Vegfa C-terminus limited its ability to induce lymphatic vessel growth when compared with wild-type Vegfc (Tammela et al., 2007). Whereas these studies suggested a possible structural role for the Vegfc C-terminal domain within the extracellular matrix, our work implicates this domain in secretion. Previous studies reported that the Vegfc VHD alone can be secreted from cells. Indeed, we observe that Vegfcum18 can eventually be...
found in the medium from transfected cells. However, there is a clear defect in secretion and a vegf<sup>mut18</sup> transgene is not capable of rescuing lymphatic development in zebrafish embryos. It is likely that eventual secretion in cell culture experiments is due to high levels of transgene expression driven by constitutively highly active promoters. Furthermore, many constructs from previous studies contain heterologous secretion signals that might mask the importance of the C-terminus. Thus, we believe that our results support an important role for the Vegf C-terminal domain in secretion, although the intervening amino acid sequences between the um18 truncation and the furin cleavage site might also be important. How these domains act to promote efficient transport through the secretory apparatus is currently unclear. Given the ease with which transient rescue experiments can be performed in the vegf<sup>mut</sup> mutants, it will be possible to further dissect the importance of this and other domains within Vegf for its function during lymphatic development.

Acknowledgements

We thank John Polli and Ed Jaskolski for excellent fish care; Julie Zhu for advice concerning statistical analyses; and Tom Smith and George Kourkoulis for technical assistance.

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This work was funded by National Institutes of Health grants [R01HL101374 and R01HL093467] from the National Heart, Lung, and Blood Institute to N.D.L. I.A.V. was supported by a Ruth L. Kirschstein pre-doctoral National Research Service Award to promote diversity in health-related research [SF31HL081927]. H.G. and K.B. are funded by Cancer Research UK, the Research Service Award to promote diversity in health-related research.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.084152/-/DC1

References


Fig. S1. Transgenic haploid screen in Tg(fli1a:egfp)y1 zebrafish to identify mutants affecting Vegfc/Flt4 signaling. (A) Tg(fli1a:egfp)y1 males were treated with N-ethyl-N-nitrosourea (ENU) to induce mutations in the pre-meiotic germline and subsequently outcrossed to wild-type Tg(fli1a:egfp)y1 females to establish F1 families. Eggs from individual F1 females were fertilized in vitro with UV-irradiated sperm from wild-type males to generate haploid embryos. (B) Epifluorescence images of cranial blood vessels in Tg(fli1a:egfp)y1 haploid embryos. (Left) Normal primordial hindbrain channel (PHBC) formation (arrows) at 28 hpf. (Right) Putative mutant embryo (‘putant’), which failed to form a PHBC (arrowheads denote location where PHBC normally forms).
Fig. S2. Decreased thoracic duct formation in \textit{vegfc}^{um18} heterozygous embryos. (A,B) Confocal microangiographs of trunk blood vessels in \textit{Tg(fli1a:egfp)y1} larvae at 5 dpf in progeny from a \textit{um18} carrier incross. Microangiography dye is pseudocolored red. (A) Normal TD formation in a wild-type embryo. (B) Loss of TD in \textit{vegfc}^{um18/+} heterozygous embryo. The normal location of the thoracic duct (TD) is indicated by arrows. Lateral view, dorsal is up and anterior to the left. DA, dorsal aorta; PCV, posterior cardinal vein. (C) Quantification for presence of TD in embryos of the indicated genotype. Scale bar: 25 μm.
Fig. S3. Vegfcum18 mutants display primary defects in lymphatic progenitor and vein sprouting. (A) Diagram describing lymphatic vessel formation in zebrafish. (1) Dorsal sprouting of parachordal lymphangioblasts (PLs) from the posterior cardinal vein (PCV) at 1.5-2 dpf. (2) Ventral migration of PLs along the horizontal myoseptum. (3) Ventral migration of PLs to a position between the dorsal aorta and posterior cardinal vein to form the TD. (B-D) Confocal microangiographs of trunk blood vessels in Tg(fli1a:egfp)y1 embryos at 2 dpf. Anterior is to the left, dorsal is up. White arrows indicate position where PLs normally form. (B) Wild-type embryo. (C) vegfcum18/+ heterozygous embryo. (D) vegfcum18 homozygous mutant embryo. (E) Quantification of PL formation. Values indicate the percentage of embryos of the indicated genotype that display either presence or absence of PLs. (F,G) Confocal microangiographs of trunk blood vessels at 3 dpf. Anterior is to the left, dorsal is up. DA, dorsal aorta; PCV, posterior cardinal vein; SegA, segmental artery; SegV, segmental vein. White arrowheads indicate SegA. (F) Wild-type sibling embryo. (G) Homozygous vegfcum18 mutant embryo. (H) Quantification of percentage artery and vein connections in wild-type and vegfcum18 mutant embryos. Mutants were identified as those lacking a PHBC at 30 hpf. At 3 dpf, embryos of both phenotypic classes were scored for the connection of intersegmental vessel to either DA or PCV based on blood flow. The values shown are based on the average of three independent experiments. Scale bar: 25 μm.
Fig. S4. A furin-resistant form of Vegfc (Vegfcss) is secreted and can partially rescue lymphatic development in vegfcum18 mutant embryos. (A) Vegfc structure showing location of the furin cleavage domain and the conserved amino acid sequences from zebrafish (Dr), human (Hs), mouse (Mm) and rat (Rn) Vegfc proteins. The amino acid substitutions to prevent furin cleavage are shown. (B) The proportion of embryos with indicated genotypes that displayed TD formation following injection of an endothelial driven egfp-2Acherry or vegfcss-2Acherry transgene. (C) Zebrafish Vegfc structure showing location of the hemagglutinin (HA) epitope tag at the C-terminus used to detect expression of furin-resistant Vegfc (Vegfcss). Western blot shows expression of wild-type zebrafish Vegfc or Vegfcss in cell lysates or extracellular media from transiently transfected NIH3T3 cells. In cell lysates, full-length Vegfc can be detected in both cases. Interestingly, we can detect a small amount of full-length wild-type Vegfc in the medium, along with the C-terminal domain that has been cleaved. Furthermore, we are able to detect furin-resistant Vegfcss in the medium, the vast majority of which remains unprocessed.

HSII RR > HSII SS = Vegfc ss
Fig. S5. Developmental angiogenesis defects in vegfc-deficient mouse and zebrafish embryos. (A,B) Confocal micrographs of mouse embryos of the indicated genotype at E9.5 stained with endomucin to visualize endothelial cells. (A) Cranial vessels (left) and quantification of branching points (right). (B) ISVs (left) and quantification of added ISV length, between somites 14-16 (right). n=3. (C-F) Confocal micrographs of Tg(fli1a:egfp)y1 wild-type zebrafish embryos at 30 hpf injected with (C) 5 ng control, (D) 2.5 ng Vegfc or (E) 5 ng Vegfc MO. Lateral views, dorsal is up, anterior to the left. (F) Quantification of ISV length in MO-injected Tg(fli1a:egfp)y1 embryos. *P<0.05; N.S., not significant. Scale bars: 100 μm in A,B; 25 μm in C-E.
**Fig. S6. um18 does not affect VEGFC stability.** (A) Visualization immunoprecipitated from VEGFC or VEGFC\textsuperscript{um18} from transfected HEK293T cell lysates following a 2-hour pulse with radiolabeled Met and Cys, and chase with non-radiolabeled amino acids for the indicated time. Arrows indicate wild type (left) and truncated (right) VEGFC. (B) Western analysis of HEK293T cells expressing either VEGFC or VEGFC\textsuperscript{um18} and treated with cycloheximide (CHX). Cells grown in 6-well plates were transfected with 360 ng pSport6hVEGFC or pCSVEGC\textsuperscript{um18} and individual wells treated for 3 or 6 hours with cycloheximide (Sigma; 50 μg/ml final concentration). (C) \textit{vegfc} expression in the dorsal aorta (DA) and intersegmental vessels (ISVs) at 24 hpf as detected by whole-mount \textit{in situ} hybridization using a DIG-labeled antisense riboprobe. Lateral view, anterior to the left, dorsal is up. Scale bar: 25 μm.
Fig. S7. Simulating a direct effect on Notch and a model for Vegfc function during vascular development. (A) ISV growth in simulations where Vegfc was predicted to have a direct effect on Notch signaling (Type 1 mechanism, see Materials and methods). (B) Model of autocrine and paracrine signaling roles for Vegfc during ISV sprouting and venous sprouting, respectively. In vegfcum18 mutants, a block in secretion prevents Vegfc from acting as a paracrine factor to promote sprouting of lymphatic progenitors from the posterior cardinal vein. The Vegfc/Flt4 autocrine loop required for ISV sprouting would likewise be affected. However, compensatory intracrine activation of Flt4 would allow normal ISV sprouting to proceed.
Movie 1. Simulation of a control tip cell migrating in a simulated ISV environment.

Movie 2. Simulation of a Vegfc MO (5 ng) tip cell migrating in a simulated ISV environment with settings that match mechanism Type 2b, which affects filopodia persistence.

Movie 3. Simulation of a wild-type vessel comprising seven cells with randomized veil advance. Color represents Vegfr2 receptor levels: pink, high; purple, low.
Movie 4. Simulation of a vessel with Vegfc MO (5 ng) using the matching mechanism for Vegfc (which increases filopodia persistence). Tip cells are selected more slowly as the cells randomly pull back migratory protrusions under the MO, affecting their position in the VEGF gradient and thus their ability to inhibit their neighbors consistently. This simulation predicts that tip cells would be seen to extend and then retract a number of times until a clear selection is made. Color represents Vegfr2 receptor levels: pink, high; purple, low.

Movie 5. Example of intersegmental vessel sprouting in a *Tg(fli1a:egfp)* embryo injected with 5 ng control MO. Images were acquired by two-photon microscopy.

Movie 6. Example of intersegmental vessel sprouting in a *Tg(fli1a:egfp)* embryo injected with 5 ng Vegfc MO. Images were acquired by two-photon microscopy.
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Table S2. Calibrated parameters for computational simulation of ISV sprouting

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Calibrated parameters for k in each of the four simulated autocrine mechanisms of Vegfc/Flt4 action giving matching results to experimental transplant data in a simulated chimera. Dashes indicate cases where no parameter could be found to closely match the experimental data.
Table S3. Vein and lymphatic vessel scoring in \textit{vegfc^{um18}} embryos

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Primordial hindbrain channel (PHBC) and thoracic duct (TD) formation in progeny of \textit{um18} heterozygous carriers. Embryos were scored at 30 hpf for the presence of the PHBC and subsequently scored at 5 dpf for the presence of a TD. Embryos were subsequently genotyped for the \textit{um18} mutation. \textit{N}, total number of embryos scored. PL, parachordal lymphangioblast.
Secondary intersegmental vein formation in progeny of um18 heterozygous carriers. Embryos were scored at 60 hpf for the presence or absence of intersegmental veins. Embryos were subsequently genotyped for the um18 mutation. ISVe, intersegmental vein.

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Table S4. Intersomitic vein formation in vegfc<sup>um18</sup> embryos