

Vegfc/Flt4 signalling is suppressed by Dll4 in developing zebrafish intersegmental arteries

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The development of arteries, veins and lymphatics from pre-existing vessels are intimately linked processes controlled by a number of well-studied reiteratively acting signalling pathways. To delineate the mechanisms governing vessel formation in vivo, we performed a forward genetic screen in zebrafish and isolated the mutant *expando*. Molecular characterisation revealed a loss-of-function mutation in the highly conserved kinase insert region of *flt4*. Consistent with previous reports, *flt4* mutants were deficient in lymphatic vascular development. Recent studies have demonstrated a role for Flt4 in blood vessels and showed that Dll4 limits angiogenic potential by limiting Flt4 function in developing blood vessels. We found that arterial angiogenesis proceeded normally, yet the *dll4* loss-of-function arterial hyperbranching phenotype was rescued, in *flt4* signalling mutants. Furthermore, we found that the Flt4 ligand Vegfc drives arterial hyperbranching in the absence of *dll4*. Upon knockdown of *dll4*, intersegmental arteries were sensitised to increased *vegfc* levels and the overexpression of *dll4* inhibited Vegfc/Flt4-dependent angiogenesis events. Taken together, these data demonstrate that *dll4* functions to suppress the ability of developing intersegmental arteries to respond to Vegfc-driven Flt4 signalling in zebrafish. We propose that this mechanism contributes to the differential response of developing arteries and veins to a constant source of Vegfc present in the embryo during angiogenesis.

KEY WORDS: Dll4, Flt4, Vegfc, Arterial, Lymphatics, Zebrafish

INTRODUCTION

During developmental angiogenesis, arteries, veins and lymphatics arise from pre-existing vessels by carefully regulated processes of sprouting, migration, proliferation and patterning of endothelial cells, collectively termed haemangiogenesis (for arteries and veins) or lymphangiogenesis (for lymphatic vessels). In the zebrafish embryonic trunk, developmental angiogenesis takes place in two distinct stages: primary sprouts form from the dorsal aorta to give rise to intersegmental vessels from ~22 hours post-fertilisation (hpf), and then secondary sprouts form from the posterior cardinal vein (PCV) to give rise to intersegmental veins and lymphatic vascular precursors from ~32 hpf (Isogai et al., 2003; Yaniv et al., 2006). Although primary sprouts will contribute to both functional arteries and veins after the later remodelling of the trunk vasculature (Isogai et al., 2003), primary sprouting is considered an arterial angiogenic process as sprouts derive exclusively from the dorsal aorta and express arterial markers (Siekman and Lawson, 2007), whereas secondary sprouting from the PCV is a venous angiogenic process. These processes are regulated by vascular endothelial growth factors and their receptors. The primary sprouting of intersegmental arteries (ISAs) is dependent on Vegfa, Kdr and Kdr-1 (Bahary et al., 2007; Bussmann et al., 2008; Covassin et al., 2009; Covassin et al., 2006;

Habeck et al., 2002) and the growth of ISAs has been reported to stall transiently upon morpholino (MO) knockdown of Flt4 or Vegfc (Covassin et al., 2006). *flt4* and *vegfc* are essential for lymphangiogenesis in the zebrafish trunk (Kuchler et al., 2006; Yaniv et al., 2006) and *vegfc* is required at the level of venous angiogenic sprouting (Hogan et al., 2009).

Mouse mutants or transgenic mice deficient for the ligand Vegfc, or for signalling by its receptor Flt4, lack lymphatic vessels and consequently display lymphedema (Karkkainen et al., 2004; Makinen et al., 2001; Veikkola et al., 2001). Vegfc-deficient mice lack lymphatic vessels owing to a block in lymphangiogenesis that occurs at the level of budding of lymphangioblasts from venous endothelium, the earliest event in lymphatic vascular differentiation (Karkkainen et al., 2004). Consistent with the established functions of these factors during development, Vegfc and Flt4 drive tumour lymphangiogenesis (Karpanen et al., 2001), their antagonists inhibit tumour metastasis (He et al., 2002) and *FLT4* mutations lead to primary (inherited) lymphedema in humans (Karkkainen et al., 2000).

A recent study has demonstrated a role for Flt4 in haemangiogenesis (Tammela et al., 2008). The inhibition of Flt4 reduced angiogenic sprouting of blood vessels during retinal or tumour-associated angiogenesis (Tammela et al., 2008). Consistent with this role, Flt4 is expressed in intersegmental vessels in the trunk of the developing mouse embryo and in the vasculature of the retina during angiogenesis, with enhanced expression in sprouting tip cells (Tammela et al., 2008). Notch signalling generally acts to robustly repress the angiogenic potential of endothelial cells during development and disease (Hellstrom et al., 2007; Noguera-Troise et al., 2006; Siekmann and Lawson, 2007; Suchting et al., 2007). Consistent with this model, Dll4 inhibition leads to dramatically increased angiogenesis that produces morphologically abnormal and non-functional blood vessels (Leslie et al., 2007; Noguera-Troise et al., 2006; Siekmann and Lawson, 2007). During retinal angiogenesis, Dll4-induced signalling suppresses Flt4 expression in

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endothelial tip cells, but when Dll4 or Notch signalling is inhibited, upregulated Flt4 activity drives ectopic sprouting and increased angiogenesis (Tammela et al., 2008).

During arterial angiogenesis in zebrafish, increased vessel sprouting induced by the loss of the Notch target transcription factor Rbpsh (Rbpja – Zebrafish Information Network) can be suppressed, at least in part, by the depletion of Flt4 using MOs, indicating that there is likely to be a conserved relationship between Notch signalling and Flt4 function in zebrafish arteries (Siekmann and Lawson, 2007). During both arterial and venous angiogenesis, there is a constant source of *vegfc* in the zebrafish trunk, expressed first in the hypochord and then in the dorsal aorta (Covassin et al., 2006). Zebrafish *flt4* expression is found at high levels in venous endothelial cells during both arterial and venous angiogenesis, but has also recently been identified in arterial cells (Covassin et al., 2009; Siekmann and Lawson, 2007). The presence of a constant source of ligand in the developing embryo, as well as receptor expression in sprouting endothelial cells during both arterial and venous angiogenesis, raises the question of how developing arteries and veins are programmed to differentially respond to Vegfc/Flt4 signalling and what role Notch signalling might play.

Here, we report the identification of a Flt4 signalling-deficient zebrafish mutant and investigate the relationship between Dll4 function in arteries and Vegfc/Flt4 signalling in the developing vasculature. As with *vegfc*, *flt4* is required for all venous-derived angiogenesis in the zebrafish trunk, both in lymphangiogenesis and for the formation of intersegmental venous sprouts. Surprisingly, arterial angiogenesis is normal in zebrafish *flt4* signalling-deficient mutants, and we show that *dll4* suppresses Vegfc/Flt4 signalling in zebrafish arteries in order to allow for normal ISA development. Analysis of the expression of *vegfc* and *flt4* in *dll4*-deficient arteries indicates that this suppression is not due to any observable changes in ligand or receptor transcription. We propose that this role of *dll4* contributes to the differential response of arteries and veins to a constant source of Vegfc in the trunk of the embryo during developmental angiogenesis.

MATERIALS AND METHODS

Zebrafish

All zebrafish strains were maintained in the Hubrecht Institute under standard husbandry conditions. Animal experiments were performed in accordance with the rules of the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences (DEC). The published transgenic lines used were *TG(fli1a:gfp)^{y1}* (Lawson and Weinstein, 2002), *TG(fli1:YFP)^{hu4624}* (Hogan et al., 2009) and *TG(kdr-l:Cherry)^{s916}* (Hogan et al., 2009). Embryos genotyped in Fig. 4 were of the *TG(fli1a:gfp)^{y1}* strain.

ENU mutagenesis was performed as previously described (Wienholds et al., 2002). The F1 progeny of mutagenised males were crossed to the *TG(fli1a:gfp)* strain to produce transgenic F2 families. Subsequent in-crossing of F2 progeny generated F3 embryos that were screened for the presence or absence of the thoracic duct.

Positional cloning of *expando*

The *flt4^{hu4602}* mutation was mapped using standard meiotic mapping with simple sequence length polymorphisms (SSLPs). The primers used for SSLP markers depicted in Fig. 2 were: z43267, 5'-CAAAGGGTGC-AAAGTGCATT-3' and 5'-AGCAAAGTGCTGGTGATCA-3'; z26376, 5'-CCTGCTAACCTGCGTTCTTC-3' and 5'-CGTTAGCATATGCGCACTCT-3'; z11725, 5'-GCTTCCAGAAGTGGAGCAAC-3' and 5'-TGA-TTTTGATTGGGGACCAT-3'. Subsequent genotyping and SNP analyses were performed by sequencing using the primer pair 5'-AGCTCTTG-ATTTGGCTTAG-3' and 5'-GAAAGTATCCTTGCTCTGC-3'. Bioinformatic construction of the genomic region was performed using the Ensembl database (<http://www.ensembl.org>), release 44, April 2007.

FLT4 kinase assay

Full-length human wild-type FLT4 and I1034S mutant FLT4 constructs were cloned into the pMXs vector (a generous gift from Dr Toshio Kitamura, University of Tokyo, Japan) and transfected into HEK 293T cells (American Type Culture Collection) grown in DMEM supplemented with 10% foetal bovine serum (FBS) (PromoCell), glutamine and antibiotics. For immunoprecipitation and immunoblotting analysis, confluent plates of the cells were serum-starved for 2 hours, stimulated with VEGF-C (100 ng/ml) and lysed in lysis buffer (50 mM HEPES pH 7.5, 1% Triton X-100, 5% glycerol, 1 mM EGTA, 150 mM NaCl, 1.5 mM MgCl₂, 100 mM NaF, 1 mM each Na₃VO₄, PMSF, aprotinin and leupeptin) (Saharinen et al., 1997). Equal amounts of protein in the lysates were used for immunoprecipitation using mouse monoclonal anti-human FLT4 antibodies (9D9) (Petrova et al., 2008). The immunocomplexes were captured using protein-G sepharose (GE Healthcare Bio-Sciences, Uppsala, Sweden), separated by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane and detected using monoclonal mouse anti-phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY, USA) or rabbit anti-human FLT4 antibodies, biotinylated secondary antibodies (Dako Denmark, Glostrup, Denmark) and streptavidin-biotinylated horseradish peroxidase complex (GE Healthcare, Little Chalfont, UK), followed by enhanced chemiluminescence detection with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA). For re-probing, the membranes were stripped for 15 minutes at room temperature using Re-Blot Plus Strong Solution (Chemicon).

mRNA and morpholino (MO) injections

MOs targeting the *vegfc* and *flt4* start codons were: *flt4* ATG MO, 5'-CTCTTCATTTCAGGTTTCAAGTCC-3' (Open Biosystems) and *vegfc* ATG MO, 5'-GAAAATCCAAATAAGTGCATTTAG-3' (Genetools); both were injected at 5 ng/embryo. The *dll4* MO targeted the exon 4/intron 4 splice-donor site (5'-TGATCTCTGATTGCTTACGTTCTTC-3') and was injected at 3 ng or 6 ng/embryo as indicated.

The *sFLT4*, *vegfc* and *vegfd* cDNA clones have been described previously (Hogan et al., 2009; Ober et al., 2004). The full-length *dll4* cDNA was subcloned into the pCS2+ vector by first amplifying from a full-length template cDNA clone (accession number BC117624; Open Biosystems) using the PCR primers 5'-GCGCGGATCCACCATG-GCAGCTTGGCTCACCTTTC-3' and 5'-GCGCTCTAGATTACCT-CAGTTGCTATGAC-3', followed by subcloning with *Bam*HI and *Xba*I. mRNA was transcribed from a *Kpn*I-linearised template using SP6 RNA polymerase and the mMessage mMachine Kit (Ambion) and injected at 200 pg/embryo. The full-length human wild-type FLT4 and I1034S mutant constructs were cloned into the pCS2+ vector using primers 5'-GCGCG-GATCCACCATGCAGCGGGGCGCCGCGCT-3' and 5'-GCGCTCTA-GACTACCTGAAGCCGCTTCTT-3' to amplify the cDNA from the templates described above, with *Bam*HI and *Xba*I digestion for cloning. mRNA for the wild-type and mutant forms of human *FLT4*, *sFLT4* and zebrafish *vegfc* and *vegfd* was transcribed from *Not*I-digested template using the SP6 RNA polymerase and the mMessage mMachine kit and injected at the concentrations indicated.

Transgenesis

The stable, germline-transmitted *TG(flt4:YFP)^{hu4881}* transgenic line was generated by recombining a citrine-neomycin cassette using Red/ET Recombination Technology (Gene Bridges) into the BAC clone DKEY-58G10 using the homology arm tagged PCR primers 5'-aacgcaggttctagaatatactaaaccccgaggactggaacctgACCATGTTGAGC-AAGGGCAGGAG-3' and 5'-aagaagggaatccaatccaatccgacagaaa-aacgtaaatctctctTCAGAAGAAGTCTCGTCAAGAAGGCG-3' (homology arms are shown in lowercase). We found that BAC transgenesis produced germline founders, but that embryos expressing YFP were highly disomorphic and died at stages prior to 24 hpf (data not shown). Hence, 6.6 kb of the 5'UTR of *flt4* as well as the YFP coding sequence was PCR amplified using Phusion high-fidelity PCR (Finnzymes) followed by cloning into the miniTol2 transgenesis vector (Kawakami, 2005). Amplification primers were 5'-GCGCGAATTCTAAGCAAAGTCAGACCTTACTG-TCC-3' and 5'-CACGTACGGTGTGGTAACG-3'. One-cell stage embryos

were co-injected with construct DNA (25 ng/μl) and transposase mRNA (25 ng/μl) and the progeny screened for germline transmission. Low-level-expressing germline-stable lines were generated, but expression was found to wane after 48 hpf, suggesting that the construct might not contain all the necessary regulatory elements for late expression.

Imaging and expression analysis

In situ hybridisation was performed as previously described (Bussmann et al., 2007). The *tie2* (Lyons et al., 1998), *flt4* (Thompson et al., 1998), *vegfc* (Hogan et al., 2009), *vegfd* (Hogan et al., 2009), *dll4* (Herpers et al., 2008) and *hey2* (Zhong et al., 2000) probes were prepared as previously described. Embryos were mounted in 1% low melting point agarose in a culture dish with a cover slip replacing the bottom. Imaging was performed with a Leica SPE or SP5 confocal microscope (Leica Microsystems) using a 10× or 40× objective with digital zoom. Angiography was performed as previously described (Kuchler et al., 2006).

RESULTS

expando is a *flt4* signalling-deficient mutant

In a screen for developmental mutants that fail to form lymphatic vessels, we identified the mutant *expando*^{hu4602} that lacked the thoracic duct at 5 days post-fertilisation (dpf) while retaining blood

circulation (Fig. 1A-D). To determine the stage at which lymphangiogenesis was first impaired in *expando* mutants, we examined the phenotype in double-transgenic *TG(fli1:GFP, kdr-l:Cherry)* embryos, in which trunk blood vessels express both GFP and Cherry but lymphatic vessels express only GFP (Hogan et al., 2009). We found that all trunk lymphatic vessels, including the intersegmental lymphatic (islv) and dorsal longitudinal lymphatic (dllv) vessels, were absent in *expando* mutants at 5 dpf and that parachordal lymphangioblasts (PLs) had failed to bud from the PCV and were absent at 54 hpf (Fig. 1E-H). We examined *tie2* expression at 48 hpf as a marker for venous-derived intersegmental vessels and found that venous sprouts were absent in *expando* mutants (Fig. 1I,J), a phenotype identical to that of *vegfc* MO-injected embryos, which lack all venous angiogenic sprouting (Hogan et al., 2009).

To identify the causative mutation for the *expando* phenotype, we used a traditional positional cloning approach and found the *expando* locus to be linked to a region of chromosome 14 containing the *flt4* gene (Fig. 2A). Sequencing of *flt4* revealed a point mutation predicted to change a conserved isoleucine residue into a serine at position 1042 in the split kinase domain (Fig. 2B,C). To examine the functional consequence of this mutation, we introduced the

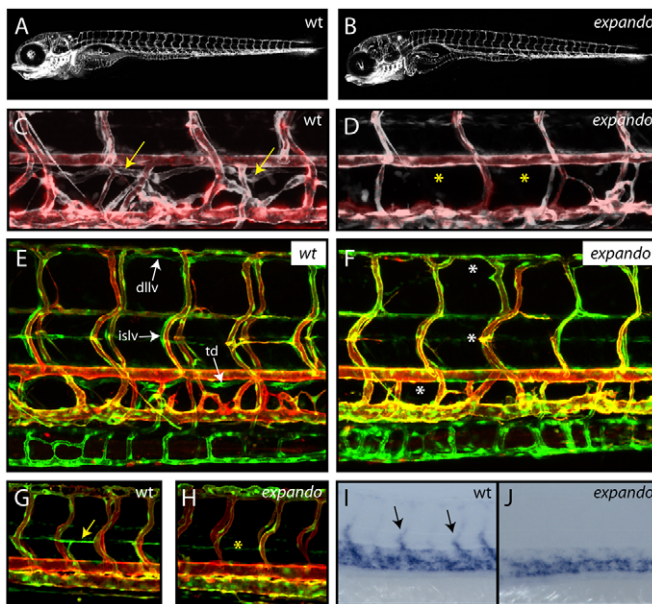


Fig. 1. Zebrafish *expando* mutants lack lymphatic vessels and venous sprouting. (A,B) Lateral views of *TG(fli1:GFP)* expression in wild-type (wt; A) and *expando* mutant (B) larvae at 5 dpf demonstrate that overall vascular patterning is not perturbed in *expando* mutants. (C,D) Overlay image of angiogram (red) and *TG(fli1:GFP)* (white) indicating the presence of blood flow in wild-type (C) and mutant (D) embryos. The thoracic duct (arrows) is present in the wild type, but absent (asterisks) from mutant larvae. (E,F) Lateral views of wild type (E) and *expando* mutant (F) in a double-transgenic *TG(fli1:GFP)*, *TG(kdr-l:Cherry)* background. All lymphatic vessels are absent in mutants at 5 dpf. Arrows indicate the thoracic duct (td), intersegmental lymphatic vessels (islv) and dorsal longitudinal lymphatic vessels (dllv) in wild-type embryos. Asterisks indicate their absence in *expando* mutants. (G,H) Parachordal lymphangioblasts (PLs) are absent from the horizontal myoseptum in *expando* mutants (asterisk in H) as compared with wild-type siblings (arrow in G indicates PL) in the double-transgenic *TG(fli1:GFP)*, *TG(kdr-l:Cherry)* background at 54 hpf. (I,J) *tie2* expression in venous sprouts (arrows in I) is absent in *expando* mutants (J). *n*=6/29 embryos scored and confirmed by genotyping.

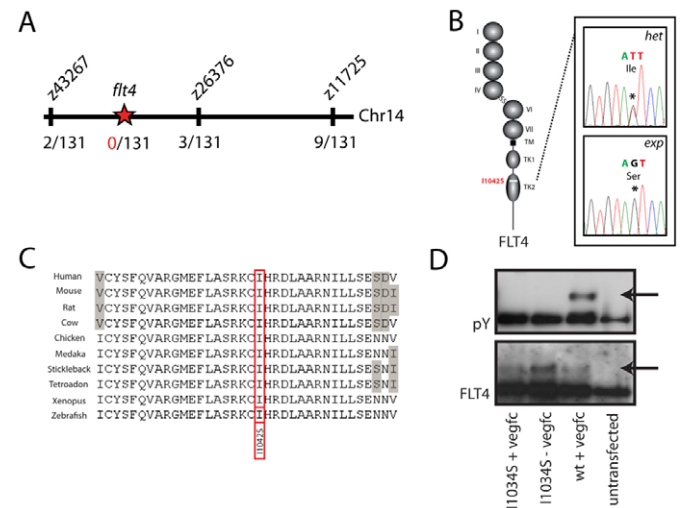


Fig. 2. A mutation in the *flt4* kinase domain is responsible for the *expando* phenotype. (A) Meiotic mapping links the *expando* locus to a region of chromosome 14 containing *flt4*. Analysis of 131 zebrafish embryos (262 meioses) identifies two recombination events at the marker z43267 and three independent recombinants at the marker z26376, thereby localising the mutation to a region containing *flt4*. (B,C) Sequencing of the coding exons of *flt4* reveals a T>G mutation (see chromatogram in B) that changes a highly conserved isoleucine to a serine at position 1042 of the predicted zebrafish Flt4 protein sequence. Flt4 receptor structure is depicted, with Ig domain (I-VII), transmembrane domain (TM) and split tyrosine kinase domain (TK1 and TK2). Alignment of conserved sequences (C) demonstrates that I1042 is a conserved residue, with grey shading indicating non-conserved residues. (D) Tyrosine phosphorylation (pY; upper panel, phosphotyrosine blot) was seen upon in vitro stimulation with VEGFC of wild-type human FLT4, but not of I1034S mutant FLT4. Lower panel (Flt4 protein blot) indicates the presence of FLT4 protein post-transfection in the assayed samples.

equivalent mutation (I1034S) into human FLT4 and compared the ability of VEGFC to stimulate kinase domain activity of the wild-type and mutant forms of the receptor. We found that the I1034S mutation led to a loss of kinase domain function in this in vitro assay (Fig. 2D). Consistent with a *flt4* mutation leading to the *expando* phenotype, the injection of either a *flt4*-targeting morpholino oligomer (MO) or an mRNA encoding the soluble Ig domain of human FLT4 (*sFLT4*) that acts as a dominant inhibitor of Flt4 signalling in zebrafish (Ober et al., 2004), phenocopied the loss of lymphatic vessels (Fig. 3G; data not shown) as previously described (Kuchler et al., 2006; Yaniv et al., 2006). We hereafter refer to the *expando* (Flt4 I1042S) mutant as *flt4^{hu4602}*.

Angiogenesis defects of the primordial hindbrain channel are observed in *flt4* morphants, *vegfc* morphants and *sFLT4* mRNA-injected embryos, but not in *flt4^{hu4602}* mutants

Previous studies have described angiogenesis defects after inhibition of *flt4* translation by MO injection (Covassin et al., 2006; Siekmann and Lawson, 2007). We injected *sFLT4* mRNA and also MOs targeting the start codons of *flt4* and *vegfc*. These injections consistently induced angiogenesis defects during the formation of the primordial hindbrain channel (PHBC) (Fig. 3C-E) in 20–40% of injected embryos. When all injected embryos from the same clutch were grown to 5 dpf, they completely lacked lymphatic vessels, phenocopying the *flt4^{hu4602}* phenotype with 100% penetrance (Fig. 3F,G). The dependency of PHBC formation upon Vegfc and Flt4 in zebrafish has been reported previously (Covassin et al., 2006). Surprisingly, in contrast to the injected embryo phenotypes, detailed examination of *flt4^{hu4602}* mutant embryos failed to reveal PHBC defects (Fig. 3B).

Taken together, these observations suggest two distinct possibilities: (1) that different thresholds of Flt4 signalling are required for distinct angiogenesis events in the zebrafish embryo,

such that normal signalling is necessary for lymphangiogenesis and venous angiogenesis, whereas very low-level, residual signalling in the presence of the I1042S mutation is sufficient for PHBC development; or (2) that a non-signalling function of Flt4 regulates PHBC angiogenesis whereas signalling is indispensable for PCV sprouting and lymphangiogenesis.

To evaluate the possibility of performing experiments to rescue the morphant PHBC defect with mutant or truncated forms of Flt4, we injected full-length mRNA encoding the human wild-type and I1034S mutant forms of FLT4 (300 pg/embryo). Upon forced expression, both of these mRNAs were capable of inhibiting thoracic duct formation (wild-type *FLT4* mRNA, 47% thoracic duct deficient, $n=42$; I1034S *FLT4* mRNA, 26% thoracic duct deficient, $n=31$; data not shown), indicating that FLT4 has mild dominant-negative activity upon overexpression by mRNA injection in zebrafish, therefore precluding an attempt to rescue PHBC defects.

Excessive arterial angiogenesis caused by the depletion of Dll4 is rescued in *flt4^{hu4602}* mutants

We next examined arterial development in *flt4^{hu4602}* mutants by taking advantage of the *TG(flt1:YFP)^{hu4624}* transgenic line, in which the arteries of the embryonic trunk are labelled by YFP (Hogan et al., 2009). We examined the presence of the venous-derived ISV components: these sprouts form during secondary sprouting from the vein, connect to ISAs in order to establish intersegmental venous vessels (Isogai et al., 2003) and are identifiable by the absence of *flt1* expression in the ventral component of functional intersegmental vessels at 3 dpf (Hogan et al., 2009). We found that like *vegfc* MO-injected embryos (Hogan et al., 2009), *flt4^{hu4602}* mutants failed to form intersegmental venous-derived vessels, and in the absence of these venous sprouts an increased number of ISA connections to the dorsal aorta were formed (Fig. 4A,B). Apart from the increased ISA connections to the dorsal aorta, the ISAs appeared otherwise normal in *flt4^{hu4602}* mutants.

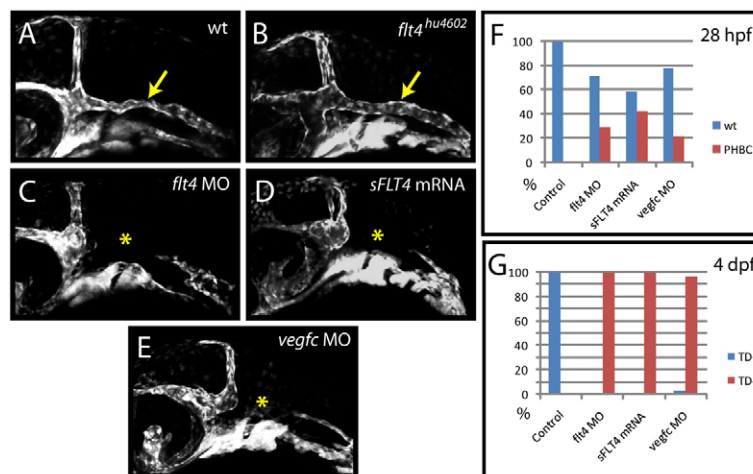


Fig. 3. Zebrafish *flt4^{hu4602}* mutants lack the PHBC defects observed upon *flt4* MO, *vegfc* MO or *sFLT4* mRNA injection. (A-E) Angiogenesis of the primordial hindbrain channel (PHBC) fails to occur in *flt4* MO-injected (5 ng/embryo) (C), *sFLT4* mRNA-injected (200 pg/embryo) (D) and *vegfc* MO-injected (E) embryos (yellow asterisk) as compared with wild-type controls (A), but is unaffected (yellow arrow) in individually genotyped *flt4^{hu4602}* mutants (B) at 28 hpf. (F,G) Angiogenesis of the PHBC and lymphangiogenesis respond differently to injection of *flt4* MO, *vegfc* MO and *sFLT4* mRNA. Injection of *flt4* MO (5 ng/embryo) inhibits PHBC development in 29% of embryos at 28 hpf but inhibits lymphangiogenesis [thoracic duct (TD) scored as readout] with 100% efficiency at 4 dpf in the same injection ($n=55$ embryos scored). *vegfc* MO (5 ng/embryo) inhibits PHBC development in 22% of embryos ($n=41$) and TD development in 97% of embryos in the same injection ($n=37$). *sFLT4* mRNA (200 pg/embryo) inhibits PHBC development in 42% of embryos and TD development in 100% of embryos in the same injection ($n=120$). Uninjected control embryos showed normal PHBC and TD development in 100% of cases ($n=20$) at 28 hpf and 4 dpf (F,G).

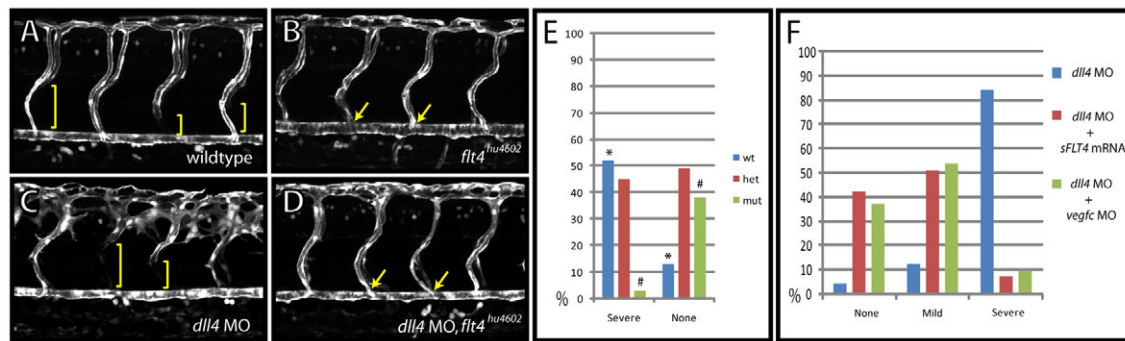


Fig. 4. The *dll4* morphant hyperbranching phenotype is rescued in *flt4*^{hu4602} mutants, *vegfc* morphants and *sFLT4* mRNA-injected embryos. (A–D) Lateral views of uninjected control (A) and *flt4*^{hu4602} mutant (B) as compared with wild-type (C) and *flt4*^{hu4602} mutant (D) zebrafish embryos injected with *dll4* MO. Images were taken in the *TG(flt1:YFP)^{hu4624}* background, labelling trunk arteries to reveal the failure to form intersegmental venous sprouts in *expando* mutants (B, yellow arrows). The *dll4* morphant arterial hyperbranching phenotype (C) is rescued in *flt4*^{hu4602} mutants (D). Venous sprouts were identified by the absence of *flt1:YFP* expression in ISVs (brackets). Embryos at 72 hpf. (E) Arterial hyperbranching phenotypes were scored post-injection of *dll4* MO and subsequently genotyped. The mutant genotype was almost completely absent from the severe category (e.g. C) (1/29 embryos, * $P < 0.01$ by χ^2 test) and was enriched in the category not displaying hyperbranching (e.g. D) (21/55 embryos, # $P < 0.01$ by χ^2 test). (F) In transgenic *TG(flt1:YFP)^{hu4624}* embryos, co-injection with *dll4* MO together with *vegfc* MO (green bars, $n=57$ embryos), or of *dll4* MO together with *sFLT4* mRNA (200 pg/embryo) (red bars, $n=67$ embryos), led to rescue of the arterial hyperbranching phenotype observed upon injection of *dll4* MO alone (blue bars, $n=81$ embryos). Arterial hyperbranching phenotypes were categorised as no hyperbranching (none), mild or severe (see Fig. S1 in the supplementary material).

Given the known relationship between Dll4 and Flt4 in angiogenesis (Siekman and Lawson, 2007; Tammela et al., 2008), we injected a *dll4* MO that recapitulated the previously described *dll4* loss-of-function arterial hyperbranching phenotype (Leslie et al., 2007) into embryos derived from *flt4*^{hu4602} heterozygous crosses (*flt4*^{hu4602+/-} × *flt4*^{hu4602+/-}). We found the hyperbranching phenotype to be variable and sorted embryos displaying severe arterial hyperbranching (e.g. Fig. 4C) from those not displaying hyperbranching phenotypes (e.g. Fig. 4D) into separate groups for genotyping. The mutant genotype was almost completely absent from the severe phenotypic category (present in 1/29 genotyped hyperbranching embryos) and was enriched in the non-hyperbranching category (present in 21/55 genotyped embryos) indicating a rescue of the arterial hyperbranching phenotype associated with the mutant allele (Fig. 4E).

Excessive arterial angiogenesis caused by the depletion of Dll4 is dependent on Vegfc

To test whether the *flt4*-dependent *dll4* hyperbranching phenotype is driven by Vegfc, we injected embryos with *dll4* MO alone, *dll4* MO with *vegfc* MO, or *dll4* MO with *sFLT4* mRNA. For all treatments, the *dll4* MO-induced arterial hyperbranching phenotype was scored as either severe, mild or wild-type (no hyperbranching) (for examples, see Fig. S1 in the supplementary material). The injection of either *vegfc* MO or *sFLT4* mRNA rescued the *dll4* excessive angiogenesis phenotype at 72 hpf (Fig. 4F). We observed no ISA defects upon injection of either *vegfc* MO or *sFLT4* mRNA at concentrations that completely inhibited lymphangiogenesis and also rescued the *dll4* phenotype.

flt4 and *vegfc* are expressed in arterial endothelial cells during angiogenesis and their expression is unaltered upon loss of *dll4*

To determine whether the phenotype could be explained by changes in gene expression during arterial angiogenesis, we analysed the expression of *flt4*, *vegfc*, *vegfd* (*figf* – Zebrafish Information Network) and *hey2* in *dll4* morphants at 24 hpf. *flt4* was expressed

in all ISAs and in the dorsal aorta at 24 hpf, but rapidly downregulated thereafter, with expression vastly reduced in arteries as compared with the PCV by 26 hpf (Fig. 5A,C). We carefully staged *dll4* MO-injected and uninjected control embryos at 24 hpf (based on head angle and tail extension) and detected no appreciable change in *flt4* expression in the ISAs at 24 hpf (Fig. 5A,B) or later, during downregulation of *flt4* expression (25, 27, 29 and 31 hpf; see Fig. S2 in the supplementary material), although we cannot exclude the possibility of mild changes for which in situ hybridisation is insufficiently sensitive to detect. Next, we generated a *flt4* promoter-driven transgenic line utilising a 6.6 kb fragment of the *flt4* 5' UTR. This promoter fragment drove expression of YFP in ISAs, the dorsal aorta and the PCV in both wild-type and *dll4* MO-injected embryos, with no apparent change in expression levels in stage-matched embryos (Fig. 5D,E). In *dll4* morphants at 24 hpf, *vegfc* expression in the dorsal aorta (Fig. 5F,G) and *vegfd* expression in the tailbud (Fig. 5H,I) were unchanged. In addition, we observed no change in the expression of *hey2*, indicating that developmental gene expression programmes in arterial endothelial cells were largely normal in *dll4* MO-injected embryos (Fig. 5J,K).

dll4 morphant arteries are sensitised to increased Vegfc during primary sprouting of intersegmental arteries

The arterial phenotype induced by the loss of *dll4* is not observed before 28–32 hpf, after which time the ISAs progressively display excessive angiogenesis and hyperbranching defects. Given that this phenotype is dependent on Vegfc/Flt4 signalling, we hypothesised that the introduction of excessive *vegfc* might increase the severity, or alter the onset, of the phenotype. We introduced mRNA encoding full-length zebrafish Vegfc into wild-type and *dll4* MO-injected embryos in the *TG(flt1:YFP)^{hu4624}* transgenic background and examined the ISAs at 28 hpf, a time point preceding secondary sprouting of vessels from the PCV and the arterial phenotype induced by the knockdown of *dll4*. *vegfc* mRNA injection in combination with *dll4* MO synergistically induced aberrant sprouting of ISAs, which turned bilaterally at approximately the level of the base of the

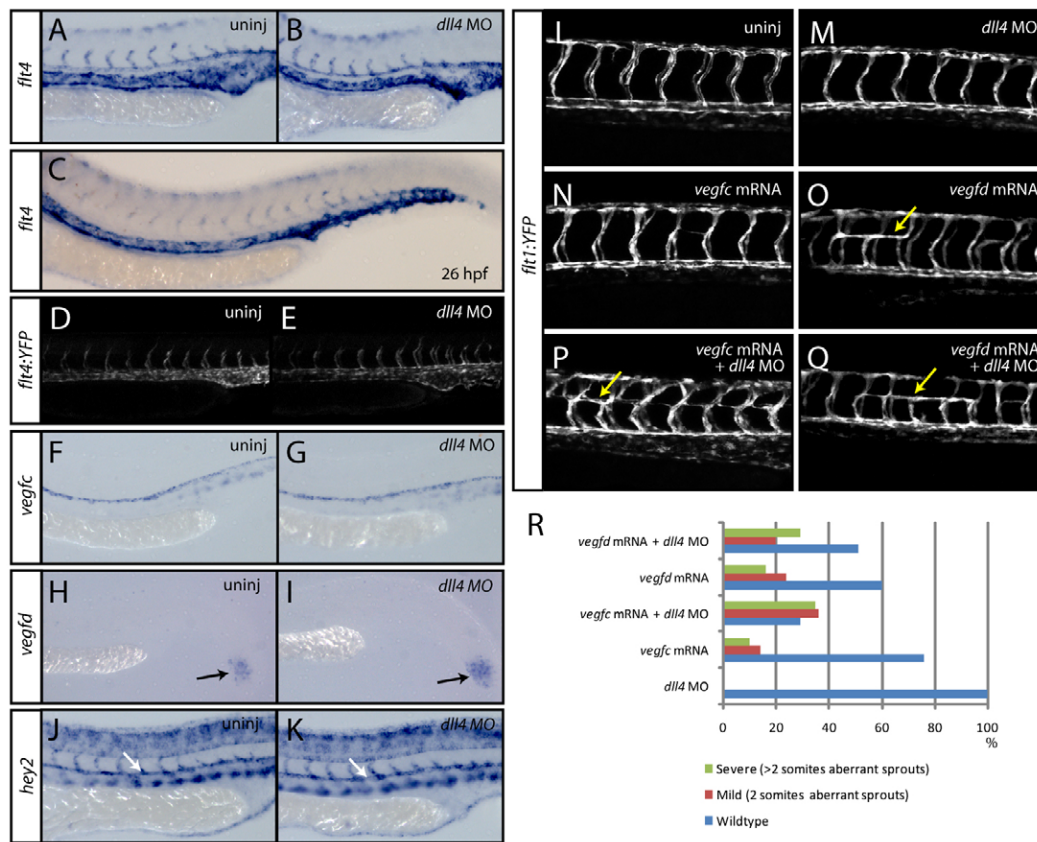


Fig. 5. Sprouting intersegmental arteries express *flt4* and are sensitised to exogenously introduced *Vegfc* in the absence of *dll4*.

(A-E) At 24 hpf, the expression of *flt4* is present in all arterial cells (A) and is unaltered upon loss of *dll4* (B) ($n=40$ uninjected control and $n=40$ *dll4* MO-injected embryos scored). By 26 hpf (C), *flt4* is downregulated in the dorsal aorta ($n=20$ embryos). Analysis of a *flt4* promoter-driven transgenic line identifies YFP expression in all arterial cells at 24 hpf (D). The expression of YFP is unaltered in *dll4* MO-injected embryos (E). (F,G) At 24 hpf, the expression of *vegfc* is present in the dorsal aorta (F) and is unaltered in *dll4* MO-injected embryos (G) ($n=20$ uninjected and $n=20$ MO-injected embryos). (H,I) The expression of *vegfd* in the tailbud is unaltered in *dll4* MO-injected (I) as compared with uninjected control (H) embryos ($n=20$ uninjected and $n=20$ MO-injected embryos). (J,K) The expression of the arterial marker gene *hey2* is unaltered in *dll4* MO-injected (K) as compared with uninjected control (J) embryos ($n=20$ uninjected and $n=20$ MO-injected embryos). (L-R) *dll4* morphants are sensitised to exogenously introduced *vegfc* mRNA during arterial angiogenesis. Forced expression by the injection of mRNA encoding full-length zebrafish *Vegfc* into *dll4* MO-injected embryos ($n=133$) (P) leads to aberrant ectopic bilateral turning of ISAs by 28 hpf (yellow arrows), which was never observed in wild-type embryos (L) or in those injected with *dll4* MO only ($n=90$) (M). ISAs rarely turn bilaterally upon injection of *vegfc* mRNA only ($n=106$) (N), whereas injection of full-length *vegfd* mRNA robustly showed this phenotype without ($n=211$) (O) or with ($n=227$) (Q) *dll4* MO. For each of the conditions tested, the percentage of embryos showing wild-type, mild (aberrant ISAs spanning two somites) or severe (aberrant ISAs spanning more than two somites) ISA phenotypes is shown in R.

neural tube in a distinctive manner (Fig. 5L-N,P). This phenotype was robustly reproducible, was not present in embryos injected with *dll4* MO only, and occurred only rarely upon injection of *vegfc* mRNA only (Fig. 5M,N,R). Interestingly, we also observed this phenotype upon injection of full-length zebrafish *vegfd* mRNA, but found no difference when *vegfd* mRNA was injected in combination with *dll4* MO (Fig. 5O,Q,R). Taken together, these data indicate that in the absence of *dll4*, arterial cells are more responsive to exogenously introduced *vegfc* at stages prior to venous sprouting, confirming an early capability as well as an ongoing suppressive role for *dll4* during *Vegfc*/Flt4 signalling in arterial angiogenesis.

Overexpression of *dll4* reduces sprouting from venous endothelium

Taken together, the above data suggest that *dll4*, which is restricted in expression to the early arteries in developing embryos (Leslie et al., 2007; Siekmann and Lawson, 2007),

functions to suppress the response of arteries to *Vegfc*-mediated Flt4 signalling. This would predict that ectopic *dll4* in embryonic veins might inhibit or reduce *Vegfc*/Flt4-driven venous sprouting. To test whether this was the case, we injected mRNA encoding full-length Dll4 into *TG(flt1:YFP)^{hu4624}* embryos and examined the formation of venous sprouts at 52 hpf. We identified venous sprouts by scoring the ventral segments of intersegmental vessels that did not express *TG(flt1:YFP)^{hu4624}* at 52 hpf. *dll4* overexpression led to cardiac defects and loss of circulation when injected at high doses, but did not lead to the ectopic venous expression of *flt1* (data not shown). We selected embryos with normal blood circulation and omitted embryos severely affected by *dll4* overexpression. When the number of venous sprouts present across the first 12 intersegmental vessels anterior to the cloaca was scored, there was a significant reduction in the number of venous sprouts in injected embryos compared with uninjected controls (Fig. 6).

DISCUSSION

Flt4 signalling is required for zebrafish lymphangiogenesis and venous sprouting

Here we describe a new zebrafish *flt4* mutant phenotype that is caused by an amino acid substitution in the conserved split kinase domain of Flt4 (I1042S). Using this *in vivo* model, we have shown a strict requirement for normal Flt4 signalling for venous angiogenesis. We find that primary angiogenesis of ISAs can occur independently of normal Flt4 signalling in zebrafish. This finding is consistent with previous studies suggesting that ISA sprouting is chiefly regulated by Vegfa, Kdr and Kdr-1 (Bahary et al., 2007; Covassin et al., 2006; Habeck et al., 2002; Lawson et al., 2003), but suggests that previously described ISA morphant phenotypes for Vegfc and Flt4 do not reflect a requirement for wild-type kinase domain activity of the Flt4 receptor (Covassin et al., 2006). The defects in secondary sprouting from the veins observed in *flt4*^{hu4602} mutants are apparently identical to the previously described phenotypes of *full of fluid* (*cbel1*) mutants and *vegfc* morphants (Hogan et al., 2009).

flt4 MO, *vegfc* MO or *sFLT4* mRNA injections led to the failure of PHBC development, but the defects were far less penetrant than the lymphangiogenesis failure seen within the same injection experiments and were not observed in *flt4*^{hu4602} mutants. These data suggest (1) that there is a differential dosage sensitivity for Flt4 signalling during different angiogenesis events and/or (2) that some degree of *flt4*-regulated angiogenesis might occur independently of kinase domain function. The scenario described here closely resembles that observed in the mouse, in which mutants for *Vegfc*, double mutants for *Vegfd* and *Vegfc*, or a hypomorphic *Flt4* allele, all produce lymphatic vascular-specific defects, whereas the complete loss of Flt4 leads to defects in haemangiogenesis and early lethality (Dumont et al., 1998; Haiko et al., 2008).

Dll4 function provides a mechanism by which arteries, but not veins, suppress the response to Vegfc-driven Flt4 signalling during development

Despite the lack of a marked arterial phenotype in *flt4*^{hu4602} mutants, we find that *flt4* is actively transcribed in the ISAs and the dorsal aorta, concurrent with sprouting arterial angiogenesis at 24 hpf.

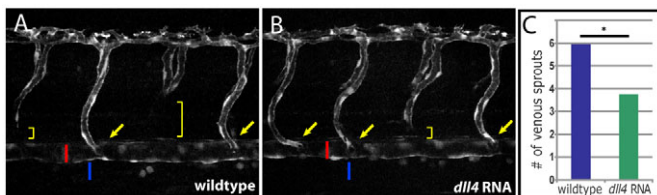


Fig. 6. Overexpression of *dll4* reduces sprouting from the PCV.

(A, B) Injection of mRNA encoding full-length Dll4 into *TG(flt1:YFP)*^{hu4624} zebrafish embryos leads to a reduction in the number of venous sprouts in injected (B) as compared with uninjected control (A) embryos at 52 hpf. ISAs that connect directly to the dorsal aorta (red bar) are indicated by yellow arrows. Venous sprouts (yellow brackets) can be readily identified by the absence of *TG(flt1:YFP)*^{hu4624} expression in the ventral component of an intersegmental vessel. Venous sprouts connect directly to the posterior cardinal vein (blue bar) from which they derive during secondary sprouting. (C) Quantification of the number of venous sprouts (y-axis) on one side of the embryo across 12 segments anterior to the cloaca ($n=23$ embryos scored for both *dll4* MO-injected and uninjected controls). *dll4* MO-injected embryos show a significant decrease in the number of intersegmental venous sprouts ($P<0.001$, Mann-Whitney rank sum test).

Furthermore, a source of Vegfc is present in the trunk derived from the dorsal aorta during this period. The severe hyperbranching phenotypes observed in the absence of *dll4* are dependent on Vegfc and on Flt4 kinase domain activity, indicating that one function of *dll4* is to suppress the arterial response to Vegfc/Flt4 signalling in developing arteries of the trunk. Consistent with this, Dll4-depleted arteries are sensitised to increased Vegfc levels supplied exogenously by mRNA injection, and the overexpression of *dll4* reduces venous sprouting, which is dependent on Vegfc/Flt4 signalling. Hence, the severe, progressive arterial hyperbranching phenotype that has been described previously upon loss of *dll4* in zebrafish is predominantly a consequence of failing to suppress the ability of developing arteries to respond to endogenous Vegfc/Flt4 signalling. A working model summarising the contribution of Dll4 in the context of arterial and venous angiogenesis and Vegfc/Flt4 signalling is outlined in Fig. 7.

Changes in *vegfc* or *flt4* expression do not explain the suppression of the arterial response to Vegfc/Flt4 signalling

We find that *flt4* transcript levels are progressively downregulated in developing arteries after 24 hpf, an event that is likely to contribute to lowering the responsiveness of arteries to endogenous

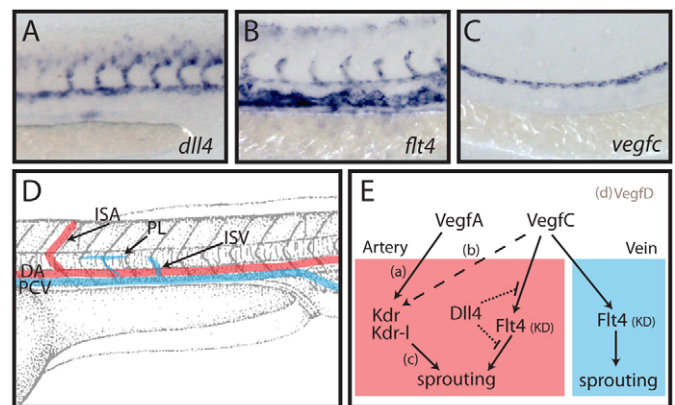


Fig. 7. Dll4 inhibits arterial Vegfc/Flt4 signalling in the developing zebrafish trunk.

(A–C) Expression analysis at 24 hpf for *dll4* (A), *flt4* (B) and *vegfc* (C) indicates the arterial restricted expression of *dll4*, expression of *flt4* in all venous and arterial cells, and expression of *vegfc* restricted to the dorsal aorta. (D) Schematic overview of the developing vasculature in the zebrafish trunk. DA, dorsal aorta; PCV, posterior cardinal vein; ISA, intersegmental artery; ISV, intersegmental vein; PL, parachordal lymphangioblast. (E) Working model of the interplay between Dll4 and Vegfc/Flt4 signalling. Previous studies have shown that *vegfa* (*vegfaa* and *vegfab* duplicates in zebrafish) is necessary for ISA development [a (Bahary et al., 2007; Nasevicius et al., 2000)], that *vegfc* influences (dashed line) ISA development [b (Covassin et al., 2006)] and that the receptors *kdr* and *kdr-1* mediate intracellular signalling to control ISA development [c (Bahary et al., 2007; Covassin et al., 2009; Covassin et al., 2006; Habeck et al., 2002; Lawson et al., 2003; Meng et al., 2008)]. We have previously shown that *vegfd* is unlikely to play a role in ISA development owing to its restricted expression [d (Hogan et al., 2009)]. Here we demonstrate that signalling through the Flt4 kinase domain (KD) does not contribute to ISA development as it is suppressed by Dll4 (dotted lines indicate that the mechanism of suppression is unknown). In the absence of Dll4, endogenous Vegfc drives a Flt4 (KD)-dependent ISA hyperbranching phenotype. In the vein (which does not express *dll4*), Vegfc/Flt4 signalling is indispensable for normal venous angiogenesis and lymphangiogenesis.

Vegfc. However, the increased responsiveness of *dll4* morphants to Vegfc/Flt4 signalling does not coincide with changes in *vegfc* or *flt4* transcription. In particular, there is no change in *vegfc* or *flt4* expression in *dll4* morphants during the formation of primary arterial sprouts (24–31 hpf), despite the fact that these sprouts are sensitised to increased levels of *vegfc* at early stages, prior to 28 hpf (Fig. 5). This indicates that *dll4* can regulate Vegfc/Flt4 signalling at a level independent of *vegfc* or *flt4* transcription. In zebrafish, it has been demonstrated previously that *notch1b* knockdown, or chemical treatment with a γ -secretase inhibitor (DAPT) that blocks the cleavage of the Notch intracellular domain (Geling et al., 2002), leads to the same increased exploratory behaviour of arterial endothelial cells that is observed upon *dll4* loss-of-function (Leslie et al., 2007). This correlation is suggestive of *dll4* acting through intracellular Notch signalling and would indicate that altered transcription should precede an altered response to Vegfc/Flt4 signalling. In this context, the unchanged expression of the key molecular players (*flt4*, *vegfc*, *vegfd*) in *dll4* morphants implies that other, as yet unidentified, molecular regulators are involved.

Interestingly, we find that *vegfd* mRNA injection alone can lead to the early ectopic turning of developing ISAs, but these defects were not enhanced upon knockdown of *dll4*. Vegfd has varying capabilities in mammals: for example, it is a Flt4-specific ligand in mouse but, like VEGFC, can bind to both VEGFR2 (KDR) and FLT4 in humans (Baldwin et al., 2001). The capability of Vegfd to stimulate aberrant angiogenesis of ISAs is likely to explain why the embryonic expression of *vegfd* is highly restricted to the tailbud during zebrafish development (Fig. 5) (Hogan et al., 2009). This restriction of *vegfd* expression would be required for normal arterial angiogenesis to occur in the absence of Flt4 activation.

Comparison of expression patterns and phenotypes for different Notch signalling components suggests combinatorial and context-dependent roles during angiogenesis

In the developing mammalian retina, both *dll4* and *flt4* display restricted expression in tip cells during angiogenesis (Hellstrom et al., 2007; Tammela et al., 2008). In zebrafish, we did not see any restricted expression of *flt4* in tip cells at 24 hpf during ISA angiogenesis, and *dll4* expression is found in all arterial cells at this stage [as discussed by Leslie et al. (Leslie et al., 2007)]. In the developing mouse trunk, Flt4 expression is also seen in all arterial cells of the intersegmental vasculature, although it is enriched in tip cells when assayed by immunohistochemistry (Tammela et al., 2008). These data suggest context-dependent differences in the expression patterns of Flt4 and Dll4.

Previous studies in zebrafish have shown that *mind bomb* mutants (which cause a block in intracellular Notch signalling) have upregulated *flt4* expression throughout the developing arterial system (Lawson et al., 2001), and *rbpsuh* morphants have upregulated expression specifically in the tip cells of arterial sprouts (Siekman and Lawson, 2007). However, we found no marked increase in *flt4* expression in *dll4* morphants by in situ hybridisation, suggesting significant differences between loss-of-function phenotypes for different Notch pathway components during angiogenesis. Furthermore, the phenotype seen upon MO knockdown of *Rbpsuh* is observed earlier than that of *dll4* morphants or mutants. Dramatically increased arterial angiogenesis is seen in *rbpsuh* morphants during the initial stages of primary sprouting (Siekman and Lawson, 2007), whereas *dll4* morphants or mutants (Leslie et al., 2007) do not display defects until hours later. Given that multiple Notch ligands are expressed and capable

of acting during angiogenesis in vertebrates (Benedito et al., 2009; Lawson et al., 2001; Smithers et al., 2000), it is likely that the combinatorial use of ligands contributes to these differences in the loss-of-function phenotypes. It seems plausible that differences in Notch ligand or receptor gene expression patterns and activities could be very widely utilised to allow for unique, context-dependent and restricted Vegf signalling outputs in order to generate diverse spatial and temporal responses during angiogenesis in the embryo or adult.

Taken together, the above findings indicate that one function of arterial Dll4 is to inhibit the responsiveness of arteries to Vegfc, whereas the development of the venous and lymphatic lineages (which do not express *dll4*) are completely dependent on Vegfc/Flt4 signalling in the developing zebrafish trunk. This mechanism contributes to the differential response of arteries and veins to a constant source of Vegfc in the embryonic trunk and further characterises a specific role of the Notch ligand Dll4 in regulating the Vegfc/Flt4 signalling axis.

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Author contributions

B.M.H. and R.H. conceived and carried out experiments and co-wrote manuscript. M.W. and H.H. carried out experiments. H.J.D. and K.A. conceived experiments. S.S.-M. conceived experiments and co-wrote manuscript.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/23/4001/DC1>

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