Vinculin controls endothelial cell junction dynamics during vascular lumen formation

Graphical abstract

Highlights
- Endothelial cells form finger-shaped junctions during angiogenic sprouting
- Junctional fingers appear in response to incoming blood pressure
- Vinculin is localized at junctional fingers during lumen inflation
- Finger formation and maintenance require vinculin

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In brief
Kotini et al. investigate junctional dynamics during angiogenic lumen formation. Finger-shaped junctions containing the mechanotransduction protein vinculin are formed in response to blood pressure. Vinculin is required for finger formation and maintenance. Junctional fingers present an in vivo paradigm to study junctional dynamics in relation to extrinsic forces.
Article

Vinculin controls endothelial cell junction dynamics during vascular lumen formation

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SUMMARY

Blood vessel morphogenesis is driven by coordinated endothelial cell behaviors. Active remodeling of cell-cell junctions promotes cellular plasticity while preserving vascular integrity. Here, we analyze the dynamics of endothelial adherens junctions during lumen formation in angiogenic sprouts in vivo. Live imaging in zebrafish reveals that lumen expansion is accompanied by the formation of transient finger-shaped junctions. Junctional fingers are positively regulated by blood pressure, whereas flow inhibition prevents their formation. Using fluorescent reporters, we show that junctional fingers contain the mechanotransduction protein vinculin. Furthermore, genetic deletion of vinculin prevents finger formation, a junctional defect that could be rescued by transient endothelial expression of vinculin. Our findings suggest a mechanism whereby lumen expansion leads to an increase in junctional tension, triggering recruitment of vinculin and formation of junctional fingers. We propose that endothelial cells employ force-dependent junctional remodeling to counteract external forces in order to maintain vascular integrity during sprouting angiogenesis.

INTRODUCTION

The cardiovascular system is essential for vertebrate development and homeostasis as it serves as a transport system to deliver and exchange gas and solutes in all parts of the body. The cardiovascular system is the first organ to form during embryonic development, and it adapts to the needs of the growing embryo by expansion of the vascular network (Hogan and Schulte-Merker, 2017; Potente et al., 2011). To a large extent, this is achieved by the formation of new vessels in a process called sprouting angiogenesis.

Blood vessel formation by angiogenesis occurs in discrete steps of sprout outgrowth, lumen formation, and sprout fusion. At the cellular level, these morphogenetic processes are achieved by numerous cell activities, such as endothelial cell migration, cell rearrangements, and cell shape changes, which in turn are driven by a complex array of intracellular activities as well as dynamic cell-cell and cell-extracellular matrix (ECM) interactions (reviewed by Betz et al., 2016; Szymborska and Gerhardt, 2018). During angiogenesis, multiple morphogenetic processes occur simultaneously, posing a particular challenge to endothelial cell-cell junctions. Fine-tuned regulation of cell-cell adhesion is required to permit endothelial cell migration and rearrangements while maintaining the vascular seal to prevent leakage or hemorrhage.

Endothelial cell-cell junctions play a central role in many aspects of endothelial cell biology. They regulate not only cell-cell adhesion and vascular permeability but also proliferation, survival, shape, and motility, and they are involved in the modulation of signaling pathways and transcriptional regulation (reviewed by Komarova et al., 2017; Lampugnani et al., 2017; Wallez and Huber, 2008). In addition to forming a structural link between neighboring endothelial cells, the junctions can act as sensors to adapt to forces that are derived from the actomyosin cytoskeleton or the vascular hemodynamic microenvironment (Huveneers et al., 2012; Lagendijk et al., 2017; Tzima et al., 2005; Liu et al., 2010). During vascular morphogenesis, endothelial cells are exposed to mechanical forces generated by matrix stiffness, fluid shear stress, blood pressure, actomyosin contractility, and junctional tension (Duchemin et al., 2019; Hoefer et al., 2013; Roman and Pekkan, 2012). Recent work has shown that endothelial cell-cell junctions play an important role...
in mechanical force sensing and force transmission, specifically by changing the organization of vascular endothelial (VE)-cadherin-based junctions (Angulo-Urarte et al., 2020; Baeyens and Schwartz, 2016; Conway et al., 2013). Tension on the VE-cadherin/β-catenin/α-catenin complex recruits the mechanotransduction protein vinculin, which in turn protects the endothelial junctions during their remodeling (Huveneers et al., 2012). Force-dependent signals are transmitted through the VE-cadherin complex and via junctional vinculin recruitment enable collective endothelial responses in vitro (Barry et al., 2015).

Recent work in zebrafish embryos showed that the adhesion molecule VE-cadherin transduces intracellular actomyosin contractility to endothelial cell-cell junctions in vivo (Lagendijk et al., 2017) and also participates in force-generating processes during endothelial cell rearrangements (Paatero et al., 2018). Here, we have studied how endothelial cells respond to mechanical stress imposed in a changing environment. To this end, we examine endothelial cell-cell junctions during angiogenic lumen expansion, which is accompanied with an acute increase in blood pressure within the sprouting intersegmental vessels (ISVs). Our studies reveal junctional structures within angiogenic sprouts, which we call junctional fingers. These junctional fingers form transiently in response to increased pressure and recruit vinculin. Genetic analyses reveal that vinculin is required for junctional finger formation. Taken together, our studies indicate that endothelial cells protect their cell-cell contact against mechanical stress by forming junctional fingers, thereby maintaining sprout integrity during lumen expansion.

RESULTS

Endothelial junctional fingers appear during vascular lumen formation in vivo

During blood vessel formation, endothelial junctions play permissive as well as active roles, driving cell rearrangements and cell elongation while maintaining vascular integrity at the same time (reviewed by Fonseca et al., 2020; Okuda and Hogan, 2020). Furthermore, endothelial junctions are thought to sense and transmit mechanical forces and thus regulate the collective endothelial response to changes in blood flow or pressure, which occur at the onset of blood flow in a newly formed vessel. To investigate the response of endothelial cell-cell junctions to changes in blood pressure, we followed junctional dynamics in zebrafish ISVs during the onset of lumen formation around 28–36 h postfertilization (hpf). Immunofluorescent analyses of the adherens junction protein VE-cadherin (Cdh5) and tight junction protein zonula occludens-1 (ZO-1/TJP1) revealed finger-shaped structures (Figure 1A), which emanated from the cell-cell contact interface, confirming our previous observations (Sauteur et al., 2014). These junctional fingers appeared perpendicular to the originating junction with an angle of around 90° (Figure 1C). To check whether these junctional fingers are specifically formed by VE-cadherin-based adhesions or represented more general junctional structures, we analyzed the localization of platelet endothelial cell adhesion molecule-1 (Pecam1) using a transgenic reporter (Tg(fli1a:pecam1a-EGFP))nco27, followed by immunofluorescent analysis of ZO-1 (Figure 1B). We found that junctional fingers also contained Pecam1 and colocalized with ZO-1, indicating that several types of cell-cell adhesion complexes localize at sites of junctional formation.

Because fixation of zebrafish embryos for immunofluorescence disrupts blood flow and can affect blood vessel morphology, we wanted to verify the existence of junctional fingers by live imaging using the above-mentioned Pecam1 reporter (Figures 1D, 1D1, and 1D2) as well as the ZO-1 reporter Tg(UAS:EGFP-hZO1)abs5 (Figures 1E and 1E1) and the VE-cadherin reporter TgBAC(ve-cad:ve-cadTS)μολθαβ (Figures 1A and 1A1). These live-imaging experiments confirmed the presence of ZO-1, Pecam1, and VE-cadherin-positive fingers in the lumenized ISVs (Figures 1A, 1B, 1D, 1E, and S1A) and that junctional fingers appear perpendicular to the blood vessel long axis (Figures 1D2, 1D’2, and 1D’’2). Features of junctional fingers, such as their size and number in the ISVs, were comparable for the various endogenous junctional molecules and the different junctional marker lines (Figures S1B–S1E).

Furthermore, junctional fingers in live embryos appeared more prominent and larger compared with those observed in fixed embryos, suggesting that they are sensitive to the loss of blood pressure or fixation. Because cell-cell junctions are physically connected to the actin cytoskeleton, we next tested whether junctional fingers colocalize with mRuby2-UCHD, which labels stabilized actin filaments.

Figure 1. Endothelial junctional fingers are formed by distinct junctional proteins

(A and B) Confocal images of intersegmental blood vessels (ISVs) in the trunk of fixed zebrafish embryos at 32 hpf (A) and 36 hpf (B). (A) Whole-mount immunofluorescence staining using anti-Cdh5 (VE-cadherin) and anti-ZO-1 in Tg(fli1a:EGFP) embryos is shown. Scale bar, 20 μm. (A1–A3) Zoom-in images of the outlined boxes show junctional proteins containing VE-cadherin and ZO-1 (black arrows). Scale bar, 5 μm. (B) Whole-mount immunofluorescence staining using anti-ZO-1 in Tg(fli1a:nls-mCherry;fli1a:pecam1a-EGFP) embryos is shown. Scale bar, 20 μm. (B1–B3) Zoom-in images of the outlined boxes in (B) show the presence of Pecam1 and ZO-1 at junctional fingers between endothelial cells. Scale bar, 5 μm.

(C) Distribution of the mean angle of junctional fingers (n = 17 embryos).

(D and E) Confocal images of ISVs in the trunk of zebrafish embryos at 36 hpf. (D) GFP* signal shows the junctional molecule Pecam1 and RFP* signal marks endothelial cell nuclei in Tg(fli1a:nls-mCherry;fli1a:pecam1a-EGFP) embryos. Scale bar, 20 μm. (E) GFP* signal marks the junctional molecule ZO-1, and RFP* signal indicates endothelial cell nuclei in Tg(fli1a:nls-mCherry;fli1a:gaia4f;UAS:EGFP-ZO-1) embryos. Scale bar, 20 μm. (D1 and E1) Zoom-in images of the outlined boxes in (D) and (E) are shown; black arrows indicate the presence of Pecam1, ZO-1, and Cdh5 at junctional fingers. Scale bars, 5 μm. (D2–D’2) XZ representation of EC junctions in (D’2) or junctional finger in (D’2) is shown.

(F) GFP* signal shows the junctional molecule Pecam1 and RFP* signal marks the F-actin cytoskeleton in Tg(fli1a:gal4ff;UAS:mRuby2-UCHD;fli1a:pecam1a-EGFP) embryos. Scale bar, 20 μm. (F1 and F2) Zoom-in images of the outlined boxes in (F) are shown. Black arrow indicates the presence of F-actin at a junctional finger. Scale bar, 5 μm.

(G) Co-localization analysis (Pearson’s coefficient, r) of Pecam-1 signal and UCHD at fingers (n = 14 embryos).

(H and I) Schematic representation of junctional fingers appearing in an ISV during lumen formation (H, lateral view; I, transverse view).

(J) Schematic representation of junctional fingers at the interface of two endothelial cells.

See also Figure S1.
F-actin fibers (Burkel et al., 2007). Live imaging revealed that Pecam1-positive junctional fingers were also marked by mRuby2-UCHD and that Pecam1 and F-actin partially colocalized at junctional fingers (Figures 1F and 1G). Taken together, this set of experiments indicates that junctional fingers are formed during angiogenic sprouting in the developing ISVs (Figures 1H–1J) and contain multiple junctional proteins, including VE-cadherin, ZO-1, and Pecam1, which can interact with the endothelial F-actin cytoskeleton.

Since junctional fingers are particularly prevalent in newly lumened blood vessels (30–36 hpf), we monitored the appearance of junctional fingers during this time window by in vivo time-lapse imaging using the Pecam1-EGFP reporter. Before ISV lumen opening, we observed the presence of dense junctional clusters but no junctional fingers (Figures 2A–2E, time points - 120 and 0 min; Video S1). By contrast, the junctional fingers readily formed upon lumen opening (black arrows, Figures 2A–2E and 2G, from time point 24 min onwards; Figure S2; Video S2). Over time, the size of the fingers fluctuated, and they eventually regressed once the ISV lumens were connected upon anastomosis, and when mostly stable, linear-oriented junctions were established (red arrows, Figures 2A–2D, 2F, and 2H, time points 48, 72, and 144 min; Figure S2; Video S1). These findings show that junctional fingers are transient structures, which form during lumen formation prior to commencement of blood flow (Figures 2E and S2). When blood flow is established, the junctional fingers disappear (Figures 2F and S2). These correlations suggest a close relationship between hemodynamic forces and junctional finger formation.

Hemodynamic forces control junctional fingers

Previous studies have shown that ISVs can lumene in different ways. Cell membrane invagination leads to the formation of a lumen within a cell (Herwig et al., 2011). This process, also called transcellular lumen formation, is driven by blood pressure and thus requires blood flow (Gebala et al., 2016; Herwig et al., 2011; Lenard et al., 2013). In the absence of blood flow, ISV lumens are formed by cord hollowing, which is driven by endothelial cell rearrangements and gives rise to multicellular tubes (Herwig et al., 2011). Because ISV sprouting is initiated just prior to the start of embryonic blood flow, both modes of lumen formation may occur in these vessels (Gebala et al., 2016; Herwig et al., 2011; reviewed by Phng and Belting, 2021).

Because of the correlation between the occurrence of junctional fingers and lumen formation on one hand and the relationship between blood flow and lumen inflation on the other, we wanted to address to what extent the formation of junctional fingers is dependent on hemodynamic forces. We applied different drug treatments to modulate blood flow in embryos expressing transgenic endothelial reporters for cell-cell junctions (Tg(fli1a: pecam1a-EGFP) and cell membranes (Tg(fli1a:mCherry-CAAX). Tricaine treatment, which reduces cardiac contraction and consequently inhibits blood flow and blood pressure (Lagendijk et al., 2017; Lenard et al., 2013), led to a decrease in size and number of fingers compared with controls (vehicle-treated embryos; Figures 3A, 3B, 3E, 3G, and 3H). By contrast, noradrenaline (NE) treatment, which raises the heart rate and increases the hemodynamic forces in zebrafish blood vessels (Chen et al., 2012; De Luca et al., 2014), increased the size and number of junctional fingers (Figures 3C, 3D, and 3F–3H).

To investigate whether changes in hemodynamic forces directly affect junctional finger dynamics, we next performed sequential treatments. Junctional clusters appeared before lumen opening of the ISV in zebrafish embryos (Figures 4A and 4A1–4A3, time point 0; Video S2), and junctional fingers were formed during ISV lumenezation (Figure 4A, time points 20 and 40 min), confirming our earlier findings (Figure 2). Subsequent treatment of the same embryo with NE to promote blood flow induced junctional clustering and finger formation in the lumenezed ISV (Figures 4B, 4B1–4B3, and 4D–4F, time points 120 and 160 min). Follow-up treatment with tricaine immediately caused lumen regression and destabilized junctional fingers (Figures 4C, 4C1–4C3, and 4D–4F, time points 240–280 min). Administration of the treatments in reverse order showed results consistent with the above observations (Figure 4) and, more specifically, first a regression of fingers in ISVs (upon tricaine addition) and subsequently a re-emergence of fingers (upon noradrenaline addition; Figures S5A–S3D; Video S3). Taken together, these experiments show that junctional fingers form in response to an increase in blood flow or blood pressure and that they represent dynamic structures that directly respond to changes in hemodynamic forces.

Junctional fingers represent regions of elevated junctional tension

The observation that junctional fingers form in response to increased blood pressure suggests that their formation may be

Figure 2. Junctional fingers assemble upon lumen opening

(A–D) Still images from a time-lapse movie of a Tg(kdrl:mCherry-CAAX;fli1a:pecam1a-EGFP) embryo starting at 32 hpf. GFP signal marks the endothelial cell-cell junctions, and mCherry signal demarcates endothelial cell plasma membranes. First two time points (-120 and 0 min) display a representative ISV before lumen opening. The following time points (24–72 min) visualize the ISV after lumen opening, and the last time point (144 min) shows the ISV after anastomosis and establishment of blood flow (see also example 3, Figure S2/C). (A) Overview images are shown. Scale bar, 50 μm. (B) Zoom-in images of the outlined boxes in (A) are shown. Scale bar, 10 μm. (C) Inverted contrast of the Pecam1a-EGFP channel from (B) is shown. Right before lumen opening, junctional accumulation is evident at future sites of junctional fingers. After lumen opening, junctional fingers assemble (black arrows) and regress (red arrows) over time. (D) Inverted contrast of mCherry-CAAX from (B) shows lumen opening. Pink pseudo-color indicates the vascular lumen.

(E and F) Junctional finger dynamics during lumen opening (E) and upon establishment of blood flow (F). Analyses show the finger size ± SE, finger number ± SE, and lumen diameter ± SE from Tg(kdrl:mCherry-CAAX;fli1a:pecam1a-EGFP) embryos (n = 7); pink boxes indicate the time period when lumen opens but without blood flow, during which junctional fingers appear.

(G) Junctional finger analysis at distinct time points before and after lumen opening (~24 and 72 min).

(H) Junctional finger analysis at distinct time points before and after establishment of blood flow (~24 and 168 min).

(G and H) n = 7 embryos; "p < 0.05; **p < 0.01 (one-way ANOVA). See also Figure S2 and Video S1.
triggered by mechanical forces. We therefore reasoned that an acute increase in blood pressure might increase tensile forces exerted on endothelial cell-cell junctions. Previous work in human umbilical vein endothelial cells (HUVECs) has shown that increased tension on the VE-cadherin complex induces unfolding of α-catenin, exposing its cryptic vinculin binding site (Huveneers et al., 2012; Yao et al., 2014). The subsequent junctional recruitment of vinculin reinforces the connection of VE-cadherin with the actin cytoskeleton and protects junctions during their force-dependent remodeling (Huveneers et al., 2012). Since junctional localization of vinculin corresponds to sites of increased tension, we wanted to test whether vinculin is localized at junctional fingers in ISVs. To achieve this, we transiently expressed an mCherry-vinculin fusion protein (kdrt:mCherry-vinculin) in the endothelium of transgenic fli1a:pecam1a-EGFP embryos. Using live imaging, we observed that vinculin localizes at the junctional fingers (black arrows, Figures 5A and 5B). Moreover, time-lapse imaging showed that vinculin localization is dynamic and follows the turnover of the Pecam1-positive junctional fingers. Although the majority of vinculin was distributed in the cytoplasm, upon formation of junctional fingers, vinculin become enriched at these structures and was most prominent once junctional fingers had reached their maximum size (Figures 5B and SC; Video S4). This close correlation between junctional finger formation and vinculin accumulation indicates that junctional fingers represent regions of elevated tension at the junctional interface. Furthermore, these observations suggest that vinculin may play a role in the formation and/or function of junctional fingers.

Vinculin is required for ISV morphogenesis

To investigate whether vinculin plays a role in endothelial junctional finger formation, we took a genetic loss-of-function approach. The zebrafish genome contains two vinculin paralogs, vinculin a (vcl) and vinculin b (vclb), which share high-sequence homology with their mammalian orthologs, including human VCL (87% and 86% identical amino acids, respectively; Han et al., 2017). In addition, both vcl and vclb paralogs are expressed in endothelial cells in zebrafish (Figure S4A; Lawson et al., 2020). Zebrafish loss-of-function mutants for vcl and vclb have previously been published (Fukuda et al., 2019; Cheng et al., 2016; El-Brolosy et al., 2019; Han et al., 2017).

To investigate the consequence of vinculin depletion for sprouting angiogenesis, we employed the vcl knockout models generated by Han et al. (2017; Figures S4B–S4D) and crossed the vclb-a10815 homozygous; vclb-a12202 heterozygous (vcl-ba/-; vclb-a/-) fish into the Tg(fli1a:EGFP) vascular reporter line. Live imaging of the offspring revealed vascular defects in angio-
genic ISV sprouts of vcl double-knockout (vcl-a/-; vclb-a/-) and vcl heterozygous (vcl-a+/--; vclb-a+/+) embryos from 28 hpf onwards (Figures S5 and S6; Videos S5 and S6). In wild-type (vcl-a+/+; vclb-a+/+) embryos, ISVs sprouted out of the dorsal aorta (DA) from and anastomosed once tip cells reached the dorsolateral roof of the neural tube (Figures S5A and S5A1). In contrast, vclb heterozygous (vcl-a+/--; vclb-a+/+; Figures S5B and S5B1) and vcl double-knockout (vcl-a/-; vclb-a/-) embryos (Figures S5C and S5C1) showed a retardation in ISV sprouting evidenced by a decreased ISV length and an increased ISV diameter at 28 hpf (Figures S5D–S5F, S6F, and S6G; Videos S5 and S6). Furthermore, we observed that increasing loss of vcl in wild-type embryo (Figures S5–S5F, S6A–S6C, S6F, and S6G). Nonetheless, vcl mutants formed a normal number of tip and stalk cells compared with wild-type embryos (Figures S5A–S5C and S6A–S6C). Furthermore, angiogenesis and anastomosis were completed in vcl/a/b mutant zebrafish (Figures S6A1–S6C1), ISVs were lumened, and blood flow was established in vcl double knockout at 36 hpf (Figures S7A–S7D; Video S7). In addition, no significant differences in heart rate or blood flow velocity between wild-type and vcl mutant embryos were observed (Figures S7E and S7F).

Overall, these data indicate that vinculin deletion results in delayed sprouting angiogenesis; however, functional blood vessels are able to form.

Endothelial vinculin is required and sufficient for the formation of junctional fingers

To assess whether vcl/a/b is required for the formation of junctional fingers, we imaged endogenous VE-cadherin in vinculin mutants expressing the reporter line Tg(fli1a:lifeact-EGFP). In vcl/a/b double heterozygotes (vcl-a+/--; vclb-a+/+), we observed a significant decrease in both the size and number of the junctional fingers upon lumen opening when compared with wild-type embryos (vcl-a+/+; vclb-a+/+; Figures 5D–5E1, 5H, and 5I). Homozygous loss of either vcl or vclb (vcl-a/-; vclb-a/- or vcl-a/-; vclb-a/-) led to a strong reduction of junctional fingers (Figures 5F, 5F1, 5H, and 5I), an effect that was even more pronounced in vcl double mutants (vcl-a/-; vclb-a/-; Figures 5G, 5G1, 5H, and 5I). Moreover, we observed gradual differences in the level of junctional finger size and number in the various genotypes corresponding with the number of vcl/a/b mutant alleles (Figures 5H and 5I).

Live imaging of vcl mutants expressing Pecam1-EGFP (Figures 5J–5N) confirmed the above observations, as deletion of the vinculin paralogs led to a reduction in finger formation in double-heterozygous and in double-knockout mutants (vcl-a/-; vclb-a/-; Figures 5J–5N).

Figure 3. Alterations in blood flow affect finger size and number

(A–D) Confocal images of representative ISVs from Tg(kdrt:mCherry-CAAX;fli1a:pecam1a-EGFP) embryos at 36 hpf before tricaine treatment (A), after treatment with 0.32% tricaine (B), before norepinephrine treatment (C), or after treatment with 60 μM norepinephrine (D). GFP+ signal marks Pecam1, and RFP+ signal marks the cell membrane. Before pharmacological additions, junctional fingers are evident (A and C, black arrows), and upon tricaine treatment, they regress (B, red arrows), while upon norepinephrine treatment, more fingers are present or they are reinforced (D, black arrows). Scale bar, 10 μm.

(E and F) Line graphs showing the junctional finger size ± SE, finger number ± SE, and lumen diameter ± SE over time after treatment with (E) tricaine or (F) norepinephrine from Tg(kdrt:mCherry-CAAX;fli1a:pecam1a-EGFP) embryos (n = 17, E, tricaine; n = 10, F, norepinephrine).

(G) Average finger size per ISV in non-treated or tricaine- or norepinephrine-treated embryos; *p < 0.05 (one-way ANOVA with Dunnett’s post-test; n = 11 ISVs from eight embryos).

(H) Number of fingers per ISV in non-treated or tricaine- or norepinephrine-treated embryos; *p < 0.05; **p < 0.001 (one-way ANOVA with Dunnett’s post-test; n = 11 ISVs from eight embryos).
Figure 4. Hemodynamic forces control junctional fingers

(A–C) Stills of time-lapse confocal imaging from a representative 36 hpf Tg(kdrl:mCherry-CAAX;fli1a:pecam1a-EGFP) embryo before treatment (A) and after sequential additions of first norepinephrine (B) and then tricaine (C). GFP* signal indicates the junctional marker Pecam-1, and RFP* signal marks the endothelial cell membrane. Scale bar, 20 μm. (A1–C1) Zoom-in images of the outlined boxes in (A)–(C) are shown. Scale bar, 10 μm. (A2–C2) Inverted contrast images of Pecam1-EGFP signal of (A1)–(C1) are shown. (A2) Before lumen opening, no junctional fingers are present; after lumen opening, Pecam1-based junctional fingers assemble (black arrows) and over time undergo regression (red arrows). (B2) Upon norepinephrine treatment, Pecam1 assembles into enlarged junctional fingers (black arrows). (C2) After tricaine treatment, remaining Pecam1-EGFP-positive fingers (black arrows) disappear over time (red arrows). (A3–C3) Inverted contrast images of mCherry-CAAX of (A1)–(C1) are shown. Pink pseudo-colored areas indicate open lumen in the ISV. (B3) After norepinephrine addition, lumen expands. (C3) Treatment with tricaine results in lumen compartmentalization and lumen collapse.

(D) Junctional dynamics analysis of junctional finger size ± SE, finger number ± SE, and lumen diameter ± SE over time before treatment (0–40 min), after treatment with norepinephrine (40–220 min), and after treatment with tricaine (240–400 min) from Tg(kdrl:mCherry-CAAX;fli1a:pecam1a-EGFP) embryos (n = 6).

(E and F) Average finger size per ISV (E) and number of fingers per ISV (F) at distinct time points before treatments (20 min), after norepinephrine treatment (60 and 220 min), and after tricaine treatment (340 min); *p < 0.05; **p < 0.01 (one-way ANOVA with Dunnett’s post-test; n = 6 embryos).

See also Figure S3 and Video S2.
Overall, loss of vclb caused stronger defects than mutations vcla, indicating a higher requirement for vclb than vcla. In particular, we observed stronger phenotypes in vcla+/−;vclb−/− than in vcla−/−;vclb−/− mutants (Figures 5H and 5I). Nevertheless, vcla−/−;vclb−/− show stronger phenotypes than double heterozygotes. Thus, both isoforms are involved in ISV formation, with a more pronounced role for vclb. Analysis of transcriptome data (Lawson et al., 2020) revealed that vclb expression, in contrast to vcla, is elevated in endothelial cells compared with non-endothelial cells (Figure S4A). This may account for a slightly stronger mutant phenotype in vcla−/−; vclb−/− compared with vcla−/−;vclb−/− mutants (Figures 5H and 5I).

Interaction between junctional proteins and the underlying actin cytoskeleton is essential endothelial maintenance and dynamic endothelial cell behaviors (reviewed by Abu Taha and Schnittler, 2014; Phng, 2018). Because vinculin provides a structural link to anchor actin to VE-cadherin, we wanted to test whether changes in the actin cytoskeleton may be responsible for defects in junctional finger formation in vcl mutants. However, analysis of Lifeact-EGFP did not reveal any obvious defects in the actin cytoskeleton of vcl mutants (Figures 5D–5G and S6H–S6J). We have previously shown that VE-cadherin is required to maintain cortical actin organization during ISV sprouting as well as for junctional elongation during endothelial cell rearrangements (Sauter et al., 2014). Moreover, junctional elongation also requires actin polymerization (Sauter et al., 2014). To test the effect of actin polymerization inhibition on finger formation, we treated embryos with latrunculin B (Figures S8A–S8F). We found that a concentration of 0.15 μM did not perturb the emergence of junctional fingers (Figures S8A–S8B), which phenotypically resemble the junctions in wild-type control group (Figures 5M and 5N). In contrast, and consistent with our earlier results, vcl double-heterozygous (vcla+/−; vclb−/−) embryos displayed reduced junctional fingers in mCherry-vinculin-negative ISVs (Figures 6B and 6B1), whereas the ISVs expressing mCherry-vinculin formed junctional fingers with their neighboring endothelial cells (Figures 6B2–6B2'). Moreover, mCherry-vinculin expression rescued junctional finger formation in homozygous vclb mutants (vcla+/−; vclb−/−), which otherwise did not form junctional fingers at all (Figures 6C–6C2'). Taken together, these results clearly show that vinculin is essential for the formation of junctional fingers and that expression of vinculin within the endothelium is sufficient to promote finger formation in a vcl-knockout background.

**Increase in blood pressure does not rescue the loss of junctional fingers in vinculin double knockouts**

As shown above, the formation of junctional fingers is triggered by an increase in blood pressure. We therefore hypothesized that loss of vinculin may reduce the junctional response to hemodynamic forces and that this reduced sensitivity may be overcome by an increase in blood pressure. To test this possibility, we treated vcl double-knockout embryos with NE to increase blood pressure at the onset of ISV lumen inflation. Notably, NE treatments did not increase the formation of junctional fingers in vcl double-heterozygous (vcla+/−; vclb−/−) or homozygous (vcla+/−; vclb−/−) mutants, in contrast to single heterozygotes (vcla+/−; vclb+/−), in which NE was able to enhance junctional fingers (Figures 6E–6I). Thus, in vcl homozygous mutants, endothelial cells appear unresponsive to increased blood pressure. Furthermore, the effect was most pronounced in vclb knockouts.

**Figure 5. Vinculin is required for the formation of junctional fingers**

(A and B) Stills of time-lapse imaging of Tg(fli1a:pecam1a-EGFP) embryos at 36 hpf with transient expression of injected kdr:mCherry-vinculin (vcl). Scale bar, 10 μm. (A1 and B1) Inverted contrast images of mCherry-vcl of (A) and (B) are shown. Black arrows point to co-localization of vinculin and Pecam1. (A2 and B2) Anti-Cdh5 of (D)–(G) are shown.

(H and I) Average finger size per ISV (H) and finger number per ISV (I) of wild-type (vcla+/−; vclb+/−), double-heterozygous (vcla+/−; vclb−/−), vcl homozygous knockout (vcla−/−; vclb−/−), vclb homozygous mutant (vcla+/−; vclb−/−), or double-knockout (vcla−/−; vclb−/−) embryos; n = 19 (vcla+/−; vclb+/−), 11 (vcla−/−; vclb+/−), 22 (vcla+/−; vclb−/−), 16 (vcla−/−; vclb−/−), and 7 (vcla−/−; vclb−/−) embryos; ns, non-significant; *p < 0.05; **p < 0.01; ***p < 0.001 (one-way ANOVA and Dunnett’s post-test).

(J–L) Confocal images of ISVs from live Tg(kdr:mCherry-CAAX;fli1a:pecam1a-EGFP) embryos at 36 hpf of (J) wild-type (vcla+/−; vclb+/−), (K) double-heterozygous (vcla+/−; vclb−/−), or (L) vclb homozygous (vcla+/−; vclb−/−). Scale bar, 5 μm. (J–L) Inverted contrast images of mCherry-CAAX of (J–L) are shown. Black arrows indicate junctional fingers; note the absence of junctional fingers in vclb homozygous knockouts.

(M and N) Average finger size per ISV (M) and finger number per ISV (N) of wild-type (vcla+/−; vclb+/−), single-heterozygous (vcla+/−; vclb−/−), double-heterozygous (vcla−/−; vclb+/−), or vclb homozygous knockout (vcla+/−; vclb−/−) embryos; n = 4 (vcla+/−; vclb+/−), 9 (vcla−/−; vclb−/−), 22 (vcla+/−; vclb−/−), and 12 (vcla−/−; vclb−/−) embryos; *p < 0.01; **p < 0.001 (one-way ANOVA and Dunnett’s post-test).

See also Videos S3 and S4.
whereas combinations of mutant and wild-type alleles displayed an intermediate phenotype (Figure 6l), supporting the notion of a dose-dependent requirement of vinculin in this process. Taken together, these results point to a mechanism in which vinculin recruitment acts as an adaptive response to elevated junctional tension during angiogenic sprouting.

**DISCUSSION**

Dynamic junctional remodeling is essential for blood vessel formation, maintenance, and function. Here, we describe the formation of junctional fingers as a novel trait of junctional remodeling that occurs during lumen formation of developing ISVs in the zebrafish embryo. Junctional fingers appear as junctional folds of up to 3 μm, which are oriented perpendicular to the originating junction and show a characteristic dynamic behavior. Junctional fingers are formed upon the intersection between the dorsal aorta and the sprouting ISV, which triggers exposure of sprouting endothelial cells to luminal pressure. However, junctional fingers disappear once ISVs have achieved patency and blood flow is firmly established. This specific response to blood pressure renders junctional fingers as a novel in vivo paradigm for the study of junctional dynamics in response to extrinsic forces.

Our studies demonstrate that formation of junctional fingers relies on the recruitment of vinculin and thus point to an interconnection between junctional tension, junctional remodeling, and blood vessel morphogenesis. Recent studies have established distinct roles for vclα and vclβ during zebrafish heart development, which reflect their expression in the myocardium and epicardium, respectively (Cheng et al., 2016; Fukuda et al., 2018; Han et al., 2017). In contrast, vclα and vclβ are both expressed in the zebrafish endothelium (Lawson et al., 2020), with vclβ being slightly higher expressed in endothelial cells than vclα (Figure S4A; Lawson et al., 2020). Correspondingly, we notice phenotypic variation in different mutant allele combinations (e.g., vclα+/−;vclβ+/− versus vclα+/−;vclβ−/−), showing a higher requirement for vclβ than vclα. Nevertheless, different genotypes display different phenotypic strength during ISV sprouting and junctional finger formation, largely depending on the number of vclα/b mutant alleles present. This indicates that the two proteins have redundant functions during blood vessel formation and that their requirement is highly dose dependent.

Dynamic changes in junctional organization have been studied previously in different contexts. In particular, perpendicular-oriented endothelial cell-cell junctions have been described as serrated junctions, discontinuous junctions, focal adherens junctions (FAJs), and VE-cadherin fingers (Bentley et al., 2014; Hayer et al., 2016; Huveneers et al., 2012). Serrated and discontinuous junctions have been associated with active or remodeling junctions (Bentley et al., 2014). The junctional fingers we describe here appear as invaginations of stable and continuous junctions and are morphologically similar to VE-cadherin fingers and FAJs that have been previously described in HUVECs (Huveneers et al., 2012; Dorland and Huveneers, 2016; Hayer et al., 2016). However, because of the presence of Pecam1 and other junctional components (Z01, VE-cadherin, and F-actin), we call them “junctional fingers” rather than “VE-cadherin fingers” in the current study.

VE-cadherin fingers and FAJs are involved in mechanical coupling between endothelial cells and are controlled by actomyosin contractility (Huveneers et al., 2012; Dorland and Huveneers, 2016; Hayer et al., 2016). In agreement with this, junctional fingers form when endothelial sprouts encounter blood pressure. Modulation of blood flow by pharmacological intervention leads to a graded response, with more and longer junctional fingers correlating with higher blood pressure, suggesting that junctional fingers are an adaptive response of endothelial cells to increased mechanical stress. In fact, the specific localization of vinculin to junctional fingers indicates that they are experiencing elevated junctional tension on the VE-cadherin-catenin complex. Our genetic analyses show that vinculin is required for the formation of junctional fingers and mosaic expression in sprouting endothelial cells can rescue defects arising from partial vinculin loss of function or complete vinculin knockout. Our previous cell culture experiments have shown that tension-induced vinculin localization to FAJs protects these junctions against mechanical stress (Huveneers et al., 2012). Our in vivo observations suggest that vinculin may have a dual role during vascular lumen formation. First, a local increase in junctional tension triggers recruitment of vinculin to the endothelial junction, which subsequently leads to the formation of junctional fingers. Alternatively, vinculin may become localized.

**Figure 6. Increase in blood pressure does not rescue the loss of junctional fingers in vinculin mutants**

(A–C) Confocal images of 36 hpf Tg(kdrl:mCherry-CAAX;fli1a:pecam1a-EGFP) embryos injected with kdr:mCherry-vinculin with genotypes (A) single-heterozygous mutant (vclα+/−;vclβ−/−), (B) double-heterozygous mutant (vclα+/−;vclβ+/−), or (C) vclβ homozygous mutant (vclα+/−;vclβ−/−). Scale bar, 20 μm. (A1–C1) Zoom-in inverted contrast images of fil1a:pecam1a-EGFP of the insets in (A–C) of an ISV without kdr:mCherry-vinculin expression (internal control) are shown. Scale bar, 20 μm. (A2–C2) Zoom-in inverted contrast images of fil1a:pecam1a-EGFP of the insets in (A–C) of an kdr:mCherry-vinculin expressing ISV are shown. (A2–C2) Merged image of (A2–C2) is shown. Arrows indicate junctional fingers.

(D) Average finger size per ISV ± SE of vinculin single-heterozygous mutant (vclα+/−;vclβ+/−), double-heterozygous mutant (vclα+/−;vclβ−/−), or vclβ homozygous mutant (vclα+/−;vclβ−/−) embryos with or without kdr:mCherry-vinculin expression (n = 2 [vclα+/−;vclβ−/−], 3 [vclα+/−;vclβ−/−], and 5 [vclα+/−;vclβ−/−] embryos); ***p < 0.001 (one-way ANOVA with Dunnett’s post-test).

(E–H) Confocal images of ISVs from Tg(kdrl:mCherry-CAAX;fil1a:pecam1a-EGFP) embryos at 36 hpf with genotypes (E) wild-type (vclα+/vclβ+/+), (F) single-heterozygous mutant (vclα+/−;vclβ−/−), (G) double-heterozygous mutant (vclα+/−;vclβ−/−), or (H) vclβ homozygous mutant (vclα+/−;vclβ−/−) after treatment with 60 μM norepinephrine (NE). GFP* signal marks Pecam-1, and RFP* signal indicates the cell membrane. Scale bar, 10 μm. (E1–H1) Inverted contrast images of Pecam1-EGFP of the outlined boxes in (E) are shown. Black arrows indicate junctional fingers. Note the absence of junctional fingers in vclβ homozygous knockouts. (E2–H2) Inverted contrast images of mCherry-CAAX of the outlined boxes in (E–H) are shown.

(l) Average finger size per ISV after NE treatment of the genotypes wild-type (vclα+/vclβ+/+), single-heterozygous mutant (vclα+/−;vclβ−/−), double-heterozygous mutant (vclα+/−;vclβ−/−), or vclβ homozygous mutant (vclα+/−;vclβ−/−); n = 7 [vclα+/−;vclβ−/−], 10 [vclα+/−;vclβ−/−], 17 [vclα+/−;vclβ−/−], and 7 [vclα+/−;vclβ−/−] ISVs; ***p < 0.001 (one-way ANOVA with Dunnett’s post-test).
upon junctional finger formation. In agreement with our study, recruitment of vinculin-a to myocardial cell-cell junctions depends on the mechanical force generated by myocardial contractility (Fukuda et al., 2019). Here, vinculin recruits actin regulators and promotes myofiber maturation. It will be of interest to discern the molecular pathways that regulate vinculin recruitment and function during vascular lumen expansion. It has recently been shown that vinculin is essential for angiogenesis in the postnatal mouse retina and that loss of vinculin leads to increased blood vessel regression downstream of non-canonical Wnt signaling (Carvalho et al., 2019). In this context, shear stress promotes VE-cadherin localization of vinculin in a Wnt5a-dependent manner. In contrast to vessel regression, which is regulated by blood-flow-mediated shear stress (reviewed by Korn and Augustin, 2015), initial lumen expansion in the ISV is driven by blood pressure, which induces circumferential stretch (reviewed by Hoefer et al., 2013). Therefore, hemodynamic forces, shear stress and circumferential stretch, promote localization of vinculin to endothelial cell-cell junctions.

Junctional fingers show a dynamic behavior. They form during lumen expansion in sprouting vessels but regress once blood flow is established. A likely scenario to explain these findings is that lumen inflation increases junctional tension, which is subsequently relieved by the commencement of blood flow. Further studies will be required to discern whether shear stress influences junctional finger maintenance or regression. However, we did not detect junctional fingers in the dorsal aorta or the posterior cardinal vein. These blood vessels are formed by vasculogenesis, which is likely to preclude junctional finger formation because of different hemodynamic conditions.

During vascular morphogenesis as well as in functional blood vessels, endothelial cells generate dynamic junctional protrusions, called junction-based lamellipodia (JBL) or junction-associated intermittent lamellipodia (JAIL), which have been observed in different vascular beds of zebrafish embryo as well as mouse embryos and the postnatal retina (reviewed by Cao and Schnittler, 2019; Fonseca et al., 2020). Live imaging of JBL showed that they are highly dynamic oscillatory structures and are associated with cell movements within the endothelium in zebrafish embryos (Pastero et al., 2018; reviewed by Okuda and Hogan, 2020). Thus, JBL form in regions of high junctional turnover and reduced junctional stability. In contrast, the junctional fingers are more stable and contribute to the formation of mature vascular structures.
fingers we describe here are transient but do not oscillate. Their dynamic behavior and association with vinculin indicate that they are strengthened junctions, which support junctional stability to maintain blood vessel integrity in response to sudden changes in extrinsic forces.

In conclusion, our results support a model (Figure 7) in which endothelial cell–cell junctions form finger-like invaginations during vascular lumen expansion in response to junctional stretch induced by an increase of blood pressure. Junctional fingers recruit vinculin and rely on it for their formation and stabilization. Taken together, junctional fingers represent a novel paradigm to study endothelial cell dynamics and junctional remodeling in response to mechanical forces during blood vessel morphogenesis in vivo.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.110658.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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Transplantation and in vivo imaging of multilaminate engraftment in zebrafish


## STAR METHODS

### KEY RESOURCES TABLE

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RESOURCE AVAILABILITY

Lead contact
Further information and request for resources and reagents should be directed and will be fulfilled by the Lead Contact; Heinz-Georg Belting (heinz-georg.belting@unibas.ch).

Materials availability
Materials and zebrafish lines generated and used in this study are available upon request to the lead contact.

Data and code availability
- Microscopy data reported in this paper will be shared by the lead contact upon request.
- All original code for blood flow measurements has been deposited at github (https://github.com/Jianmin-YIN/Blood-flow-measurement) and is publicly available. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish lines and maintenance
Zebrafish were maintained in standard conditions (Alestrom et al., 2019). All experiments were performed in accordance with federal guidance and approved by the Kantonales Veterinäramt of Kanton Basel-Stadt. The vclbhu10818;vclbh11202 (Han et al., 2017) zebrafish lines were crossed into the following transgenic lines: Tg(fli1a:EGFP)ku1 (Lawson and Weinstein, 2002); Tg(gata1a:dsRed)sd2 (Traver et al., 2003); Tg(fli1a:pecam1a-EGFP)ucv27 (Ando et al., 2016); Tg(fli1a:GFF)ub3 and Tg(UAS:EGFP-hZO1)ubs3 (Herwig et al., 2011); Tg(UAS:mRuby2-UCHD)ubs20 (Paatero et al., 2018); Tg(kdrl:EGFP-nls)ubs1 (Blum et al., 2008); Tg(kdrl:mCherry-CAAX)ubs3 (Hogan et al., 2009); Tg(fli1a:Lifeact-EGFP) (Phng et al., 2013), TgBAC(ve-cad:ve-cadTS)unn (Lagendijk et al., 2017).

Experiments were performed between 24 h postfertilization (hpf) and 48 hpf. Before imaging, embryos were selected for fluorescent signal. After imaging of the vinculin mutants, genotype was assessed by DNA extraction and PCR.

METHOD DETAILS

Genotyping of the vcl mutant lines
Genomic DNA from fin biopsies from adult fish or whole embryos was extracted by incubating the biopsies with NaOH at a final concentration of 50 mM and incubated for 10 min at 95 °C. Subsequently, 1/10th of 1M Tris HCl pH 8 was added (Meeker et al., 2007). Finally, samples were treated with proteinase K for 1 h at 37 °C and then for 5 min at 95 °C to stop the proteinase K activity. Genomic DNA was then used to genotype the vcl and vclb loci. For genotyping analysis, four different primers were used to distinguish between wild-type, heterozygous or homozygous vcl or vclb alleles. For vcl, a combination of four primers (FP 5'-AGCTGATGATCTGACCTGGTG-3', RP 5'-TCGGTTCAATCTGTGACACAG-3', Fwd-wt 5'-ACCATGGAGGACTTGTACACT-3', Rev-mut 5'--GGTCCAGGTTTGTAGTAGT-3') and a PCR protocol including denaturation at 95 °C for 30s, annealing at 52 °C for 15s, extension at 68 °C for 50s, 35 cycles and OneTaq DNA polymerase (NEB), generated PCR products of 497 bp and 203 bp for the wild-type allele, three PCR products of 497 bp, 282 bp and 203 bp for the heterozygous vcl mutants and for two PCR products of 497 bp and...
282 bp for vcla homozygous mutants. For vclb, a combination of four primers (FP 5’-CGGTAGTGTGTGAGGGA GTC-3’, RP 5’-AATGAGAAAGCCTGAATGCG-3’, Rev-wt 5’-GCATGATCACCAGATGGG-3’, Fwd-mut 5’-CCCAGCTCTGG TGAT-3’) and a PCR protocol, including denaturation at 95°C for 30s, annealing at 49°C for 15s, extension at 68°C for 50s, 35 cycles and OneTag DNA polymerase (NEB), generated two PCR products of 490 bp and 201 bp for the wild-type allele, PCR products of 490 bp, 319 bp and 201 for the heterozygous vclb mutant allele and PCR products of 490 bp and 319 bp for homozygous vclb mutant allele. PCR products were then loaded on a 1% agarose gel to assess the genotype (see Figures S4D and S4E).

Generation of kdrl:mCherry-vinculin expression plasmid
The kdrl promoter (Genbank accession number: AY045468) was subcloned in the tol2 transposon plasmid pDB739 (Balciunas et al., 2006) using the restriction sites BamHI and EcoRV. The N-terminal tagged mCherry-vinculin open reading frame (Twiss et al., 2012) was then inserted into the EcoRV restriction site adjacent to the kdrl promoter.

Embryo microinjections
For transient expression of the kdrl:mCherry-vinculin, plasmid DNA was injected into Tg(fli1a:pecam1a-EGFP) or vclb into embryos using a standard microinjection protocol (Lenard et al., 2013). Briefly, 1- to 2-cell stage embryos were placed on agarose matrices in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, pH 7.4) containing 5ppm methylene blue (Sigma) and were injected using glass microcapillaries (Biomedical instruments). Upon PBST washes, embryos were incubated with secondary antibodies, Alexa Fluor 405 goat anti-rabbit IgG (Thermo Fisher Scientific), Alexa Fluor 568 goat anti-guinea pig IgG (Thermo Fisher Scientific). Zebrafish embryos were fixed in 2% PFA in PBST (0.2% tween in PBS) and incubated overnight at 4°C in 1% BSA, 0.2% Triton, 5% Goat Serum, 0.01% sodium azide in PBST overnight at 4°C. Embryos were stained with mouse-anti-human-ZO-1 1:500 (Thermo Fisher Scientific, 33-9100), guinea pig-anti-zebrafish-VE-cadherin 1:500 (Paatero et al., 2018) or mouse anti-vinculin 1:500 antibodies (Sigma, V9131, Huveneers et al., 2012) diluted in Pierce Immunostain Enhancer medium (Thermo Fisher Scientific). Upon PBST washes, embryos were incubated with secondary antibodies, Alexa Fluor 405 goat anti-mouse immunoglobulin (IgG) 1:500 (Thermo Fisher Scientific), Alexa Fluor 568 goat anti-guinea pig IgG 1:500 (Thermo Fisher Scientific) or Alexa Fluor 405 goat anti-rabbit IgG 1:500 (Thermo Fisher Scientific).

Immunofluorescence
Immunofluorescence staining of embryos was performed as previously described (Herwig et al., 2011) with few modifications. Briefly, embryos were fixed in 2% PFA in PBST (0.2% tween in PBS) and incubated overnight at 4°C. Embryos were washed in PBST and consecutively permeabilized in 0.5% Triton/PBST for 30-45 min at room temperature, and subsequently incubated in blocking solution (1% BSA, 0.2% Triton, 5% Goat Serum, 0.01% sodium azide in PBST) overnight at 4°C. Embryos were stained with mouse-anti-human-ZO-1 1:500 (Thermo Fisher Scientific, 33-9100), guinea pig-anti-zebrafish-VE-cadherin 1:500 (Paatero et al., 2018) or mouse anti-vinculin 1:500 antibodies (Sigma, V9131, Huveneers et al., 2012) diluted in Pierce Immunostain Enhancer medium (Thermo Fisher Scientific). Upon PBST washes, embryos were incubated with secondary antibodies, Alexa Fluor 405 goat anti-mouse immunoglobulin (IgG) 1:500 (Thermo Fisher Scientific), Alexa Fluor 568 goat anti-guinea pig IgG 1:500 (Thermo Fisher Scientific) or Alexa Fluor 405 goat anti-rabbit IgG 1:500 (Thermo Fisher Scientific).

Pharmacological treatments
To reduce heartbeat and inhibit blood circulation, we treated zebrafish embryos with 4x tricaine (0.32%, Sigma) in E3 embryo medium with 0.003% PTU (Sigma), as previously described (Lenard et al., 2013). To increase heartbeat and blood flow, we treated zebrafish embryos with 60 µM norepinephrine (Sigma) in E3 containing 0.003% PTU, as previously described (Chen et al., 2012; De Luca et al., 2014). Treatment of tricaine or norepinephrine started directly during imaging as indicated in the corresponding figures at 30-32 hpf. To perturb the actin cytoskeleton, embryos were treated with Latrunculin B (Sigma) using concentrations between 0.15 µg/mL to 0.6 µg/mL. A concentration of 0.15 µg/mL is sufficient to disrupt filopodia formation as previously described (Phng et al., 2013).

Imaging and image analysis
Fixed or live embryos were selected for fluorescence signal, anesthetized in E3 with 1x tricaine (0.08%, pH 7, Sigma) and mounted in glass bottom Petri dishes (MatTek) using 0.7% low-melting-point agarose (Sigma) containing 1x tricaine. For live imaging, E3 with 1x tricaine and 0.003% 1-phenyl-2-thiourea (PTU, Sigma) to avoid pigmentation, was added to the dish. A Zeiss LSM880 Airy scan inverted confocal microscope was used for live-imaging and imaging of fixed samples. For fixed samples, the 40x (NA = 1.2) silicon oil immersion objective was used. Z-stacks were made with a step size of 0.5 µm. For live imaging, the 25x oil (NA = 0.8) or 40x silicon oil (NA = 1.2) immersion objectives were used. Images were acquired with a zoom of 1-1.6 and z-stacks were made with a step size of 0.5–1.0 µm. Frames were acquired every 5-30 min. High-resolution time-lapse images were acquired with an Olympus SpinSR spinning disc microscope using a 30x (NA = 1.05) oil objective (Photometrics). Frames were acquired every 1-2 min with a z stack step size of 0.7 µm. Confocal and Spinning disc images were analyzed using the Fiji/ImageJ software. For heartbeat and blood flow velocity, high-speed image acquisition was achieved using an iPhone 7 Camera at 120fps in conjunction with a Labcam adaptor connected to a Zeiss stereomicroscope (Stemi SV 11). Then velocity of blood flow was measured by tracing the movement of blood cells at the dorsal aorta using Particle Image Velocimetry (PIV) in combination with a homemade MATLAB code. The heart beat cycle was determined by the periodical oscillation of blood flow velocity at the dorsal aorta.
QUANTIFICATION AND STATISTICAL ANALYSIS

For analysis of blood vessel length, an average of three measurements was taken for each ISV and for analysis of blood vessel diameter, an average of three measurements was taken at different levels of each ISV. For analysis of the finger number, averages were taken for each ISV and junctional finger size was traced in three measurements per finger and averaged per ISV. For co-localization analysis, we subtracted the background (rolling ball radius 25) and selected regions of interests at junctional fingers. Co-localization analysis was performed using the Fiji software with the JACoP plugin. For gene expression levels of vcla, vclb and ve-cadherin in ECs versus non-ECs, analysis was performed using the transcriptome dataset from Lawson et al. (Lawson et al., 2020). Microsoft Excel was used for data analysis and Prism Graphpad V6 was used for statistical analysis and data visualization. Statistical parameters are reported in the figure legends. Violin plots represent median ± quartiles. When two groups were compared, a Student’s T and one-way ANOVA (Kruskal-Wallis) tests were used. An analysis of variance (ANOVA) was used when more than two groups were compared to the control, in combination with a Dunnett’s test for multiple comparisons and a D’Agostino-Pearson test for normality. Asterisks indicate p values, and are defined as ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001.