Distinct and redundant functions of Esam and VE-cadherin during vascular morphogenesis

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Summary:
Genetic analyses of Esama function in esama single and esama/ve-cad double mutant zebrafish embryos reveal specific and redundant functions of both proteins in endothelial cell recognition and blood vessel morphogenesis.

Abstract:
The cardiovascular system forms during early embryogenesis and adapts to embryonic growth by sprouting angiogenesis and vascular remodeling. These processes require fine-tuning of cell-cell adhesion to maintain and reestablish endothelial contacts, while allowing cell motility. We have compared the contribution of two endothelial cell specific adhesion proteins - VE-cadherin (VE-cad/Cdh5) and Esama (Endothelial cell-selective adhesion molecule a) - during angiogenic sprouting and blood vessel fusion (anastomosis) in the zebrafish embryo by genetic analyses. Different combinations of mutant alleles can be placed into a phenotypic series with increasing defects in filopodial contact formation. Contact formation in esama mutants appear wild-type like, while esama+/ ve-cad+ and ve-cad single mutants exhibit intermediate phenotypes. The lack of both proteins interrupts filopodial interaction completely. Furthermore, double mutants do not form a stable endothelial monolayer, display intrajunctional gaps, dislocalization of Zo-1 and defects in apical-basal polarization. In summary, VE-cadherin and Esama have distinct and redundant functions during blood vessel morphogenesis and both adhesion proteins are central to endothelial cell recognition during anastomosis.
Introduction:

Establishment and expansion of vascular beds is essential for embryonic development and growth. Vascular networks are formed by the outgrowth of new blood vessels in a process called sprouting angiogenesis and their subsequent connection to another vessel or sprout by anastomosis. At the cellular level, sprouting angiogenesis and anastomosis are characterized by a series of dynamic behaviors, which are tightly coordinated (reviewed in Betz et al., 2016; Wacker and Gerhardt, 2011). In response to pro-angiogenic signals, endothelial cells (ECs) are activated. They remodel their actin cytoskeletons and down-regulate their junctional adhesion molecules thereby permitting cell motility, while maintaining cell contacts to each other and to the parental blood vessel. Thus, fine-tuning the cellular responses to angiogenic cues is essential to maintain vascular integrity and apical-basal cell polarization during angiogenic sprouting and anastomosis.

The cellular mechanisms, which underlie blood vessel morphogenesis have been analyzed in detail by in vivo time-lapse microscopy in the zebrafish embryo (reviewed by Betz et al., 2016; Schuermann et al., 2014). During the formation of segmental arteries (SeAs), two or more cells emigrate from the dorsal aorta (DA) and form an angiogenic sprout. This sprout grows out to the dorsal side of the embryo (Blum et al., 2008; Siekmann and Lawson, 2007), where the tips of two adjacent sprouts interconnect and form the dorsal longitudinal anastomotic vessel (DLAV) (Blum et al., 2008; Herwig et al., 2011). Upon anastomosis the ECs switch back to a more quiescent state (Leslie et al., 2007).

During sprouting and anastomosis, ECs show very diverse behaviors. Tip cells in the nascent sprout migrate extensively and form numerous filopodia, whereas the following stalk cells undergo extensive cell and junctional elongation (Gerhardt et al., 2003; Phng et al., 2013; Sauter et al., 2014). This characteristic stalk cell behavior extends the sprouts towards the dorsal direction and leads to the formation of a multicellular tube. At the tip of the sprout, filopodia of neighboring tip cells establish contact and initiate anastomosis. Anastomosis entails a number of dynamic behaviors, which ensure proper apical-basal polarization, establishment of a continuous endothelium and formation of a patent tube (Herwig et al., 2011; Lenard et al., 2013).
Many of the mentioned cellular activities, such as formation of filopodia and cell shape changes, require dynamic regulation of the actin cytoskeleton (Phng et al., 2013). These activities need to be coordinated by endothelial cell-cell interactions likely to be mediated by adhesion proteins. We have previously shown that the endothelial specific adhesion protein VE-cadherin (VE-cad/Cdh5) is required for coordinated stalk cell elongation during extension of the SeA sprout, and that this function requires the interaction of VE-cad with the cortical F-actin network (Phng et al., 2015; Sauteur et al., 2014). Furthermore, it was shown that VE-cad is also important for anastomosis by facilitating the formation interfilopodial contacts at the onset of anastomosis (Lenard et al., 2013). However, the lack of VE-cad does not prevent EC interactions, suggesting that additional adhesion molecules cooperate with VE-cad during EC recognition and adhesion.

One of the adhesion proteins expressed in an endothelial specific manner is Esam (Endothelial cell-Selective Adhesion Molecule), which belongs to the JAM (Junction Adhesion Molecules) family (Hirata Ki et al., 2001; Nasdala et al., 2002). Unlike its JAM homologues, Esam has been shown to exhibit considerable adhesion properties and to be sufficient to generate efficient cell contacts, when expressed in heterologous cell aggregation assays (Hirata Ki et al., 2001; Nasdala et al., 2002). Although the mouse knock-out of Esam does not cause vascular defects during embryonic development (Ishida et al., 2003), we considered Esam a good candidate as a cooperative partner of VE-cad for cell-type specific adhesion during angiogenic sprouting and anastomosis.

We have generated a frame shift mutation in the zebrafish esama gene using TALEN technology (Cermak et al., 2011). Zebrafish esama mutants are homozygous viable and do not exhibit overt defects during vascular development. In contrast, by combining of ve-cad and esama mutant alleles, we show that loss Esama aggravates the ve-cad phenotype and that both proteins function in different aspects of blood vessel morphogenesis.
**Results:**

*Single filopodial contacts are sufficient to initiate interendothelial contacts*

To analyze the cell behaviors at the onset of anastomosis in detail, we performed live-imaging using transgenic reporters to visualize filamentous actin (F-actin, *fli:Galffubs3; UAS:EGFP-UCHDubs18*, Sauteur et al., 2014) and cell membranes (*kdrl:mCherry-CAAXs916*, Hogan et al., 2009), thus allowing to follow filopodial dynamics at high temporal and spatial resolution (Figure 1A and Movie S1). DLAV formation is initiated at around 30hpf, when tip cells of SeAs send out filopodia in anterior and posterior directions and start to engage with each other. At the same time, the tip cells maintain motility and their cell bodies come into close proximity.

Our analysis showed that filopodia (visualized with mCherry-CAAX) of adjacent cells touched one to three times, but did not stabilize the contact at the first encounter (n=14 movies, Figure 1A-C and Movie S1). Eventually, a filopodial contact was maintained and cytoskeletal components (EGFP-UCHD) were quickly recruited, presumably stabilizing the contact (Figure 1D and E). The cells subsequently expanded their contact area and formed junctions (Figure 1F). In some occasions, tip cells formed contacts over two filopodial touching sites (data not shown), which were then fused to a single, continuous anastomotic contact.

*VE-cad is required for efficient contact formation*

The establishment of properly patterned vascular networks via sprouting angiogenesis and anastomosis requires cell-type specific recognition as well as adhesion. In principle, both tasks can be accomplished by cell-type specific adhesion molecules. ECs express a number of general and cell-type specific adhesion proteins. Among them, VE-cad has been shown to be the major component of endothelial adherens junctions (AJs) (reviewed by Dejana and Vestweber, 2013; Lagendijk and Hogan, 2015). We therefore decided to analyze the behavior of tip cell filopodia in the absence of VE-cad function.

In agreement with earlier studies (Lenard et al., 2013; Montero-Balaguer et al., 2009), tip cells lacking VE-cad still extended filopodia towards each other (Figure 2A, Movie S2). However, we observed that filopodial contacts were more transient than in wild-type embryos, and that VE-cad deficient tip cells required more than 10 filopodial
interactions to establish firm contacts ($n=8$ movies, Figure 2B and C), compared to wild-type siblings: 1-3 filopodial interactions ($n=14$ movies). We were able to observe two scenarios in VE-cad morphants: while single filopodial contacts were not sufficient to produce definitive contacts, the tip cells continued their forward movements and established contacts via their cell bodies. Alternatively, in instances when two or more filopodial contacts occurred simultaneously, these contacts were maintained and ultimately led to anastomosis (Figure 2D-F). These observations indicate that lack of VE-cad reduces filopodial adhesion, rendering single contacts ineffective. However, when the initial contact areas are enlarged, either by multiple filopodial contacts or by direct cell body contacts, anastomosis eventually occurred, suggesting that the available adhesion proteins were sufficient to trigger the process.

Esama is expressed in the endothelium and sprouting SeAs

Since in the absence of VE-cad, ECs are still able to form new cell-cell contacts during anastomosis in a cell-type specific manner, we reasoned that other adhesive components must contribute to this process. Furthermore, these proteins should be restricted to the endothelium, in order to be able to confer cell-type specificity. Among the reported endothelial adhesion proteins, Esam is expressed mostly in the endothelium, but is also found in platelets and megakaryocytes (Hirata Ki et al., 2001; Nasdala et al., 2002). The zebrafish genome encodes two paralogs of Esam, *esama* and *esamb*. In zebrafish, *esama* expression has been reported in ECs, the endocardium and also in a subset of neurons in the brain, including the epiphysis/pineal gland (Wong et al., 2009). We confirmed the endothelial specific expression of *esama*, whereas we observed only weak expression of *esamb* in the vasculature (Figure S1A and B).

Although no obvious vascular defects have been reported for murine Esam mutants (Ishida et al., 2003), we reasoned that Esam may cooperate with VE-cad during cell type specific recognition, even if VE-cad may be sufficient for endothelial specific adhesion in the absence of Esam. To assess the individual and combined contribution of both proteins to endothelial contact formation we generated targeted mutations in the zebrafish *esama* gene using TALEN technology (Cermak et al., 2011). The isolated *esama*<sub>1bs19</sub> allele harbors a 10bp deletion in exon 2, which leads to a premature stop codon leaving only the first 37 amino acids intact (see Figure S2A and B). We confirmed
the absence of the protein by immunohistochemistry in homozygous esama mutant embryos, using Esama specific antibody (Sauteur et al., 2014), which targets the intracellular domain of the protein (compare Figure S2D and E).

**esama/ve-cad double mutants exhibit aggravated anastomosis defects**

Heterozygous esama<sub>abst19</sub> carriers were crossed to obtain homozygous mutants for phenotypic analysis. Initial analyses of esama mutants did not reveal any obvious defects (compare Figure 3A and B) and anastomosis occurs comparable to wild-type conditions, with 2 to 5 transient filopodial contacts before contact stabilization (n=26 movies, Figure S3 and Movie S3). Furthermore, homozygotes developed to fertile adults. To elucidate a potential synergism of VE-cad and Esama in endothelial contact formation, we generated esama/ve-cad double mutant embryos (dKO) and examined the developing vasculature during angiogenic sprouting and anastomosis by time-lapse imaging. While the DA did not display any obvious additional defects compared to ve-cad single mutants, we found that the defects in angiogenesis and anastomosis were strongly aggravated in dKO (Figure 3 and Movies S4-S7). The most obvious defects we observed concerned angiogenic cell behavior, cell adhesion and the establishment of a continuous endothelium during DLAV formation. (1) dKO ECs showed enhanced hyper-protrusive activity with an increase of filopodia on tip and also on stalk cells (Figure 3E and F). (2) We also found that interendothelial adhesion was further decreased in dKO, when compared to ve-cad single mutants. This adhesion defect was reflected by a high incidence of tip cell detachments during SeA sprouting (43% of 190 SeAs in 38 movies of dKO embryos, 34% of 65 SeAs in 13 movies of ve-cad mutant embryos Figure 3C and D and Sauteur et al., 2014). Remarkably, ve-cad mutant stalk cells, which had detached from their leading tip cells, were generally able to reattach to the DLAV within 8h after detachment. In contrast, only 37% of detached stalk cells of dKO reattached to the DLAV (38 stalk cell detachments in 19 movies). Moreover, the tip cells normally making up the DLAV rather formed loose aggregates in dKO embryos (Figure 3D”).

The latter defect suggests that esama/ve-cad dKO ECs are incapable to generate stable interendothelial contacts and to form an organized endothelium. To address this possibility, we crossed the ve-cad and esama mutant alleles into a transgenic reporter line, which simultaneously labels the cell membrane and F-actin in ECs. This allowed us
to perform high-resolution time-lapse analyses of filopodial dynamics and EC interactions during anastomosis in dKO embryos (Figure 4 and Movie S8). Sprouting tip cells showed normal protrusive filopodial activity. However, and in contrast with wild-type embryos, the interactions between filopodia of neighboring tip cells did not lead to the formation of stable contacts. Instead, filopodia showed repetitive cycles of contact and retraction (n=20 movies). During transient filopodial contacts, we usually did not observe an accumulation of F-actin at these sites. Occasionally, when F-actin did accumulate, the tip cells were still not able to maintain these connections (Figure 4B and C). Moreover, even when the two tip cell bodies came into direct contact, the connections were again only transient and the tip cells later dissociated from each other (Figure 4D and E). This behavior could occur several times between the same tip cells. Even when the tip cell eventually did overlap extensively and apparently joined more stably, the actin cytoskeleton did not form junctional ring structures and consequently the cytoskeleton appeared disorganized compared to wild-type (compare green arrowheads in Figure 4F with 1F).

Taken together, these observations show that VE-cad and Esama are required to generate and maintain filopodial contacts, as well as general endothelial cell contacts during sprouting and in particular during anastomosis. Furthermore, our observation that remodeling of the F-actin network is disturbed in the dKO suggests that Esama/VE-cad-mediated adhesion is essential for the assembly of ECs into an organized endothelium.

Our results show that even though the loss of Esama alone has no effect on the ability of tip cell anastomosis, it drastically affects tip cell behavior in the absence of VE-cad. This phenotypic discrepancy between esama, ve-cad and dKO suggests a partially redundant role of both proteins during angiogenesis. Alternatively, the loss of esama function may be compensated by upregulation of ve-cad or esamb. We therefore compared the expression levels of esama, esamb and ve-cad in wild-type, esama and ve-cad mutant embryos (see Figure S1C). RT qPCR analysis on 32 hpf embryos revealed that esamb mRNA levels were unchanged in esama, ve-cad and in dKO mutants. For ve-cad mRNA, we observed a 2-fold increase in esama mutants and similarly, in ve-cad mutants the levels of esama mRNA were increased 2-fold. Thus, elevated ve-cad mRNA levels may compensate for loss of Esama function in esama mutants.
To further test whether the ve-cad gene dose may modify the anastomosis process in the context of loss of Esama function, we performed time-lapse imaging on ve-cad<sup>+/−</sup>; esama<sup>−/−</sup> mutants. Although these embryos are viable and fertile, they do show mild defects in cell-cell contact formation (Figure S4 and Movie S9). On average, these tip cells require 7 filopodial contacts prior to achieve stable contacts (n=9 movies) compared to 2 to 5 in esama mutants and more than 10 in ve-cad mutants as shown above. These observations support our notion that VE-cad and Esama act redundantly during filopodial contact formation and that different levels of VE-cad influence the efficacy of filopodia to establish stable interendothelial contacts.

The role of Esama and VE-cad in DA formation and angiogenic sprouting

Since we observed decreased cell-cell adhesion between stalk and tip cells as well as defects during cell-cell contact formation in dKO embryos, we addressed the junctional morphology in these embryos by examining the distribution of the scaffold protein Zo-1 during SeA sprouting. Furthermore, we examined apical-basal polarization at EC contacts based on the localization of the apical protein Podocalyxin (Pdxl). Wild-type ECs in the DA showed a regular junctional pattern (Figure S5A), and cross-sections through the DA showed a clear apical localization of Pdxl (Figure S5A'). Furthermore, we observed the highest concentration of apical Pdxl in close proximity to cell junctions (red arrowheads in Figure S5A). The junctional pattern of the DA in esama mutant embryos was indistinguishable from those in wild-type embryos (Figure S5B), whereas the DA of ve-cad mutant embryos showed discontinuous lumens. The cell junctions of the DA in ve-cad mutants appeared irregular, indicating that the cellular organization of the tube was disturbed (red arrowheads in Figure S5C). However, even in non-inflated regions, intercellular junctions were formed and Pdxl localized apically between endothelial junctions (Figure S5C'). Additional loss of Esama did not lead to obvious additional morphological defect of the DA when compared to ve-cad single mutants (Figure S5D and D').

In striking contrast to the DA, the cellular architecture of SeA sprouts was much more strongly affected in dKO. It is generally acknowledged that the activation of ECs by angiogenic stimuli leads to a decrease in endothelial cell adhesion and an increase in cell motility (Chaki et al., 2015; Dejana, 2004). Nevertheless, junctional localization of VE-cad, Esama and Zo-1 is maintained during SeA sprouting (Blum et al., 2008; Sauteur
et al., 2014). When we examined Zo-1 distribution in sprouting SeAs in the absence of VE-cad and Esama, we found that Zo-1 was often lacking at cell borders, even between two obviously connected ECs (Figure 5D). Moreover, the junctions, which did form, were often discontinuous and exhibited large intrajunctional gaps (red arrowheads in Figure 5D”). Frequently, Zo-1 appeared delocalized in these cells and accumulated in the cytoplasm, suggesting a lack of available binding partners for Zo-1 at EC junctions (compare Figure 5A” and D”, green arrowheads).

We have previously shown that the dorsal extension of SeAs is to a large extent accomplished by stalk cell elongation and that this processes requires VE-cad (Sauteur et al., 2014). Examination of junctional patterns revealed that this process was not affected in esama mutants (compare Figure 5B and 5C). Despite this, esama single mutants showed small intrajunctional gaps, albeit much smaller than those we observed in dKO (red arrowheads Figure 5B”). These gaps were seen mostly in early steps of sprout extension, while they were absent in sprouts that had undergone lumen formation (see also Figure 6B). When we compared the occurrence and extent of intrajunctional gaps in wild-type, esama and ve-cad mutants (Figure 5E), we found that they were more frequent in esama than in ve-cad mutant embryos. In the absence of VE-cad, we counted approximately one intrajunctional gap per SeA, while they were almost twice as abundant in esama mutants. These differences point to non-redundant roles of Esama and VE-cad in sprouting SeAs. Esama is required during earlier steps of junction formation and maturation, while it is expendable at later stages of blood vessel formation. In contrast, VE-cad appears to be primarily required for dynamic junctional rearrangements.

**Intrajunctional gaps are associated with defects in apical polarization**

Because cell junctions are thought to be essential for the separation of apical and basal membrane compartments of ECs (Lampugnani et al., 2010; Strilic et al., 2009; Strilić et al., 2010), we wondered whether the appearance of intrajunctional gaps in esama and ve-cad mutants impacts apical-basal polarization. When we stained wild-type embryos for Zo-1 and the apical membrane marker Pdxl, we observed Pdxl to be contained within junctional rings (Figure 6A). Similarly, in single mutants, Pdxl was confined to those membrane compartments, which were enclosed by intact (i.e. not-interrupted) junctional rings (see base of sprouts in Figure 6B and C, respectively). In contrast, in the
context of intrajunctional gaps, Pdxl became delocalized and was also found in basal membrane compartments. Furthermore, the size of the junctional gaps correlated with the degree of basal Pdxl localization (Figure 6B” and C”) and became most obvious in dKO (Figure 6D). In dKO, these junctional gaps were maintained during the entire process of SeA formation and were associated with defects in apical-basal polarization. By contrast, in ve-cad and esama single mutants, these gaps appeared only transiently and were not observed in later stage SeAs; in these vessels, Pdxl was restricted within the junctional rings.
Discussion:

Angiogenesis requires the tight regulation and coordination of interendothelial cell adhesion and motility. In this study we have analyzed the individual and combined requirements of VE-cad and Esama during angiogenic sprouting and anastomosis. While the importance of VE-cad for embryonic blood vessel formation is well documented (Carmeliet et al., 1999; Gory-Fauré et al., 1999; Montero-Balaguer et al., 2009; Sauteur et al., 2014 and reviewed by Lagendijk and Hogan, 2015), the role of Esam in this process is not clear. Previously, Esam has been implicated in neutrophil diapedesis and the etiology of several human diseases (Khandoga et al., 2009; Wegmann et al., 2006 and reviewed by Luissint et al., 2014). However, murine Esam null mutants are homozygous viable and no vascular defects have been described (Ishida et al., 2003). This is in contrast to in vitro studies, which show that Esam has strong adhesive properties and suggest an important role in EC junction formation and maturation (Kimura et al., 2010; Nasdala et al., 2002). We therefore reasoned that the role of Esam in vivo may be obscured by the presence of other adhesion molecules, which function at least partially redundant with Esam during embryonic angiogenesis. To explore this possibility, we generated a putative null allele of esama in zebrafish. In agreement with data from mouse mutants, homozygous esama mutants do not display overt vascular defects, are viable and fertile. Here we show, however, that when bred into the ve-cad mutant background, the additional loss of esama strongly aggravates the ve-cad single mutant phenotype, suggesting that Esama and VE-cad act at least partially redundant during blood vessel morphogenesis (see also Figure S6).

Synergistic functions of Esama and VE-cad during blood vessel anastomosis

Cell type-specific recognition and adhesion between ECs are a prerequisite for efficient blood vessel anastomosis. Both processes are mediated by the intercellular engagement of tip cell filopodia. However, recent in vivo studies have shown that lack of filopodia does not prevent sprout outgrowth and anastomosis and that tip cells can generate contacts between their cell bodies in the absence of filopodia (Phng et al., 2013). These findings are in agreement with our observations of filopodial and cellular dynamics in ve-cad and esama mutants, as discussed below.

The lack of VE-cad and the additional loss of Esama in tip cell filopodia leads to an increasing disability to generate intercellular contacts. Especially in dKO, the filopodia
are going through repetitive cycles of connection. This suggests that in wild-type both proteins are present in tip cell and mediate EC recognition and adhesion at the onset of anastomosis. The different combinations of mutant alleles can be placed into a phenotypic series with increasing anastomosis defects. esama mutants appear wild-type like, while esama^{-/-}; ve-cad^{+/+} and ve-cad single mutants exhibit an intermediate phenotype. Lack of both proteins finally disrupts anastomosis almost completely. In this situation, cells accumulate as loose aggregates, indicating that double mutant ECs still retain some cell-type specific adhesiveness. Remarkably, loss of a single copy of ve-cad induces mild filopodial defects in homozygous esama mutants, suggesting that esama mutants are sensitized to ve-cad levels. This is in agreement with our qPCR analyses, which show that mRNA levels of ve-cad are increased by two-fold in esama mutants. Taken together, these observations indicate that VE-cad and Esama act partly redundant during anastomosis and that VE-cad can compensate for the loss of Esama, but not vice-versa (Figure S6). Moreover, these observations suggest that the degree of cell-type specific adhesiveness determines the efficacy of anastomosis and thus cell-cell recognition.

Synergistic functions of Esama and VE-cad during angiogenic sprouting
The formation of SeAs is initiated by the activation of ECs in the DA by VEGF-A (Habeck et al., 2002 and reviewed by Siekmann et al., 2013). In general, EC activation is accompanied by a remodeling of the actin cytoskeleton, a reduction in inter-endothelial adhesion and an increase in motility (Gerhardt et al., 2003 and reviewed by Herbert and Stainier, 2011; van Buul et al., 2014). Nevertheless, junctional proteins, such as Zo-1, Esama and VE-cad are maintained at cell junctions during SeA sprouting (Blum et al., 2008; Sauteur et al., 2014). In esama mutants, SeA formation is almost indistinguishable from wild-type. However, the loss of Esama strongly aggravates the defects of ve-cad mutants. In ve-cad mutants, we have observed two defects within the angiogenic sprout: a detachment of the tip cell from the sprout, which can be attributed to a reduction in interendothelial cell adhesion, and a defect in junction/cell elongation within the sprout (Sauteur et al., 2014). The additional loss of Esam appears to enhance primarily the adhesion defects within the sprout. In dKO, ECs in the sprout attain a mesenchymal appearance and their junctional contacts are severely disrupted.
By comparison, the axial vessels, which form by vasculogenesis, are less severely affected by the loss of both proteins. This indicates that dKO ECs still possess cell-type specific adhesive properties, which may be mediated by other endothelial specific adhesion molecules such as Claudin 5. However, once dKO ECs have initiated angiogenic sprouting and emigrate from the DA, they start to dissociate. And after the ECs have reduced their contacts, they are not able to reestablish an organized endothelium.

In general, our observations support the findings from the mouse Esam knock-out, which showed that Esam is not essential for physiological angiogenesis (Ishida et al., 2003). Notably, however, this study also showed an essential role for Esam in tumor angiogenesis and tumor growth. The endothelium of tumor vessels is often disturbed and ECs are exposed to high levels pro-angiogenic cues (Baluk et al., 2005; Hida et al., 2016). It is therefore conceivable that the tumor environment may affect ECs in a way that mimics the loss of VE-cad and thus makes them susceptible to the additional loss of Esam. In agreement with this notion, knock-down of ve-cad in zebrafish can inhibit tumor angiogenesis in xenografts (Nicoli et al., 2007).

**Differential roles for Esama and VE-cad in junction formation and apical polarization**

In general, our phenotypic analyses indicate that VE-cad function is more vital to cardiovascular development than Esama and that VE-cad can compensate for the loss of Esama during embryonic angiogenesis. We noticed however, a transient appearance of intrajunctional gaps, which was more pronounced in esama than in ve-cad mutants (Figure S6). These gaps were frequent during the early sprouting stages, when junctions are thought to be remodeling extensively, than at later stages, when the lumen was forming. The intrajunctional gaps are associated with a delocalization of Zo-1 from the junction to the cytoplasm and of Pdxl from the apical to the basal cell membrane compartment, which is most obvious in dKOs. The delocalization of Zo-1 is already apparent in esama; ve-cad mutants (data not shown), but is strongly enhanced in dKOs. This finding may be surprising, because Zo-1 has been reported to directly bind to Claudins and JAMs (Fanning and Anderson, 2009), but not to Esam, which binds MAGI-1 (Wegmann et al., 2004). However, cell culture experiments suggest that Esam/MAGI-1 interaction activates RhoA, which in turn leads to an accumulation of Zo-1 and F-actin at newly formed junctions (Kimura et al., 2010). Our observations of cytoplasmic Zo-1 accumulation support the view that Esama may have essential
function for the recruiting Zo-1 to newly formed or remodeling EC junctions. We cannot exclude alternative explanations, such as that the loss of Esama (plus VE-cad) may lead to more widespread defects in the organization of junctional proteins, which may lead to a loss of direct Zo-1 binding partners at the cell junction.

The redundant functions of VE-cad and Esama during vascular morphogenesis raise the question to what extent these proteins may interact in the same or in separate molecular pathways. Whether Esama and VE-cad physically interact remains to be investigated. Although Esama and VE-cad are localized in different junctional compartments, they may be in close proximity, since tight junctions and adherens junctions of ECs are not strictly segregated (Rüffer et al., 2004 and reviewed in Bazzoni and Dejana, 2004). Both proteins may interact via MAGI-1 and ß-catenin, since Esama has been shown to directly bind to MAGI-1 (Kimura et al., 2010; Wegmann et al., 2004) and VE-cad can bind MAGI-1 through ß-catenin (Sakurai et al., 2006).

Taken together, our findings uncover unique and redundant functions of Esama and VE-cad during angiogenic sprouting and anastomosis. VE-cad is essential for contact formation and junctional dynamics during stalk cell elongation and anastomosis. In esama mutants, most of the Esama functions can be compensated for by VE-cad. However, the subtle defects in esama single mutants point to a unique role of Esama in de novo junction formation and maturation.
Materials and Methods:

Zebrafish lines and maintenance and morpholinos:
Zebrafish were maintained at standard conditions (Westerfield, 2000). All experiments were conducted in accordance with federal guidelines and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt. The used zebrafish lines were Tg(fli1a:EGFP)v1 (Lawson and Weinstein, 2002), Tg(kdrl:mCherry-CAAX)5916 (Hogan et al., 2009), Tg(fli1ep:gal4ff)ubs3 and Tg(UAS:EGFP-hsZO-1, cmlc:EGFP)ubs5 (Herwig et al., 2011), Tg(UAS:EGFP-UCHD)ubs18 and ve-cadubs8 (Sauteur et al., 2014) and esamaubs19 (this study).

Morpholinos (GeneTools) used were as follows: VE-cadherin splice 5’-TTTACAAGACCGTCTACCTTTCCA-3’ (Nicoli et al., 2007) and standard control 5’-CCTCTTACCTCAGTTACAATTTATA-3’. After imaging, the morphant embryos were stained for VE-cad to verify efficient knock down. Embryos showing incomplete knockdown were excluded from the analysis.

In situ hybridization:

In situ hybridization was performed as described by (Thisse and Thisse, 2014). To increase the signal, the hybridization mixture was supplemented with 5% dextran sulfate, as described in the protocol.

Images were acquired with a Microphot-FXA (Nikon) microscope, equipped with a Nex-5R (Sony) digital camera, using 20x dry objective (NA = 0.5).

The esama antisense template was generated by PCR amplification from the vector cssl:d0254 (Thisse et al., 2008) using T7 polymerase (Roche) for transcription, as described by (Thisse et al., 2008).

The esamb probe vector was cloned from the cDNA EST clone IMAGp998L2215582Q (GenomeCube, Source BioScience), which was subcloned into the pBluescript II KS backbone. The vector was linearized by Acc65I (NEB) for antisense transcription (T7 polymerase, Roche).

Generation and genotyping of the esamaubs19 mutant line:

For the generation of esamaubs19 the second exon of the esama gene was targeted for mutagenesis. For targeted mutagenesis TALEN technology was used as described by (Cermak et al., 2011). The forward TALEN binds 5’-T-GTTGCCTTATGAAAA-3’ and the
reverse TALEN binds 5’-AGATGGTGTTGCTGC-A-3’ and are spaced by 20bp (5’-TGTGATGTGATCAAGGGA-3’).

The repeat-variable diresidues (RVDs) of the forward TALEN (NH NG NH HD HD NG NG NI NG NH NI NI NI NI) and the reverse TALEN (NH HD NI NH HD NI HD HD NI HD NI NG HD NG) were assembled as described by (Cermak et al., 2011). Subsequently, the RVD clones were cloned into RCIscript_GoldyTALEN (Bedell et al., 2012), where the T3 promoter was converted to a T7 one. The TALEN scaffold vectors were linearized with BstEII (NEB) and BsaI (NEB), gel extracted and transcribed in vitro using mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Ambion), according to the manufactures protocol. Approximately 600pg of RNA of each TALEN were coinjected into one-cell stage wild-type eggs. The injected embryos were raised to adult hood. G0 fish were crossed out to wild-type fish, the genomic DNA was extracted by alkaline lysis (Meeker et al., 2007) and the region of interest was amplified by PCR (using primers Pesama_I1_fwd 5’-ATGGTCTTTCAGTCAGCGAG-3’ and Pesama_I2_rev 5’-GTGTGGCAGTTTAATTCAAATC-3’). To screen for mutations, the PCR amplicon was digested with StyI (NEB), which cuts in the spacer region flanked by the TALEN binding sites. Uncut product was sent for sequencing (Microsynth, Switzerland), which revealed a 10bp deletion. This 10bp deletion in exon 2 of the esama gene leaves the first 37 amino acids of the protein intact and leads to a premature stop after another (altered) 33 amino acids (see Figure S2A).

For genotyping adult fish or embryos the genomic DNA from fin biopsies (Meeker et al., 2007) was extracted and genotyped using four primers to discriminate wild-type from heterozygous or homozygous mutant individuals. The four primers (P30 5’-ATGGTCTTTTCAGTCAGCGAG-3’, P55 5’-GTGTGGCAGTTTAATTCAAATC-3’, P99 5’-GGATGTGATCAAGGGAAG-3’ and P101 5’-CCACCATCTTCCCTCCA-3’) combination generates products of 356bp for wild-type alleles and 142bp for mutant alleles (see Figure S2B and C).

Immunofluorescence
Immunofluorescence was performed as previously described (Herwig et al., 2011). Following antibodies were used: rabbit anti-Esama 1:200 (Sauteur et al., 2014), mouse anti-human-Zo-1 1:100 (Zymed), rabbit anti-VE-cad 1:200 (Blum et al., 2008), rabbit anti-Pdxl 1:200 (Herwig et al., 2011), Alexa 568 goat anti-rabbit immunoglobulin (IgG)
1:1000, Alexa 633 goat anti-rabbit IgG 1:1000 and Alexa 633 goat anti-mouse IgG 1:1000 (all secondary antibodies from Invitrogen).

Imaging and image analysis
Fixed or live samples were selected for fluorescence signal, anaesthetized in E3 supplemented with 1x tricaine (0.08%) and mounted in a 35 mm glass-bottom Petri dish (0.17 mm, MatTek), using 0.7% low melting agarose (Sigma) containing 1x tricaine. For live imaging, the mounting agarose was additionally supplemented with 0.003% 1-phenyl-2-thiourea (PTU, Sigma). Leica TCS SP5 confocal microscope was used for time-lapse and images of fixed samples, using 40x (NA=1.1) or 63x (NA=1.2) water immersion objectives. Routinely, z stacks were acquired with a step size of 0.25-0.8 µm and stacks for time-lapse imaging were acquired every 6-10 min.

High time resolution movies were acquired with a Perkin Elmer (Ultraview VoX) spinning disk confocal microscope, using a 63x (NA=1.2) water immersion objective. Z stacks were acquired with a step size of 0.3 µm every minute. Subsequently, images were deconvolved using Huygens (SIV).

Images and movies were analyzed with ImageJ software (http://fiji.sc/).

Quantification of filopodial protrusions and intra-junctional gaps
Filopodial protrusions were counted in projected confocal stacks of Tg(fli1ep:gal4ff)ubs3, Tg(UAS:EGFP-UCHD)ubs18, Tg(kdrl:mCherry-CAAX)s916 embryos of different genotypes, fixed around 32hpf.
Junctional gaps were measured in projected confocal stacks of embryos of different genotypes, fixed around 32hpf and stained for Zo-1.

Statistical analysis was performed using Prism software (GraphPad) and ordinary one-way ANOVA with multiple comparisons, corrected with Tukey test.

RNA extraction from embryos and RT qPCR analysis
At 32 hpf, wild-type and esamaubs19 embryos were collected from incrosses, while ve-cadubs8 and dKO embryos were selected according to their phenotype. Three samples of 15 embryos and of each genotype were pooled for RNA extraction. For each genotype, the RNA was extracted using ReliaPrep™ RNA Cell Miniprep System (Promega™) following the manufacturer’s instructions.
cDNA was transcribed for 2 h at 37°C from 1µg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturers protocol. Subsequently, the cDNA was diluted 1:10.

qPCR was performed using FastStart Universal SYBR green Master (Rox) (Roche) and the ABI 7500 Fast Real-time PCR System (Applied Biosystems). Briefly, 5µl of the diluted cDNA was mixed with 0.5µM primes in final 20µl SYBR green master mix. The cycling program of the qPCR included 10 min initial denaturation at 95°C, followed by 45 cycles of denaturation at 95°C for 10 sec and annealing plus extension at 60°C for 30 sec.

Experiments were performed for each genotype in 3 biological, and 3 technical replicates. RPS11 and beta-actin were chosen as housekeeping genes and the fold change of a specific gene was calculated for each genotype. A list of primers is found in Supplemental Table 1.
Acknowledgments:
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Competing Interest:
The authors declare no competing interest.

Author Contributions:
L.S., M.A. and H.G.B. conceived the project. L.S. and H.G.B. designed the experiments. L.S. carried out the experiments. L.S., M.A. and H.G.B. analyzed the data. L.S. and H.G.B. wrote the manuscript.

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References:


Sakurai, A., Fukuhara, S., Yamagishi, A., Sako, K., Kamioka, Y., Masuda, M.,


Figure 1: Anastomosis occurs over a single filopodial contact.

(A-F) Still images from Movie S1 of a Tg(fli1ep:gal4ff)ubs3, Tg(UAS:EGFP-UCHD)ubs18, Tg(kdrl:mCherry-CAAX)embryo at around 32hpf, anterior to the left. Single channels are shown in inversed contrast (green is EGFP-UCHD and red is mCherry-CAAX on left and right, respectively) and the merge is shown in the middle. Red arrowheads point to filopodial interactions; green arrowheads point to cell-cell bridges or a junctional ring in (F); scale bar, 10µm.
Figure 2: In the absence of VE-cad several filopodial contacts are required to initiate cell-cell contact formation

(A-F) Still images from Movie S2 of a VE-cad morphant Tg(fli1ep:gal4ff)ubs3, Tg(UAS:EGFP-UCHD)ubs18, Tg(kdrl:mCherry-CAAX)916 embryo at around 32hpf, anterior
to the left. Single channels are shown in inversed contrast (green is EGFP-UCHD and red is mCherry-CAAX on left and right, respectively) and the merge is shown in the middle. Red arrowheads point to filopodial interactions or cell bodies touching in (E); green arrowheads point to cell-cell bridges and forming junctions in (E and F); scale bar, 10µm.
Figure 3: Loss of both, VE-cad and Esama, enhances the defects observed in ve-cad\textsuperscript{ubs8} mutants

(A-D) Still images from Movie S4 (A), Movie S5 (B), Movie S6 (C) and Movie S7 (D) of Tg(fli1a:EGFP)\textsuperscript{y1} wild-type, esama\textsuperscript{ubs19}, ve-cad\textsuperscript{ubs8} and esama\textsuperscript{ubs19}; ve-cad\textsuperscript{ubs8} double mutant (dKO) embryos, respectively. Images are shown in inversed contrast at 3 different stages of angiogenesis: ~32hpf, ~38hpf and ~44hpf. Red arrowheads at 32hpf point to anastomotic contacts; red arrowheads at 38hpf point to forming lumen; green
arrowheads point to stalk cells that detached from the tip cell; scale bars, 20µm. (E and F) Quantification of filopodial protrusions on SeA stalks (E) and tip cells (F). (E) The number of filopodia on angiogenic stalks (comprising 1-3 ECs) is comparable between wild-type (77 SeAs in n=20 embryos), \textit{esama}^{ubs19} (114 SeAs in n=30 embryos) and \textit{ve-cad}^{ubs8} (31 SeAs in n=9 embryos) genotypes. Significantly more filopodia were counted on dKO stalks (31 SeAs in n=8 embryos). (F) Tip cells generally generate more filopodial protrusions (average of 50 filopodia per tip cell) than stalk cells. Significantly more filopodia were counted on tip cells of dKO compared to wild-type. Bars in the plots represent mean and standard deviation. For the statistical analysis unpaired, two tailed t tests were performed. dKO, double mutant; DA, dorsal aorta; SA, segmental artery; DLAV, dorsal longitudinal anastomotic vessel; ns, not significant; n, number of analyzed embryos; ***, p-value < 0.0001, with one-way analysis of variance.
Figure 4: *esama*\textsuperscript{ubs19}; *ve-cad*\textsuperscript{ubs8} double mutant tip cells can not maintain cell-cell contacts

(A-F) Still images from Movie S8 of a *esama*\textsuperscript{ubs19}; *ve-cad*\textsuperscript{ubs8} double mutant Tg(*fli1ep:gal4ff*)\textsuperscript{ubs3}, Tg(UAS:EGFP-UCHD)\textsuperscript{ubs18}, Tg(kdrl:mCherry-CAAX)\textsuperscript{s916} embryo at around 32hpf, anterior to the left. Single channels are shown in inversed contrast (green is EGFP-UCHD and red is mCherry-CAAX on left and right, respectively) and the merge is shown in the middle. Red arrowheads point to filopodial interactions (B) or resolved...
contacts (E); green arrowheads point to cell-cell bridges or forming junctions in (F); scale bar, 10\(\mu\)m.
Figure 5: Defects in junctional continuity in the absence of Esama, which are greatly increased in double mutants

(A-D) Confocal images of SeAs of Tg(fli1a:EGFP)$^v1$ (green) wt (A), esama$^{ubs19}$ (B), ve-cad$^{abs8}$ (C) and esama$^{ubs19}$; ve-cad$^{abs8}$ double mutant (dKO, D) embryos stained for Zo-1 (red), around 32hpf. Single channels are shown in inversed contrast. Red arrowheads point to intrajunctional gaps or missing junctions between the red arrowheads in (D''); green arrowheads indicate cytoplasmic localization of Zo-1. Asterisk, nucleus; scale bars, 20µm. (E) Intrajunctional gaps (µm) measured in confocal projections of embryos stained for Zo-1 around 32hpf. One-way analysis of variance revealed significance for esama$^{ubs19}$ (p=0.0449), ve-cad$^{abs8}$ (p=0.42) and double mutant (p<0.0001). Tukey boxplot shows boxes with lower quartile, median and upper quartile (Tukey whiskers). Medians are 0.4µm, 0.9µm, 0.7µm and 1.3µm for wild-type, esama$^{ubs19}$, ve-cad$^{abs8}$ and dKO respectively. The number of observed intrajunctional gaps (total gaps), the number of analyzed SeAs (total SeAs) and the calculated number of intrajunctional gaps per SeA
(gaps/SeA) are indicated below the boxplot for every genotype. The number of intrajunctional gaps per SeA is omitted for the double mutant genotype, because strong junctional defects did not allow a quantitative analysis. SeA, segmental artery; dKO, double mutant; N/A, not available (omitted); n, number of embryos analyzed; *, p<0.05; **, p<0.01; ***, p<0.001.
Figure 6: Intrajunctional gaps correlate with defects in apical polarization
(A-D) Deconvolved projections of SAs of Tg(fli1a:EGFP)y1 (blue) wt (A), esamaabs19 (B), ve-cadubs8 (C) and esamaabs19; ve-cadubs8 double mutant (dKO, D) embryos stained for Zo-1 (red) and Pdxl (green), around 32hpf. SAs are shown either in EGFP plus Zo-1 or Pdxl plus Zo-1 channels, while insets and sections are merges of all three colors, except for
B” where the blue channel was omitted. The panels on the right hand side represent magnifications and cross-sections of the insets and dotted lines, respectively. Red arrowheads point to junctions; white arrowheads point to intrajunctional gaps or missing junctions between the white arrowheads in (D’); blue arrowheads point to basal localized Pdx1; L, lumen; asterisk, nucleus; scale bars, 20μm.
Supplementary Information.

Supplemental Figures.

Figure S1

Figure S1: Esama is specifically expressed in the trunk vasculature

In situ hybridization for esama (A) and its paralog esamb (B) expression in embryos at 28 hpf. (A) esama is expressed in the DA, PCV and the growing SeAs. (B) esamb expression appears to be very weak in the trunk vasculature. The colorimetric reaction was prolonged, but stopped to prevent too strong background. DA, dorsal aorta; SeA, segmental artery; PCV, posterior cardinal vein. (C) Quantitative RT-PCR analysis of ve-cad, esama and esamb transcripts in different mutant genotypes. mRNA levels are compared relative to wild-type embryos. Experiments were performed in 3 biological and 3 technical replicates. dKO, double mutant.
Figure S2: Generation and verification of an esama mutant allele

(A) Schematic representation of the wild-type and \textit{esama}^{ubs19} mutant allele. Top: the amino acid sequence of wild-type Esama (total of 428aa) flanking the region of mutagenesis and schematic drawing of wild-type Esama. Esama harbors two N-terminal extracellular Ig-like domains (one V- and one C2-type), a single transmembrane domain and a long cytoplasmic tail harboring a MAGI-1 binding domain. Bottom: the 10bp deletion leads to a frame shift, which alters Esama’s sequence...
after the first 37 aa and leads to a premature stop after a total of 70 aa (altered amino acid sequence highlighted in red). This premature stop leaves only a very short peptide.

(B and C) Genotyping PCR strategy (B) and examples of amplicons produced by wild-type, esama\textsuperscript{ubs19} heterozygous or homozygous mutants. (A) The external primers (P30 and P55) flank exon 2 and amplify both, mutant and wild-type alleles (482 bp). This amplicon is outcompeted by the smaller wild-type and ubs19 products. P99 specifically anneals to the wild-type sequence and produces a band of 356 bp with P55. P101 is specific for the mutation and together with P30 generates a 142 bp product.

(D and E) Confocal images of wild-type (D) and esama\textsuperscript{ubs19} (E) Tg(fli1a:EGFP)\textsuperscript{y1} (blue) embryos stained for Esama (green) and Zo-1 (red), single channels are shown in inversed contrast, anterior to the left. The architecture of SeAs looks similar between wild-type and esama\textsuperscript{ubs19} (compare the EGFP channels D and E); there are no obvious angiogenic defects in the mutant. The staining for Esama confirms the loss of protein in the mutant background (E'). Importantly, the Esama antibody recognizes epitopes in the cytosolic C-terminal portion of the protein (Sauteur et al., 2014), excluding the possibility of splice variants in the esama\textsuperscript{ubs19} mutant background. The Esama antibody shows cross-reactivity with the myotome, which remains visible in the mutant (demarcated by red asterisks in D’ and E’). aa, amino acid; TM, transmembrane domain, scale bars, 20 μm.
Figure S3: Loss of Esama does not lead to defects in anastomosis

(A-F) Still images from Movie S3 of an esamaubs19 mutant Tg(fli1ep:gal4ff)ubs3, Tg(UAS:EGFP-UCHD)ubs18, Tg(kdrl:mCherry-CAAX)s916 embryo at around 32hpf, anterior to the left. Single channels are shown in inversed contrast (green is EGFP-UCHD and red is mCherry-CAAX on left and right, respectively) and the merge is shown in the middle. (A) Two tip cells are extending filopodia (red arrowheads) towards each other. (B) A filopodial contact is established (red arrowhead), but actin cytoskeleton (green arrowhead) has not accumulated at the cell-cell bridge yet. (C) The cell-cell bridge is quickly stabilized with actin cytoskeleton (green arrowhead). (D and E) A second protrusion is established between the two tip cells (red arrowhead) and quickly stabilized with actin cytoskeleton (compare green arrowheads in C and D). (F) During contact maturation, the two cell-cell contact sites are fused to a single one (red arrowhead). Scale bar, 10µm.
Figure S4: *esama*/*; ve-cad*+/ embryos display an intermediate filopodial phenotype.

(A-F) Still images from Movie S9 of an *esama*/*; ve-cad*+/ embryo. Tg(*fli1ep:gal4ff*)/*; Tg(UAS:EGFP-UCHD)/*; Tg(*kdrl:mCherry-CAAX*) embryo at around 32hpf, anterior to the left. Single channels are shown in inversed contrast (green is EGFP-UCHD and red is mCherry-CAAX on left and right, respectively) and the merge is shown in the middle. (A) Two tip cells are extending filopodia towards each other. (B) Two filopodia touch (red arrowhead). (C) A filopodial contact is
established (red arrowhead) and actin cytoskeleton (green arrowhead) is being recruited to the cell-cell contact site. (D) The cell-cell bridge (red arrowhead) is quickly stabilized with actin cytoskeleton (green arrowhead). (E) Anastomosis led to the formation of a junctional ring between the two tip cells (green arrowhead). Scale bar, 10µm.
Figure S5:
The DA appears to form normally in the absence of VE-cad and Esama

(A-D) Deconvolved projections of DAs of Tg(fli1a:EGFP)y1 (blue) wt (A), esamaubs19 (B), ve-cadubs8 (C) and esamaubs19; ve-cadubs8 double mutant (dKO, D) embryos stained for Zo-1 (red) and Pdxl (green), around 32hpf. Single channels are shown in inversed contrast. (A) In wild-type embryos the DA is lumenized and the strongest signal for apical Pdxl is usually observed close to junctions (Zo-1, and see red arrowheads in A). (A’) shows a cross-section through the DA. Several ECs surround the lumen (L); apical staining (green) is seen between junctions (arrowheads) and on the luminal side of ECs. (B) The DA in esamaubs19 mutant embryos forms similar to wild-type ones. (B’
shows lumen (L) surrounded by several cells and also here the apical Pdxl is located between junctions (arrowheads) and on the luminal side of cells. (C) In the absence of VE-cad, the DA is lumenized only partially. Cell-cell junctions look more disorganized compared to wild-type siblings (red arrowheads in the Zo-1 panel). (C’) But even in collapsed portions of the DA, apical signal (green) is observed between junctions, where lumen would be expected. Section was chosen where three cells (two nuclei and one cell body form the DA; only two junctions are observed (red arrowheads), because two junctions overlap between the two cell nuclei (n). (D) Similar to ve-cadnull mutants, the DA of dKO embryos is only partially inflated and the junctions appear disorganized (red arrowheads in the Zo-1 panel). (D’) However, apical polarization appears normal with signal (green) between junctions and on the luminal (L) side of the endothelial cells. Section shows lumenized portion of the DA surrounded by 4 cells. DA, dorsal aorta; L, lumen; asterisk, nucleus; c, cell body; white arrowheads, junctions; Pdxl, Podocalyxin; cross-section is located at the dotted line; scale bars, 10µm.
Figure S6: Schematic summary of ve-cad and esama mutant phenotypes

**Top:** tip cell interaction during anastomosis. In wild-type and esama mutants cell contacts are generated by filopodial contacts. In ve-cad mutants filopodial contacts are inefficient and contacts are established by endothelial cell bodies. In esama; ve-cad mutant embryos (dKOs), cell contact formation by filopodia, as well as cell bodies is basically inhibited.

**Bottom:** junctional dynamics during tube formation. During angiogenic sprouting of SeAs, wild-type junctions stretch over the whole SeA and apical Podocalyxin (Pdxl) is contained within the junctional ring. esama mutants show small gaps in their junctions (based on Zo-1 distribution), which correlates with mislocalized Pdxl (outside of the junctional ring). ve-cad mutants stalk cells show reduced cell rearrangement and elongation, and junctions occasionally show small gaps (smaller compared to esama mutants). dKO show an enhanced phenotype with reduced cell-cell adhesion, accompanied by enlarged gaps of Zo-1 at cell junctions. The size of junctional gaps correlates with the mislocalization of Pdxl.
### Supplemental Table 1: qPCR primer list

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<th>Primer name</th>
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<td>RPS11-rev</td>
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Supplemental movies.

Movie S1: Anastomosis in a wild-type embryo
Deconvolved spinning disc confocal movie of a Tg(fli1ep:gal4ff)ubs3, Tg(UAS:EGFP-UCHD)ubs18, Tg(kdrl:mCherry-CAAX)s916 embryo at around 32hpf, anterior to the left. Confocal stacks were acquired every minute. Single channels are shown in inversed contrast (green is EGFP-UCHD and red is mCherry-CAAX on left and right, respectively) and the merged channels are shown in the middle. Two tip cells expand filopodia towards each other. Few transient filopodial interactions are observed before a single filopodial contact is maintained and reinforced by the recruitment of actin cytoskeletal components (around the 84th minute of the movie). The cell-cell contact matures and the actin cytoskeleton reorganizes to form junctional ring between the two tip cells (around 129th minute of the movie).
Movie S2: Anastomosis in a VE-cad morphant embryo

Deconvolved spinning disc confocal movie of a Tg(fli1ep:gal4ff)ub3, Tg(UAS:EGFP-UCHD)ub318, Tg(kdrl:mCherry-CAAX)s916 embryo injected with VE-cad morpholino. Movie was recorded around 32hpf, anterior to the left. Confocal stacks were acquired every minute. Single channels are shown in inversed contrast (green is EGFP-UCHD and red is mCherry-CAAX on left and right, respectively) and the merged channels are shown in the middle. Two tip cells extend filopodia towards each other. Many transient filopodial interactions are observed as the cell bodies of the two tip cells get closer to each other. Around the 89th minute, the tip cell on the right hand side establishes contact to the left one over two filopodia simultaneously. These two filopodia appear to be maintained and at the 92nd minute the two cell bodies touch. As the cell-cell contact expands junctions start to form between the two cells (seen in the EGFP-UCHD channel around the 165th minute). Cell-cell contact formation appears less efficient compared to wild-type siblings, and moreover, takes generally longer.
Movie S3: Anastomosis in an esamaabbs19 mutant embryo

Deconvolved spinning disc confocal movie of a Tg(fli1ep:gal4ff)absc3, Tg(UAS:EGFP-UCHDaabsc18, Tg(kdrl:mCherry-CAAX)cs916 esamaabbs19 mutant embryo at around 32hpf, anterior to the left. Confocal stacks were acquired every minute. Single channels are shown in inversed contrast (green is EGFP-UCHD and red is mCherry-CAAX on left and right, respectively) and the merged channels are shown in the middle. Similar to wild-type embryos, two tip cells expand filopodia towards each other. Few transient filopodial interactions are observed before a single filopodial contact is maintained and reinforced by the recruitment of actin cytoskeletal components (around the 24th minute of the movie). Shortly after, a second protrusion, made by the tip cell on the right hand side, establishes contact to the left tip cell. This second contact is quickly reinforced by actin cytoskeletal components (around the 45th minute of the movie). As anastomosis progresses, the two contacts are fused to a single one (around 75th minute of the movie). We also observe the establishment of two anastomotic contacts, which are then fused to a single one, in wild-type embryos. Therefore, anastomosis in the absence of Esama is comparable to wild-type.
**Movie S4: Angiogenesis in a wild-type embryo**

Confocal movie of a Tg(*fli1a:EGFP*)y1 embryo starting around 26hpf (= 0min) shown in inversed contrast, anterior to the left. Confocal stacks were acquired every 10 minutes. The angiogenic sprouts expand dorsally and the first anastomotic contacts are observed around 31hpf (5h of the movie). As angiogenesis progresses lumen is pushed through the SAs into the DLAV (12h of the movie). At the end of the movie (~44hpf), all the SAs and the DLAV are lumenized.
Movie S5: Angiogenesis in an esama^{ub19} mutant embryo

Confocal movie of a Tg(fli1a:EGFP)^y1 embryo starting around 26hpf (= 0min) shown in inverted contrast, anterior to the left. Confocal stacks were acquired every 10 minutes. Angiogenesis in esama^{ub19} mutant embryo progresses indistinguishable from wild-type embryos. The angiogenic sprouts expand dorsally and the first anastomotic contacts are observed around 30hpf (4h of the movie). As angiogenesis progresses lumen is pushed through the SAs into the DLAV (12h of the movie). At the end of the movie (~44hpf), all the SAs and the DLAV are lumenized.
Movie S6: Angiogenesis in a ve-cad<sup>hub8</sup> mutant embryo

Confocal movie of a Tg(fli1a:EGFP)<sup>y1</sup> embryo starting around 29hpf (26hpf = 0min) shown in inverted contrast, anterior to the left. Confocal stacks were acquired every 8 minutes. Tip cells of angiogenic sprouts start to anastomose around 30hpf (around 4h of the movie), which appears less efficient compared to wild-type embryos. In 34% of the SAs we observe detachments of tip cells from stalk cells, this examples however does not show this phenotype. Nevertheless, a basic vascular network, i.e. SAs and the DLAV, is formed. No lumen is pushed through the vessels due to the absence of blood pressure. However, sometimes lumen is observed in SAs up to the level of the horizontal myoseptum. The DA is mostly collapsed but may also show inflated portions.
Movie S7: Angiogenesis in an esama^{abs19}; ve-cad^{abs8} double mutant embryo

Confocal movie of a Tg(fli1a:EGFP)^{y1} embryo starting around 29hpf (26hpf = 0min) shown in inversed contrast, anterior to the left. Confocal stacks were acquired every 8 minutes. Tip cells of angiogenic sprouts start to anastomose around 30hpf (around 4h of the movie), which is even less efficient compared to ve-cad^{abs8} mutant embryos. In two SAs, the tip cells detach from the stalk (around 5h and 8h of the movie). The stalks of these two SAs do not manage to reattach to the tip cells in the DLAV within the time the embryo was imaged. This would be different in ve-cad^{abs8} mutant embryos, where stalk cells that detached from their tip cell manage to reconnect to the DLAV within 8h after detachment. As angiogenesis progresses, cells in the DLAV appear not to integrate into a proper endothelium and retain their protrusive activity.
Movie S8: Anastomosis in an \textit{esama}^{ubs19}; \textit{ve-cad}^{ubs8} double mutant embryo

Deconvolved spinning disc confocal movie of a Tg(\textit{fli1ep:gal4ff})^{ubs3}, Tg(\textit{UAS:EGFP-UCHD})^{ubs18}, Tg(\textit{kdrl:mCherry-CAAX})^{^{916}} esama^{ubs19}; ve-cad^{ubs8} double mutant embryo at around 32hpf, anterior to the left. Confocal stacks were acquired every minute. Single channels are shown in inversed contrast (green is EGFP-UCHD and red is mCherry-CAAX on left and right, respectively) and the merged channels are shown in the middle. As the two tip cells migrate towards each other, they probe the environment for neighboring tip cells. Filopodia of the adjacent tip cells touch occasionally. At the 59th minute we observe a filopodial contact between the two tip cells, which appears to be reinforced by the actin cytoskeleton, but is resolved shortly after. As the two cell bodies get closer to each other, they start to touch and retract again. This happens several times and even when the two cell bodies overlap extensively (see 97th minute), they resolve the contact made. Eventually, the two tip cells appear to form a cell-cell contact and to maintain it (around the 201st minute of the movie). We imaged this embryo over 3h and anastomosis takes significantly longer compared to wild-type and even \textit{ve-cad}^{ubs8} mutant embryos. In the last 20 minutes of the movie, we observe junction-like patterns with the EGPF-UCHD marker. However, in many other instances, we observed that some tip cells remain isolated, even after they tried to establish contact to neighbors.
Movie S9: Anastomosis in an esamaubs19; ve-cad+/ubs8 mutant embryo

Deconvolved spinning disc confocal movie of a Tg(fli1ep:gal4ff)ubs3, Tg(UAS:EGFP-UCHD)ubs18, Tg(kdrl:mCherry-CAAX)s916 esamaubs19; ve-cad+/ubs8 mutant embryo at around 32hpf, anterior to the left. Confocal stacks were acquired every minute. Single channels are shown in inversed contrast (green is EGFP-UCHD and red is mCherry-CAAX on left and right, respectively) and the merged channels are shown in the middle. Similar to ve-cad mutant embryos, many transient filopodial interactions are observed. Only as the tip cell bodies are getting closer to each other and start to overlap (around the 66th minute) the filopodia/cell bodies do not retract and form an anastomotic contact.