Endothelial cell–cell adhesion during zebrafish vascular development

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**Keywords:** vascular, zebrafish, adhesion, adherens junction, angiogenesis, anastomosis, cadherin, cytoskeleton, transgenic, time-lapse

**Abbreviations:** CCM, cerebral cavernous malformations; CCV, common cardinal vein; DLAV, dorsal longitudinal anastomosing vessel; dpf, days post-fertilization; hpf, hours post-fertilization; PLA, palatocerebral artery; TILLING, targeting induced local lesions in genomes; VE-cadherin, vascular endothelial cadherin

The vertebrate vasculature is an essential organ network with major roles in health and disease. The establishment of balanced cell–cell adhesion in the endothelium is crucial for the functionality of the vascular system. Furthermore, the correct patterning and integration of vascular endothelial cell–cell adhesion drives the morphogenesis of new vessels, and is thought to couple physical forces with signaling outcomes during development. Here, we review insights into this process that have come from studies in zebrafish. First, we describe mutants in which endothelial adhesion is perturbed, second we describe recent progress using in vivo cell biological approaches that allow the visualization of endothelial cell–cell junctions. These studies underline the profound potential of this model system to dissect in great detail the function of both known and novel regulators of endothelial cell–cell adhesion.

**Introduction**

Delivery of blood and oxygen to all organs in the body and subsequent recycling of deoxygenated blood requires a fully functioning cardiovascular system. Adhesion of endothelial cells is crucial for establishment and maintenance of structure and functionality in mature vessels. Different types of functional vessels require a distinctive balance between vessel permeability and vessel integrity (for a review, see refs. 1–3). For example, the endothelial cells lining the arterial vasculature establish strong intercellular junctions since these cells need to resist high-pressure blood flow. On the contrary, the lymphatic vasculature is a highly permeable system that is required to maintain fluid homeostasis, and consequently, lymphatic endothelial cells form more loose cell-to-cell contacts. Diversity of endothelial architecture during development and adult life therefore is continuously regulated by factors that either promote the strengthening of endothelial junctions, or that increase their permeability (for a review, see refs. 5 and 6).

While much progress has been made into understanding the role of adhesion in cultured cell systems as well as in mature vessel function and vessel stabilization, the role of cell–cell adhesion during vessel development and morphogenesis has received less attention. One notable system that has helped to progress knowledge of many processes in vascular development has been the zebrafish model for genetic and cellular biological studies (for a review, see ref. 7). While zebrafish has not yet fulfilled its full promise for understanding vascular adhesion pathways, recent studies have begun to demonstrate the great potential of the model to shed light on endothelial cell adhesion in development. In this review, we will discuss the use of the zebrafish model for vascular biology, and in particular, will outline recent discoveries in vascular endothelial adhesion, especially cell–cell adhesion. We will first discuss zebrafish mutant models and genetic studies. Second, we will review insights from live imaging of endothelial junctional phenomenon in vivo.

**Endothelial Cell–Cell Adhesion in Development: Lessons from Zebrafish Mutants**

**Mutants from forward genetics**

Large-scale forward genetics for developmental processes was pioneered using *Drosophila* and *C. elegans*, but has remained challenging in vertebrate model systems. It was largely the need for a vertebrate forward genetics model to study development that led to the introduction of the zebrafish (*Danio rerio*) in the early 1990s and has since sparked a rapid growth of laboratories using this model for gene discovery. High-throughput forward genetic “big screens” used zebrafish embryonic morphology as a readout and led to the isolation of a series of mutants with defects in cardiovascular development. Many of these mutants influenced key transcriptional or growth factor signaling pathways (for a review, see refs. 7 and 15), but several were subsequently found to be caused by mutations in important genes in endothelial cell–cell adhesion. The insights from this first generation of mutants are summarized below (Table 1 and Fig. 1).
Table 1. Zebrafish mutants identified with genetic lesions in genes involved in vascular adhesion pathways.

| Zebrafish mutant allele | Gene affected | Vascular phenotype | References |
|------------------------|---------------|--------------------|------------|
| santa                  | ccm1          | Dilation of the major vessels | 17,28,29 |
| valentine              | ccm2          | Dilation of the major vessels | 17,28,29 |
| heart of glass         | heg           | Circulatory block     | 16,25     |
| bubblehead             | βpix          | Cranial hemorrhaging  | 42        |
| redhead                | pak2a         | Cranial hemorrhaging  | 42,43     |
| glass onion/parachute  | cdh2 (N-cadherin) | Dysmorphic vascular network | 59  |
| ve-cadherin<sup>val</sup> | cdh5 (VE-cadherin) | Failure to form established junctions during anastomosis | 64,65 |
| tie2<sup>tie2<sup>mutant</sup> | tie2          | Enhancement of junctional integrity via VE-cadherin | 67  |

Santa (santa), Valentine (vtn), and Heart of glass (heg)

These mutants were isolated on the basis of dramatic cardiac development defects.12,13,16,17 Positional cloning of santa and vtn mutants led to the identification of mutations in the zebrafish homologs of the human CCM genes, ccm1 and ccm2, respectively.17 Mutations in human CCMs (CCM1/KRIT-1, CCM2/MGC4607, and CCM3/PDCD10) are causative for the autosomal dominant disease cerebral cavernous malformations (CCM). CCM patients develop enlarged thin-walled capillaries in the brain. The severity of the disease varies, causing health problems ranging from headaches to severe brain hemorrhages and death.

CCM proteins regulate cell adhesion and have been described to form a complex that can associate with cadherins and strengthen cell–cell junctions in cell culture systems.18-20 The PTB and FERM protein domains affected in the original santa and vtn Ccm mutants have been shown to be important for the interaction between CCM proteins and Integrin or cadherin complexes. A PTB domain-facilitated interaction of CCM1 and CCM2 has been shown to drive CCM1 binding to Integrin cytoplasmic domain-associated protein-2 (ICAP-1) and the formation of ICAP-1-β1-Integrin complexes in vitro.21,22 CCM1–CCM2 interaction is essential for maintenance of junctional integrity.23 The FERM domain of CCM1 controls junctional localization of the protein via activation by Rap1, a central regulator of cytoskeletal–junctional interactions.24 Also, CCM1 stabilizes VE-cadherin-bound β-catenin, thereby strengthening the adherens junctions.24 A phenotypically similar mutant heart of glass (heg) encodes for a membrane-localized receptor, which has been shown both in zebrafish and mice to genetically interact with members of the CCM complex, together enforcing junctional integrity.16,25,26 Hence, Ccm–Heg complexes have been suggested to regulate cell–cell and cell–matrix interactions and this is consistent with mutations in these genes leading to perhaps the most dramatic morphological cardiovascular defects observed in zebrafish mutants.

While much of our understanding of the role of CCMs in adhesion does not come from zebrafish (for a review, see ref. 27), particularly informative findings came from a handful of zebrafish loss-of-function studies that expand our knowledge of CCM function at the gross phenotypic level during vascular development. One study investigated the interaction of ccm1 with rap1b, which is a central regulator of the adherens junction–cytoskeletal relationship.28 Transient knockdown of both genes simultaneously, using morpholino oligomers, was used to show genetic interaction of the two factors during the pathogenesis of cranial hemorrhage.28 This study suggested a mechanistic link between Rap1 and CCMs in the pathogenesis of cavernous malformations, which can lead to stroke. Another study investigated the vessels in both ccm1 and ccm2 mutants, identifying a novel ccm1 allele in a forward genetic screen.29 It was found that the major vessels are progressively, quantifiably dilated during development in the absence of CCMs; this is not due to a loss of blood flow in these mutants as a loss of blood flow by knockdown of silent heart (sih)10 led to distinct progressive vascular phenotypes in comparative experiments.29 The endothelial cells within the enlarged vessels were properly differentiated and the number of endothelial cells populating ccm1-deficient vessels was not altered. Interestingly, ultrastructural analysis of the cell–cell junctions in both ccm1 and ccm2 mutants indicated that junctional integrity was not disrupted. Since junctions are established but endothelial morphology is affected it is likely that CCMs act more at the level of cadherin–cytoskeleton interactions, which is supported by extensive cell culture data (for a review, see ref. 31). This study and others investigating gene expression17 suggested a likely cell autonomous role for Ccm1 in endothelium and endocardium. Additional analysis of other Ccm family members have gone on to support a critical role for this gene family in adhesion and morphogenesis during zebrafish cardiovascular development.25,26,32,35 Studies in mice have since definitively confirmed and significantly extended these findings.25,34–40

Bubblehead (bbh) and red head (rhd)

These mutants represent a class of zebrafish vascular mutants isolated from forward genetic screens for defects in vascular integrity. Mutants in this broader class displayed brain hemorrhaging with mild additional cardiovascular defects and several of these mutants remain to be characterized.14,41 Genetic mapping of bubblehead (bbh) and red head (rhd) revealed that the genetic lesions were positioned in p21-activated kinase (Pak)-interacting exchange factor protein β (βpix)42 and p21-activated kinase2a (pak2a),43 respectively. In vitro studies had previously provided evidence of a βPix–Pak interaction acting upstream of the small GTPase Rac to induce cell motility.44,45 Analysis of bhh and rhd mutants has led to the discovery of a novel pathway regulating vascular integrity.
where the Pak2a kinase functions downstream of βPix in stabilization of the brain vasculature in vivo. This βPix signaling was suggested to function non-cell autonomously in peri-vascular mural cells, however this remains to be fully shown and the timing of appearance of mural cells in the brain may suggest an endothelial role (Yuying Wang Y, et al. Notch3 establishes brain vascular integrity by regulating pericyte number. Development). The vascular network in bbh mutants and βPix morphants was described to remain in an immature state and it was suggested that this might be due to defective migration in these loss-of-function scenarios. In addition to vascular stabilization, βpix was shown to promote cerebral angiogenesis and that for both functions βPix linkage to Integrins via Git1 is essential. Interestingly, a proportion of the bbh and rhd mutants are viable, suggesting that the necessity of this pathway is restricted to the cerebral vasculature during a short time window in development. Both in vivo and in vitro observations have indicated roles of Rac and Integrins in not just migration but also in modulating key junctional complexes (for a review, see refs. 48–50), it is likely that defects in adhesion might be secondary to the severe neuronal abnormalities in this mutant. Outstanding questions are how βPix–Pak drives vascular integrity and whether βPix–Pak signaling is cell-autonomous.

**Glass onion (glo) and parachute (pac)**

Neuronal-cadherin (N-cadherin/Cdh2)-deficient glass onion (glo) and parachute (pac) zebrafish embryos were independently identified in forward genetic screens concentrating on either neural or retinal defects and suffer from multi-organ developmental defects. Although vascular endothelial–cadherin (VE-cadherin/ Cdh5) is known as the major cadherin that connects all endothelial cells, N-cadherin has also been shown to be expressed in cultured endothelial cells. Distribution of N-cadherin proteins in vitro however was proven to be mostly non-junctional with exclusion of N-cadherin from endothelial junctions caused by a competition with VE-cadherin. Nevertheless, endothelial-specific deletion of N-cadherin in mice results in early embryonic lethality due to severe vascular defects underlining the importance of N-cadherin in the endothelium. Analysis of the cardiovascular phenotypes in zebrafish glo mutants revealed that even though the morphology of the heart tube is highly distorted, cardiomyocytes had differentiated and cardiac contraction was present. Although N-cadherin was not detected in endothelial cells, the mutants display a dysmorphic vascular network with reduced numbers of intersegmental vessels and a disorganized non-perfused trunk vasculature. The underlying causes of the vascular phenotypes have been suggested to be either the lack of vascular flow or disruption of the surrounding skeletal muscles. Noteworthy, both the Vegfa ligand and Vegfr-1 (Flt1) have been described to be expressed in the neural tube in zebrafish. Therefore, vascular defects observed in glo mutants might be secondary to the severe neuronal abnormalities in this mutant. In addition, it will be important to examine N-cadherin expression in endothelial cells in vivo and determine if it functions cell-autonomously in vascular development.

**Mutants from reverse genetics**

Another genetic approach that has led to insights into the role of vascular endothelial cell–cell adhesion in zebrafish is the use of gene targeting approaches or reverse genetics. Mutants generated through TILLING (Targeting Induced Local Lesions in Genomes) or through Zinc Finger genome editing approaches include zebrafish mutants in Tie2 and VE-cadherin.

**VE-cadherin (cdh5)**

The most notable endothelial adhesion gene studied to date in mutant zebrafish is VE-cadherin. Recently, a novel VE-cadherin loss-of-function allele (ve-cadherin<sup>shh</sup>, further referred to as ve-cad) was described in which a premature stop mutation was induced in exon 3 of the zebrafish re-cadherin (cdh5) gene by using Zinc Finger Nuclease-mediated genome editing technology. VE-cadherin (Cdh5) belongs to the large cadherin superfamily sharing great structural homology with other cadherins, like E-cadherin (Cdh1) and N-cadherin (Cdh2). Loss of VE-cadherin function in the mouse causes embryonic lethality at around E9.5 due to severe cardiovascular defects. Perhaps surprisingly, zebrafish ve-cad mutants are morphologically

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*Figure 1.* Interaction of proteins analyzed in zebrafish loss-of-function models and their role in endothelial adhesion. (A) Endothelial cells lining the wall of a blood vessel. (B) Enlargement of two endothelial cells from A. VE-cadherin proteins are the major cadherins that connect the endothelial cells. Endothelial N-cadherin expression in zebrafish and its interaction with VE-cadherin remains to be explored. VE-cadherin is connected to the actin cytoskeleton via linker proteins β-catenin, α-catenin, and Vinculin. Ccm complexes formed by at least Ccm1 and Ccm2 bind to the cytoplasmic domains of Integrins (via ICAP-1) and VE-cadherin. These complexes have been suggested to stabilize the connections between junctional proteins and the actin cytoskeleton by interacting with Rap1 and β-catenin. The membrane receptor Heg facilitates the Ccm complex in promoting junctional integrity. βPix–Pak signaling can activate Rac in the cerebral endothelium, thereby promoting junctional integrity. βPix binding to Integrins (via Git1) was shown to be required for βPix regulation of junctional stability and angiogenesis of the cerebral vasculature, although cell autonomy remains unclear. Analysis of Tie2-deficient zebrafish revealed that loss of Tie2 strengthens endothelial junctions when challenged by atorvastatin in a VE-cadherin-dependent manner.
indistinguishable from sibling embryos at 32hpf and lumenised vessels have developed in a pattern comparable to wild-type embryos at that stage. However, the mutant vasculature is not perfused and ve-cad mutant hearts are severely malformed causing a lack of blood circulation.

In the prominent recent study by Lenard et al., the authors investigated in unprecedented detail the role of VE-cadherin in vascular anastomosis. Using high power time-lapse imaging, the formation of the palatocerebral artery (PLA), a vessel that develops as a part of the zebrafish head vasculature, was analysed in depth. This vessel forms by the defined anastomosis of the internal carotid arteries. At the leading edge, these tip cells display EGFP-ZO1 and VE-cadherinAC-EGFP positive filopodia. (B) Connecting filopodial extensions of the sprouts initiate concentrated expression of EGFP-ZO1 and VE-cadherinAC-EGFP at the stable contact point. A dead ended unicellular lumen (TL) is established from the internal carotid arteries. (C) The unicellular EGFP-ZO1 and VE-cadherinAC-EGFP proteins at the contact point rapidly form a ring shaped apical membrane. Extensive junctional remodeling and cellular rearrangements lead to the formation of a perfused multicellular tube at ~40 hpf (D).

Figure 2. Summary of junctional changes during vascular morphogenesis based on Lenard et al. to highlight anastomosis of the palatocerebral artery. (A) At ~32 hpf tip cells migrate medially from the bilateral internal carotid arteries. At the leading edge, these tip cells display EGFP-ZO1 and VE-cadherinAC-EGFP positive filopodia. (B) Connecting filopodial extensions of the sprouts initiate concentrated expression of EGFP-ZO1 and VE-cadherinAC-EGFP at the stable contact point. A dead ended unicellular lumen (TL) is established from the internal carotid arteries. (C) The unicellular EGFP-ZO1 and VE-cadherinAC-EGFP proteins at the contact point rapidly form a ring shaped apical membrane. Extensive junctional remodeling and cellular rearrangements lead to the formation of a perfused multicellular tube at ~40 hpf (D).

contact between filopodia of these tip cells results in an increased deposition of junctional material, such as Zona Occludens 1 (ZO1) and VE-cadherin at the contact site (Fig. 2B), preceding the formation of a polarized junction between the sprouts (Fig. 2C). In ve-cad mutants, tip cells migrate medially and form a similar number of filopodial protrusions. However, the first contact point that is established between the two sprouts does not lead to the formation of a stable junction. Instead, multiple exploratory filopodia remain and fusion of these remaining filopodia leads to the formation of multiple junctions between the VE-cadherin-deficient PLA sprouts. These results indicate that although sprouting behavior appears to be normal, VE-cadherin is required for the tip cells to recognize each other upon contact and to subsequently induce the formation of a polarized junction. Notably, in ve-cad mutants the vessels are not perfused due to cardiac defects. However, in sib morphants, which also lack blood flow, the PLA developed in a similar fashion to wild-type, forming a single junction at an established contact point. Although lumensisation is likely flow-dependent, the ve-cad junctional defects appear to occur independent of the loss of flow. The study therefore led to the conclusion that the process of anastomosis requires the presence of functional VE-cadherin. Moreover, this study highlighted the potential of the model to study the role of endothelial junctions in vascular development with unprecedented resolution.

A second study examined this ve-cad mutant in the context of a novel mechanism of vascular lumen formation referred to as “lumen ensheathment.” This process occurs in the development of the common cardinal veins (CCVs), large collecting vessels that connect the vasculature to the inflow pole of the heart. Using time-lapse imaging, it was shown that single cell migration of angioblasts at 20 hpf initiates the formation of the CCVs. These angioblasts populate the space between the epidermis and the yolk syncytial layer (YSL) where they form a monolayer at the side of the YSL. From this layer, individual angioblasts delaminate and migrate toward the epidermal pole leading to the formation of a circular sheet of cells that line a luminal space between the YSL and the epidermis, which is first observed at 26 hpf. VE-cadherin was shown to be necessary for delamination of endothelial cells in the YSL sheet and also for later collective migration of the cells in the direction of the heart. Interestingly, the authors show that the process of CCV formation is independent of blood flow. Therefore, in ve-cad mutants that lack flow, the described behavior of VE-cadherin mutant cells during CCV formation is not secondary to a loss of circulation.

Hence, while perhaps far more has been garnered about VE-cadherin and its role in vascular adhesion from cultured cells and mouse model studies, these analyses in the zebrafish define contextual roles for VE-cadherin in cellular and tissue level morphogenesis events that shape vascular development. It is likely that further extensive analyses of the zebrafish VE-cadherin mutant will yield many more such insights.

Tie1 and Tie2

Generated by TILLING (Targeting Induced Local Lesions in Genomes), tie2<sup>Δ1067</sup> mutants (further referred to as tie2<sup>−</sup> mutants) have led to a better understanding of the role of Tie receptor.
tyrosine kinases in vascular integrity in the context of the developing vertebrate embryo. Gjini et al. have shown that in zebrafish, Tie1 and Tie2 function redundantly during the development of the heart to establish normal adhesion between both myocardial and endocardial cells. In single tie2 mutants however the heart develops comparably to wild-type siblings despite the fact that mouse Tie2 knockouts suffer from severe cardiac defects causing embryonic lethality. This mutant model therefore was used to further explore the function of Tie2 in the developing vasculature. The authors show that both blood and lymphatic vessels are present and functional in tie2 mutants. Interestingly, when challenging the permeability of the endothelium using the chemical atorvastatin, a potent inducer of hemorrhaging in the brain, tie2 deficiency was shown to prevent the onset of hemorrhaging. More detailed analysis of the process of atorvastatin-induced hemorrhaging revealed that the cranial vessels become leaky upon endothelial rupture during angiogenesis. Adhesion via VE-cadherin was shown to be required for preventing hemorrhaging in tie2 mutants since morpholino knockdown of ve-cadherin resulted in an incidence of hemorrhaging that was comparable between tie2 mutant and wild-type embryos. These data suggest that during angiogenesis, continuous changes in permeability are maintained by a balance between functional Tie2 and VE-cadherin proteins.

**Endothelial Cell–Cell Adhesion in Development: Lessons from in vivo Cell Biology in Zebrafish**

Since the introduction of transgenic reporter lines driven by endothelial-specific promoter regions, the zebrafish model has been used to analyze in great detail the onset of endothelial differentiation, endothelial cell behavior, and vessel morphogenesis. Time-lapse imaging has been a very powerful technique in the field, mainly due to the inherent optical transparency of zebrafish embryos. More recently, transgenic models expressing fluorescently tagged proteins have refined imaging approaches, making it possible to visualize live where and when proteins are active, providing a great utility to the zebrafish model when compared with other vertebrate model systems. Here we will discuss the observations made using live imaging techniques to visualize proteins involved in endothelial adhesion (Table 2).

**C. elegans** (for a review, see refs. 77 and 78). To visualize endothelial membranes during lumen formation, both zebrafish Moesin1 and β-catenin were fluorescently tagged and driven by the endothelial *kdrl* promoter. These proteins are hypothesized to localize to actin rich apical membranes and adherens junctions, making these ideal polarity markers. Time-lapse imaging of Moesin1-EGFP and mCherry-β-catenin in sprouting intersegmental vessels confirmed that these proteins co-localize at the apical/lumenal membrane before the onset of blood flow. Interestingly, Moesin1-EGFP localization did not fully overlap with VE-cadherin by immunofluorescence on fixed samples. This localization suggested that Moesin1 is targeted to polarized junctions to determine regions of lumen formation.

Table 2. Zebrafish transgenic tools to visualize endothelial adhesion

| Zebrasfish transgene | Labeled protein | References |
|----------------------|----------------|------------|
| Tg(fli1a:EGFP-Cdc42) | Cdc42         | 74         |
| Tg(kdrl:msna-EGFP)  | Moesin1       | 76         |
| Tg(kdrl:mCherry-actb1) | β-actin1     | 76         |
| Tg(kdrl:ctnna-EGFP) | α-catenin1    | 76         |
| Tg(14xUAS:EGFP-hZO1) | hZO1          | 64, 81     |
| Tg(5xUAS:VE-cadherinα-EGFP) | VE-cadherin | 64         |
| Tg(4xUAS:Lifeact-EGFP) | F-actin      | 65         |
| Tg(fli1a:Lifeact-EGFP) | F-actin      | 91         |
| Tg(fli1a:mCherry-hZO1) | hZO1          | 91         |

imaging of the endothelial cell-to-cell junctions in conjunction with the vacuoles was not performed. Vacuole fusion has not been described in detail in different vessel types in the zebrafish embryo.

Interestingly, a later study by Wang et al. provided evidence that endothelial junctions are required for the process of tubulogenesis. Moesin is a member of the Ezrin/Radixin/Moesin (ERM) family of proteins that link the actin cytoskeleton to proteins localized at the apical plasma membrane both in vitro and in vivo in *Drosophila* and *C. elegans* (for a review, see refs. 77 and 78). To visualize endothelial membranes during lumen formation, both zebrafish Moesin1 and β-catenin were fluorescently tagged and driven by the endothelial *kdrl* promoter. These proteins are hypothesized to localize to actin rich apical membranes and adherens junctions, making these ideal polarity markers.
membrane rather than establishing a vascular lumen as such. By mosaic analysis of Moesin1 and VE-cadherin-knockdown cells in a wild-type environment, the authors went on to find that cell–cell junctions are required cell autonomously for lumenisation to occur, refining our understanding of the junctional contribution to lumen formation.

**ZO1 and VE-cadherin**

The adaptation of the Gal4-UAS expression system in fish has generated a suite of UAS-driven lines expressing labeled proteins. This method was applied to drive the expression of an EGFP-tagged human ZO1 protein in zebrafish. Although ZO1 is conventionally thought of as a tight junction protein, it can associate with α-catennin, therefore potentially with cadherin complexes. This fusion protein linking ZO1 to the C terminus of GFP was previously shown to recapitulate endogenous ZO1 behavior in cultured cells. When crossed to the fli1a:Gal4FF transgene, the EGFP-ZO1 protein was enriched in endothelium. Although this method applied forced expression of ZO1, essentially overexpressing it in addition to endogenous protein, phenotypic abnormalities were not described. Furthermore, expression of EGFP–ZO1 was shown to recapitulate the expression of endogenous ZO1, suggesting that this transgene was a faithful tool to follow ZO1 dynamics at endothelial junctions.

Co-expression analysis of the labeled ZO1 protein with zebrafish VE-cadherin in the dorsal longitudinal anastomotic vessel (DLAV) protein by immunofluorescence labeling revealed that the junctional architecture of these vessels was highly heterogeneous. There are well-defined ring shaped junctions but also more complex junctions with an undefined shape. As the name suggests, the DLAV forms by an event of tip cell anastomosis. EGFP–ZO1 localizes to the contact point where two tip cells meet and rapidly (within 40 min) a ring-shaped junction is formed at this site. These initial stages of DLAV anastomosis share great similarity with PLA anastomosis (Fig. 2). Remodeling of the initial DLAV junction however was shown to be quite complex and occurs via two alternate cellular mechanisms, either via cellular extrusion or, more frequently, via a morphogenetic mechanism of cord hollowing. Interestingly, upon loss of flow (sih morpholino inhibition of flow), initial DLAV anastomosis upon contact of tip cells occurs normally but the vessels fail to inflate and lumenise.

In addition to the UAS-controlled EGFP–ZO1 transgene, the same group later developed a UAS-driven truncated form of the zebrafish VE-cadherin protein. In this transgenic line, the VE-cadherin cytoplasmic domain has been replaced by EGFP. Due to this truncation, the protein is most likely not fully functional since it can no longer connect to actin inside the cell (for a review, see refs. 87 and 88). However, the VE-cadherinΔC-EGFP protein gets targeted to the junctions and can dock in the membrane. Also, the expression pattern of VE-cadherinΔC-EGFP overlaps with previous VE-cadherin localization studies in zebrafish, making this line a useful tool to image endothelial junctional dynamics. With the VE-cadherinΔC-EGFP transgene, the authors show that, as seen previously for ZO1 in DLAV anastomosis in the trunk, junctional material rapidly accumulates at the contact point between tip cells in the context of the anastomosis of the cranial PLA vessel (Fig. 2). Interestingly, during tip cell sprouting, both EGFP-ZO1 and VE-cadherinΔC-EGFP are present in the tip cell protrusions that sense the environment (Fig. 2A). This suggests that the protein may interact with components of the extracellular matrix during angiogenic sprouting or that junctional proteins are pre-emptively deposited in filopodia so that immediately upon first cell–cell contact, junctions can be rapidly established. In previous immunofluorescence localization studies, endogenous zebrafish VE-cadherin and ZO1 proteins were detected in the junctions between the leading cells and the connecting stalk cell but not at the distal part of the leading cells. This raises the question whether endogenous, low levels of junctional proteins are expressed and functional in filopodia or whether the forced expression of transgenically encoded proteins induces ectopic localization.

**F-actin dynamics**

VE-cadherin at the adherens junctions connects, via linker proteins like Vinculin, α-catennin, and β-catennin, to the actin cytoskeleton (for a review, see ref. 79). The intracellular network of actin is crucial since it strengthens the cell body but it is also required to drive migration of cells. Cadherins may potentially function as receptors that signal to regulate actin dynamics (for a review, see ref. 89). In actively migrating cells, filamentous F-actin has been shown to localize to filopodia or lamellipodia (for a review, see ref. 90). Two recent studies have made use of fluorescently tagged forms of F-actin to study actin dynamics in endothelial cells during sprouting angiogenesis and CCV formation. Both transgenes express a GFP-labeled version of the previously published Lifeact peptide that has been proven to bind F-actin both in vitro and in vivo. The fluorescently labeled Lifeact peptide was placed behind either the endothelial fli1a promoter or UAS repetitive elements to image changes in endothelial actin dynamics. With regards to CCV formation, the UAS-driven F-actin was used solely as a readout to prove that the sheet of endothelial cells that form the CCV are actively migrating, and hence, contain F-actin-rich lamellipodia at the leading cell front. While it needs to be considered that actin fusion modules can affect cell morphology and the dynamics of actin microfilaments in vitro, these studies do not such phenotypes were observed.

More detailed analysis of endothelial F-actin was performed in the study by Phng et al. where they show that using their version of the fli1a-driven Lifeact, GFP localizes to the hypothesized cellular compartments, the cell cortex, membranous, and cytoplasmic clusters and filopodia. In fixed tissues, the Lifeact signal co-localized with the well-established F-actin marker phalloidin, indicating that the fusion protein serves as a suitable readout for F-actin localization. Clusters of F-actin were also observed in endothelial filopodia, co-expressing mCherry-ZO1, indicating a function for this complex outside cell–cell junctions. Simultaneous imaging of EGFP-Lifeact with a membrane-targeted mCherry protein showed that F-actin polymerization does not occur throughout the circumference of the filopodia. Low levels of Latrunculin B, which sequesters G-actin to prevent actin polymerization, were used to significantly reduce EGFP-Lifeact expression and morphologically inhibit filopodia formation.
Quite remarkably, time-lapse imaging of Latrunculin B-treated embryos revealed that despite the loss of filopodia, endothelial sprouts still migrate. However, the migratory velocity of the sprouts is significantly reduced when filopodia are not present. This reduced velocity was proven not to be due to changes in cell number in the migrating sprout. Using the F-actin transgene, the authors could observe lamellipodia formation at the leading front of the tip cell, which were suggested to be sufficient to drive migration in the absence of filopodia. When looking at the process of DLAV anastomosis however, filopodia were proven to be essential. As described previously with ZO1 and VE-cadherin transgenes, these junctional proteins are required at the contact point of two filopodia from opposing tip cells. For the establishment of a junction, an intact F-actin deposition appears to be crucial since Latrunculin B-treated embryos fail to establish DLAV anastomoses completely throughout the embryo. Overall, this study demonstrated the relative contribution of different cellular extensions to distinct processes during angiogenic sprouting.

This first generation of fusion protein studies described above show that when carefully controlled, the live imaging of cellular morphogenesis events and the relative contributions of junctional components can be exquisitely dissected using the zebrafish model.

Future Perspectives

As highlighted in this review, the live imaging of cell biological events during zebrafish development is now moving from the imaging of cellular events to the individual proteins and molecular interactions that drive morphogenesis. This opens up new avenues in the study and understanding of vascular adhesion pathways during development.

From a technical perspective, thus far, protein fusion transgenic models have most commonly made use of endothelial promoters that induce overexpression of the protein of interest. Recently, the application of tol2-mediated Bacterial Artificial Chromosome (BAC) transgenesis in zebrafish has led to a new generation of transgenes, where fusion proteins can be driven by their own promoter elements. This approach is perhaps less artificial because the endogenous regulatory elements of a gene drive its expression; however, genomic insertion of a BAC construct still results in an exogenous copy of the protein. Emerging techniques include TALEN and CRISPR-mediated gene mutation or integration. This can now be used to overcome overexpression of proteins in transgenics by knocking out the endogenous copy of the gene. Furthermore, fusion proteins can be generated by inserting fluorescent proteins in the genome directly, fused to the endogenous protein. Hence, as has been done extensively in cultured cell systems, zebrafish can also now serve as a host for protein reconstitution experiments to study fluorescent fusion proteins as they perform the function of their wild-type counterparts.

Concomitantly to protein localization, an exciting new imaging route will be the visualization of protein activity. In cell culture systems, FRET-based biosensors have been developed that can be used to measure protein activity. A handful of such biosensors have been proven to work in zebrafish embryos, including Rac1, PI3 kinase, and ERK biosensors. In vitro, FRET sensors can be used as a readout for mechanical tension across junctional proteins. These tension sensors may also be applied in vivo, and due to the versatile imaging possibilities of zebrafish, this would be a suitable system to implement such techniques. However, it should be noted that the functional state of these fusion protein sensors in vivo remains to be demonstrated. Combining such emerging approaches with conventional manipulation, such as cell and subcellular ablations, FRAP and the generation of cellular mosaic animals stands to serve a host of possible applications.

At the level of genetics, since most loss-of-function alleles described in this review were isolated in forward genetic screens, progress in studying loss-of-function phenotypes in endothelial adhesion has been relatively slow. However, recent breakthroughs in genome editing tools now enable extremely rapid and efficient reverse genetics in zebrafish. Therefore, we expect acceleration in the phenotypic analysis of full zebrafish knockouts to a much larger proportion of the genome. Importantly, for broadly acting cellular factors, the fact that these technologies allow for tissue-specific knockouts by genomic insertion of LoxP sites hold great potential.

For the study of endothelial cell–cell junctions during development, the combination of rapid, flexible genetic manipulation and increasingly high-resolution sub-cellular in vivo imaging, this model seems likely to continue to rise in prominence.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We would like to thank Dr Katarzyna Kołtowska for critical reading of the manuscript.

Funding
Yap AS is supported by NHMRC fellowship APP1044041. Lagendijk AK is supported by NHMRC APP1010489 and a University of Queensland Postdoctoral Fellowship. Hogan BM is supported by an ARC Future Fellowship FT100100165.
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