Angiomotin-Like Protein 1 Controls Endothelial Polarity and Junction Stability During Sprouting Angiogenesis

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Rationale: We have previously shown that angiomotin (Amot) is essential for endothelial cell migration during mouse embryogenesis. However, ~5% of Amot knockout mice survived without any detectable vascular defects. Angiomotin-like protein 1 (AmotL1) potentially compensates for the absence of Amot as it is 62% homologous to Amot and exhibits similar expression pattern in endothelial cells.

Objective: Here, we report the identification of a novel isoform of AmotL1 that controls endothelial cell polarization and directional migration.

Methods and Results: Small interfering RNA–mediated silencing of AmotL1 in mouse aortic endothelial cells caused a significant reduction in migration. In confluent mouse pancreatic islet endothelial cells (MS-1), AmotL1 colocalized with Amot to tight junctions. Small interfering RNA knockdown of both Amot and AmotL1 in MS-1 cells exhibited an additive effect on increasing paracellular permeability compared to that of knocking down either Amot or AmotL1, indicating both proteins were required for proper tight junction activity. Moreover, as visualized using high-resolution 2-photon microscopy, the morpholino-mediated knockdown of amotl1 during zebrafish embryogenesis resulted in vascular migratory defect of intersegmental vessels with strikingly decreased junction stability between the stalk cells and the aorta. However, the phenotype was quite distinct from that of amot knockdown which affected polarization of the tip cells of intersegmental vessels. Double knockdown resulted in an additive phenotype of depolarized tip cells with no or decreased connection of the stalk cells to the dorsal aorta.

Conclusions: These results cumulatively validate that Amot and AmotL1 have similar effects on endothelial migration and tight junction formation in vitro. However, in vivo Amot appears to control the polarity of vascular tip cells whereas AmotL1 mainly affects the stability of cell–cell junctions of the stalk cells. (Circ Res. 2009;105:260-270.)

Key Words: AmotL1 ■ polarity ■ migration ■ junction stability ■ zebrafish

The mechanisms of blood vessel development have received much attention because of their involvement in pathological processes such as neoplasia and ocular diseases.1–3 The major blood vessels, such as the aorta, are initially formed through the process of vasculogenesis in which precursor cells migrate, differentiate, and polarize to form tube-like structures.4 Once the central vessels are formed they extend, sprout and migrate to form a secondary capillary bed that supports the developing tissues with oxygen and nutrients. The migration trajectories are guided by attractive and repulsive signals that control the precise formation of a circulatory network in time and space.5 During angiogenesis, the leading endothelial cells (ECs), tip cells, extend filopodia that detect migratory cues in the microenvironment. The stalk cells form lumens and maintain cell–cell contacts to form a seamless vessel.6 Although a number of promigratory molecules have been identified little is yet known about how the extracellular signals exert their control on cell polarity, cell–cell junction stability and lumen formation.

Angiomotin (Amot) is a coiled-coil protein that is expressed in ECs and controls migration, tight junction (TJ) formation and cell polarity in vitro via its C-terminal PDZ-binding motif.7–9 Consistent with these in vitro findings, Amot-deficient mice of C57/B6-background showed severe vascular insufficiency in the intersomitic region as well as dilated vessels in the brain resulting in embryonic lethality.
between embryonic day (E)11 and E11.5. In vitro studies also revealed that Amot-deficient ECs lost polarity and had reduced directional migration in response to chemoattractants. In addition, DNA vaccination or antibody treatment against Amot significantly inhibited tumor angiogenesis and tumor growth, indicating that Amot is a potential target for cancer therapy. Despite the significant contribution of tumor growth, it was noted that ≈5% of Amot-deficient mice with C57/B6 genetic background did survive without any detectable vascular defects. This led to the speculation that other members of the same protein family may compensate for the loss of Amot.

Amot together with angiomotin-like protein (AmotL1) (AmotL1, also referred to as JEAP) and AmotL2 (also referred to as LCCP or MASCOT) belong to a novel protein family characterized by their conserved glutamine rich domain, coiled-coil domains and PDZ-binding motif. AmotL1 was initially cloned from MS-1 EC line as a TJ-enriched and associated protein (JEAP). However, the biological function of AmotL1 has yet to be characterized. Here, we report the identification of a novel isoform of AmotL1 that controls endothelial migration and cell polarity. We provide evidence that AmotL1 regulates sprouting angiogenesis by affecting tip cell migration as well as controlling cell–cell adhesions in vivo.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results
Identification of the Short Isoform of AmotL1
In collaboration with the Human Protein Atlas consortium, we have analyzed AmotL1 protein expression in human tissue arrays. Immunohistochemical analysis showed that AmotL1 was detectable in ECs of human placenta, ovary, and cancer tissues (http://www.proteinatlas.org). We further examined the expression pattern of AmotL1 in EC lines including human umbilical vein ECs, bovine capillary ECs (BCEs), mouse pancreatic islet ECs (MS-1), mouse aortic ECs (MAEs), polyoma middle T ECs (derived from embryonic stem cells), and tumor ECs by Western blot. In addition to the band with a molecular mass more than 100 kDa, which has been reported as JEAP previously, we could detect another band with an apparent molecular mass of 90 kDa (Figure 1A).

To assess the sequence of the 90-kDa band, AmotL1 was immunoprecipitated from MAE lysate and separated by SDS-PAGE. Two bands with respective molecular mass of ≈100 and 90 kDa were excised for mass spectrometric analysis. AmotL1 was unambiguously identified by Peptides Mass Fingerprint in both bands, indicating the existence of 2 AmotL1 isoforms. The N-terminal initiation site of the 90-kDa band was identified by Edman degradation (Figure 1B and Online Table I). Taken together, these data show that the 2 isoforms of AmotL1 only differ in the N-terminus. We subsequently cloned the short isoform from the DNA sequence corresponding to the 86th methionine of the original AmotL1 amino acid sequence (Figure 1C). Western blot analysis revealed that when transiently transfected into CHO cells, the cloned AmotL1 of 90 kDa was of the same molecular mass as the endogenous one from MAEs (Figure 1D). Thus, we denominated the 2 isoforms of AmotL1 as p90 and p100 AmotL1 according to their molecular mass.

AmotL1 Localizes to Endothelial Lamellipodia and Tight Junctions
Next, we investigated the subcellular localization of endogenous AmotL1 in ECs. Immunofluorescence staining of migrating MAEs showed that endogenous AmotL1 localized to the cell leading front edge lamellipodia and overlapped with F-actin as visualized by phalloidin staining, suggesting that AmotL1 might regulate EC migration (Figure 2A). In addition, AmotL1-positive staining was also detected in the cell–cell contacts of confluent MS-1 ECs where it colocalized with the TJ marker zonula occludens (ZO)-1 (Figure 2B). To further investigate whether Amot and AmotL1 colocalized to TJs, MS-1 cells were double stained with rat anti-mouse AmotL1 monoclonal antibody and rabbit anti-human Amot polyclonal antibodies. Immunofluorescence signals with the respective antibody showed perfect overlap in cell–cell junctions, indicating that the 2 proteins may interact directly with each other (Figure 2C).

The PDZ-Binding Motifs Are Not Required for the Direct Interaction Between Amot and AmotL1
We have previously reported that Amot and AmotL1 bind to the scaffold protein Patj (or Mupp1) through distinct PDZ domain and PDZ-binding motif, which opens up the possibility that Amot and AmotL1 could be part of the same protein complex. However, there is no evidence that Amot and AmotL1 directly bind to each other. All of the members in the angiomotin family share the conserved coiled-coil domains, through which they form homooligomers. Based on their colocalization in ECs, it might be speculated
that Amot and AmotL1 could either form heterooligomers through the conserved coiled-coil domains or by binding to distinct PDZ domains of Patj (or Mupp1) to stabilize the polarity protein complex Amot:AmotL1:Patj. To verify this hypothesis, p80 Amot and p100 AmotL1 or their mutants at PDZ-binding motif (H9004) were cotransfected into CHO cells, which lack endogenous expression of both Amot and AmotL1. The immunoprecipitation results revealed that Amot and AmotL1 coprecipitated with each other independent of whether the PDZ-binding motifs were intact or not (Figure 3). Thus, Amot and AmotL1 can form heterooligomers most likely through the direct binding of coiled-coil domains and their PDZ-binding motifs are not required during the direct interaction between Amot and AmotL1.

**AmotL1 Controls Cell Polarity and Promotes Endothelial Cell Migration**

To investigate the biological functions of AmotL1, SMARTpool small interfering (si)RNA specifically targeting AmotL1 was transfected into MAEs (which only express AmotL1; Figure 1A). Western blot analysis confirmed that both p90 and p100 AmotL1 were efficiently knocked down by using siRNA (Figure 4A). Cell polarity was studied by analyzing Golgi positioning. Reorientation of the Golgi is thought to facilitate polarized secretion thereby providing membrane and secreted products directly to the most proximate plasma membrane such as the leading edge in migrating cells. Visualization of Golgi apparatus with a GM130 antibody revealed that the cloned p90 AmotL1 was the same molecular mass to the endogenous AmotL1 short isoform in MAEs.
MAEs surrounded the nucleus, whereas in the majority of control siRNA-treated MAEs the Golgi located within one 120° sector relative to the nucleus (Figure 4B). Compared to that transfected with control siRNA, the polarity of AmotL1 siRNA-treated MAEs showed ~50% reduction in Golgi reorientation (Figure 4C).

Because the polarization of the cell is related to the directional migration, we further examined the migration of AmotL1 siRNA-treated MAEs with the Boyden chamber assay. The results indicated that both basal and fibroblast growth factor (FGF)2-induced migration were significantly decreased when the MAEs were treated with AmotL1 siRNA for 72 hours (Figure 4D). We then constructed MAE lines stably expressing the p90 or p100 AmotL1 isoforms. Stable expression of the p100 AmotL1 in MAEs also resulted in overexpression of p90 AmotL1 as compared to the control cell line transfected with vector alone (Figure 4E). Consistent with our findings that AmotL1 controls Golgi positioning, p90 and p100 AmotL1 MAEs exhibited increased polarity (Figure 4F and 4G) and upregulated migration toward FGF2 in comparison with MAE vector cells. However, p90 AmotL1 MAEs possessed an even higher migratory attributable to the expression level of p90 AmotL1 (Figure 4H).

AmotL1 Regulates Paracellular Permeability

The results of the immunofluorescence staining demonstrated that stably transfected p90 and p100 AmotL1 localized to cell–cell junctions in the confluent CHO cells (Figure 5A). Although CHO cells do not form properly organized TJIs, they are commonly used in an in vitro permeability assay to study the function of proteins, which may promote TJ formation. According to their junctional localization in CHO cells, we hypothesized that both AmotL1 isoforms might affect TJ function and paracellular permeability. To verify this hypothesis, CHO cells stably transfected with p100 and p90 AmotL1 were used in an in vitro paracellular permeability assay where the diffusion of fluorescein isothiocyanate (FITC)-labeled dextran across a monolayer of cells grown on a permeable membrane was measured. FITC-dextran was added to the upper chamber and aliquots from the bottom chamber were removed for quantitation of the FITC-dextran by fluorimetry. A significant difference in monolayer permeability was observed between CHO vector and AmotL1-transfected CHO cells. The diffusion of FITC-dextran to the bottom chamber gradually increased from p90 or p100 AmotL1 CHO cells monolayer which was comparable to that of p130 Amot and after 8 hours, the fluorescence intensity was 50% reduced compared with that of CHO vector cells (Figure 5B). In contrast, using the same approach, we also found that the paracellular permeability of Amot or AmotL1 siRNA-treated MS-1 ECs significantly increased compared with that of control siRNA-treated MS-1 cells. Double knockdown of Amot and AmotL1 via siRNA resulted in an additive effect on increasing the paracellular permeability of MS-1 cells (Figure 5C and 5D).
results indicated that Amot and AmotL1 not only colocalized at the cell–cell junctions but also regulated proper TJ activity.

**Knockdown of Zebrafish amotl1 Causes Vascular Deficiency During Embryogenesis**

The zebrafish is an important vertebrate model for the studies of gene functions in vivo because of its unique advantages, such as large numbers of embryos, rapid and external embryonic development, and optical clarity, which allows the direct visualization of organogenesis.\(^{21,22}\) We identified the zebrafish amotl1 ortholog by BLAST searches against the Ensembl and NCBI databases. Zebrafish amotl1 is present in one copy on chromosome 15 and shows overall 48% amino acid identity with human AmotL1 (Online Figure I). During embryogenesis, the zebrafish fltl gene is expressed in the hemangioblast and ECs.\(^{23}\) To address the expression of amot and amotl1 in vivo, fltl:EGFP transgenic zebrafish in which green fluorescent protein (GFP) was expressed in the forming vasculature was used for collecting GFP\(^+\) cells at 26 hours postfertilization (hpf) by fluorescence-activated cell sorting (Figure 6A). Zebrafish amot and amotl1 expression levels were examined by quantitative real-time polymerase chain reaction (PCR) with the standard curve method (User Bulletin 2, Applied Biosystems, 2001). The results revealed that similar to zebrafish endothelial-specific gene VE-cadherin (CDH5), the expression of both amot and amotl1 were 9-fold increased in GFP\(^+\) ECs as compared to the unsorted control (Figure 6B). This indicates that during early stage of zebrafish development amot and amotl1 are primarily expressed in vascular ECs.

To determine whether amotl1 was required for vessel development in vivo, 3 different antisense morpholinos (MOs) targeting exon 6, exon 10, or exon 12 of zebrafish amotl1 were individually injected into embryos of fli1:EGFP transgenic zebrafish at the 1 to 2 cell stage. None of the injected-MOs induced any gross morphological defects as examined under bright field microscopy (Online Figure II). The injection of 3 different amotl1 MOs resulted in similar vascular defects in intersegmental vessels (ISVs) migration (Online Figure III). We then focused on MO1 and the knockdown efficiency was confirmed by RT-PCR at 30 hpf. Injection of amotl1 MO1 caused the appearance of shorter mRNAs as detected by RT-PCR, demonstrating that the amotl1 transcript was affected in these morphants (Figure 7A). Sequencing and conceptual translation of the RT-PCR products showed that both amotl1 morphant mRNAs encoded the prematurely truncated proteins (Figure 7B and Online Figure IV).

Compared to zebrafish treated with the mismatched control MO1 at 32 hpf, amotl1 morphants exhibited defective ISV migration and most of the ISVs were typically arrested at the horizontal myoseptum, a phenotype shared in common with the amot morphants. However, in comparison to the phenotype of amot morphants, the penetrance and severity of the amotl1 phenotype were lower (Figure 7C and 7E). To determine whether amot and amotl1 have overlapping functions during endothelial migration, we simultaneously knocked down both amot and amotl1. Quantification revealed that injection of amotl1 MO1 caused 33% of zebrafish embryos with ISV defective phenotype at 40 hpf and the average number of defective ISVs per embryo was 4.2±1.8. Whereas coinjection of amot and amotl1 MOs resulted in 78% of the embryos showing an ISV phenotype at 40 hpf. The average number of defective ISVs per embryo with the dual knockdown was increased to 12±3.1, which was significantly higher than that of either amot or amotl1 single MO injection. The results indicated that amot and amotl1 had overlapping roles in controlling EC migration and angiogenesis (Figure 7C and 7F).

To further verify that the defect in ISV migration was specifically caused by amotl1 knockdown, as well as further validate the potential overlap function between amot and amotl1, rescue experiments were carried out with the coinjection of amotl1 MO1/mouse p90 AmotL1 mRNA, amotl1 MO1/mouse p100 AmotL1 mRNA, or amotl1 MO1/human p80 Amot mRNA, respectively. As shown in Figure 7G, at 36 hpf, both p90 mAmotL1 and p80 hAmot mRNA had very similar effects in rescuing the ISV defective phenotype of...
amotl1 morphants. In contrast, p100 mAmotL1 mRNA could not rescue the ISV migration defects. These results demonstrated that the vascular phenotype was specifically caused by amotl1 gene knockdown and provided additional substantiation that primarily the p90 isoform of AmotL1 promotes EC migration.

Examination with high-resolution 2-photon microscopy revealed distinct differences of ISV defects between amot and amotl1 morphants. When amot was knocked down, the tip cells of ISVS exhibited a depolarized phenotype, spread horizontally, and extended filopodia in an unpolarized manner (Figure 7D and Online Movie II). In contrast, the ISVs of amotl1 morphants became arrested at the horizontal myoseptum and the polarity of some tip cells was also affected. However, the striking and distinct phenotype of amotl1 morphants was that the cell–cell junctions between stalk cells and the dorsal aorta appeared perturbed (Figure 7D and Online Movie III). In addition, the double knockdown of both amot and amotl1 combined these phenotypes with depolarized tip cells as well as inhibited connection to the aorta (Figure 7D and Online Movie IV).

The endothelial junctional defect of amotl1 morphants was further visualized by whole mount immunofluorescence staining of claudin-5. The claudin-5 together with the GFP signal of ISVs from control fish showed stable connection of stalk cell to the dorsal aorta (Figure 8A, left), whereas weaker connection between ISV stalk cells and the dorsal aorta was detected in amotl1 morphants (Figure 8A, right). The ze-
brafish ISV is composed of 3 ECs by 30 hpf. The tip cell (designated as No.3 EC in Figure 8A), stalk cell (No.2 EC), and inverted T-shaped EC (No.1 EC). Because of the procedures of claudin-5 whole mount immunofluorescence staining, the GFP signal in the cytoplasm of zebrafish vascular EC became weaker than that in the nucleus, which allowed us to visualize the nuclei of 3 different ISV ECs. As shown in Figure 8A, both the tip cell and stalk cell in the ISV of amotll morphant stayed at the horizontal myoseptum, indicating the tip cell migration was halted compared to that of the control ISVS. In addition, we confirm that the cell–cell junction defect of amotll morphant is between stalk cell and inverted T-shaped EC by the combination of visualization of EC nucleus, GFP signal, and claudin-5 staining.

Discussion

Here, we report the characterization of a novel isoform of AmotL1 which controls endothelial migration and TJ formation in vitro. We further show that AmotL1 and Amot have overlapping but distinct roles in vertebrate angiogenesis in vivo.
We have previously shown that angiomotin is essential for directional migration of ECs by the formation of a signaling scaffold.\textsuperscript{9} This signaling complex consists in part of the Pals:Patj:Lin7 polarity proteins that are evolutionary conserved and promotes cell polarity in epithelial cells. In ECs, this complex is also involved in determining cell polarity of migrating tip cells, as judged by the lack of polarized extensions of filopodia in vivo.\textsuperscript{10} In this report, we have studied the roles of AmotL1 in blood vessel formation. Because AmotL1, like Amot, associates to the Pals:Patj:Lin7 polarity complex, we propose that AmotL1 promotes migration by controlling cell polarity. One possible effector may be the RhoGEF Syx (PLEKHG5/TECH) that regulates RhoA activity in a spatiotemporal manner in ECs and forms a ternary complex with AmotL1 via the interaction of Mupp1/Patj.\textsuperscript{9}

AmotL1 was initially identified as a TJ enriched protein consisting of the coiled-coil domains and PDZ-binding motif.\textsuperscript{14} Coiled-coil domains have been identified in a variety of cytoskeletal proteins and are involved in inter- or intramolecular protein–protein interactions. Indeed, all members of the Amot family have been shown to oligomerize via the coiled-coil domains. Here, we further show that Amot and AmotL1 could form heterooligomers and colocalized in endothelial TJs in vitro. Both proteins interact with individual PDZ domains of Patj/Mupp1, but this interaction is not required for oligomerizations because Amot:AmotL1 formed a complex even when the C-terminal PDZ-binding motifs were mutated. Whether the Amot:AmotL1 heterooligomers activate different signaling pathways than the homooligomers remains to be shown. Functional analysis of Amot and AmotL1 in TJ formation showed that exogenously overexpression of both proteins decreases paracellular permeability. Interestingly, siRNA knockdown of both Amot and AmotL1 in MS-1 ECs exhibited an additive effect on paracellular permeability, suggesting that both proteins may be required for proper TJ activity in ECs.

The dorsal aorta of zebrafish is derived from angioblasts migration out of the lateral plate mesoderm, and this process is an early event in vasculogenesis.\textsuperscript{26–28} The phenotypes of zebrafish \textit{amotl1} knockdown showed that the formation of dorsal aorta and the subsequent ECs sprouting from dorsal aorta to the horizontal myoseptum were not affected, suggesting that \textit{amotl1} is not required during vasculogenesis. In contrast, the endothelial tip cell migration from the horizontal myoseptum to the dorsal neural tube was greatly hindered when zebrafish \textit{amotl1} was knocked down, indicating that \textit{amotl1} is essential for ECs migration during sprouting angiogenesis. However, the ISV sprouting from dorsal aorta to the horizontal myoseptum occurred in an \textit{amotl1}-independent manner. This might be attributable to the existence of alternative signaling pathway during this period or the expression of another protein with a function analogous to \textit{amotl1} but only active before the expression of functional \textit{amotl1}.

There appeared to be a distinct difference between the vessels that were arrested because of either \textit{amot} or \textit{amotl1} knockdown. When \textit{amot} expression was inhibited, the sprouting tip cells was arrested and spread horizontally along the myoseptum and extended filopodia in a multi-directional manner (Figure 7D). In \textit{amotl1} knockdown vessels, sprouting vessels were arrested but horizontal spreading of tip cells was...
less observed than \textit{amot} knockout. However, the connections between the stalk cells and the aorta appeared to be destabilized as observed by high-resolution 2-photon imaging, time-lapse photography, and visualizing endothelial junctions with claudin-5 staining. The double knockdown phenotype adds these 2 phenotypes together as the tip cells appeared depolarized and the connection to the aorta is destabilized (Figure 7D and Online Movie IV).

There are several possible explanations for why \textit{amot} and \textit{amotl1} exert different effects on ISV formation. Firstly, the...
spatiotemporal expression pattern may differ. This has been shown by analysis of protein expression during different stages of retinal angiogenesis in newborn mice (Y Zheng, unpublished data, 2009).16 Secondly, both Amot and AmotL1 are associated to the Patj/Mupp1 scaffold proteins. However, these protein complexes differ in that Pals2, filamin A, and PTN13 are associated to Amot but not to the other proteins of the Amot family.9 It is also possible that level oligomerization may locally affect signal in the developing vessels.

In conclusion, our findings show that AmotL1 functions as a key regulator of EC migration and cell–cell junction stability during zebrafish embryogenesis. The results of this study emphasize the role of AmotL1 in angiogenesis, and we speculate that the combination of Amot and AmotL1 might be a potential future targets for antiangiogenic therapy.

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Disclosures
None.

References


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Materials and Methods

Cell Lines

Human umbilical vascular endothelial cells (HUVEC) (BD Biosciences, Franklin Lakes, NJ) were cultured in endothelial cell culture medium (Promocell, GmbH, Heidelberg, Germany). Bovine capillary endothelial hTERT+ cells (BCE-hTERT)\(^1\) were grown in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS (GIBCO, Grand Island, NY) and 2 ng/mL FGF2. A murine endothelial cell line, MS-1, was cultured in RPMI-1640 (GIBCO, Grand Island, NY) with 5% FCS. Ecotropic retrovirus producing Phoenix Eco cells (provided by G. Nolan, Stanford University, Palo Alto, CA) and mouse aortic endothelial cells (MAE)\(^2\) were cultured in DMEM supplemented with 10% FBS. PmT-EC (endothelial cells differentiated from embryonic stem cells and immortalized with polyoma middle T virus)\(^3, 4\) and tumor endothelial cells (TEC)\(^5\) were grown in DMEM with 10% FBS, 50 μg/mL endothelial cell growth supplement (Sigma-Aldrich, St. Louis, MO) and 100 μg/mL heparin (Sigma-Aldrich, St. Louis, MO)\(^3\). CHO and CHO stably expressing mouse AmotL1 cells were grown in DMEM Ham-12 medium (Sigma-Aldrich, St. Louis, MO) with 10% FCS. All cell lines were supplemented with 1% glutamine and 1% penicillin/streptomycin (GIBCO, Grand Island, NY).

Western Blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane (Waterman, GmbH, Heidelberg, Germany). Non-specific antibody binding was blocked
with 5% milk in PBS buffer containing 0.1% Tween 20 and membranes were incubated with primary antibody for 2 hours at room temperature. Species-specific HRP-conjugated secondary antibodies were used for detection of the primary antibodies and developed with a chemiluminescent substrate as per manufacturer’s instructions (Perkin Elmer, Zaventem, Belgium).

**Transfection**

pcDNA3, pcDNA3-p100 or pcDNA3-p90 mouse AmotL1 was transfected to CHO cells using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen, Carlsbad, Calif). For retrofection, phoenix ecotropic packaging cell line was transfected with the pBABE, pBABE-p100, or pBABE-p90 mouse AmotL1 using standard calcium phosphate coprecipitation method. The culture supernatant was added to MAE cells and incubated for 72 hours and then selected with 5 μg/mL puromycin.

**Immunoprecipitation and Mass Spectrometry**

MAE cell lysates were immunoprecipitated with AmotL1 polyclonal antibodies (reactive to murine AmotL1 C-terminal peptide, GKASEHRGRVSNLLHKPEFP). Immunocomplexes bound to protein A Sepharose beads were gently washed five times with lysis buffer (50 mM HEPES buffer, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM EGTA and protease inhibitors) and separated by SDS-PAGE. The gel was stained with coomassie brilliant blue and bands around 100 and 90 kDa were excised, digested with trypsin (Promega, Madison, WI, USA) and subjected to mass spectrometric analysis for identification.
Protein identification was performed by nanoLC-MS/MS using a nano-LC Ultimate system (Dionex, LC Packing) on line with an ion trap analyser (HCT, Ultraflex, Bruker Daltonics Inc. Bremen, Germany). MS/MS Raw data were processed using DataAnalysis 3.1 and Biotools 3.1 softwares (Bruker Daltonics Inc.). Protein search were done using a local Mascot search engine against SwissProt database 57.2 (Matrix Science Ltd., London UK). Parameters used were: Mouse for taxonomy, a 0.75 Da mass tolerance for parent and MS/MS fragments and one allowed miss cleavage. Fixed carbamidomethylated cystein and potential methionine oxidation were selected as modification.

**Protein N-terminal Sequencing (Edman Degradation)**

AmotL1 was pulled down with rabbit anti-AmotL1 polyclonal antibodies from MS-1 endothelial cell lysates. The immunoprecipitated complex was resolved by SDS-PAGE and then was blotted to a PVDF membrane. The proteins in PVDF membrane was visualized by Coomassie Brilliant Blue R-250 staining and the p90 AmotL1 was cut off for five cycles of Edman degradation. The N-terminal sequencing was performed with a Procise cLC sequencer according the manufacture’s instruction.

**Immunofluorescence Staining**

Cells were cultured in chamber slides, fixed with 4% paraformaldehyde for 10 minutes at room temperature and permeabilized in 0.1% Triton X-100 for 30 seconds. Non-specific reactivity was blocked by incubating with 5% horse serum in PBS for 1 hour. Primary antibody in blocking buffer was added for 1 hour and subsequently incubated with the fluorescent-conjugated secondary antibody. Digital images were taken by a
Zeiss Axioplan 2 microscope, collected using an AxioCam HRm Camera and axiovision 4.2 software.

Gene Silencing via siRNA
A pool of 4 distinct siRNA directed against Amot or AmotL1 was designed using a proprietary algorithm and commercially purchased (siGENOME Smartpool™, Dharmacon Inc. Lafayette, CO, USA). MAE cells were transfected with the AmotL1 siRNA using the Trans IT-TKO transfection reagent (Mirus Bio, Madison, WI). MS-1 cells were transfected with Amot or AmotL1 siRNA using FECT1 transfection reagent (Dharmacon Inc. Lafayette, CO, USA). Cells treated in an identical manner with non-silencing siRNA were included as controls in all experiments. Specific gene silencing was examined by western blotting 72 hours after siRNA transfection.

Endothelial Cell Migration Assay
Cells were loaded to 48-wells Boyden chamber at a density of 30,000 cells/well in DMEM containing 0.1% BSA and allowed to migrate toward 50 ng/mL FGF2 for 5 hours. The filter was fixed in ice-cold methanol and stained with Giemsa. Cells that had not migrated were dislodged with a cotton swab and migrated cells were counted using microscopy at 20X magnification. Six replicates were quantitated for each sample.

In vitro Permeability Assay
The In Vitro Vascular Permeability assay kit (Chemicon Inc., Billerica, MA, USA) was used according to the manufacturer’s instructions. Briefly, 12,000 of CHO cells stably expressing AmotL1 or 200,000 of siRNA treated MS-1 cells were added to the upper
chamber and allowed to form a monolayer. Then FITC-dextran was added to the upper chamber and 100 μL sample from the lower chamber was withdrawn at different time points. Fluorescence intensity was measured on a Bio-Tek FL 600 plate reader using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The background fluorescence of cell culture medium was subtracted. The diffusion of FITC-dextran across a membrane insert without cells was measured in parallel to ensure integrity of cell monolayers.

**Isolation of GFP+ Cells from fli1:EGFP Transgenic Zebrafish**

3000 embryos of fli1:EGFP transgenic zebrafish were collected at 26 hours post fertilization (hpf) and dechorionated with pronase (Sigma-Aldrich, St. Louis, MO) at the concentration of 2 mg/mL. After wash with PBS, the embryos were treated with protease solution (0.25% trypsin, 1 mM EDTA in PBS, pH 8.0) to get single cell suspension. The trypsinization was stopped by adding CaCl$_2$ to the final concentration of 2 mM as well as 10% FCS to the reaction. Cells were suspended at the concentration of 10×10$^6$ cells/mL in PBS containing 0.8 mM CaCl$_2$, 1% FCS and 1% penicillin/streptomycin. GFP+ cells were FACS sorted and collected for RNA isolation.

**Quantitative Real-time PCR**

Real-time PCR was performed to quantify amot, amotl1, and CDH5 (zebrafish VE-Cadherin) expression in fli1:EGFP transgenic zebrafish. Total RNA was extracted either from the whole zebrafish or from FACS-sorted GFP+ cells of fli1:EGFP transgenic zebrafish at 26 hpf by RNeasy mini kit (Qiagen, Inc, Valencia, Calif) according to the manufacture’s instructions. Purified RNA was treated with RNase-free
DNaseI to ensure complete degradation of potential genomic DNA contamination, followed by the addition of 25 mM EDTA and heat denaturation of the enzyme. Different RNA samples were reverse transcribed to cDNA with random hexamer primers using a SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, Calif). Real-time PCR primers were designed with RealTimeDesign software (http://www.biosearchtech.com) and were custom made by Thermo.

The real-time PCR was carried out on an ABI PRISM 7500 System (Applied Biosystems) according to the manufacture’s instructions in a 96-well microtiter plate with a 25 μL reaction volume containing 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems), 5 pmol of each primer, 25 ng of cDNA templates and the following primers: zebrafish actin, 5'-ATTGCTGACAGGATGCAGAAG-3' (forward), and 5'-GATGGTCAGACTCATCGTACTC-3' (reverse); zebrafish amot, 5’-ATCAG CGCTCGCGAAAGA-3’ (forward) and 5’-TGGCAGATGGACATTAGGGAT-3’ (reverse); zebrafish amotl1, 5’-CGGAACAGCTCGAACA-3’ (forward) and 5’-CGGTGCGGGAAACCGATA-3’ (reverse); zebrafish CDH5, 5’-ACACAAGATCCA CACGCTGG-3’ (forward) and 5’-GAACATACACTCAGAGCGTG-3’ (reverse). Each sample was run in triplicate. Zebrafish CDH5 gene was used as an endothelial specific positive control and amplification of housekeeping gene β-actin was used as a control to normalize the expression level of gene of interested. Negative controls without template were performed in each run. Dissociation analysis was performed at the end of each run to confirm the specificity of the reaction. Plasmids with an insert of zebrafish amot, amotl1 or CDH5 were used for generation of standard curve. Absolute copy number of individual gene per 25 ng total RNA was calculated from the corresponding standard curve.
**Morpholino-Mediated Gene Knockdown in Zebrafish**

*fli1*:EGFP transgenic zebrafish were maintained under standard condition. Zebrafish *amotl1* was identified by BLAST searches within the Ensembl database (gene accession number: ENSDART00000060229). Morpholinos were obtained from Gene Tools LLC and injected into zebrafish embryos at the 1-2 cell stage at the concentration of 250 μmol/L. Morpholino-injected zebrafish embryos were maintained at 28°C in standard E3 water supplemented with 0.003% PTU (phenyl-2-thiourea). MO1 (5’-CCTCGATCTCCAACTGCAAATGTTC-3’) targets exon 6 of *amotl1* by interfering with the splice donor site thereby resulting in a 145 nt deletion in the mRNA which disrupts the reading frame before the coiled-coil repeats. The corresponding mismatched MO1 (5’-CgTCGATgTCgAAC TcCAAATcTTC-3’) was used as the negative control. MO2 (5’-GATATACCAGCAGCGTACACCAGAG-3’) targets exon 10 and MO3 (5’-AGTCCTTTATCCTGTGACATGGAGA-3’) targets exon 12 of *amotl1*. In order to demonstrate the specificity of the morpholino defect, rescue experiments were performed with human p80 Amot, mouse p90 or p100 AmotL1 mRNAs. The mRNAs were synthesized from linearized templates *in vitro* using Ambion’s Message Machine kits (Ambion, Austin, TX, USA), mixed with MO1 and injected at concentration of 100 pg / embryo. The efficiency of *amotl1* knockdown with MO1 was confirmed by RT-PCR using the primers 5’-AGACAGCACCACAGAGAG GAA-3’ (forward) and 5’-TACCCGCTCCACATTTCTG-3’ (reverse). For *amot* knockdown, MO (5’-CCACTGACACAATCCACCACAGAGTG-3’) targeting exon 2 was used and the ISV defects were counted as previously described.12
**Zebrafish Whole Mount Immunofluorescence Staining**

Different MO-injected *fli1*:EGFP transgenic zebrafish were fixed at 30 hpf with 2% paraformaldehyde at room temperature for 45 min, permeabilized with 0.1% Triton X-100 in PBS overnight at 4°C and followed by blocking with 2% sheep serum in PBS containing 0.1% Triton X-100 at room temperature for 2h. The control fishes and morphants were stained with mouse anti-claudin-5 antibody (1:25 dilution, Zymed) and Alexa Fluor 635-conjugated anti-mouse IgG (Molecular Probes) were used as secondary antibodies. Z-stack images were taken and projected using a Leica TCS SP5 confocal microscope.

**Live Imaging and Time-lapse movie with 2-photon Laser Scanning Microscopy**

Two-photon laser scanning microscopy was performed for *in vivo* studies of *fli1*:EGFP transgenic fish. Zebrafishes were anaesthetized by tricaine and in-bedded in low-melting agar in a Petri’s dish. The Petri’s dish was covered with E3 water supplemented with 0.003% PTU (phenyl-2-thiourea) and mounted on a computer-controlled stage bolted to a 2-photon laser scanning microscope (Zeiss LSM510 META NLO, Germany) equipped with a 20X/1.0 dipping lens (Zeiss, Germany) and Ti:Sapphire tunable Chameleon Ultra2 laser (Coherent, CA, USA). The laser wavelength was tuned to 910 nm and laser power was kept at a minimum to ensure tissue viability. Time-lapse movies were generated with the 2-photon microscope at indicated time-points. Movies were rendered from Z-stack time-lapse series where each frame is a maximum projections of the optical slices. All experiments were carried out in room temperature.
Statistical Analysis

Data from at least 5 independent experiments are presented as mean ± SD. Comparisons between different groups were analyzed for statistical significance with the student unpaired $t$-test. A value of $p<0.05$ was considered as statistically significant.
References for Materials and Methods


Online Table

Online Table I. N-terminal amino acid sequences of mouse p90 AmotL1 identified by Edman degradation.

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Online Figure Legends

Online Figure I. Alignment of AmotL1 sequences of different origins. The homologous of human AmotL1 (accession number: Q8IY63), mouse AmotL1 (accession number: Q9D4H4) and zebrafish amotl1 (accession number: XP_690202) was analyzed with DNAssist2.0 software. Identical amino acids were shown in pink and conserved amino acids were shown in green.

Online Figure II. Morpholino injection targeting amot or amotl1 does not affect the morphogenesis of zebrafish embryos. The morphology of uninjected control flil1:EGFP zebrafish embryo and different morphants was examined under bright field microscopy at 30 hpf.

Online Figure III. Vascular defect phenotype of knocking down zebrafish amotl1 at 28 hpf (A), 32 hpf (B) and 36 hpf (C) with three different morpholinos (MO).

Online Figure IV. Verification of the specificity of knocking down amotl1 in zebrafish. (A) Sequencing results showed that two PCR products amplified from cDNA of amotl1 morphants (injected with MO1) are specific. Band 1 got the deletion at exon 6 (framed in red) and band 2 got both exon 6 and exon 8 (framed in blue) deleted. (B) Alignment of the amotl1 mutant proteins according to the sequencing results showed that the two amotl1 transcripts from morphants encoded the same mutant protein which contain the peptide encoded by amotl1 exon 1 to 5 and a peptide, RA FGNSQT AG K SGARGTR, encoded by the shifted reading frame.
Online Movies

**Online Movie I.** Time-lapse movie of sprouting ISVs of control zebrafish embryo from 29 to 31 hpf.

**Online Movie II.** Time-lapse movie of sprouting ISVs of *amot* morphant from 29 to 31 hpf.

**Online Movie III.** Time-lapse movie of sprouting ISVs of *amotl1* morphant from 29 to 31 hpf.

**Online Movie IV.** Time-lapse movie of sprouting ISVs of *amot* and *amotl1* double morphant from 29 to 31 hpf.
Online Figure II

control

amot

amotl1

amot+amotl1