Blood flow drives lumen formation by inverse membrane blebbing during angiogenesis in vivo

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How vascular tubes build, maintain and adapt continuously perfused lumens to meet local metabolic needs remains poorly understood. Recent studies showed that blood flow itself plays a critical role in the remodelling of vascular networks1,2, and suggested it is also required for the lumenization of new vascular connections3,4. However, it is still unknown how haemodynamic forces contribute to the formation of new vascular lumens during blood vessel morphogenesis. Here we report that blood flow drives lumen expansion during sprouting angiogenesis in vivo by inducing spherical deformations of the apical membrane of endothelial cells, in a process that we have termed inverse blebbing. We show that endothelial cells react to these membrane intrusions by local and transient recruitment and contraction of actomyosin, and that this mechanism is required for single, unidirectional lumen expansion in angiogenic sprouts. Our work identifies inverse membrane blebbing as a cellular response to high external pressure. We show that in the case of blood vessels such membrane dynamics can drive local cell shape changes required for global tissue morphogenesis, shedding light on a pressure-driven mechanism of lumen formation in vertebrates.

Blood vessels form a vast but highly structured network that pervades all organs in vertebrates. During development as well as in pathological settings in adults, vascular networks expand through a process known as sprouting angiogenesis. New blood vessels form from the coordinated migration and proliferation of endothelial cells into vascular sprouts. Subsequent fusion of neighbouring sprouts, defined as anastomosis, then leads to the formation of new vascular loops, whose functionality relies on their successful lumenization and perfusion1. During anastomosis, endothelial lumens form both through apical membrane invagination into single anastomosing cells (unicellular lumen formation), and through de novo apical membrane formation at their nascent junction (multicellular lumen formation)3,4. As the tip of endothelial sprouts can be occupied by either one or several cells as they compete for the tip position6,7, we asked whether similar mechanisms of lumen formation apply to unicellular and multicellular endothelial sprouts before anastomosis.

Using a zebrafish transgenic line expressing an mCherry–CAAX reporter for endothelial plasma membrane (Tg(kdr-l:rasCherry)9,10), we imaged lumen formation in tip cells as they sprout from the dorsal aorta to form the intersegmental vessels (ISVs) from 30 h post-fertilization (hpf). We found that lumens expand in spraying ISVs before anastomosis, and do so by invagination of the apical membrane either into single tip cells, or along cell junctions when the tip of a sprouting ISV is shared between several cells (Fig. 1a,b).

To test whether this mechanism of lumen formation is conserved in other vertebrates, we performed immunolabelling of the apical membrane (ICAM-2, intercellular adhesion molecule 2) and cell junctions (ZO-1, zona occludens 1) in developing mouse retinae at postnatal day 6 (P6). As in zebrafish ISVs, we observed that lumens are present either as membrane invaginations into single tip cells, or between cells when they share the tip position (Fig. 1c,d), suggesting that endothelial sprouts undergo both unicellular and multicellular lumen formation in the mouse retina.

Whereas lumens form independently of blood flow during dorsal aorta formation8–10, previous studies suggested both flow-independent and flow-dependent lumen formation in ISVs (ref. 11) and during anastomosis3,4. To test whether lumen expansion in angiogenic sprouts requires blood perfusion, we treated Tg(kdr-l:rasCherry)9,10 embryos with a fourfold higher dose of tricaine methanesulfonate (4× tricaine) than the dose normally used for anaesthesia. Under these conditions, embryos show lower heart rate, loss of blood flow and decreased blood pressure4. On the addition of 4× tricaine midway through ISV

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Figure 1 Blood pressure drives unicellular and multicellular lumen expansion in angiogenic sprouts. (a) Schematic illustration of unicellular and multicellular lumen formation in angiogenic sprouts. (b) Tg(kdr-l:ras–Cherry)ző16 embryos were imaged from 32 h post-fertilization (hpf). Black arrows, cell junction. Magenta arrows, apical membrane. Time is in hours:minutes:seconds. Scale bar, 10 μm. Images are representative of ten embryos analysed. (c) Mouse retinae were collected at P6 and stained for ICAM-2, ZO-1 and isolectin IB4. Isolectin IB4 staining was used to draw the cell outline (white dotted line). White arrow, unicellular membrane invagination. Scale bar, 10 μm. (d) The number of endothelial sprouts with unicellular or multicellular lumens was quantified in P6 mouse retinae stained for ICAM-2 and ZO-1 (n = 487 sprouts from 9 retinae). (e) Tg(kdr-l:ras–Cherry)zoek embryos were imaged from 33 hpf after the addition of 4× tricaine. Blood flow stopped after 20–25 min of treatment, leading to a decrease in blood pressure noticeable through the decrease in diameter of the dorsal aorta (double-headed arrow). At 48 hpf, embryos were returned to 1× tricaine and imaged further. Magenta arrows, apical membrane. Magenta filling, lumen. Times are in hours:minutes:seconds and correspond to the times after addition of 4× tricaine (left panels) and after washout (right panels). Scale bar, 20 μm. Images are representative of seven embryos analysed.
**Figure 2** Apical membrane undergoes inverse blebbing during lumen expansion. (a) Embryos with mosaic expression of EGFP–CAAX were imaged from 36 hpf. Arrow in B, retracting bleb. Arrowheads in C, bleb necks. Arrow in D, lumen collapse. Time is in hours:minutes:seconds. Scale bars, 10 \( \mu \)m (A,C,D) and 5 \( \mu \)m (B). Images are representative of seven embryos analysed. (b) A kymograph was generated along the magenta line in a, panel A. t, time; d, distance. Black arrowheads, retracting blebs. White arrowheads, non-retracting blebs. (c) Multicellular sprouts were imaged in \( Tg(kdr-lras-Cherry) \) embryos with mosaic expression of EGFP–CAAX from 32 hpf. Arrowheads, inverse blebs. Time is in hours:minutes:seconds. Scale bar, 10 \( \mu \)m. Images are representative of four embryos analysed. (d) Mouse retinae were collected at P6 and stained for ICAM-2, ZO-1 and isolectin IB4. Isolectin IB4 staining was used to draw the cell outline (white dotted line). Arrow, constricted apical membrane. Arrowhead, lumen fragment. Scale bar, 10 \( \mu \)m. (e) The number of lumenized unicellular sprouts showing expanded, constricted or disconnected apical membrane was quantified in P6 mouse retinae stained for ICAM-2 and ZO-1 (\( n = 57 \) sprouts from 9 retinae).
lumenization, lumens did not expand further and eventually collapsed (Fig. 1e). However, when placed back in 1× tricaine at 2 days post-fertilization (dpf), the embryos recovered normal heartbeat, blood flow was re-established (as assessed by the presence of circulating red blood cells) and lumens expanded within the ISVs (Fig. 1e). Together, these data show that lumen expansion in angiogenic sprouts in vivo is dependent on cardiac activity and thus on haemodynamics.

Using mosaic expression of an endothelial-specific EGFPC-AAX reporter for plasma membrane (fli1ep:EGFP-CAX) and high spatial and temporal resolution imaging, we discovered that apical membranes undergo rapid expansion through a process reminiscent of membrane blebbing (Fig. 2a, panels B,C). Membrane blebs are plasma membrane protrusions caused by local disruption of the actomyosin cortex or its detachment from the plasma membrane12–16. Under cytoplasmic pressure, the membrane in such actomyosin-free regions inflates from a neck into a spherical protrusion. Depending on the context, blebs are resolved by detachment (as seen in apoptosis), by forward movement of the cell (during cell migration), or through recruitment and contraction of the actomyosin cortex on the inner side of the bleb (bleb retraction, as seen in cell division)16. In endothelial cells, we observed blebbing of the apical membrane during lumen expansion (Fig. 2a and Supplementary Video 1). These blebs, however, showed inverted polarity compared with previously described blebs, with the apical membrane protruding into the cell body. Hence, we propose to name this process ‘inverse membrane blebbing’. Following expansion, the inverse blebs either retracted (Fig. 2a, panel B and black arrowheads in Fig. 2b) or persisted, in particular as larger structures, leading to an expansion of the luminal compartment (Fig. 2a, panel C and white arrowheads in Fig. 2b). Interestingly, persisting blebs were found only at the tip of the growing lumen, therefore restricting lumen expansion to this region of the cell. In contrast, the blebs arising on the lateral sides of the lumen always retracted (Supplementary Video 1). Quantitative morphometric analysis of inverse blebs showed that their size, expansion time and speed, as well as retraction time and speed, are of the same order of magnitude as those of classical blebs12,15 (Supplementary Fig. 1a–c). Similar membrane dynamics were observed using a PLCδ2–PH–RFP reporter for phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P₂), an early apical determinant in epithelia17, confirming that inverse blebbing occurs specifically at the apical membrane of endothelial cells (Supplementary Fig. 1f and Supplementary Video 2).

Inverse blebs were observed at the apical membrane of both unicellular (Fig. 2a) and multicellular (Fig. 2c and Supplementary Video 3) sprouts during lumen expansion. However, because endothelial cell junctions are highly dynamic6,18,19 and accumulate apical markers during lumenization7 (Supplementary Video 2), we chose for clarity to focus our subsequent analysis on unicellular lumens where non-junctional apical membrane can clearly be distinguished.

In the mouse retina, stainings for ICAM-2 revealed the presence of two major lumen configurations in angiogenic sprouts where the apical membrane was either expanded (Fig. 2d, top panels, and Fig. 2e) or constricted (Fig. 2d, middle panels, and Fig. 2e), suggesting that a similar mechanism of apical membrane blebbing might take place during sprouting angiogenesis in mice.

To assess whether inverse blebbing is driven by blood pressure, blood flow was stopped in single ISVs by laser ablating the connection of the sprouts to the dorsal aorta (Fig. 3a). The loss of blood flow resulted in an immediate stop of apical membrane blebbing and a gradual regression of the lumen (Fig. 3a and Supplementary Video 4). Similar results were obtained by treating embryos with 4× tricaine (Fig. 3b). Following 15–20 min of treatment, blood flow stopped (as assessed by the absence of circulating red blood cells) and blebs could no longer be observed at the apical membrane of lumenizing cells (Fig. 3b, kymograph and panel B, and Supplementary Video 5). When returned to 1× tricaine, embryos recovered blood flow and re-expanded lumens by inverse blebbing (Fig. 3b, kymograph and panel E, and Supplementary Video 5). Together, these experiments suggest that the generation of inverse blebs depends on the positive pressure difference existing between the luminal and the cytoplasmic sides of the apical membrane.
Figure 4 Endothelial cells retract inverse blebs by recruiting and contracting actomyosin at the apical membrane. (a) Tg(kdr-l:ras–Cherry)s916 embryos with mosaic expression of Lifeact–EGFP were imaged from 35 hpf. Dotted line, apical membrane. Arrow, expanding apical membrane. Arrowhead, onset of F-actin polymerization. C, cytoplasm; L, lumen. Time is in hours:minutes:seconds. Scale bar, 5 μm. Images are representative of five embryos analysed. (b) Kymograph generated along the magenta line in a. t, time; d, distance. Dotted line, apical membrane. (c) Embryos with mosaic expression of Myl9b–EGFP and Lifeact–mCherry were imaged from 35 hpf. Arrow, onset of F-actin polymerization. Arrowhead, onset of myosin II recruitment. E, extracellular space. Scale bar, 5 μm. Images are representative of five embryos analysed. (d) Kymograph generated along the magenta line in c. (e) Embryos with mosaic expression of Myl9b–EGFP or Myl9bAA–EGFP and Lifeact–mCherry were imaged from 34 hpf. Blebs growing on the lateral sides of expanding lumens were assessed for their ability to retract within the maximum time necessary for expansion and retraction (approximately 10 min; see Supplementary Fig. 1a,b). A multinomial log-linear model was used to test for association of bleb count in the different categories with the mutation status. WT, n = 102 blebs from five cells; AA, n = 161 blebs from five cells; data pooled from three independent experiments; \( P = 2.1 \times 10^{-13}; \quad **** P < 0.0001. \) (f) Tg(kdr-l:ras–Cherry)s916;fli1ep:Lifeact–EGFP) embryos were imaged from 33 hpf. Laser ablation was performed along a line spanning the entire thickness of the apical membrane and its underlying cortex, at the tip of the growing lumen. Arrowhead, site of ablation. Arrow, inverse bleb. Scale bar, 5 μm. Images are representative of five embryos analysed. (g–i) Lifeact–EGFP \( ^{wt} \) (g) and wild-type (h,i) mouse retinae were collected at P6 and stained for ICAM-2 (g–i), non-muscle (nm) myosin IIA (h), and phospho-myosin light chain 2 (pMLC2; i). Arrows show localization of F-actin, nm myosin IIA and pMLC2 at the apical membrane. Images correspond to single confocal planes. Scale bars, 10 μm. (j) Schematic illustration of inverse membrane blebbing. P, pressure.
Figure 5 Apical membrane contractility regulates lumen formation during sprouting angiogenesis. (a) Tg(kdr-l:ras-Cherry) embryos with mosaic expression of Myl9b–EGFP or Myl9bAA–EGFP were analysed at 2 dpf. EGFP-positive ISVs were classified by eye into three categories according to their level of EGFP expression (low, moderate, strong) and screened for the presence of a lumen. A multinomial log-linear model was used to test for association of cell count in the different categories with the mutation status. WT, n = 55 ISVs from 24 embryos; AA, n = 31 ISVs from 9 embryos; data pooled from three independent experiments; P = 0.30 (low), P = 0.11 (moderate), P = 0.0002 (high), P < 0.01 (total). EGFP-positive cells where the presence or absence of a lumen could not be appreciated were referenced as undetermined. (b) Tg(kdr-l:ras–Cherry) embryos with mosaic expression of Myl9bAA–EGFP were imaged from 35 hpf. Arrowheads, lumen. Time is in hours:minutes:seconds. Scale bar, 10 μm. Images are representative of three embryos analysed. (c) Schematic model of lumen formation by inverse membrane blebbing during sprouting angiogenesis in vivo. Haemodynamic forces generate a positive pressure difference between the luminal and the cytoplasmic sides of the apical membrane (1). Consequently, inverse blebs expand along the apical membrane at sites of weak attachment of the cortex to the membrane (2). Following bleb expansion, F-actin polymerizes and myosin II is recruited at the apical membrane of growing blebs (3). Actomyosin contraction leads to bleb retraction (4), and selective bleb retraction ensures unidirectional lumen expansion (5).

Importantly, unlike previous reports suggesting that lumens form in sprouting ISVs through the fusion of intracellular vacuoles, we could not observe the formation of any vacuolar structure in the cytoplasm of endothelial cells during phases of lumen expansion (Fig. 2a and Supplementary Video 1). Isolated lumen fragments were seen arising only from the local collapse of the lumen, and rapidly reconnected to the growing lumen (Fig. 2a, panel D and Supplementary Video 1). The fact that such collapse and regrowth events can be reproduced experimentally by stopping then restarting blood flow (Fig. 3b and Supplementary Video 5) suggests that these events occur during normal development following local variations in blood pressure. The observation of a low number of large disconnected lumen fragments in angiogenic sprouts in mouse retinae (Fig. 2d, bottom panels, and Fig. 2e) suggests that the apical membrane undergoes similar dynamics during mouse retina development.

To identify the molecular mechanism underlying bleb retraction, fluorescent reporters for F-actin (Lifeact–EGFP and...
Lifeact–mCherry) and for the regulatory light chain of non-muscle myosin II (Myl9b–EGFP) were expressed in wild-type or Tg(kdr-l:rasCherry) embryos. At 2 dpf, both reporters co-localized at the apical membrane in perfused ISVs (Supplementary Fig. 2a, panel B), indicating that an actomyosin cortex supports the apical membrane in small vessels. During lumen formation, blebs expanded devoid of any F-actin or myosin II (Fig. 4a–d and Supplementary Fig. 2b,c). In the event of retraction, F-actin polymerization was observed at the apical membrane all around the bleb surface, from the initiation of retraction until its completion (Fig. 4a,b and Supplementary Video 6). Similarly, myosin II was recruited to the cytoplasmic surface of the bleb during retraction (Fig. 4c,d and Supplementary Fig. 2b,c). Co-expression of F-actin and myosin II reporters showed that myosin II is recruited to the apical membrane shortly after the initiation of F-actin polymerization (Fig. 4c,d). Together, these data suggest that the recruitment and contraction of an actomyosin cortex at the apical membrane drives bleb retraction during lumen expansion (Fig. 4j).

To test this hypothesis, we generated a non-phosphorylatable form of the myosin II regulatory light chain (Myl9bAA) previously shown to act as a dominant-negative of the myosin II regulatory light chain (Myl9bAA) previously shown to act as a dominant-negative of the myosin II regulatory light chain (Myl9bAA) previously shown to act as a dominant-negative of the myosin II regulatory light chain (Myl9bAA) previously shown to act as a dominant-negative of the myosin II regulatory light chain (Myl9bAA) previously shown to act as a dominant-negative of the myosin II regulatory light chain (Myl9bAA) previously shown to act as a dominant-negative of the myosin II regulatory light chain (Myl9bAA) previously shown to act as a dominant-negative of the myosin II regulatory light chain (Myl9bAA) previously shown to act as a dominant-negative of the myosin II regulatory light chain (Myl9bAA). On expression of Myl9bAA, we observed a significant difference in the frequency of bleb retraction compared with control cells expressing the wild-type form of Myl9b (Myl9b–EGFP), with a larger proportion of blebs showing no or partial retraction (Fig. 4e). These data therefore confirm that actomyosin contraction drives bleb retraction during lumen formation.

To test whether inverse membrane blebbing is, similarly to classical blebbing, the result of the local detachment of the membrane from its underlying cortex, we performed local laser ablation of the cortex at the apical membrane of lumenizing sprouts in Tg(kdr-l:rasCherry) embryos. By doing so, we could induce the expansion of inverse blebs at the apical membrane of lumenizing vessels (Fig. 4f and Supplementary Video 7). This result suggests that local detachment of the cortex from the apical membrane, in conjunction with blood pressure, could be the trigger of inverse blebbing (Fig. 4j).

In mice, the imaging of retinae from Lifeact–EGFP+/−/− pups and of wild-type retinae stained for non-muscle myosin IIA or phosphorylated myosin light chain 2 (pMLC2) showed accumulation of actomyosin at the apical membrane in sprouting cells (Fig. 4g–i), suggesting that a similar recruitment and contraction of actomyosin could take place during lumen formation in angiogenic vessels in mice.

To assess whether apical membrane contractility is required for proper lumenization, we inhibited actomyosin contraction by expressing Myl9bAA from 30 hpf and checked ISVs for the presence of a lumen at 2 dpf. Quantification of ISVs with Myl9bAA expression revealed a significant difference compared with control embryos (Fig. 5a), with a decrease in the proportion of cells showing normal lumens. Depending on their level of Myl9bAA expression, abnormal ISVs were either found to be unlumenized or exhibited diluted lumens (Fig. 5ab). Live imaging from 30 hpf showed that the absence of lumen was due to an inability of Myl9bAA-expressing cells to expand lumens (Fig. 5c and Supplementary Video 8).

To gain a deeper mechanistic understanding of the effects of Myl9bAA expression on the apical membrane dynamics, we performed fast imaging of both unlumenized and dilated cells at 2 dpf (Fig. 5b and Supplementary Videos 9 and 10). In both cases, the membrane dynamics was visibly affected by the expression of Myl9bAA. In unlumenized ISVs, lumen initially expanded into Myl9bAA-expressing cells but the apical membrane showed excessive and uncoordinated blebbing with frequent disconnections of blebs from the membrane (Fig. 5b, arrowhead, and Supplementary Video 9), therefore preventing lumen expansion. On the other hand, dilated, partially lumenized cells were unable to fully retract blebs growing on the lateral sides of the lumen (Figs 4e and 5b and Supplementary Video 10), leading to the formation of side lumen branches (Fig. 5b, arrow, and Supplementary Video 10). Together, these data show that sprouting cells require actomyosin contraction at the apical membrane to control membrane deformations and ensure single, unidirectional lumen expansion in response to blood pressure (Fig. 5d).

Our present results challenge the previous idea that sprouting cells expand lumens independently of blood flow during angiogenesis in vitro through the generation and fusion of intracellular vacuoles. Although endothelial cells are able to generate lumens independently of blood flow in vitro and during vasculogenesis, we show here that haemodynamic forces dynamically shape the apical membrane of single or groups of endothelial cells during angiogenesis in vivo to form and expand new lumenized vascular tubes. We find that this process relies on a tight balance between the forces applied on the membrane and the local contractile responses from the endothelial cells, as impairing this balance either way leads to lumen defects.

Our finding of inverse blebbing suggests that the process of blebbing, best studied in cell migration and cytokinesis, does not require a specific polarity, but is likely to be generally applicable to situations in which external versus internal pressure differences challenge the stability and elasticity of the actin cortex. In the case of endothelial cells, we describe a role for inverse blebbing in expanding the apical membrane under pressure while ensuring unidirectional expansion of a single lumen in angiogenic sprouts.

Our work more generally raises the question of the role of apical membrane contractility in the adaptation to varying haemodynamic environments, both during blood vessel morphogenesis, as connections form or remodel, and in pathological settings. Our present work and previous studies highlight the importance of balanced endothelial cell contractility in allowing the expansion and maintenance of endothelial lumens during blood vessel development. Future work will need to elucidate how the contractile properties of the apical membrane evolve as vessels mature and are exposed to higher levels of blood pressure and shear stress. The transition towards a multilayered organization of endothelial tubes, and the observed changes in cell shape and junction stability imply adaptations in the structure and dynamics of the actin cytoskeleton. Understanding whether and how this plasticity of the apical membrane and its underlying cortex is challenged in pathological conditions, where vessels exhibit altered perfusion and lack organized structure, has the potential to provide deeper insights into mechanisms of vascular adaptation and maladaptation.
METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

V.G., L.-K.P. and H.G. designed the experiments. V.G. and L.-K.P. performed the experiments and analyzed the data. R.C. generated the Tg(fli1ep:PLC V.AX) zebrafish line. V.G. and L.-K.P. and H.G. designed the experiments. V.G. and L.-K.P. performed the experiments and analyzed the data. R.C. generated the Tg(fli1ep:PLC V.AX) zebrafish line. V.G. and L.-K.P. and H.G. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Mouse care and procedures. The following mouse (Mus musculus) strains were used in this study: C57BL/6 and Lifeact–EGFP (ref. 28). Animal procedures were performed in accordance with the United Kingdom’s Home Office Animal Act 1986 under the authority of project licence PPL 80/2391. Animals were analysed regardless of sex.

Retina dissection, immunofluorescence staining and imaging. Eyes were collected at postnatal day 6 (P6) and fixed in 4% paraformaldehyde (PFA) in phosphate-buffer saline (PBS) for 1 h at 4°C. Retinas were dissected, blocked in CBB buffer (0.5% Triton X-100, 1% bovine serum albumin (BSA), 2% sheep serum, 0.01% sodium deoxycholate, 0.02% sodium azide) for 2 h at 4°C and incubated overnight at 4°C with the following primary antibodies diluted in 1:1 PBS/CBB as indicated: ICAM-2 (1:400; RD Biosciences, Cat. no. 553326, lot. no. 4213932), phosopho-myosin light chain 2 (1:100; Cell Signaling, Cat. no. 3671), non-muscle myosin heavy chain-IIA (1:100; Covance, Cat. no. PRB-440P), ZO-1 (1:400; Life Technologies, Cat. no. D-7300). Retinas were then washed three times for 10 min in PBS supplemented with 0.1% Tween-20 (PBST), and incubated for 2 h at room temperature with secondary antibodies diluted in 1:1 PBS/CBB as indicated: goat anti-rabbit Alexa Fluor 488 (1:1,000; Life Technologies, Cat. no. A-11008) and goat anti-rat Alexa Fluor 555 (1:1,000; Life Technologies, Cat. no. A-21434). Retinas were finally washed three times for 10 min with PBST, fixed for 10 min at room temperature in 4% PFA, and mounted in Vectashield (Vector Laboratories, H-1000). When needed, isolectin staining was performed by incubating the retinae overnight at 4°C with isolectin GS-IB, Alexa Fluor 647 (Life Technologies, Cat. no. I32450) diluted 1:400 in PBlec buer (1% Tween-20, 0.1 mM CaCl$_2$, 0.1 mM MgCl$_2$, 0.1 mM MnCl$_2$ in PBS, pH 6.8). Samples were imaged with an upright Carl Zeiss LSM 780 microscope using an Alpha Plan-Apochromat ×63/1.46 NA oil objective.

Fish maintenance and stocks. Zebrafish (Danio rerio) were raised and staged as previously described30. The following transgenic lines were used: Tg(kdr-eras-Cherry)$^{30}$ (ref. 30), Tg(fli1ep:EGFP)$^{31}$ (ref. 31), Tg(fli1ep:PLC$\gamma$1-PH-RFP), Tg(fli1ep:EGFP–CAAX) and Tg(fli1ep:LifectEGFP) (ref. 32).

Cloning, constructs and mosaic expression in zebrafish. All constructs were generated using the Tol2Kit (ref. 33) and the Multisite Gateway system (Life Technologies, Cloning, constructs and mosaic expression in zebrafish.

Fish maintenance and stocks. Zebrafish (Danio rerio) were raised and staged as previously described30. The following transgenic lines were used: Tg(kdr-eras-Cherry)$^{30}$ (ref. 30), Tg(fli1ep:EGFP)$^{31}$ (ref. 31), Tg(fli1ep:PLC$\gamma$1-PH-RFP), Tg(fli1ep:EGFP–CAAX) and Tg(fli1ep:LifectEGFP) (ref. 32).

Cloning, constructs and mosaic expression in zebrafish. All constructs were generated using the Tol2Kit (ref. 33) and the Multisite Gateway system (Life Technologies). The coding sequence of EGFPCAAX was provided in the Tol2Kit; the generation of EGFPCcbe1 is required for embryonic development of the zebrafish. Embryos were dechorionated and anaesthetized with 0.16 mg ml$^{-1}$ (1×) tricaine methanesulfonate (Sigma). Embryos were then mounted in 0.8% low-melting-point agarose (Life Technologies) and immersed in E3 buffer with 1× tricaine. When needed, heartbeat was inhibited by changing the medium for E3 buffer with 4× tricaine. Live imaging was performed on an inverted 3i spinning-disc confocal using a Zeiss C-Apochromat ×63/1.2 NA water immersion objective, on an upright 3i spinning-disc confocal using a Zeiss Plan–Apochromat ×63/1.0 NA water-dipping objective, and on an inverted Andor Revolution 500 spinning-disc confocal using a Nikon Plan Apo ×60/1.24 NA water-immersion objective.

Laser ablation. Laser ablations were performed on an upright 3i spinning-disc confocal fitted with a Zeiss Plan-Apochromat ×63/1.0 NA water-dipping objective using an Ablate 532 nm pulse laser. Ablations were performed in single confocal planes along lines spanning the entire thickness of the structures to be ablated (cell body, or membrane and underlying cortex). Laser was applied for 10 ms at 10–20% laser power. Ablation of the structures of interest was obtained by performing sequential laser cuts using increasing laser power (starting from 10% with 1% increments, up to 20%) at 5–10 s intervals.

Image analysis. Images were analysed using the Fiji software.$^{27}$ Z-stacks were flattened by maximum intensity projection. XY drifts were corrected using the MultiStackReg plugin (B. Busse, NICHD). Fluorescence bleaching was corrected by Histogram Matching. Kymographs were generated using the MultipleKymograph plugin (J. Rietdorf and A. Setz, EMBL). Contrast in all images was adjusted in Adobe Photoshop CS5.1 for visualization purposes. All images are maximum intensity projections, except when otherwise stated in the figure captions. All images are representative of the analysed data.

Statistical analysis. A multinomial log-linear model was used to test for association of bleb or cell count in different defined phenotypic categories with the cell mutation status (WT or AA). The null model was that count variation was due only to experimental batch. No statistical method was used to predetermine sample size. Zebrafish embryos were selected on the following pre-established criteria: normal morphology, beating heart, and presence of circulating red blood cells suggestive of blood flow. The experiments were not randomized. The investigators were not blinded to allocation during experiment and outcome assessment.

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Supplementary Figure 1 (related to Figure 2). a-e) Tg(kdr-l:ras-Cherry) s916 embryos with mosaic expression of Lifeact-EGFP were used to measure expansion time (a), retraction time (b), expansion speed (c) and retraction speed (d) in relation to bleb size (n=31 blebs from 3 cells). Dots in (a-d) correspond to single blebs. (e) shows mean and standard deviation for each property. Values for classical blebs come from ^1. f) Tg(fli1ep:EGFP;fli1ep:PLCδ-PH-RFP) embryos were imaged from 35 hpf. Arrowhead, inverse bleb. C, cytoplasm. E, extracellular space. L, lumen. Time is in hours:minutes:seconds. Scale bar is 5 μm. Images are representative of 3 embryos analysed.
Supplementary Figure 2 (related to Figure 4). a) Embryos with mosaic expression of Myl9b-EGFP and Lifeact-mCherry were imaged at 2 dpf. Arrowheads show co-localisation of F-actin and Myosin-II at cell junctions (A), at the apical membrane (B), and at the base of filopodia (C). Scale bars are 10 μm. Images are representative of 6 embryos analysed. b) Tg(kdr-lras-Cherry)916 embryos with mosaic expression of Myl9b-EGFP were imaged from 34 hpf. Dotted line, apical membrane. Arrow, expanding apical membrane. Arrowhead, onset of Myosin-II recruitment. C, cytoplasm. E, extracellular space. L, lumen. Time is in hours:minutes:seconds. Scale bar is 5 μm. Images are representative of 3 embryos analysed. c) Kymograph generated along the magenta line in b. X axis, time (t) in seconds. Y axis, distance (d) in μm.
Supplementary Video legends

**Supplementary Video 1** (related to Figure 2a). Apical membrane undergoes inverse blebbing during lumen expansion in sprouting ISVs. Time-lapse series of an endothelial sprout with mosaic expression of EGFP-CAAX imaged from 36 hpf. The apical membrane shows inverse blebs as the lumen expands into the sprout (black arrow). The red arrow shows a disconnected lumen fragment originating from the collapse of the lumen. Time is in hours:minutes:seconds.

**Supplementary Video 2** (related to Supplementary Figure 1f). Early apical determinants localise at the apical membrane during inverse blebbing. Time-lapse series of an endothelial sprout expressing cytoplasmic EGFP (left panel, green) and a PLCδ-PH-RFP reporter for PIP₂ (right panel, magenta) imaged from 35 hpf. The apical membrane retains apical markers (PIP₂) as it expands (white arrow). Time is in hours:minutes:seconds.

**Supplementary Video 3** (related to Figure 2c). Inverse membrane blebbing drives multicellular lumen expansion in sprouting ISVs. Time-lapse series of an endothelial sprout with mosaic expression of EGFP-CAAX (left panel, green) and expression of mCherry-CAAX (right panel, magenta) imaged from 32 hpf. Inverse blebbing occurs simultaneously in both cells forming the ISV as the lumen expands (white arrows). Time is in hours:minutes:seconds.

**Supplementary Video 4** (related to Figure 3a). Interruption of blood flow by laser ablation inhibits inverse blebbing at the apical membrane of sprouting ISVs. Time-lapse series of an endothelial sprout expressing EGFP-CAAX imaged from 33 hpf. Laser ablation was performed along a line spanning the entire thickness of the vessel at the place indicated by the red arrow, and at the time indicated. Ablation led to an immediate loss of the inverse blebs at the apical membrane and to gradual regression of the lumen (black arrow). Time is in hours:minutes:seconds.

**Supplementary Video 5** (related to Figure 3b). Interruption of blood flow by tricaine treatment inhibits inverse blebbing at the apical membrane of sprouting ISVs. Time-lapse series of an endothelial sprout expressing mCherry-CAAX imaged from 34 hpf, before, during and after treatment with 4x tricaine. Blood flow stops about 15-20 minutes after addition of 4x tricaine, leading to a loss of the inverse blebs at the apical membrane. Black arrows show expansion of the apical membrane by inverse blebbing before treatment with 4x tricaine and after washout. Time is in hours:minutes:seconds.

**Supplementary Video 6** (related to Figure 4a,b). F-actin polymerises around inverse blebs as they retract.
Time-lapse series of an endothelial sprout with mosaic expression of Lifeact-EGFP (left panel, green) and mCherry-CAAX (right panel, magenta) imaged from 35 hpf. F-actin polymerises around inverse blebs as they retract. Time is in hours:minutes:seconds.

**Supplementary Video 7** (related to Figure 4f). Laser ablation of the cell cortex at the apical membrane of growing lumens leads to the expansion of inverse blebs.
Time-lapse series of an endothelial sprout expressing Lifeact-EGFP (left panel, green) and mCherry-CAAX (right panel, magenta) imaged from 33 hpf. Laser ablation of the cell cortex was performed along the indicated black/white line and led to the expansion of a bleb that later retracted (white arrow). Time is in hours:minutes:seconds.

**Supplementary Video 8** (related to Supplementary Figure 5c). Apical contractility is required for lumen expansion in sprouting ISVs.
Time-lapse series of an endothelial sprout with mosaic expression of Myl9bAA-EGFP (left panel, green) and mCherry-CAAX (right panel, magenta) imaged from 35 hpf. The cell expressing Myl9bAA fails to lumenise from the ventral part of the ISV. Lumen pushes into the cell from the dorsal longitudinal anastomotic vessel (DLAV) but fails to expand (white arrow). Time is in hours:minutes:seconds.

**Supplementary Video 9** (related to Figure 5b). Endothelial cells with decreased apical contractility show uncontrolled blebbing.
Time-lapse series of an endothelial sprout with mosaic expression of Myl9bAA-EGFP (left panel, green) and mCherry-CAAX (right panel, magenta) imaged from 48 hpf. The apical membrane undergoes excessive and uncoordinated blebbing and fails to expand. Time is in hours:minutes:seconds.

**Supplementary Video 10** (related to Figure 5b). Partially lumenised endothelial cells with decreased apical contractility show side lumen branches.
Time-lapse series of an endothelial sprout with mosaic expression of Myl9bAA-EGFP (left panel, green) and mCherry-CAAX (right panel, magenta) imaged from 52 hpf. The ISV is dilated and shows side lumen branches that fail to retract (white arrows). Time is in hours:minutes:seconds.

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