The GEF Trio controls endothelial cell size and arterial remodeling downstream of Vegf signaling in both zebrafish and cell models

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Arterial networks enlarge in response to increase in tissue metabolism to facilitate flow and nutrient delivery. Typically, the transition of a growing artery with a small diameter into a large caliber artery with a sizeable diameter occurs upon the blood flow driven change in number and shape of endothelial cells lining the arterial lumen. Here, using zebrafish embryos and endothelial cell models, we describe an alternative, flow independent model, involving enlargement of arterial endothelial cells, which results in the formation of large diameter arteries. Endothelial enlargement requires the GEF1 domain of the guanine nucleotide exchange factor Trio and activation of Rho-GTPases Rac1 and RhoG in the cell periphery, inducing F-actin cytoskeleton remodeling, myosin based tension at junction regions and focal adhesions. Activation of Trio in developing arteries in vivo involves precise titration of the Vegf signaling strength in the arterial wall, which is controlled by the soluble Vegf receptor Flt1.
The arterial vascular network distributes blood flow through the body, a process crucial for sustaining organ function and homeostasis. During embryonic development, arterial networks enlarge and expand to meet the increasing metabolic demand of the growing and differentiating tissue. Also in ischemic cardiovascular diseases, revascularization, and regeneration of hypoperfused organs involve adaptations of the arterial system. Selective targeting of the arterial growth process, and in particular creating arteries with a structurally large diameter to enhance flow conductance and relief hypoxia, is considered therapeutically relevant for treating patients with ischemic cardiovascular diseases. The molecular and cellular mechanisms controlling arterial diameter are partly understood but involve interactions between blood flow and endothelial cells, and BMP-Smad signaling controlling distinct endothelial cell behaviors during the vascular remodeling process.

An increase in flow promotes endothelial cell proliferation and migration collectively facilitating the transition of a small caliber vessel segment, with only few endothelial cells, into a larger caliber arterial segment, with many endothelial cells. Flow activates the Akt-PI3-kinase signaling pathway responsible for endothelial proliferation. In addition, flow activates the BMP9-ALK1-Eng-Smad4 signaling pathway, which restricts flow-induced Akt activation and promotes endothelial quiescence. The extent of diameter remodeling of arteries is determined by the amount of flowing blood and the shear stress setpoint. Blood flow creates frictional forces exerted on endothelial cells, termed shear stress. Shear stress level has to be kept within strict boundaries to maintain endothelial quiescence and vessel stability. A sustained increase in blood flow and shear stress promotes outward remodeling of the arterial lumen, resulting in a return of shear stress levels to the original setpoint. VEGF receptor-3/Fli1 is a component of the endothelial junctional mechanosensory complex, and acts as a determinant of the shear stress setpoint. Lowering Fli1 expression and increasing the shear stress setpoint causes arteries to narrow thereby allowing shear stress to return to the setpoint. In addition, increases in flow, after causing an initial diameter increase, have been shown to induce vessel contraction mediated via endothelial cell shape changes. The transforming growth factor beta co-receptor Endoglin regulates vessel contraction, and loss of endoglin results in enlarged endothelial cells and blood vessel diameter in response to flow increases. In adult pre-existing collateral arteries, flow-dependent arterial outward remodeling also involves activation of NFkB and inflammatory pathways in endothelial cells, leading to the recruitment of monocytes/macrophages that assist with remodeling by secretion of matrix metalloproteinases, and growth factors. In gridlock mutant zebrafish embryos, macrophages contribute to flow driven outward remodeling of aortic collaterals, suggesting a developmentally conserved role for macrophages in arterial remodeling.

Cell shape is determined by the activity of small Rho GTPases. Their activity contributes to maintaining an equilibrium between the forces providing centripetal tension and forces ensuring cell spreading and avoidance of cell collapse. Here we show that in endothelial cells, the guanine nucleotide exchange factor Trio, and activation of the small GTPases Rac1 and RhoG, trigger F-actin remodeling events in the endothelial cell periphery, increasing endothelial cell size. Arterial-specific expression of Trio in vivo augments endothelial cell size, resulting in functional arteries with a structurally larger lumen diameter, without change in endothelial cell numbers. Activation of Trio in vivo requires subtle fine-tuning of local arterial VEGF-KDR signaling levels, which is achieved by arterial Fli1 acting as a VEGF trap. Genetic targeting of the local arterial Fli1-VEGF balance results in endothelial cell enlargement, and significant outward arterial diameter remodeling, even during low flow conditions. Increases in vessel diameter reduce the resistance to flow. Trio-induced endothelial shape changes, and diameter remodeling in response to VEGF, may therefore aid to fine-tune local flow distribution in response to tissue metabolism or hypoxia.

**Results**

**Arterial Fli1 determines arterial diameter.** VEGF is an attractive candidate for targeting arterial caliber as it controls key aspects of arterial development, and is capable of activating small Rho-GTPases. Yet, beyond a narrow therapeutic window, VEGF may induce adverse side-effects such as vessel overgrowth, e.g., hemangioma formation, and increased vessel permeability. How to deliver VEGF without deleterious side-effects is still an outstanding issue. To fine-tune the spatio-temporal delivery of VEGF needed to obtain large arteries, we examined formation of arterial networks in the trunk of developing zebrafish embryos using different vegfa gain of function scenarios.

We first employed transgenic embryos with constitutive or inducible expression of vegfaa under control of the somite muscle-specific 503unc promoter. Constitutive vegfaa overexpression increased endothelial cell number and disrupted the arterial vasculature, abruptly breaking blood flow perfusion when compared to wild-type (WT). Similar results were obtained upon inducible vegfaa overexpression (Supplementary Fig. 1b, d, e–w). Because such vegfaa transgenic approaches resulted in Vegfaa overdose inappropriate for targeting the diameter of arteries, we next aimed at obtaining more subtle changes by manipulating Vegf protein bioavailability, which is determined by the Vegfr3 decay receptor Fli1.

Fli1 has a very high affinity for VEGF and mainly acts as a VEGF trapping receptor, limiting VEGF bioavailability and signaling through KDR. Conversely, ablating fli1 or displacing Fli1-trapped Vegf produces a Vegf gain-of-function scenario, with endogenous production of Vegf. We hypothesized that Fli1 can be used as a vehicle to deliver Vegf directly into growing arteries. For this approach to work efficiently, Fli1 protein must be expressed in close proximity to the Vegf signaling receptor KDR on arterial endothelium. We found that this is indeed the case (Fig. 1c–g). The developing arterial intersegmental vessels (aISV) displayed fli1 promoter activity (Supplementary Fig. 2a–c) and expressed both fli1 isoforms; the membrane bound fli1 (mli1) and soluble fli1 (sli1) (Supplementary Fig. 2d, e). To determine Fli1 protein distribution, we generated a knockin line, harboring two HA tags in exon 3 of the endogenous fli1 locus, which labeled both mFli1 and sFli1 (Supplementary Fig. 2f). Immune staining of the transgene demonstrated Fli1 protein expression in the developing aISVs (Fig. 1c, d). To substantiate the distribution of the secreted, soluble Fli1 isoform around developing arteries, we furthermore examined protein distribution in a transgenic line expressing an engineered HA-tagged sli1 transgene with an inactive Vegfaa binding domain (Tg[fli1hins:sli1Δ7-HA]) and Supplementary Fig. 2g–n. Immune staining showed sFli1 protein co-localizing with KDR in arterial endothelium (Fig. 1e–g). Transgensics with tagged wild-type sli1-HA could not be used, because gain of wt-sli1-HA decreased local Vegf and inhibited aISV formation, thus rendering this experiment uninterpretable (Supplementary Fig. 2i–k).

Arterial ISVs, which express the Fli1 protein, are juxtaposed to Vegf-producing cells in developing somites. Ablation of fli1, thereby creating a Vegfaa gain of function scenario, resulted in...
GOF transgenic embryos (Fig. 1h, i, j, k, l) to show Flt1 protein distribution. Arrows indicate aISVs and box shows zoom. e-g Immunestaining with anti-HA antibody showing sFlt1 protein distribution in WT (a) and vegfa_musc transgenic embryos. aISV express sFlt1 protein (green; for indicated scenario, ***p < 0.001). Immunestaining with anti-HA antibody showing sFlt1 protein distribution in Tg(fli1a:lifeactEGFP)mu240 (WT, flt1−/−) to show Flt1 protein distribution. Arrows indicate aISVs and box shows zoom. h–k Confocal imaging of aISV in WT injected with flt1 targeting morpholino. Area indicated by the red dotted box in the upper panel is displayed at higher magnification in the lower panel. n Relative aISV diameter change in embryos injected with flt1 targeting morpholino (WT = 100%). mean ± s.e.m, unpaired two-sided t-test, n = 18, 16, 16, 10 aISV s per genotype. ***p < 0.001. m Confocal imaging of aISV in WT injected with flt1 targeting morpholino. Arrows indicate radial Flt1 binding. Area indicated by the red dotted box in the upper panel is displayed at higher magnification in the lower panel. n Relative aISV diameter change in embryos injected with flt1 targeting morpholino (WT = 100%). mean ± s.e.m, unpaired two-sided t-test, n = 10, 25 aISV s for indicated scenario, **p < 0.001. o, p Imaging of plasma extravasation in WT and vegfb_musc (n = 14, 16 embryos) injected with 70kD Dextran Texas-Red from 3 biologically independent experiments. q Quantification of images in o, p, mean ± s.e.m; unpaired two-sided t-test, n = 6, 8 embryos per indicated group. ns, not significantly different. Scale bar, 50 μm in a–g, o, p; 25 μm in h–k, m. aISV intersegmental artery, DA dorsal aorta, PCV posterior cardinal vein. hpf hours post fertilization, MO morpholino, GOF gain of function.

A significant increase of aISV diameter (Fig. 1h–l). A similar result was obtained with a flt1 targeting morpholino (Fig. 1m, n).

High levels of the Flt1 ligands Plgf or Vegfb cause displacement of Vegf from Flt1 to Kdrl, creating a local Vegf gain of function scenario. In line with this competition model, while both loss of plgf and not leaky (Fig. 1o) showed significantly increased arterial lumen diameters (Fig. 1j, k, l; Supplementary Fig. 2o, p). On average aISV diameter increased 1.9 and 2.0 fold in plgfmusc and vegfb_musc embryos respectively (Fig. 1). The larger aISVs were functional and not leaky (Fig. 1o–q; Supplementary Fig. 2q–t; Supplementary Movies 1–3). Membrane bound mFlt1 signaling was not required for diameter growth in this setting: aISVs were significantly larger in mflt1−/− mutants (Fig. 2a–d), and overexpression of plgf in mflt1−/− mutants still resulted in a pronounced increase in arterial diameter when compared to WT or mflt1−/− diameter (Fig. 2a–d).

While both loss of flt1 and plgf gain of function induce a Vegf gain of function scenario, one component that may contribute to the difference in arterial diameter between plgfmusc embryos and flt1−/− mutants involves the spatial distribution of arterial Flt1 and Kdrl receptors. In plgfmusc, Flt1, and Kdrl are both expressed in the same arterial ECs (Fig. 1g, Supplement Fig. 2a). Such expression pattern is absent in flt1−/− mutants as Flt1 is not expressed. If Flt1-Kdrl co-expression in arterial EC indeed
accounts for the observed difference, the model predicts that reducing flt1 expression in plgfmusc, should annihilate the difference between plgfmusc and flt1−/− mutants. Nevertheless, arteries in plgfmusc transgenics injected with a flt1 targeting morpholino should still show some degree of arterial diameter growth as the knockdown of flt1 itself induces a vegfaa gain-of-function scenario, similar as in flt1−/− mutants. We tested these assumptions, and in-line with our hypothesis we indeed found that knockdown of flt1 in plgfmusc embryos reduced arterial diameter growth, to the levels observed in flt1−/− mutants (Fig. 2e–h).

Arterial diameter growth requires Vegf receptor-2 signaling. Our data support the model in which excessive Plgf displaces Vegf receptor-2 signaling from vegfaa to bind arterial Kdrl receptors to induce signaling. Intrapped Vegfaa from arterial Flt1, thereby allowing the released vegfaa/Ang1 to bind arterial Kdrl receptors to induce signaling. In vivo confocal imaging of aISV in WT (a); mflt1−/− mutant, mflt1+/+GFP (b); mflt1−/− combined with plgf gain of function plgfmusc (c); n = 58,64 and 57 aISVs per genotype derived from three autonomous experiments. The areas indicated by the red dotted boxes are displayed at higher magnification in the lower panels. d Quantification of images in a–c. Mean ± s.e.m, unpaired two-sided students t-test, n = 7,13, and 24 aISVs for indicated scenario. **p = 0.0051, ***p < 0.001. Note: significantly increased aISV diameter in mflt1−/−/plgfmusc scenario.

e–g In vivo confocal imaging of aISV in plgfmusc (e), plgfmusc concomitant with morpholino knock-down of flt1 (f, plgfmusc + flt1-MO), flt1−/− mutant, flt1MO (g). The areas indicated by the red boxes are displayed at higher magnification in the lower panels. h Quantification of images in e–g. Mean ± s.e.m, unpaired two-sided students t-test, n = 40,19, and 13 aISVs for indicated scenario. ns not significant. **p < 0.01. Scale bar; 25 μm in all panels. hpf hours post fertilization, aISV intersegmental artery, MO morpholino.
developing aISV can be achieved in three ways: by having more endothelial cells, larger endothelial cells or a combination of both. We detected the combination of both: more and enlarged endothelial cells in aISVs upon plgf gain-of-function when compared to WT (Fig. 4a–e; Supplementary Fig. 6a–f; Supplementary Movies 4 and 5). Analyses of life-act reporter embryos showed a significant increase in total endothelial cell surface area and in surface area expansion rate, when comparing plgfmusc embryos to WT (Fig. 4e; Supplementary Fig. 6g; Supplementary Movies 4 and 5). We reasoned that RhoGTPase activity and changes in F-actin remodeling could contribute to the in vivo phenotypic changes in endothelial cell shape.

To block F-actin polymerization, we administered the actin polymerization inhibitor Latrunculin B (LatB) in vivo. LatB significantly reduced aISV diameter growth in plgfmusc embryos when compared to vehicle treated plgfmusc embryos (Fig. 4f; Supplementary Fig. 6h–p). Major aspects of intracellular F-actin dynamics are regulated through the small Rho GTPases family, including Rac1 and RhoG. The Rac1 inhibitor CAS 1177865-17-6 significantly inhibited aISV diameter expansion in plgfmusc embryos (Fig. 4f; Supplementary Fig. 6h–p). The RhoGEF (Rho-Guanine nucleotide Exchange Factor) Trio is well recognized for its ability to activate Rac1 and RhoG through its GEF1 domain. ITX3, a selective inhibitor of the Trio N-terminal RhoGEF
Fig. 3 Vegfaa—Vegf receptor-2/Kdr signaling drives outward arterial lumen remodeling. a-d aISV in WT (upper panels) and plgfmusc embryos (lower panels) treated with DMSto vehicle - control (a), low dose Vegf-R2 inhibitor (b), high dose Vegf-R2 inhibitor (c), or vegfa ATG targeting morpholino (d). Red dotted box is displayed at higher magnification in panel below. e Quantification of aISV diameter at 50 hpf upon R2 inhibition. Mean ± s.e.m, one-way ANOVA, and post-hoc bonferroni, n = 18, 20, 15, 15 aISVs for indicated scenarios. **p = 0.0026, ***p = 0.001. f Quantification of aISV diameter at 48 hpf upon morpholino-mediated knockdown of vegfaa. Mean ± s.e.m, one-way ANOVA, and post-hoc bonferroni, n = 15, 22, 14, 28 aISVs for indicated scenarios. **p < 0.001. g–j in vivo confocal imaging of WT (g), fl/t4 −/− mutant, fl/nnt2 (h), fl/t4 −/− mutant injected with fl ATG targeting morpholino (i), fl/t4 −/− mutant injected with plgfmusc plasmid (j). k Quantification of images in g–j. Left panel, absolute aISV diameter (n = 21, 16, 42, 34 aISVs per indicated genotype). Right panel, aISV diameter change relative to WT. Mean ± s.e.m, unpaired two-sided students t-test, ns not significant, **p < 0.001. 

Endothelial enlargement requires the GEF1 domain of Trio. Rho GTPases control actin reorganization, and GEFs like Trio activate Rho GTPases by promoting their exchange of GDP for GTP. Trio is a unique Rho GEF, because it has two separate GEF domains, GEF1 and GEF2, that control the GTPases RhoG/Rac1 activate Rho GTPases by promoting their exchange of GDP for GTP. Trio activates Rac1 in particular at positioning of GEFs, and activation of RhoGTPases in junctional regions. That one part of cell enlargement may require careful spatial positioning of GEFs, and activation of RhoGTPases in junctional regions.

Trio activates Rac1 in junctional regions. Using a Rac1 FRET-based biosensor we found that Trio activated Rac1 in particular at endothelial cell junctional regions (Fig. 6a, b). TrioG and Tiam1 were equally potent in activating Rac1 at junction regions (Fig. 6c, d; Supplementary Fig. 9a–c). Mutating the functional GEF1 domain in TrioN (TrioNmut40) abrogated Rac1 activation (Fig. 6c, d; Supplementary Fig. 9d). TrioN-transfected cells showed formation of lamellipodia, and enlargement of the endothelial cells along the front of the lamellipodia extensions (Supplementary Fig. 9e–g; Supplementary Movie 6). Control-transfected cells also showed lamellipodia formation, but in control cells these lamellipodia more often retracted and no subsequent enlargement was observed (Supplementary Fig. 9b–j; Supplementary Movie 7). TrioN significantly increased the lifetime of lateral lamellipodia when compared to transfected cells; accordingly the number of protrusions per hour was reduced in TrioN-transfected cells (Fig. 6c, f). After 17 h post-TrioN transfection, no additional enlargement was observed and the VE-Cadherin expression pattern was reminiscent of a honeycomb structure, suggestive of a force-equilibrium between the interacting cells41.

Actomyosin complexes and myosin-II-mediated contractility contribute to tension development42,43. Using GFP-tagged Myosin II we found that Myosin II localized on F-actin bundles throughout the cell but was most prominently present on junctional F-actin bundles upon TrioN transfection (Fig. 6g, h).
Also, the majority of active myosin, distinguished by staining for mono-phosphorylated myosin light chain S19, localized strongly at the junction region upon TrioN overexpression (Fig. 6i, j). In addition, we observed that fully di-phosphorylated myosin light chain (T18/S19) localizes even more specifically to junctional F-actin bundles (Fig. 6k, l). Inhibition of myosin activity from 1 h after TrioN transfection significantly abrogated Trio-induced endothelial cell size enlargement (Supplementary Fig. 9k–m). Inhibiting myosin activity in already enlarged endothelial cells resulted in the formation of gaps in particular at tri-cellular junctions (Supplementary Fig. 9n, o).

We next reasoned that Trio-induced changes in myosin-II and cytoskeletal remodeling affect the tension distribution along the F-actin cytoskeleton. To test this, we examined local tension at...
junctional, cortical, and central F-actin bundles, in TrioN and control-transfected endothelial cells by laser-ablating single F-actin bundles (Fig. 6m–s). As a read out for local tension, we measured the recoil of the actin structures upon ablation (Fig. 6p)\^44. Transfection of TrioN only increased the tension on junctional actin bundles, and not on cortical or central bundles (Fig. 6q–s). Taken together, these findings suggest that TrioN gain-of-function preferentially augmented tension at junctional F-actin bundles.

VE-Cadherin is not required for Trio-induced EC enlargement. Trio interacts with VE-Cadherin\^45, and endothelial cells gain stability by making contacts with neighboring cells through VE-Cadherin–based cell–cell junctions. Endothelial monolayers transfected with TrioN in vitro showed augmented junctional integrity as indicated by increased electrical cell-substrate impedance (ECIS) (Fig. 7a). Interestingly, this increase in junctional strength was independent of VE-Cadherin, as TrioN overexpression augmented electrical cell-substrate impedance even in the absence of VE-Cadherin (Fig. 7b, c). Other junctional molecules may compensate for loss of VE-Cadherin. We found no substantial changes in N-Cadherin expression at junctions in endothelial cells in which overexpression of TrioN was combined with silencing of VE-Cadherin (Supplementary Fig. 10a–l).

To test if VE-Cadherin-based cell–cell junctions are functionally relevant for the TrioN-induced enlargement of endothelial cells, we overexpressed TrioN while simultaneously silencing endothelial VE-Cadherin (Fig. 7d–m). Loss of VE-Cadherin had no significant impact on TrioN-induced endothelial cell enlargement (Fig. 7m). To test if coordination between adjacent endothelial cells is critical for endothelial enlargement in vitro, we performed mosaic experiments by mixing TrioN overexpressing endothelial cells with control-transfected endothelial cells (Supplementary Fig. 10m), and compared cell size with a scenario in which all (neighboring) cells were expressing TrioN (homozygous expression scenario). In both the mosaic and in the homogeneous scenario we found increased EC size in TrioN overexpressing cells (Fig. 7n). The magnitude of cell size increase was not significantly different between the two scenarios (Fig. 7n).

Endothelial cells anchor to the extracellular matrix (ECM) through focal adhesions. In endothelial lamellipodia extensions, anchoring to the ECM is necessary for the buildup of tractional forces that regulate forward extension and cell shape. We hypothesized that to maintain their shape, the enlarged endothelial cells have to be able to stably anchor to the ECM. In line with this, we found that TrioN massively increased the number of focal adhesions, in particular near cell–cell junctions, as judged by phosphorylated Paxillin staining (Fig. 7o–s). TrioN overexpressing cells furthermore showed a shift in the expression of a5 Integrin and b1 Integrin toward junctional regions (Fig. 7t–w).

Trio augments EC size and arterial diameter in vivo. To test if Trio can augment endothelial cell size and arterial dimensions in vivo in zebrafish embryos, we generated a transgenic line and overexpressed TrioN under the control of the fltl\^enh promoter (Tg (fltl\^enh:GFP-TRION) here termed fltl\^enh:TrioN) (Fig. 8a–g). The fltl\^enh promoter is mainly active in arterial ISVs. Overexpression of TrioN in vivo resulted in significantly larger aISVs (Fig. 8e) without affecting endothelial cell numbers (Fig. 8g). Instead, in-line with our in vitro observations, TrioN increased endothelial surface area in aISVs (Fig. 8f). The aISV diameter increase was therefore solely attributed to larger endothelial cells. The larger aISVs upon TrioN overexpression were perfused and nonleaky as evidenced from dextran extravasation analyses (Supplementary Fig. 11a–d).

We next overexpressed TrioN under control of the fltl\^enh promoter in plgfn fus embryos (Fig. 8h–o). Overexpression of TrioN in plgfn fus embryos resulted in significantly larger aISVs and endothelial surface area, when compared to plgfn fus alone (Fig. 8m, n, o, u). On an average, combining plgf and TrioN resulted in 2.5-fold larger aISV diameters (Fig. 8o); whereas in plgfn fus embryos the increase was 2-fold (Fig. 8o). The enlarged aISVs in plgfn fus + TrioN gain of function embryos were actively perfused (Supplementary Movie 8). Overexpression of TrioNmut (TrioN with inactive GEF1 domain) in plgfn fus transgenic embryos failed to produce any additive increase in aISV diameter or endothelial surface area (Fig. 8o). Taken together, these data suggest that combining plgf with artery specific Trio transgenic gain-of-function results in arteries having multiple enlarged endothelial cells, which additively facilitate the arterial outward lumen remodeling.

We furthermore found that VE-Cadherin was not required for TrioN-induced endothelial cell size increase in vivo. Overexpression of TrioN in aISVs of ve-cadherin mutants (cdh5-MO) or ve-cadherin\^\textsuperscript{−/−} mutants augmented endothelial area at a similar magnitude as observed in TrioN gain-of-function embryos with intact ve-cadherin expression (Fig. 8p–t; Supplementary Fig. 11e).

We next examined if the effect of Trio on arterial diameter in vivo and cell size in vitro is conserved when using alternative vascular promoters or endothelial cells derived from different anatomical origins, respectively (Supplementary Fig. 11f–h). The kdr and flila promoter are ubiquitously expressed in endothelial cells of the zebrafish embryo trunk vasculature. For both kdr and flila we observed significantly increased aorta and aISV formation in WT (a) and in plgfn fus embryos (b). ECs derived from migration events numbered in yellow, from proliferation events in purple. c Quantification of migration (yellow) and proliferation (purple) events during aISV formation; mean ± s.e.m, unpaired two-sided students t-test, n = 10, 11 aISVs for indicated genotype. *p = 0.0190, **p < 0.001. d Time-lapse imaging in Tg(iIFlac:lifeactGFP)\textsuperscript{pm240} showing endothelial actin in aISV of WT (upper panels) and plgfn fus embryos (lower panels). Red box in the left panel at highest magnification in the right panels. Arrowheads at 41hpf delineate an individual EC. e EC surface area changes in WT (blue circles) and plgfn fus embryos (red squares); mean ± s.e.m, unpaired two-sided students t-test, n = 6 ECs for each genotype. f Left panel, arterial ISV diameter in plgfn fus embryos (magenta bar) treated with the actin polymerization inhibitor Latrunculin B (LatB, pink bar), Rac1 inhibitor CAS 1177865-17-6 (green bar), Trio inhibitor ITX3 (yellow bar). Right panel, RI upon indicated inhibitor treatment. Mean ± s.e.m, one-way ANOVA, post-hoc bonferroni, (LatB, pink bar), Rac1 inhibitor CAS 1177865-17-6, Trio inhibitor ITX3. g-h) Quantification of images in c–i. Mean ± s.e.m, ANOVA & post-hoc bonferroni, n = 17, 18, 15, 16 and 17 aISVs/group. ***p < 0.001. i–p aISV in WT (n), WT injected with 1.7 ng Trio targeting morpholino (k), and plgfn fus injected with 5 ng Trio targeting morpholino (l). m Quantification of images in a–i. Mean ± s.e.m, ANOVA & post-hoc bonferroni, n = 25, 38, 30, 60, 59 aISVs for indicated condition. ***p < 0.001. q–s aISV in plgfn fus (q), plgfn fus injected with 1.7 ng Trio targeting morpholino (r), and plgfn fus injected with 5 ng Trio targeting morpholino (s). t Quantification of images in n–s. Mean ± s.e.m, ANOVA & post-hoc bonferroni, n = 18, 14, 16, 18, and 18 aISVs per group. ns not significant, **p < 0.01, ***p < 0.001. Scale bar, 25 μm in all panels. aISV intersegmental artery, MO morpholino.

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diameters, and EC size, in WT and in perfmuc embryos (Fig. 8v, w, Supplementary Fig. 11g, h). In vitro, human aorta (HAEC) and human umbilical artery derived endothelial cells (HUAEC) showed a significantly increased size upon TrioN overexpression (Supplementary Fig. 11f). As Vegf is the major driver of Trio activation in our setting, we hypothesized that our findings regarding enlargement may be conserved in other vegf gain of function scenarios. Von Hippel Lindau (Vhl) is a protein relevant for probing Hypoxia Inducible Factor-1α (HIF-1α), the main driver of vegf expression, for proteasomal degradation. Vhl−/− mutants show increased vegfaa expression, in particular in developing neuronal tissue. Accordingly, vhl−/− mutants showed increased cerebral arterial diameters, without significant change in endothelial cell numbers (Supplementary Fig. 11i–p). Vhl morphants also showed increased vessel diameter, concurrently with increased EC surface area (Supplementary
Fig. 5 Trio-GEF1 domain targets Rac1 and RhoG increase endothelial cell size. a–f Confocal images showing mCherry (a, c, e) and F-actin distribution (b, d, f) in ECs transfected with mCherry, mCherry-TrioN (TrioN), or mCherry-TrioGEF1 (TrioGEF1). Scale bar, 25 μm. g Endothelial cell surface area in ECs expressing mCherry, mCherry-TrioN, or mCherry-TrioGEF1. Mean ± s.e.m, unpaired two-sided students t-test, n = 59, 69, 63 cells per indicated group. ***p < 0.001. h–k Confocal images of ECs transfected with mCherry, photo-activatable Rac1 (mCherry-Rac1-PA-WT), mutated photo-activatable Rac1 (mCherry-Rac1-PA-C450A), or constitutively active Rac1 (mCherry-Rac1-Q61L). Red arrowheads indicate cell–cell junctions. Scale bar, 25 μm. l Quantification of images in h–k. Mean ± s.e.m, two-sided Mann–Whitney U test, n = 67, 56, 65, 74 cells per group. *p = 0.0477, ***p < 0.001. m Surface area of ECs transfected with control plasmid or constitutive active RhoG Q61L. Mean ± s.e.m, unpaired two-sided students t-test, n = 89, 83 cells per group, from three independent experiments. **p < 0.01. n Western blots for Rac1 and RhoG; control of knockdown efficiency for shRNA shRac1 and shRhoG as used in O, P and actin for protein loading control, as indicated. o Change in EC size upon TrioN overexpression after silencing of Rac1. Mean ± s.e.m, unpaired two-sided students t-test, n = 73, 97 cells from three independent experiments. **p < 0.01. p Change in EC size upon TrioN overexpression after silencing of RhoG. Mean ± s.e.m, unpaired two-sided students t-test, n = 101, 146 cells per group from three independent experiments. **p < 0.01. q s Endothelial cells transfected with control plasmid (ctrl) and stained for Tiam1 (q), F-actin with phalloidin (r) and VE-Cadherin as junction marker (s). Tiam1 localizes cytosolic and to a lesser extent to junction regions. Scale bar, 20 μm. t–u Endothelial cells transfected with TrioN and stained for Tiam1 (t), F-actin with phalloidin (u) and VE-Cadherin as junction marker (v). Tiam1 localizes at junction regions (arrowheads). Scale bar, 20 μm. w Endothelial cell size of ECs transfected with TrioN, TrioN, or transfected with both Tiam1 and TrioN. Cell size was measured of n = 78, 82, 103 and 80 cells derived from three separate experiments. Mean ± s.e.m, unpaired two-sided students t-Test. ns not significant, **p < 0.001.

Fig. 11q–u). Blocking Trio function using ITX3 inhibited the diameter increase in vhl morphants (Supplementary Fig. 11s, u). This suggests that Trio-mediated cell and vessel dimension changes is conserved in this Vegf gain of function scenario.

In zebrafish embryos, loss of the TGFβ co-receptor endoglin augments aortic diameter in response to flow increases10. Morpholino-mediated loss of endoglin increased aorta diameter at 3dpf, not 2dpf, in-line with the endoglin mutant phenotype (Supplementary Fig. 12a–u). We found no evidence for loss of endoglin and gain of Trio acting synergistically to influence aISV or aortic diameters in 2dpf embryos (Supplementary Fig. 12a–i; Supplementary Fig. 12j–l). In vitro, cell size of TrioN over-expressing cells was not significantly different from the size of TrioN expressing cells in which endoglin was silenced (Supplementary Fig. 12s, t). At 3dpf endoglin morphants showed an increased aorta diameter similar to the phenotype reported in endoglin mutants (Supplementary Fig. 12u)10. Since in the endoglin mutant the change in aorta diameter was attributed to an increase in EC size and not EC number, we considered a potential contribution of Trio in mediating the diameter increase. Accordingly, loss of Trio in endoglin morphants, or inhibiting Trio function using ITX3 in endoglin morphants inhibited the aorta diameter increase and reduced EC surface area (Fig. 9a, b). This suggests that at 3dpf the TGFβ pathway may act as an inhibitor of Trio function and diameter control.

Discussion

Here we identified the GEF Trio as a central signaling hub in driving endothelial cell enlargement and outward arterial lumen diameter remodeling, downstream of Vegf receptor-2 signaling. Endothelial cell enlargement requires the GEF1 domain of Trio, and activation of the Trio-GEF1 targets Rac1 and RhoG, inducing F-actin remodeling events, focal adhesions, and actomyosin tension specifically at the cell periphery. Actomyosin-activity induced tension at extracellular matrix anchors in lamellipodia extensions is subsequently conveyed into a homoeostatic expansion of the endothelial cell. Actin remodeling in the endothelial cell periphery requires activation of Rho GTPases specifically in junctional regions. In line with this, we show that Trio activates Rac1 in the cell periphery. Active Trio is expressed at junctional regions, and Trio also directs other GEFs including Tiam1 to the junctions. Tiam1 has thus far not been linked to Vegf receptor signaling. Tiam1 is a known activator of Rac1, and we show that Tiam1 promotes endothelial cell size, similar to Trio. Combining Trio and Tiam1 overexpression has no additive effect on augmenting endothelial cell size, thus leaving open the option that Trio and Tiam1 may act in a linear way to induce Rac1 activity at junctional regions. Trio and Tiam1 are not the only GEFs that can activate Rac1 and it is conceivable that activation of additional Rac1 pools, at different intracellular locations and coupled to other downstream effectors, also contributes to the complex process of cell enlargement.

Besides activation of Rac1, the GEF1 domain of Trio is also recognized for its ability to exchange GTP on the related small GTPase RhoG. Similar to Rac1, we show that RhoG gain-of-function results in larger endothelial cells, while loss of RhoG reduces Trio-induced cell size. How RhoG contributes to endothelial cell size increase may however differ from Rac1, as RhoG is dispensable for lamellipodia formation37. We noticed that upon Trio activation, the distribution of the Integrins β1 and α5 shifted from a well-known focal adhesion pattern towards a more junctional pattern. Also Paxillin, a well-recognized marker for focal adhesions shifted to the junction regions. Integrins may be involved in the regulation of Trio-mediated increase in cell size by controlling ECM contacts near junctions as a means to keep the enlarged cell in its shape and preventing it from collapsing. RhoG may contribute herein as it has been shown to modulate focal adhesion turnover46 and microtubule dynamics, at least in cancer cells and epidermal keratinocytes46–48. Vegf has also been shown to activate RhoG independently of Trio19. Interestingly, here RhoG subsequently activated Rac1 through the atypical GEF DOCK4, whereas SGEF, instead of Trio, was the responsible upstream activator of RhoG. Thus, downstream from VEGF, SGEF, and Trio may work in parallel, to activate RhoG and Rac1, respectively. Both pathways may also function as a reciprocal backup, as it was reported that SGEF knock out animals do not show any vascular malformations30.

Increasing Vegf—Trio signalling strength in developing arteries and augmenting both arterial endothelial cell size and their number allows for a 2–2.5-fold structural increase of arterial lumen diameter in vivo (Fig. 10a, b). Using Vegf to obtain arterial diameter growth in aISVs in vivo requires precise fine-tuning of local Vegf signaling strength in arterial endothelium. This can be achieved by targeting the arterial Flt1-Vegf binding equilibrium with the Flt1 specific ligands Plgf and Vegfb. We demonstrate that developing arteries express Flt1 protein, and analyses of f1l1 mutants and f1l1 morphants shows that arterial Flt1 traps sufficient endogenous Vegf to modulate arterial diameter growth. Overexpression of Plgf or Vegfr, and displacing the arterial Flt1-trapped Vegf, results in an artery specific Vegf gain of function scenario. This triggers an increase in arterial endothelial cell size and number, culminating in a two-fold increase in arterial diameter. Plgf-induced arterial diameter growth requires Vegfaa-Kdr signaling, but is independent of mFlt1 signaling, as the
growth effect of Plgf is maintained in mflt1 mutants. Arterial endothelial cells of alSVs enlarge considerably in plgfmusc transgenics. Consistent with Trio driving endothelial enlargement in this Vegf gain of function scenario, we find that inhibiting Trio reduces endothelial cell size, and alSV diameter in plgfmusc. Our model suggests that arterial diameter growth in plgfmusc involves a combination of both increased cell number and enlarged size, enabling the shaping of a structurally larger lumen (Fig. 10a, b).
autonomous manner. Trio furthermore augments junctional integrity and stabilizes endothelial–endothelial cell–cell junctions. Such increased junctional stability would be beneficial to keep expanding vessels sealed and prevent plasma leakage during the outward remodeling process.45,51. The TGFβ co-receptor Endoglin controls arterial diameter in response to shear stress through modulation of endothelial cell shape.6,10. With respect to the regulation of 2dpf aSLV and aorta diameter, we find no interaction between Vegf-Trio signaling and Endoglin. However, observations in 3dpf aorta suggest that Trio may be required for mediating aorta diameter increase upon loss of endoglin. How loss of endoglin couples to Trio remains to be determined but human endothelial cells devoid of Endoglin show altered VEGFR-2 kinetics, and exhibit differential activation of VEGFR-2 downstream pathways, including AKT.6 We hypothesize that Vegf can activate Trio during early stages of arterial network remodeling, whereas the TGF beta pathway may act to restrict Trio function once flow becomes more dominant during the maturation of the arterial network. Although we originally investigated activation of Vegf-Trio as a means to augment arterial diameter in the context of improving perfusion for ischemic cardiovascular disease, the notion that inhibiting Trio and reducing EC and arterial size, may have therapeutic relevance as well for preventing shunt formation and vascular complications in, for example hereditary hemorrhagic telangiectasia (HHT).10,11

In early zebrafish embryos, mural cells are absent and vessel size is determined solely by the endothelium. In adult resistance sized arteries the presence of several layers of smooth muscle cells, and sympathetic nerve induced vascular tone may restrict the impact of Trio on diameter remodeling. It therefore seems unlikely that activating Trio and promoting EC size, can directly affect arterial diameter independent of vascular smooth muscle function in mature resistance sized arteries. Flt1 and its ligands PIGF and VEGF-B can regulate vascular adaptation in ischemic cardiovascular conditions in mouse, rat and pig models.52–56. In rats, constitutively cardiomyocyte specific expression of full length Vegf-b results in enlargement of all coronary arteries.55 In the coronary arteries, Flt1 and Kdr only colocalize in the most distal areas and capillaries, whereas coronary arteries in the proximal part express only Flt1. Based on the juxtapositioning of Flt1 and Kdr, it is therefore conceivable that arterial enlargement commences in precapillary arteries and that this is propagated retrogradely to the proximal areas.53 We previously showed that such retrograde communication mechanism between micro- and macrocirculation, exists in an ischemic setting and promotes arterial remodeling.57. In a more physiological context, increases in metabolism or tissue hypoxia can trigger the local release of Vegf. Vegf-Trio-induced endothelial enlargement and diameter increase reduce the resistance to flow. At a given pressure gradient this results in a redistribution of flow toward the enlarged vessel segment in the Vegf-producing or hypoxic segment. We hypothesize that such vessel adaptations allow matching of changes in metabolism with flow delivery. We propose that this mechanism may operate in particular in vessel segments that lack the ability to actively regulate vascular tone by smooth muscle cells, which is typically the case in the most distal parts of the microcirculation, the areas where Flt1 and Kdr are co-expressed.

**Methods**

**Ethics statement.** Zebrafish husbandry and experimental procedures were performed in accordance with local and national German animal welfare standards and were approved by the government of Baden-Württemberg, Regierungsspräsidium Karlsruhe, Germany (Akz.: 35-9185.17 G/93-13; Akz.: 35-9185.17 G/93-19), the government of Berlin, Regierungsspräsidium Berlin, Germany (Akz.: Reg0318/13) and the government of Nordrhein-Westfalen, Germany (Akz.: 81-02.05.40.19.044).

**Zebrafish maintenance and strains.** Zebrafish were maintained at standard conditions.58. Embryos were incubated at 28.5 °C and staged by hours or days post fertilization (hpf or dpf, respectively). The following zebrafish lines were used: Tg(flhMrfEGFP1), Tg(Mf1RFPYFP2), Tg(flhRasTomato34), and TgR:flhtdTomato44,55. Zebrafish background is in (mCiRNI:eea1335, Tg(flhRasEGFP1) and Tg(flhMrfEGFP1), Tg(flhRasEGFP1)s330 Tg(flhMrfEGFP1)s331, Tg(flhRasEGFP1)n67, Tg(UAS:VE-cadherinAC-EGFPRs6), Tg(mpeg1-GAL4VP16)24, Tg(UAS:EkAedes)m1999, flh1201, flh14001, vdh2114, cdh5ho1b and nrp1ahu10012 as published.59–68.

**Generation of mutant and transgenic lines.** Zebrafish embryos were injected at a one-cell stage with a glass microneedle and a microinjector (World Precision Instruments). For transgenesis tol2 mRNA, transcribed from pCS2FA with the MAXIscript T7 transcription kit (Thermo Fisher Scientific); sgRNA target sequences were cloned into DR274 (Addgene plasmid # 42250) and transcribed with the MAXIScript T7 transcription kit (Thermo Fisher Scientific). Subsequently, 1 nl of a mixture of 600 ng ml$^{-1}$ Cas9 mRNA and 50 ng ml$^{-1}$ sgRNA were co-injected into one-cell stage embryos.59.60

**Generation of Vegfaa gain of function lines.** In order to generate a constitutive overexpression construct for vegfaa165 in skeletal muscle tissue, a Gateway reaction was performed (Supplementary Fig. 1a). For constitutive overexpression 25 ng ml$^{-1}$ of 500unceGFP-p2a_vegfaa was co-injected with tol2 transposase mRNA. Embryos were identified by means of the transgenesis marker cmil2EGFP as well as green fluorescence in the skeletal muscle tissue. For inducible vegfaa165 gain of function, an inducible, muscle-specific expression construct was generated in a Gateway reaction with p5E_503unc, pME_galER1T2, p3E_polA, and pDestTol2CG2; the other expression construct

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**Fig. 6 Trio activates Rac1 and induces tension in endothelial junctional regions.** a EC transfected with mCherry and Rac1 biosensor (Cer3, cerulean3 channel) and stained for VE-Cadherin. FRET signals detected as warm colors. b EC transfection mCherry-3N and Rac1 biosensor (Cer3) and stained for VE-Cadherin. FRET signals detected as warm colors. Arrowheads indicate FRET signal in junctional regions. c, d Rac1 activation for indicated scenario. Mean ± s.e.m, two-sided Mann-Whitney U test, n = 69, 59, 65, 69 cells per indicated group (c). Ratio junctional to cytoplasmic Rac1 activation (d): mean ± s.e.m. two-sided Mann-Whitney-test, n = 69, 59, 65, 69 cells per group, respectively. ***p < 0.001. e Kymograph illustrating the actin cytoskeleton dynamics along indicated line in control and Trio-transfected cells. f Quantification of protrusion lifetime (left panel). Mean ± s.e.m, unpaired two-sided t-test, n = 50, 110 cells. (right panel) Quantification of protrusion dynamics expressed as protrusion number per hour from data in left panel. ***p < 0.001. g EC transfected with mCherry and GFP-Myosin II, stained for F-actin and VE-Cadherin. Myosin-II localizes at junction actin bundles (arrowheads). h EC transfected with mCherry-TrioN and GFP-Myosin II, stained for F-actin and VE-Cadherin. Myosin-II localizes at junction actin bundles (arrowheads). i EC transfected with GFP and pMLC-519, F-actin, and VE-cadherin. j EC transfected with GFP-TrioN, stained for pMLC-519, F-actin and VE-cadherin. Arrowheads show pMLC-19 at junctional actin bundles. k EC transfected with GFP, stained for pMLC-T18519 and F-actin. l EC transfected with GFP-TrioN, stained for pMLC-T18519 and F-actin. Arrowheads show pMLC-T18519 at junctional regions. m, n distribution of F-actin (m) and VE-Cadherin (n) in Trio overexpressing EC. o Intracellular distribution of central, cortical and junctional F-actin bundlere in ECs. p F-actin recol response upon laser ablation. Shown are pre-ablation (left panel) and post-ablation states (right panel); yellow arrowheads indicate the extent of recol. q-s Recol distance in central (q), cortical (r) and junctional (s) F-actin bundles, in GFP and GFP-TrioN-transfected ECs; mean ± s.e.m, unpaired two-sided students t-test, n = 11, 7, 13, 11, 25 cells per indicated group. ns not significant ***p < 0.001. Scale bar, 25 μm in a, b, m, n, 20 μm in e, g-l, 10 μm in o, 5 μm in p.
was produced using p5E_UAS, pME_GFP-p2A-vegfaa165, p3E_polyA and pDestTol2CG2 (Supplementary Fig. 1b). 12.5 ng µl\(^{-1}\) of each of these two constructs were co-injected with \(\text{tol2}\) transposase mRNA. Vegfaa expression was induced at 28 hpf by addition of 0.5 \(\mu\)M endoxifen in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl\(_2\), 0.33 mM MgSO\(_4\)) and incubation of the embryos in the dark.

**Generation of an early stop \(\text{flt4}\) mutant allele.** For the generation of the \(\text{flt4}^{\text{flmnt}}\) allele via CRISPR-Cas9 genome editing, sgRNA synthesis was performed according to the standard protocol\(^7\). The target sequence 5\’-GGCTGTTATTGATGGC ACCA-3\’ resides in exon 3 and expanded a BsaI restriction site, which was used for screening and efficacy testing after PCR amplification using the primer pair 5\’-GAAGGAGTGTCAAGGGGTGG-3\’ and 5\’-CGGTGTCACATTCCCAAG-3\’.

A mix containing 300 pg of zebrafish codon-optimized Cas9 mRNA and 20 pg of
flt1 sgRNA was injected into the cytoplasm of one-cell stage embryos. An 8 bp deletion was identified by sequencing using previously indicated primers. The deletion introduces a predicted premature stop codon within the flt1 genomic region surrounding the DSB (Supplementary Fig. 2f). Founders were identified by the presence of an additional 60 bp larger PCR product, amplified with flt1_E3_gDNA_fw and flt1_E3_gDNA_rev, and in-frame integration of the double HA tag was verified by Sanger sequencing of the PCR product. The knockin line was viable, showed no vascular defects compatible with HA-tagged flt1 being functional.

Generation of the Tg(fltl1_Δ7-HAHA)katfl1 knockin line. The knockin line Tg(fltl1_Δ7-HAHA katfl1) was generated by injecting 600 ng μl−1 Cas9 mRNA and 50 ng μl−1 sgRNA(Δ7) to induce a double strand break (DSB), together with 12.5 pg μl−1 oligonucleotide sFlt1_HAHA_ODN_1 (oligonucleotide sequences are listed in Supplementary Table 2) for homologous recombination. The oligonucleotide encodes two HA tags that are flanked by two 33 nt homology arms for the flt1 genomic region surrounding the DSB (Supplementary Fig. 2f). Founders were identified by the presence of an additional 60 bp larger PCR product, amplified with flt1_E3_gDNA_fw and flt1_E3_gDNA_rev, and in-frame integration of the double HA tag was verified by Sanger sequencing of the PCR product. The knockin line was viable, showed no vascular defects compatible with HA-tagged flt1 being functional.

Generation of the Tg(fltl1enht-fltl1_Δ7-HAHA)katfl1 overexpression construct. For overexpression of fltl1 tagged with two HA tags, sfltl1 was amplified with Phusion High-Fidelity DNA polymerase (NEB) from the primers Fltl1_Xhol_fw and sFltl1_HAHA_Xba and adapted into the MiniTo2 vector containing fltl1 promoter/enhancer (ref. 72) using the restriction enzymes Xhol and XbaI. The resulting sfltl1 overexpression construct sfltl1_enht-sfltl1_Δ7-HAHA (Supplementary Fig. 2g, upper panel) was injected at a concentration of 12.5 ng μl−1 together with 25 ng μl−1 tol2 mRNA. For generation of the stable transgenic line Tg(fltl1enht-GFP-TRION) katfl1 (referred to as fltl1enht:TrioN), injected fish were raised and founders were identified by means of the transgenesis marker cmlc2:EGFP. Overexpression of TriN under the kdrl and fltl1 promoter in zebrafish embryos was performed likewise via Gateway recombination reactions using the respective pSE_kdrl and pSE_fltl1 plasmids. The mutant GFP-TrioNmut construct (TrioNmut) was generated by site-directed mutagenesis of nucleotides 4399–4405 (NM007118) AAT-GAC to GCTGCC. This results in the amino acid mutations N1406A and D1407A, located in the C-terminus of the DH1 domain. The mutated GEF domain is still able to bind Rac1, but unable to induce guanine nucleotide exchange. Over-expression of TrioNmut under the fltl1 promoter was performed similarly to TriN. For TriN overexpression in vitro, primary HUVECs, which were acquired from Lonza (Verviers, Belgium) and regularly checked for mycoplasma contamination, were seeded on culture flasks. HUVECs were grown in EGM-2 medium (Promocell, Heidelberg, Germany). Cells were cultured on fibronectin-coated glass coverslips and transfected with human GFP-TrioN, mCherry-TrioN, mCherry-TrioNmut, mCherry-TrioGef or the isoform TrioB. The mCherry-TrioNmut/TrioNmut/TrioGef1 constructs were generated by replacing GFP in GFP-TrioNmut/TrioGef1 with mCherry using AgeI and Kpn2I restriction enzymes. After treatment, cells were washed with warm PBS, containing 1 mM CaCl2 and 0.5 mM MgCl2, and fixed in 4% (v/v) formaldehyde for 10 min. After fixation, cells were permeabilized in PBS supplemented with 0.1% (v/v) Triton X-100 for 10 min followed by a blocking step in PBS supplemented with 2% (w/v) BSA and stained as indicated.

Rac1 gain of function. In vitro Rac1 gain of function experiments were performed similarly to Trio gain of function approaches. pTriEx-mCherry-PA-Rac1 and pTriEx-mCherry-PG-A-rac1 were a kind gift from Klaus Hahn (Addgene plasmid # 22037: https://n2t.net/addgene/22037, RRID:Addgene_22037 and Addgene plasmid # 22028: https://n2t.net/addgene/22028, RRID:Addgene_22028). Cloning of mCherry-TrioC1-Q61L and Rac1B was performed as previously described.

RhoG gain of function. In vitro RhoG gain of function experiments were performed similarly to Trio & Rac1 gain of function approaches. GFP-RhoG Q61L was a kind gift from Dr. Keith Burridge (UNC Chapel Hill, USA).
**Fig. 8** Trio augments endothelial cell size and arterial diameter in vivo. a, b Imaging of aISV in WT (a) and in \textit{flt}^{\text{GOF}}:TrioN embryos (b). c, d EC nuclei distribution in WT (c) and \textit{flt}^{\text{GOF}}:TrioN embryos (d). Note: six ECs in both scenarios and larger aISV diameter upon TrioN. e Quantification of aISV diameter for indicated scenario. Mean ± s.e.m, two-sided Mann-Whitney U test, \( n = 35 \) aISVs per genotype. **\( p < 0.001 \). f Quantification of EC surface area in aISVs for indicated scenario. Mean ± s.e.m, two-sided Mann-Whitney U test, \( n = 34, 48 \) aISVs per indicated genotype. ***\( p < 0.001 \). g Quantification of EC nuclei number in aISVs for indicated scenario. mean ± s.e.m, two-sided Mann-Whitney U test, \( n = 51, 70 \) aISVs per genotype. ns not significant. h-k aISV in WT (h), in \textit{plgf}^{\text{GOF}} (i), \textit{plgf}^{\text{GOF}} combined with \textit{flt}^{\text{GOF}}:TrioN (j), and \textit{plgf}^{\text{GOF}} combined with \textit{flt}^{\text{LMO}}:TrioNmut (k). l-n Transverse optical section of aISV vessel lumen in WT (l), \textit{plgf}^{\text{GOF}} (m), and \textit{plgf}^{\text{GOF}} + \textit{flt}^{\text{GOF}}:TrioN scenario (n). The lumen area is color indicated (lower panels). o Left panel: Quantification of aISV diameter in WT, \textit{plgf}^{\text{GOF}}, \textit{plgf}^{\text{GOF}} + \textit{flt}^{\text{GOF}}:TrioN, and \textit{plgf}^{\text{GOF}} + \textit{flt}^{\text{GOF}}:TrioNmut scenario. Mean ± s.e.m, unpaired two-sided students t-test, \( n = 18, 19, 13, 14 \) aISVs per group. ns not significant; ***\( p < 0.001 \). Right panel: Quantification of EC size in WT, \textit{plgf}^{\text{GOF}}, \textit{plgf}^{\text{GOF}} + \textit{flt}^{\text{GOF}}:TrioN, and \textit{plgf}^{\text{GOF}} + \textit{flt}^{\text{GOF}}:TrioNmut scenario. Mean ± s.e.m, unpaired two-sided students t-test, \( n = 21, 24, 15, 12 \) cells per group. ns not significant; ***\( p < 0.001 \). p-s aISV in WT embryo (p), \textit{flt}^{\text{GOF}}:TrioN embryo (q), WT embryo injected with ve-cadherin (cdh5) targeting morpholino (r), \textit{flt}^{\text{GOF}}:TrioN embryo injected with ve-cadherin (cdh5) targeting morpholino (s). t Quantification of images in p-s, mean ± s.e.m, unpaired two-sided students t-test, \( n = 15, 21, 16, 13 \) cells per indicated genotype. ***\( p < 0.001 \). u Dynamic changes in EC surface area in \textit{plgf}^{\text{GOF}} + \textit{flt}^{\text{GOF}}:TrioN embryo, \textit{plgf}^{\text{GOF}}:TrioN embryo, \textit{plgf}^{\text{GOF}} + \textit{flt}^{\text{GOF}}:TrioN embryo, and WT embryo injected with \textit{flt} targeting morpholino, and WT embryo. \( n = 12, 12, 10, 18 \) cells per indicated genotype, mean ± s.e.m. v, w Aorta diameter (v) and EC surface area (w) in \textit{kdrl}^{\text{GOF}}:TrioN or \textit{flh2a}:TrioN scenario. Mean ± s.e.m, unpaired two-sided students t-test, \( n = 8, 10, 10 \) (v) and \( n = 8, 16, 14 \) (w) biologically independent embryos/group. *\( p < 0.05 \), **\( p = 0.0061 \). Scale bars: a-d, h-k, p-s, 25 μm, l-n 10 μm.
Fig. 9 Trio and endoglin interact to determine 3dpf aortic EC size. a Dorsal aorta diameter at 3dpf in endoglin morphants (magenta bar), endoglin morphants injected with 0.8 ng Trio targeting morpholino (blue bar), endoglin morphants treated with Trio inhibitor ITX3 (dark green bar) and WT treated with ITX3 (light green bar). Note that loss of Trio or inhibiting Trio reduced reduced diameter growth in endoglin morphants. Mean ± s.e.m, unpaired two-sided students t-test, n = 8, 9, 8, 10 independent embryos per group. **p = 0.0011, ***p = 0.001. b Aorta EC surface area at 3dpf in endoglin morphants (magenta bar), endoglin morphants injected with 0.8 ng Trio targeting morpholino (blue bar), endoglin morphants treated with Trio inhibitor ITX3 (dark green bar) and WT treated with ITX3 (light green bar). Note that loss of Trio or inhibitor reduced EC surface area in endoglin morphants. Mean ± s.e.m, unpaired two-sided students t-test, n = 18, 20, 22, 16 cells derived from six independent embryos per group; **p < 0.001; **p = 0.0055; *p = 0.0100.

Aorta diameter at 3 dpf [μm] 125

Promoting endothelial cell enlargement results in larger arterial diameter. Simultaneously promoting endothelial cell number and size, and creating arteries with multiple enlarged ECs results in even more pronounced arterial diameter increase.

**Tiam gain of function.** In vitro Tiam1 gain of function experiments were performed similarly to Rhog gain of function approaches. HA-TIAM C1199 was a kind gift from Dr. Ana Pasapera (NHLBI Bethesda, USA).

Further plasmids. Myosin IIA-GFP was a kind gift from Dr. Ana Pasapera (NHLBI Bethesda, USA).

**Morpholino knockdown.** The following morpholino antisense oligomers (MOs; Gene Tools) were injected into the yolk of one-cell stage embryos: 1 ng flt1 ATG MO (5′-ATATCGAACATTCTCTTGGTCTTGC-3′); 4 ng flt4 ATG MO (5′-CTCTCATTTCCAGTTTCAAGTCC-3′); 0.3 ng vegfaa ATG MO (5′-GTATCAAATGCCAACACCAGTGATCA-3′); 2.7 ng tnnt2 ATG MO (5′-AATGTTTCGGTACTGTTTC-3′); 1.7 ng irf8 splice-blocking MO (5′-AATGTTTCGGTACTGTTTC-3′); a combination of 8.5 ng plgf musc-1 ATG MO (5′-GTATCAAATGCCAACACCAGTGATCA-3′) and 8.5 ng plgf musc-2 ATG MO (5′-GTATCAAATGCCAACACCAGTGATCA-3′); 8.5 ng gcsfr ATG MO (5′-GATCTGACACGCA-3′); 5 ng endoglin (eng) ATG MO-1 (5′-AACACACAGCATGCTCTCCTCATGTC-3′); 5 ng eng ATG MO-2 (5′-GATGAACTCAACACTCGTGTCTGAT-3′); 6 ng, 3 ng vhl ATG MO (5′-GCTATAATTTTCAGAAACCACAAAG-3′); 10 ng standard control MO (5′-CCTCTTTACCTACGTTACAATTTATATA-3′), all as described[56,74,75].

**RNA interference.** Inhibitory shRNA constructs (Supplementary Table 3) as well as a non-targeting shCtrl (shC002) were packaged into lentivirus in HEK293T cells by means of third generation lentiviral packaging plasmids. Lentivirus-containing supernatant was harvested on day 2 and 3 after transfection. Lentivirus was concentrated via Lenti-X concentrator (Clontech). Target cells were infected and cells were used for assays 3 days after virus infection.

**Chemical and inhibitor treatments.** Embryos were dechorionated prior to chemical and inhibitor treatments using 1 mg ml−1 Pronase (Roche, Basel, Switzerland). The chemicals/inhibitors were added at 32 hpf, followed by incubation at...
28.5°C in the dark until the embryos were analysed at 50 hpf. For chemicals/ inhibitors dissolved in DMSO, control embryos were mock treated with DMSO (Sigma). Inhibitor and chemical concentrations are listed in Supplementary Table 5 and 6. Embryos were randomly assigned to experimental groups. Investigators were blinded to inhibitor treatment. In vitro chemical treatments were performed at indicated time points. Inhibitor concentrations are further specified in Supplementary Table 5.

Immunohistochemistry of zebrafish embryos. Fixed embryos were permeabilized for 1 h in PBT (1% (v/v) Triton X-100 in 1x PBS). In order to reduce unspecific background, samples were subsequently incubated for 2 h in blocking buffer (1% (v/v) Triton X-100, 3% (w/v) BSA in 1x PBS). The primary antibody (Supplementary Table 7) was diluted in 0.5% (v/v) Triton X-100, 1% (w/v) BSA in 1x PBS, and samples were incubated overnight at 4°C. Embryos were washed five times for 20 min with PBT before the secondary antibody (Supplementary Table 7) was added for 2 h at room temperature. Before imaging, samples were washed for five times for 20 min in PBT.

Cell lines and immunofluorescent stainings in vitro. Antibodies used for immunofluorescent staining of human endothelial cells in culture are indicated in Supplementary Table 7. The following cell lines were used: Primary Human Umbilical Vein Endothelial Cells (HUVECs, C2519A, Lonza); Primary Human Umbilical Artery Endothelial Cells (HUAEs, isolated as described previously79) and Primary Human Aortic Endothelial Cells (HAEcs, CC-2535, Lonza). Cells were routinely checked for mycoplasma contamination and authenticated by immunoblotting for expression of standard endothelial cell markers (VE-Cadherin, CD31, ICAM-1, VCAM-1, etc.). Endothelial cells were cultured in EGM-2 medium (Promocell, Heidelberg, Germany) up to passage 5.

Whole mount in situ hybridization. Whole mount in situ hybridization was performed with DIG labelled RNA probes. For generating the antisense ISH probe of I−plasmin, the plasmid, kindly provided by Philippe Herbomel, was linearized with Spl and transcribed with T7 RNA polymerase (Promega) and DIG labelling mix (Roche). sFltl and sFlt1 antisense ISH probes were both linearized with Spl and transcribed with T7 RNA polymerase and DIG labelling mix. Embryos for whole mount in situ hybridization were fixed in 4% (w/v) paraformaldehyde in 1x PBS for 2 h at room temperature or overnight at 4°C. After dehydration in 100% methanol overnight at −20°C, the embryos were rehydrated in a decreasing methanol series, washed twice in PBT (1x PBS, 0.1% (v/v) Tween 20) and permeabilized using proteinase K at a concentration of 10 µg ml−1 in PBT. In order to minimise unspecific background, embryos were prehybridized in hybridization mix (50% (v/v) formamide; 5x SSC; 0.1% (v/v) Tween 20; 50 µg ml−1 heparin; 500 µg ml−1 RNase-free tRNA) for 1 h at 65°C. Embryos were then incubated with the RNA probe in hybridization mix overnight at 65°C. Next, the removal of the formamide was obtained by a decreasing series of hybridization mix in 2x SSC at 65°C and a final washing step in 0.2x SSC at room temperature. Non-hybridized RNA was removed by DNase A (Roth) in RNase free buffer (HEPE 0.1 M pH 7.5; NaCl 0.15 M; 0.1% (v/v) Tween 20) for 45 min at room temperature, followed by a washing step in 1x MAB-T (maleic acid buffer; 0.1% (v/v) Tween 20). Subsequently, non-specific antibody binding sites were blocked by incubation in 2% (w/v) Bovine Serum Albumin (Roche) in 1x MAB-T, followed by incubation with alkaline phosphatase-conjugated antibody (Anti-DIG, Fab fragments, 1:2,000, Roche) overnight at 4°C. Afterwards, embryos were washed once with 1x MAB-T and two times for 15 min with NTMT (Tris-HCl 0.1 M pH 9.5; NaCl 0.1 M; 1% (v/v) Tween 20; MgCl2, 50 mM). The in situ hybridization was developed with BM Purple (Roche), a chromogenic substrate for alkaline phosphatase. When the staining reaction was completed, embryos were washed with PBT and transferred to 80% glycerol in 1x PBS for microscopy and long-term storage.

Rac1 activity pull-down assay. Classical biochemical pull-down assays were performed as described87. Briefly, a confluent monolayer of HUVECs was washed with ice-cold PBS++ and subsequently lysed in lysis buffer (50 mM Tris, pH 7.4, 20 mM MgCl2, 500 mM NaCl, 1% (v/v) Triton X-100) supplemented with protease inhibitors. Lysates were cleared at 14,000 × g for 5 min. GST-bound Rac1 was isolated with biotinylated PAK1-Crib peptide coupled to streptavidin agarose. Beads were washed five times in wash buffer (50 mM Tris, pH 7.4, 10 mM MgCl2, 150 mM NaCl, 1% (v/v) Triton X-100) supplemented with protease inhibitors. Pull downs and total cell lysates were immunoblotted with monoclonal Rac1, Trio and beta-actin antibodies (Supplementary Table 7).

Western blotting. SDS-PAGE samples were analysed on 7.5, 10, or 15% (w/v) polyacrylamide gels, depending on the size of the proteins of interest, and transferred onto nitrocellulose membranes (Whatman, Pitchaway, NJ). Subsequently of blocking in 5% (w/v) low-fat milk in Tris-buffered saline Tween 20 (TBST), the blots were incubated with respective primary antibodies (Supplementary Table 7) for 1 h at room temperature. The blots were washed with TBST and incubated with respective HRP-coupled secondary antibodies (Supplementary Table 7) for 1 h at room temperature. Blots were developed via enhanced chemiluminescence (ECL) (Thermo-Scientific, Etten-Leur, The Netherlands). For Trio protein expression, 3–8% (w/v) Tris-acetate precast gels (ThermoFisher) were used according to the manufacturer’s instructions and rinsed onto nitrocellulose by blotting for 18 h at 20 mA. Full Western blots in Supplementary Fig. 13.

Laser ablation. For laser ablation experiments HUVECs were transduced with LifeAct-mScarlet expressing lentiviral particles. The 442 nm laser was used to locally cut specific F-actin bundles. F-actin bundles were ablated for 10 seconds using full laser power and retracted beam expander to concentrate the laser beam intensity. Initial recut of the F-actin bundles was measured immediately after ablation.

Confocal microscopy. Zebrafish larvae were embedded in 0.7% (w/v) low-melting agarose (NuSieve GTG Agarose, Lonza) in 35 mm glass bottom microscopy dishes (MatTek). The agarose was covered with E3 medium supplemented with 0.112 mg ml−1 Tricine and 0.003% (w/v) PTU (Sigma). Confocal t- and z-stack image acquisition was performed on a confocal laser scanning microscope (LaserSharp NLO; Leica Microsystems) equipped with a 400 µm oil immersion objective and 0.14 oil immersion objective and LAS X software. Maximum intensity projections were also generated and analyzed using ImageJ/Fiji software.

Transmitted light microscopy. Zebrafish larvae were anaesthetized at 0.112 mg ml−1 Tricine and transferred to E3 medium in a glass bottom microscopy dish. Red blood cell perfusion was visualized by imaging a single sagittal plane in the middle of two ISVs using a Zeiss Axioskop 5 microscope with ×40 objectives and ×4 optical gain for 40 ms exposure time.

Microangiography. Embryos were anaesthetized with 0.112 mg ml−1 Tricine in E3 medium and mounted in 0.7% (w/v) low-melting agarose (NuSieve GTG Agarose, Lonza) in glass bottom microscopy dishes (MatTek). For the microangiography Texas Red-dextran with a molecular weight of 70 kDa (Thermo Fisher Scientific, Supplementary Table 6) was solubilized in E3 medium to a concentration of 2 mg ml−1. Using a glass microneedle and a microinjecor (World Precision Instruments), the dextran was injected into the sinus venosus. Subsequently, images were taken with an SP8 confocal microscope (Leica).

Measurements of aSV diameter, DA diameter, endothelial cell sizes, and numbers. For vessel parameter measurements, confocal z-stacks of ISVs plus dorsal aorta were acquired of up to five aSVs per animal in the middle of the yolk sac angiography region and parameters were measured with Fiji using maximum projection images of the z-stacks. For aSV diameter analysis, lateral lines were drawn at seven evenly distributed positions along the aSV perpendicularly to the vessel wall between the dorsal aorta and the DLAV, approximately every 18 µm. The average of these seven lines was calculated as the average aSV diameter. The remodeling index (RI) was calculated as follows: RI = (aSV diameter of the respective treatment group in µm)/(average aSV diameter of the WT control group).

Rac1 FRET biosensor analysis. The DORA Rac1 FRET-based biosensor was a kind gift from Y. Wu (University of Connecticut Health Center, Farmington, CT). Development and characterization of the DORA single-chain Rac1 biosensor are described in more detail elsewhere45. Briefly, dimeric cerulean3 coupled to the Rac1 effector p21-activated protein kinase (PAK) is linked via ribosomal protein-linker (LH2) to circular-permutated Venus coupled to Rac1. HUVECs were transfected with the Rac1 biosensor via electroporation using the Neon transfection system (Life Technologies, one pulse, 1300 V, 30 ms) and used 24 h post transfection. The Zeiss Observer Z1 microscope equipped with a ×40/n numerical aperture 1.3 oil immersion objective, an HXP 120-V excitation light source, a Chroma 510
Resistance measurements. Monolayer integrity was determined by measuring the electrical resistance using ECIS as published\(^\text{37}\). Electrode-arrays (8x10E; IBIDI, Planegg, Germany) were treated with 10 mM l-cysteine (Sigma) for 10 min and subsequently coated with 10 μg/mL fibronectin (Sigma) in 0.9% NaCl for 1 h at 37°C. Cells were seeded at 0.009 cells per well (100 μM) and grown to confluence. Electrical resistance of the continuous monolayer was measured at 4000 Hz at 37°C under 5% CO\(_2\) using ECIS model 9600 (Applied BioPhysics, New York, MA).

Focal adhesion quantification. Phalloidin-Pallxin-positive (pY118, Supplementary Table 7) focal adhesions were quantified on thresholded images by analysing particles sized between 0.5–10 μm².

Statistical analysis and reproducibility. Statistical analysis was performed using GraphPad Prism 6. Each dataset was tested for normal distribution with the D’Agostino-Pearson test. Only if the data were normally distributed, a parametric method (unpaired two-sided Student’s t-test) was applied. A nonparametric test (two-sided Mann–Whitney U test) was applied if data were non-normally distributed. In case of multiple comparisons, one-way ANOVA plus Bonferroni correction or two-way ANOVA were applied. P values <0.05 were considered significant. Data are represented as mean ± s.e.m, unless otherwise indicated. *p < 0.05, **p < 0.01 and ***p < 0.001. *p < 0.05 to indicated ctrl group. No data were excluded from statistical analysis. No statistical method was used to predetermine sample size. For every treatment, treated and control embryos were derived from the same egg lay; embryo were selected on the following pre-established criteria: normal morphol,ogy, a beating heart and circulating red blood cells. All images shown in the figures are representative examples of the respective phenotypes and expression patterns. The four embryos shown in each figure have been used independently with similar results. Figure 1a, b, representative micrographs from n = 48, and n = 32 independent embryos by indicated condition. Figure 1c, d, images are representative for n = 24 embryos by condition examined over three independent experiments. Figure 1h–k, n = 112, 86, 126, 93 aISVs per genotype derived from four independent experiments. Figure 1l, mean ± s.e.m, unpaired two-sided students t-test, t = 18, 18, 16, 10 aISVs per genotype. ***p < 0.001. Figure 1m, n = 56 aISVs examined over three independent experiments. Figure 1n, mean ± s.e.m, unpaired two-sided students t-test, n = 20, 25 aISVs for indicated scenario. ***p < 0.001. Figure 1o, p, plasma extravasation in WT (unpaired two-sided students t-test, n = 40, 19, 13, respectively).

DCSP dichroic splitter, and two Hamamatsu ORCA-R2 digital charge-coupled device cameras was used for simultaneous monitoring of Cer3 and Venus emission. Zeiss 20× objective was used. ImageJ software was used for analysis. ImageJ analyses between Cer3 and Venus images were processed using the MFB Image collection. Image stacks were background corrected, subsequently aligned, and a smooth filter was applied to both image stacks to improve image quality by noise reduction. An image threshold was applied exclusively to the Venus image stack, converting background pixels to "not a number (NaN)" eliminating artifacts in ratio image derived from the background noise. Finally, the Venus/Cer3 ratio was calculated with high activation shown in red/white and low activity in blue/black. For FRET quantification, data were analysed using a MatLab script (MATLAB, The MathWorks, Inc., Natick, Massachusetts, United States). Prior to the analysis, the ratiometric FRET analyses, donor GFP and acceptor YFP channels were background corrected by subtracting the modal pixel value, and both channels were aligned to each other. Prior to watershed segmentation, cells were manually selected by adding seed points and touching cells were separated by manually drawing boundaries between them. A local threshold was applied to the watershed region, excluding pixels that were higher than 15% of the maximum intensity in that region. For all channels the mean fluorescence intensity was calculated for each segmented region. Ratiometric FRET analysis was applied to each segmented region in the cell. The endothelial junction marker VE-cadherin was used as positive control. Quantification of FRET signal overlapping with VE-cadherin was done between the cells edge and 10 pixels inward. These areas were defined as junction regions.

Table 7) focal adhesions were quantified using ECIS model 9600 (Applied BioPhysics, New York, MA). Monolayer integrity was determined by measuring the electrical resistance using ECIS as published\(^\text{37}\). Electrode-arrays (8x10E; IBIDI, Planegg, Germany) were treated with 10 mM l-cysteine (Sigma) for 10 min and subsequently coated with 10 μg/mL fibronectin (Sigma) in 0.9% NaCl for 1 h at 37°C. Cells were seeded at 0.009 cells per well (100 μM) and grown to confluence. Electrical resistance of the continuous monolayer was measured at 4000 Hz at 37°C under 5% CO\(_2\) using ECIS model 9600 (Applied BioPhysics, New York, MA).
are representative for n = 130 cells examined over three independent experiments. Figure 8a, b, images are representative for n = 80 aISVs per genotype examined over four separate experiments. Figure 8c, d, images are representative for n = 76,755 aISVs per genotype examined over three independent experiments. Figure 8e, g, mean ± s.e.m, two-sided Mann–Whitney U test, n = 35 aISVs per genotype, ***p < 0.001. Figure 8f, mean ± s.e.m, two-sided Mann–Whitney U test, n = 54, 48 aISVs per indicated genotype, ***p < 0.001. Figure 8g, mean ± s.e.m, two-sided Mann–Whitney U test, n = 51, 70 aISVs/genotype, ns, not significant. Figure 8h–k, images are representative for n = 96, 83, 72 examined aISVs per genotype derived from three independent experiments. Figure 8n–u, images are representative for n = 116, 103, 112 examined aISVs per genotype. Figure 8o, (left panel) mean ± s.e.m, unpaired two-sided students t-test, n = 18, 15, 14 aISVs per indicated genotype, ns, not significant; ***p < 0.001; (right panel) mean ± s.e.m, unpaired two-sided students t-test, n = 21, 24, 15, 12 cells per genotype, ns, not significant; ***p < 0.001. Figure 8p–s, images are representative for n = 84, 78, 89, 92 aISVs per condition. Figure 8t, mean ± s.e.m, unpaired two-sided students t-test, n = 15, 21, 16, and 13 cells per indicated genotype, ***p < 0.001. Figure 8u, n = 12, 10, 12, 10 examined aISVs per genotype, mean ± s.e.m. Figure 8v, w, mean ± s.e.m, unpaired two-sided students t-test, n = 8, 10, 10 (v) and n = 8, 16, 14 (w) embryos per group. **p < 0.01; **p < 0.001. Figure 9a, mean ± s.e.m, unpaired two-sided students t-test, n = 8, 9, 8, 10 embryos per group. **p < 0.01; ***p < 0.001. Figure 9b, mean ± s.e.m, unpaired two-sided students t-test, n = 18, 20, 22, 16 cells derived from six biologically independent embryos per group; ***p < 0.001; **p = 0.0055; *p = 0.0100.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary files or from the corresponding author upon reasonable request.

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Author contributions

A.K., J.v.R., and A.S.R. designed and performed experiments, interpreted experimental data, and participated in manuscript preparation. J.H., M.M., L.D., and L.P. performed experiments and analyzed data. R.W. designed and analyzed Flt1-HA knockin and transgenesis experiments, and interpreted experimental data. R.H., C.K., R.K., and A.A. participated in the conceptual development, manuscript preparation, and editing. R.V. and S.S. performed computational analyses, S.S-M. and I.S-M. generated the bfl mutant and performed experiments. J.D.v.R.B. supervised the in vitro experiments, provided conceptual input, and contributed to editing of the manuscript. F.L.N. conceived and designed the project, performed experiments, analyzed data, supervised the overall project, and wrote the manuscript, with input from all authors.

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Competing interests

The authors declare no competing interests.

Additional information

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