In vivo modulation of endothelial polarization by Apelin receptor signalling

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Endothelial cells (ECs) respond to shear stress by aligning in the direction of flow. However, how ECs respond to flow in complex in vivo environments is less clear. Here we describe an endothelial-specific transgenic zebrafish line, whereby the Golgi apparatus is labelled to allow for in vivo analysis of endothelial polarization. We find that most ECs polarize within 4.5 h after the onset of vigorous blood flow and, by manipulating cardiac function, observe that flow-induced EC polarization is a dynamic and reversible process. Based on its role in EC migration, we analyse the role of Apelin signalling in EC polarization and find that it is critical for this process. Knocking down Apelin receptor function in human primary ECs also affects their polarization. Our study provides new tools to analyse the mechanisms of EC polarization in vivo and reveals an important role in this process for a signalling pathway implicated in cardiovascular disease.
vascular endothelial cells (ECs) form a continuous cell layer that lines blood vessels. ECs are constantly subjected to a variety of shear stresses from the flow of blood and display profound morphological adaptations to their environmental conditions. These morphological adaptations include planar cell polarization, cell elongation and alignment of microtubules in the direction of blood flow. Recent studies have shown that defects in ECs’ adaption to blood flow are highly correlated with cardiovascular diseases including atherosclerosis. The alignment of ECs in the direction of flow requires the sensing of mechanical shear stress and the conversion of these stimuli to biochemical signals that mediate cytoskeletal remodelling. A number of mechanosensitive molecules have been identified including mechanically gated channels, mechanosensitive receptors, G-protein-coupled receptors (GPCRs), mechanosensitive enzymes and cilia. The observation that certain G-proteins are activated within seconds following mechanical perturbation by shear stress suggests that GPCRs are involved in mediating mechanochemical signal transduction in ECs.

Here we generated a new zebrafish transgenic line in which the Golgi apparatus is labelled with a fluorescent protein. Using this line in combination with an endothelial nuclear line allows one to follow EC polarization in real time. We analyzed EC polarization during development and investigated the role of blood flow in this process. We further found that the Apelin receptor (Aplnr), a GPCR involved in EC migration, also regulates EC polarization.

**Result**

**EC polarization during development.** To investigate the dynamics of endothelial polarization by blood flow in vivo, we generated a new zebrafish transgenic line that labels the Golgi apparatus specifically in ECs. We fused the complementary DNA of human β-1, 4-galactosyltransferase1 (B4GALT1), which is mainly localized to the trans Golgi network, to mCherry and expressed it under the control of the flila endothelial promoter.

To validate this transgenic line, we performed confocal imaging during vascular development of Tg(kdrl:NLS-EGFP);Tg(flila:B4GALT1-mCherry) embryos, which express nuclear enhanced green fluorescent protein (EGFP) and Golgi-specific mCherry specifically in ECs. We found that during EC migration the Golgi was localized at the leading edge of cells (Supplementary Fig. 1a,b and Supplementary Movie 1). However, when ECs stopped migrating, the Golgi quickly moved to a middle position relative to the nucleus (Supplementary Fig. 1b,d). In addition, the Golgi localized to the luminal side of ECs during lumen formation (Supplementary Fig. 1c,c’). These data indicate that this reporter line allows real-time monitoring of EC polarity in vivo and can be used to track changes in EC polarization.

We next examined Tg(kdrl:NLS-EGFP);Tg(flila:B4GALT1-mCherry) embryos to determine whether and when ECs become polarized in response to blood flow during development. In accordance with a previous report, we classify ECs as polarized when the Golgi apparatus lies upstream of the nucleus with respect to the direction of flow (Fig. 1(iii)). At 30 hours post fertilization (h.p.f.), when blood circulation has just started (Fig. 1a-b’-c’-c’-j(j)), ECs in the dorsal aorta (DA) and the posterior cardinal vein (PCV) remained unpolarized. However, the ECs in the DA (Fig. 1d-e-g-h-j(iii)) and the intersegmental vessels (ISVs) (Fig. 1f-i,i’i’) became polarized between 48 and 72 h.p.f., a time when vigorous blood flow was established. Consistent with observations from confocal images, the percentage of polarized ECs in the DA (6.3% at 30 h.p.f., 72.9% at 48 h.p.f. and 86.5% at 72 h.p.f.) and ISVs (arterial ISVs: 77.2% at 48 h.p.f. and 80.3% at 72 h.p.f.; venous ISVs (vISVs): 43.9% at 48 h.p.f. and 76.1% at 72 h.p.f.) significantly increased as blood circulation became more vigorous (Fig. 1k,l). In contrast, most ECs in the PCV (98.2% at 30 h.p.f., 96.4% at 48 h.p.f. and 91.4% at 72 h.p.f.) failed to polarize during this time (Fig. 1l).

**EC polarization by blood flow.** To better understand the dynamics of EC polarization by flow, we performed time-lapse confocal imaging of ECs starting at 30 h.p.f., that is, shortly after the onset of blood flow (Fig. 2a-d). Interestingly, the Golgi apparatus in DA ECs gradually moved from a downstream to an upstream position on initiation of vigorous blood flow (Fig. 2d, Supplementary Fig. 3a-c(ii) and Supplementary Movie 2) and it took ~4.5 h for them to become polarized after the onset of blood flow (Fig. 2e). As blood flow gradually increases in speed after the heart starts contracting, we decided to look at dividing ECs at a time when vigorous blood flow was established, to exclude this variable. EC division plane is normally oriented perpendicular to the long axis of the vessel. By measuring the time required for ECs to become polarized after cell division, we observed that most resulting daughter cells became polarized in the direction of blood flow within 105 min following division (Supplementary Fig. 3d,e,f(ii) and Supplementary Movie 3). For comparison, we also analysed the correlation of EC division and polarization in the PCV by time-lapse confocal imaging. ECs within the PCV did not polarize under established blood flow, even though they divided as the DA ECs do (Supplementary Fig. 3g,h). Arterial and venous ECs have been reported to exhibit different polarization patterns in vivo. To investigate whether arterial or venous identity plays a role in the polarization response to blood flow, we examined ECs in the arterial ISVs and vISVs, respectively. Interestingly, ECs in both vessel types polarized (Fig. 2f-j) within 30 min after the onset of blood flow, suggesting that flow-induced EC polarization may not simply be dependent on arterial or venous identity.

**EC polarization by blood flow is reversible.** To further investigate the role of blood flow in EC polarization, we eliminated cardiac function by injecting morpholinos (MOs) for tntt2a, a gene encoding the thin-filament contractile protein cardiac troponin T, into one-cell stage Tg(kdrl:NLS-EGFP);Tg(flila:B4GALT1-mCherry) embryos. Most ECs in the DA of 48 h.p.f. tntt2a morphants failed to polarize (89.1% non-polarized), as they displayed a random Golgi orientation, in contrast to ECs within the DA of uninjected embryos (76.6% polarized) (Fig. 3a-g). Likewise, when we stopped the heartbeat of 96 h.p.f. larvae with a 10 mM 2, 3-butanedione monoxime (BDM) treatment, we observed the gradual movement of the Golgi apparatus in DA ECs (Fig. 3a–g). Interestingly, venous ECs were usually less polarized than arterial ECs. These data reveal that there is a clear heterogeneity between vascular beds in terms of EC polarization and especially between arteries and veins.

**Discussion**

The Golgi apparatus is a key organelle in cellular physiology, playing a central role in intracellular transport and biogenesis. In ECs, the Golgi apparatus is involved in the transduction of biochemical signals that mediate cytoskeletal remodelling. The alignment of ECs in the direction of flow requires the sensing of mechanical shear stress and the conversion of these stimuli to biochemical signals that mediate cytoskeletal remodelling. A number of mechanosensitive molecules have been identified, including mechanically gated channels, mechanosensitive receptors, G-protein-coupled receptors (GPCRs), mechanosensitive enzymes and cilia. The observation that certain G-proteins are activated within seconds following mechanical perturbation by shear stress suggests that GPCRs are involved in mediating mechanochemical signal transduction in ECs.

To investigate the dynamics of endothelial polarization by blood flow, we generated a new zebrafish transgenic line in which the Golgi apparatus is labelled with a fluorescent protein. Using this line in combination with an endothelial nuclear line allows one to follow EC polarization in real time. We analyzed EC polarization during development and investigated the role of blood flow in this process. We further found that the Apelin receptor (Aplnr), a GPCR involved in EC migration, also regulates EC polarization.

**Conclusion**

In conclusion, our study provides new insights into the dynamics of EC polarization by blood flow. We observed that ECs in the arterial ISVs become polarized within 30 min after the onset of blood flow, whereas ECs in the venous ISVs do not. Our findings suggest that arterial and venous ECs may exhibit different responses to blood flow, and that the mechanosensory molecules involved in EC polarization may be different in arterial and venous ECs. These findings have important implications for understanding the pathogenesis of cardiovascular diseases and for developing novel therapeutic strategies.
Figure 1 | EC polarization during development. (a–i') Three-dimensional-rendered confocal stack images of the trunk region of 30, 48 and 72 h.p.f. Tg(kdrl:NLS-EGFP);Tg(fli1a:B4GALT1-mCherry) animals injected intravascularly with qdot 655. The white and red boxes in the left panels (a,d,g) are enlarged in the middle (b,e,h) and right (c,f,i) panels, respectively. (b',e',f',h',i') Surface-rendered images of boxed regions. White dashed arrows indicate the direction of blood flow. White arrowheads point to polarized ECs and yellow arrowheads point to non-polarized ECs. (j) Schematic representation of EC polarization during development in the trunk region (i and ii) and illustration of the various polarization patterns (iii). (k,l) Quantification of EC polarization in the DA, PCV (k) and ISVs (l). The numbers of larvae (n) and ECs (n') are indicated above the graph. Anterior to the left, dorsal to the top. Scale bars, 20 μm. alSV, arterial intersegmental vessel; DA, dorsal aorta; PCV, posterior cardinal vein; viSV, venous intersegmental vessel; 1°, primary sprouts; 2°, secondary sprouts. Error bars, s.e.m.
started flow again, to analyse whether ECs were able to repolarize (Fig. 3k). We found that most ECs in the DA completely depolarized in larvae treated with BDM for 12 h (86.4%; ) or 24 h (93.5%; Fig. 3p,q,t). However, 12 h after BDM removal to reinitiate blood flow, ECs repolarized to an extent (72.5%; Fig. 3n,o,t) similar to control larvae treated with dimethyl-sulfoxide (84.0%; Fig. 3r–t). These data indicate that EC polarization can be dynamically modulated by blood flow in vivo. We also investigated the role of red blood cells (RBCs) in EC polarization and depleted RBCs by injecting a gata1 MO32,33. We found no significant differences in EC polarization between gata1 morphants and uninjected controls at 96 h.p.f. (Supplementary Fig. 4d–g). These data indicate that blood flow, but not the presence of RBCs, induces EC polarization.
Aplnr signalling modulates EC polarization. Based on our in vivo observations, EC migration (Supplementary Fig. 1a,b,d) and blood flow (Fig. 2d,i–k) induce the same polarization response. Thus, we decided to investigate the role of signalling pathways known to regulate EC migration during EC polarization. The Aplnr is a GPCR implicated in the migration of ECs and cardiac precursor cells. In addition, Aplnr is strongly expressed in the cardiovascular system of mouse, frog and zebrafish, and it plays an important role in cardiovascular function. In zebrafish, there are two paralogues of aplnr, aplnrα and aplnrβ, and two ligand-encoding genes, apelin (apln) and apela. We examined the expression of these genes by in situ hybridization and found that aplnrα, aplnrβ and apln were highly expressed in the zebrafish vasculature during development (Supplementary Fig. 5a–c), whereas apela was not (Supplementary Fig. 5d), consistent with previous reports. As we did not detect apela expression in developing blood vessels, we decided to focus on aplnrα, aplnrβ and apln. We first analysed aplnrα, aplnrβ and apln mutant larvae. We did not observe any obvious phenotype in aplnrα+/− or aplnrβ−/− larvae (Supplementary Fig. 5e). As previously reported, aplnrβ−/− animals develop pericardial oedema (red arrow, Supplementary Fig. 5e), whereas aplnrα+/− animals exhibit no obvious phenotype (Supplementary Fig. 5e). However, analysis of Golgi localization in aplnrβ−/− larvae at 72 h.p.f. revealed defects in EC polarization (23.1%) despite vigorous blood flow (Fig. 4g–i) and Supplementary Fig. 5f–i). In contrast, EC polarization in aplnrα−/− larvae (59.6%; Fig. 4d–l) appeared similar to wild-type siblings (66.7%; Fig. 4a–c,j). These data suggest that aplnrβ modulates EC polarization. To test whether Apnrn signalling modulates EC polarization cell autonomously, we generated mosaic embryos by cell transplantation (Fig. 4k). We observed a reduction in the polarization frequency of mosaic embryos by cell transplantation (Fig. 4k). We observed a reduction in the polarization frequency of apln−/−/C0 embryos transfected into wild-type larvae (63.4%; n′n=9/41) (Fig. 4l–o,p), indicating that Aplnrβ−/− embryos and wild-type siblings in the expression of the arterial reporter line Tg(EPV.TP1-Mmu.Hbb: hist2h2l-mCherry) were observed between apln−/− embryos and wild-type siblings (Supplementary Fig. 7j–k).

Figure 2 | EC polarization by blood flow. (a–d) Three-dimensional-rendered confocal stack images of the trunk region of 30 h.p.f. Tg(kdrl:NLS-EGFP);Tg(fli1a: B4GALT1-mCherry) embryos. White box in a is enlarged in b and single plane images of red boxed region in a is enlarged in c and c'. (c,c') At t = 60 min, RBCs (red arrows) in the DA are mainly stationary, whereas by 90 min they are moving too fast to be clearly distinct (white arrow). Red asterisks indicate the time when vigorous circulation starts. (d) Confocal time-lapse images of ECs in the white box in b. Surface-rendered images are displayed below. Yellow asters indicate the time when the EC is polarized. (e) Quantification of time required for DA ECs to become polarized starting at 30 h.p.f. and at 28.5°C. Numbers of larvae (n) and ECs (n′) indicated above the graph. Error bars represent s.e.m. (f–j) Three-dimensional-rendered confocal stack images of the trunk region of 30 h.p.f. Tg(kdrl:NLS-EGFP);Tg(fli1a:B4GALT1-mCherry) embryos. (g,h) Red and yellow boxes in f is enlarged in g and h. White dashed arrows indicate direction of blood flow. White dashed lines indicate ISV boundaries. (g) Red arrow points to stationary RBCs in the Sea; white arrow denotes apparent absence of RBCs. (h) At t = 1:49, lumen formation does not appear to be completed (red arrow), whereas at t = 2:17, a lumen appears to be formed (white arrow). (i,j) Time-lapse images of ECs marked with numbers (1 (i) and 2 (j)) in f. White dashed arrows indicate direction of blood flow. White arrowheads point to polarized Golgi apparatus. Red asterisks indicate the time when vigorous circulation starts. Yellow asterisks indicate the time when the EC is polarized. (k) Schematic model showing the process of EC polarization by blood flow. Anterior to the left, dorsal to the top. Scale bars, 20 μm (a–c,f–h), 7 μm (d,i,j). aISV, arterial intersegmental vessel; DA, dorsal aorta; PCV, posterior cardinal vein; vISV, venous intersegmental vessel. Error bars, s.e.m.
control cells, which show stress fibres aligned along the direction of flow (Fig. 5a,b). We observed similar defects in Golgi localization and stress fibre assembly when we knocked down APLNR in human arterial ECs (HUAECs) (Supplementary Fig. 8a–b,c). These results suggest that the role of Aplnr in EC polarization is conserved in humans.

The APLNR has been reported to function in mouse cardiomyocytes as a dual receptor for Apln via G-protein (Gαi) and mechanical stretch via β-Arrestin (Arrb)\(^{46}\). To analyse whether the role of APLNR in laminar flow-induced EC polarization was dependent on its ligand APLN and Gαi, we knocked down APLN (Fig. 5e–g) and also inhibited Gαi.
signalling by treatment with the Gs2 inhibitor pertussis toxin (PTX) (Fig. 5h–j). We did not find a significant effect on EC polarization or the actin cytoskeleton in APLN KD HUVECs (56.5%) under laminar flow, compared with controls (59.6%) (Fig. 5e–g). However, we observed a moderate decrease in EC polarization under laminar flow in PTX-treated HUVECs (36.6%) compared with controls (56.6%) (Fig. 5h–i). Given that the exposure to PTX was relatively long (>30 h due to the experimental design), it is likely to be that the effect of PTX is not specific to inhibiting APLNR downstream signalling. Next, we investigated whether laminar flow-induced EC polarization was dependent on ARRB. We found depolarized Golgi localization as well as disrupted actin cytoskeleton when both ARRB1 and ARRB2, were knocked down compared with controls (Fig. 5k–m). These data suggest that EC polarization may be modulated in an APLN-independent manner, and that APLNR-dependent EC polarization may function, at least in part, via ARRB. We also analysed whether ARRB is an effector of APLNR in laminar flow-induced signalling by transfecting ARRB-GFP into HUAECs. We found that ARRB-GFP translocated to the membrane when HUAECs were exposed to laminar flow, but not after APLNR KD (Supplementary Fig. 8d–g). These data suggest that ARRB functions downstream of APLNR in a flow-dependent signalling.

**KLF2 regulation by APLNR and EC polarization in klf2 mutants.**

Aplnr mutant mice show decreased endothelial expression of Krüppel-like factor 2 (Klf2), which encodes a master regulator of vascular adaptation to blood flow. Thus, we examined whether KLF2 expression was reduced in APLNR KD HUVECs under laminar flow and in aplnrb heterozygous and homozygous mutant zebrafish embryos. We found that KLF2 expression was reduced in APLNR KD HUVECs under laminar flow compared with controls (Supplementary Fig. 9a). However, there did not appear to be a dramatic difference in klf2a expression between wild-type and aplnrb+/− zebrafish embryos, and although klf2a expression in aplnrb−/− is clearly reduced compared with wild type, these animals have no blood flow that probably accounts, at least in part, for this reduction in expression (Supplementary Fig. 9b–d). To investigate whether loss of Klf2 function leads to endothelial polarization defects to a similar extent as that observed in aplnrb+/− larvae, we examined zebrafish mutants during their migration (Supplementary Fig. 1a–d) and also after exposure to flow (Fig. 2a–d,k). Aplnr is known to regulate EC migration during development. Previously, Scimia et al. reported that in mouse cardiomyocytes, the Aplnr transduces Aplin binding or mechanical stretch in different ways. The

**Discussion**

In this study, we found that EC polarization was heterogeneous between different vascular beds (Fig. 1a–i,k and Supplementary Fig. 2a–k). Previous studies have reported different polarization patterns in arterial and venous ECs in fixed tissue samples. Consistent with these reports, we observed that ECs in the DA polarize shortly after the onset of vigorous blood flow (Fig. 2a–d), whereas ECs in the PCV do not (Supplementary Fig. 3g,h). Interestingly, some non-polarized ECs in the PCV became polarized after migrating dorsally to form the vISVs (Fig. 2h,i). These data suggest that ECs’ ability to respond to flow is not fixed by arterial or venous identity, but dynamically adjusted according to their environment. Factors such as the velocity and pulse patterns of flow are known to influence EC polarization in vivo and in vitro. Anton et al. and Goetz et al. analysed the differences in blood cell migration in the DA, PCV, and ISVs of zebrafish embryos. Blood cell motion in the DA (mean velocity of ~300 μm s−1; shear stress of ~0.43 dynes cm−2) reflects the contractions of the heart, that is, with regular intermitting propagation of fluid and almost no flow between pulses. ISV flow is also pulsatile, but with a profile significantly different from that of the DA. Its peak flow is slower than that of the DA. By contrast, blood flow in the PCV (mean velocity of ~100 μm s−1; shear stress of ~0.14 dynes cm−2) is relatively steady with a 57% reduction in pulse amplitude. Thus, changes in blood flow velocity and pulse patterns may affect endothelial response to flow, as ECs move from the PCV into vISVs. In addition, Baeyens et al. reported that Vegfr3 signalling regulates the shear stress response of ECs in different blood vessels including arteries, veins and lymphatics. However, the relative contribution of different extrinsic and intrinsic signals in ECs’ response to shear stress remains to be investigated. Using live imaging, we observed that ECs become polarized during their migration (Supplementary Fig. 1a–d) and also after exposure to flow (Fig. 2a–d,k). Aplnr is known to regulate EC migration during development. Previously, Scimia et al. reported that in mouse cardiomyocytes, the Aplnr transduces Aplin binding or mechanical stretch in different ways. The
response to Apln seems to involve G-proteins, as it is PTX sensitive, whereas that induced by stretch occurs in the absence of Apln, which is PTX insensitive and G-protein independent but Arrb dependent. However, whether the Aplnr acts in ECs as a dual receptor for Apln and mechanical stimuli as it does in cardiomyocytes is unclear. Our studies on aplnr mutants reveal that ECs in aplnrb+/− larvae fail to polarize under blood flow (Fig. 4g–i). Consistently, APLNR depleted primary human ECs...
showed polarization defects under laminar flow (Fig. 5a–b’,d), indicating that the role of Apln in EC polarization is conserved. Interestingly, ECs in apln mutant and apln morphant larvae did not show a significant difference in polarization compared with controls (Fig. 4q–u and Supplementary Fig. 6k–p,q). Similarly, APLN KD HUVECs did not show a significant reduction in polarization compared with controls (Fig. 5e–f,g). Furthermore, HUVECs deficient for ARRBs showed a reduction in EC polarization under laminar flow (Fig. 5k–n). Altogether, these data suggest an Apln-independent function of Apln in flow-induced EC polarization and one that requires at least in part Arrb function. However, even though EC polarization appeared mostly unaffected in apln mutants and morphants, as well as in APLN KD HUVECs under laminar flow, we cannot exclude the possibility of ligand dependency, for example, via Apela or an unknown ligand. Ligand dependency could still be the case if the residual levels of Apela in apln mutants and morphants or in APLN KD HUVECs are sufficient to induce EC polarity, or if another ligand, even possibly a heretofore unknown ligand, can activate Apln during EC polarization.

**Methods**

**Zebrafish.** All zebrafish husbandry was performed under standard conditions in accordance with institutional (Max Planck Society) and national ethical and welfare guidelines. Embryos were staged by h.p.f. at 28.5°C. *aplna*mutant lines were previously reported56, 7*klf2a*mutant alleles were generated by TALENs. We used wild-type (AB), Tg(kdrl:NLS-EGFP)506 (ref. 55), Tg(fli1a:B4GALT1-mCherry)099, Tg(gata1:B4GALT1-mCherry)099, Tg(0.881fli1:EPF)00333 (ref. 56) and Tg(TFP1-MMM-HbH;hu2b2l-mCherry)039 (ref. 45), Tg(BAC(eltz2EGFP))07 (ref. 57) and Tg(fli1a:EGFP)07 (ref. 58) embryos.

**Assessment of EC polarization.** Polarization patterns of ECs were defined as follows: (1) upstream, if the Golgi was located on the upstream side of the nucleus; (2) none, if the Golgi was located anywhere else; (3) downstream, if the Golgi was located on the downstream side of the nucleus. For quantitative analyses of EC polarization, we analysed ECs of the DA, PCV and ISVs at the 5th and 6th somites at 30, 48 and 72 h.p.f. (Fig. 1k,l). To assess EC number, we counted the number of ECs located in the upper half of the DA. For quantitative analyses of EC polarization in apln heterozygous mutant larvae, we analysed ECs of the DA between, and including, the 5th and 11th somites at 72 h.p.f.

**Plasmid construction and transgenesis.** The pTol I sel filtmCherry plasmid was constructed by modifying a pTol2 vector and inserting the fli1a promoter, to drive expression of the reporter protein and 1-scel meganuclease recognition sequences (5'-TAGGTTAAACGCGTAAT-3') and mCherry cDNA. The pTol I sel fli1a:B4GALT1-mCherry plasmid was constructed by inserting a CDNA encoding amino acids 1–60 of the human B4GALT1 into the pTol I sel fli1amCherry plasmid. Tg(fli1a:B4GALT1-mCherry)099 fish were generated by injecting pTol I sel fli1a:B4GALT1-mCherry (25 ng), along with Tol2 transposase mRNA (25 ng) into one-cell stage AB embryos. Larvae were selected at 3 days post fertilization for high expression and grown to adulthood. Germine founders were identified by specific expression of B4GALT1-mCherry in the blood vessels of their progeny.

**MO oligonucleotides and quantum dot injections.** MOs (apln MO (4.5 ng), ttn2a MO (1 ng), gata1 MO (1 ng) and klf2a (2 ng)) were injected into one-cell stage embryos. The sequences of MOs used were as follows: apln MO, 5'-AACAGC CGTACACGGTCTCGACTAC-3'; ttn2a MO, 5'-CATGTTTGCTCTGATGC TACGACGCA-3' (ref. 30); gata1 MO, 5'-CCGAAGTGTAGTATTGAAGAT GTCG-3' (ref. 32); and klf2a MO, 5'-ggaaagtctgctcaacctgagct-3' (ref. 32) (Gene Tools). One to two nanoliters of Quantum dot 655 (qdot 655) (Molecular Probes) was injected into the CCV of Tg(kdrl:NLS-EGFP); Tg(fli1a:B4GALT1-mCherry) embryos and larvae at 48 and 72 h.p.f.

**In vivo imaging and image processing.** Pigmentation of embryos and larvae was inhibited by 1-phenyl-2-thiourea (Sigma). The embryos were treated with 100 µM tricaine (Sigma), a mount of a 1.5% low melting agarose in water and placed onto a glass dish. Fluorescence images were obtained using a Zeiss LSM 780 confocal microscope. Three-dimensional-rendered z-stack images and three-dimensional surface-rendered images and movies were analysed and assembled using the IMARIS software (BITPLANE).

**Chemical treatment.** Forty-eight, 60 and 72 h.p.f. Tg(kdrl:NLS-EGFP); Tg(fli1a:B4GALT1-mCherry) zebrafish embryos and larvae were incubated for 12 or 24 h in egg water containing 10 mM BDM (Sigma).

**Cells and reagents.** HUVECs and human umbilical artery ECs (HUAECs) were obtained from Lonza. HUVECs were cultured on collagen-I-coated dishes and
Figure 5 | APLNR signalling modulates the polarization of human ECs. (a–b’, e–f, h–i’, k–m’) Immunofluorescence staining of HUVECs transfected with si SC or si APLNR (a–b’), transfected with si SC or si APLN (e–f’) treated with water (MOCK) or the Gαi inhibitor PTX (100 ng ml\(^{-1}\)) (h–i’) and transfected with si SC, si ARRB1 or si ARRB2 (k–m’) subjected to laminar flow at 12 dynes cm\(^{-2}\) for 18 h. Cells were fixed and stained with the GM130 Golgi antibody (green), Phalloidin (red) and 4,6-diamidino-2-phenylindole (DAPI, blue). White arrowheads point to polarized ECs (that is, Golgi apparatus positioned within ±45° against the direction of laminar flow (G1)). Yellow arrowheads point to depolarized ECs (that is, Golgi positioned within +135° to +180° and +180° to −45° against the direction of laminar flow (G2–G4)). (c) Schematic representation showing the classification of polarization: The orientation of the Golgi apparatus relative to the nucleus was assessed to define cell polarization under laminar flow. (d, g, j, n) Quantitative analysis of polarization of HUVECs subjected to laminar flow at 12 dynes cm\(^{-2}\) for 18 h treated with si SC or si APLNR (d) transfected with si SC or si APLN (g) treated with water (MOCK) or the Gαi inhibitor PTX (100 ng ml\(^{-1}\)) (j) transfected with si SC, si ARRB1 or si ARRB2 (n). n > 300 cells, from at least three independent experiments. Scale bars, 20 μm. Error bars, s.e.m.
supplied with the endothelial growth medium EGM-2 (Lonza). Confluent cells at passages <P3 were used for all experiments. Pre-designed small interfering RNAs (siRNAs) against human APLNR (si AP LN R), human AP LN (si AP LN)46 (Sigma-Aldrich), human ARRB1 (si AR RB1) and human ARRB2 (si AR RB2) (Dharmacon), were used as non-targeting control scrambled siRNA (si SC) (Invitrogen) were obtained from commercial sources. HUVECs were transfected with siRNAs using Opti-MEM and Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. A repeated KD was performed the next day and assays were started at 72h after the first KD. RNA isolation was performed using Qiagen RNeasy kit. Reverse transcription was done using the Transcriptor high-fidelity cDNA synthesis kit from Roche. Primers were designed with the online tool provided by Roche and quantification was performed using the LightCycler 480 Probe Master System (Roche). For ARRB localization assays, HUAECs were transfected with pre-designed siRNAs against human APLNR (si AP LN R) or non-targeting control scrambled siRNA (si SC) or si AP LN R to lowar mass to 20 dynes cm⁻² for 15 min. We then analysed the plasma membrane of the GFP-positive cells.

**Immunofluorescence staining.** HUVEC and HUAEC cells were fixed for 10 min with 2% paraformaldehyde (PFA), permeabilized for 10 min with 0.5% Triton X-100 in PBS, blocked for 30 min with 5% sheep serum and then probed with mouse anti-β1-integrin (1:100 dilution, BD Biosciences). Alexa Fluor 568 Phalloidin (1:500 dilution, Molecular Probes) and 4 and 6-diamidino-2-phenylindole (1:1,000 dilution). Images were captured with a ×25 objective mounted on an LSM800 confocal microscope.

**Flow experiments and PTX treatment.** HUVEC cells were grown in a μ-slide VI 0.4 flow chamber (Ibidi, Germany) until confluence. Unidirectional laminar flow was generated by a computer-controlled setup containing an air-pressure pump and a two-way switching valve (Ibidi pump system). For quantitative analysis of blood vessel diameter in these blood vessels. For quantitative analysis of blood vessel diameter in these blood vessels.

**Whole mount in situ hybridization.** For in situ hybridization, zebrafish embryos and larvae were fixed by 4% PFA overnight at 4 °C and subsequently dehydrated in methanol and stored at −20 °C until required. Before hybridization, embryos were rehydrated to PBS/0.1% Tween, pre-incubated in hybridization buffer at 70 °C for 4h and then incubated with Dig-labelled RNA probes in hybridization buffer at 70 °C overnight. After washing, the hybridized probes were detected with alkaline-phosphatase-conjugated, alkaline-phosphatase-labelled anti-digoxigenin antibody (11093274970, Roche, dilution 1:1,000) at 4 °C overnight. To assess blood flow velocity, we performed time-lapse imaging of the trunk region of 84 h.p.f. wild type. klf2a⁻/⁻ and klf2a⁻/⁻ klf2b⁻/⁻ larvae using a Zeiss Spinning disc CSU-X1 confocal microscope with a high-speed camera and calculated blood flow velocity in the DA by measuring the time needed by RBCs to move 100 μm at the level of the fifth and sixth somites. A statistical significance for paired samples and for multiple comparisons was determined by Student’s t-test and one-way analysis of variance with Tukey’s test, respectively. Data were considered statistically significant if the P-value <0.05 (**).

**Data availability.** The authors declare that data supporting the findings of this study are available within the article and its Supplementary Information files.

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H.-B.K. and D.Y.R.S. conceived and designed experiments. H.-B.K., C.S.M.H. and H.-M.M. performed the zebrafish experiments. H.-B.K. and S.W. performed the in vitro experiments using cell lines. C.S.M.H., S.J.R. and W.H. provided unpublished reagents. H.-B.K., C.S.M.H., S.J.R., S.O. and D.Y.R.S. and W.H. H.-B.K. and D.Y.R.S. wrote the manuscript. All authors commented on the manuscript.

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