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Wnt/β-catenin signaling regulates VE-cadherin-mediated anastomosis of brain capillaries by counteracting S1pr1 signaling

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Canonical Wnt signaling is crucial for vascularization of the central nervous system and blood-brain barrier (BBB) formation. BBB formation and modulation are not only important for development, but also relevant for vascular and neurodegenerative diseases. However, there is little understanding of how Wnt signaling contributes to brain angiogenesis and BBB formation. Here we show, using high resolution in vivo imaging and temporal and spatial manipulation of Wnt signaling, different requirements for Wnt signaling during brain angiogenesis and BBB formation. In the absence of Wnt signaling, premature Sphingosine-1-phosphate receptor (S1pr) signaling reduces VE-cadherin and Esama at cell-cell junctions. We suggest that Wnt signaling suppresses S1pr signaling during angiogenesis to enable the dynamic junction formation during anastomosis, whereas later S1pr signaling regulates BBB maturation and VE-cadherin stabilization. Our data provides a link between brain angiogenesis and BBB formation and identifies Wnt signaling as coordinator of the timing and as regulator of anastomosis.
The central nervous system (CNS) depends on nutrient and oxygen delivery from blood vessels during the development and homeostasis, but also requires protection from blood-borne toxins and pathogens. Endothelial cells (ECs) of CNS blood vessels acquire characteristic properties in order to fulfill the tasks of this blood-brain barrier (BBB), such as expression of a specific subset of junction molecules and nutrient transporters, down-regulation of vesicular transport and establishment of cell–cell interactions within the neurovascular unit. During a defined time window of embryonic development, molecular cues from neuronal and perineuronal tissues orchestrate CNS angiogenesis and barrierogenesis. The process of brain angiogenesis is well conserved in vertebrates: After acquiring a pre-sprouting signature (hereafter called pre-tip cell), these specified cells migrate out from the resident vessel, with tip cells guiding sprout formation, and invade into the neural tissue, where they form cell–cell contacts and anastomose with other sprouts or extra-cerebral vessels in order to establish circulatory loops. In zebrafish (Danio rerio), hindbrain capillaries (intra-cerebral central arteries, CtAs) invade the brain parenchyma at around 32 h post fertilization (hpf) from the primordial hindbrain channel (PHBC), extend dorsally and connect ventrally to the basilar artery (BA) or laterally to other CtAs (Fig. 1a–d). At 48 hpf, most CtAs carry blood flow and by 72 hpf most of the respective BBB properties are established.

Studies in mice and zebrafish demonstrate β-catenin-dependent Wnt signaling through Wnt7a–Wnt7b to be essential for brain vascularization. Additionally, Wnt signaling has been shown to be involved in the establishment of BBB characteristics, such as upregulation of tight junction components (e.g., Claudin 1 and 3) or nutrient transporters (e.g., Glut1) and decrease of transcellular transport processes (e.g., Plvap) in a transcription-independent manner. During a developmental time window of embryonic development, molecular cues from neuronal and perineuronal tissues orchestrate CNS angiogenesis and barrierogenesis. The process of brain angiogenesis is generally conserved in vertebrates: After acquiring a pre-sprouting signature (hereafter called pre-tip cell), these specified cells migrate out from the resident vessel, with tip cells guiding sprout formation, and invade into the neural tissue, where they form cell–cell contacts and anastomose with other sprouts or extra-cerebral vessels in order to establish circulatory loops. In zebrafish, hindbrain capillaries (intra-cerebral central arteries, CtAs) invade the brain parenchyma at around 32 h post fertilization (hpf) from the primordial hindbrain channel (PHBC), extend dorsally and connect ventrally to the basilar artery (BA) or laterally to other CtAs (Fig. 1a–d). At 48 hpf, most CtAs carry blood flow and by 72 hpf most of the respective BBB properties are established.

In this study, we clarify the distinct requirements for Wnt signaling during brain angiogenesis. Whereas Wnt signaling is essential before sprouting to regulate yet to be defined early function of the future tip cells (pre-tip cells) within the parental vessels, it is surprisingly dispensable during sprout elongation and migration, although it remains continuously active in the invading sprout. We show that Wnt signaling is regulating brain capillary anastomosis and lumen formation. Interestingly, Wnt signaling is crucial for VE-cadherin and Esama localization at cell–cell junctions in a transcription-independent manner. During active brain capillary angiogenesis, Wnt signaling counteracts S1pr signaling, which enables VE-cadherin-dependent anastomosis and lumen formation. In contrast, when angiogenesis is completed at later stages, S1pr signaling regulates BBB formation. Our data therefore reveal an important functional link between the intertwined processes of brain capillary angiogenesis and BBB formation.

**Results**

Wnt signaling is required for hindbrain capillary patterning. Studies in mice have shown that Wnt signaling is essential for hindbrain capillary angiogenesis. Studies using zebrafish revealed that this control by Wnt signaling reflects an essential function in defining the pre-tip cell during early, pre-invasive angiogenic events within the parental vessels. As a result, no hindbrain capillaries (central arteries, CtA) are formed in the absence of Wnt signaling. In order to bypass this early requirement, we blocked Wnt signaling pharmacologically using IWR-1 and generated transgenic lines, which allow for temporally and spatially controlled expression of a dominant-negative Tcf transcription factor.

Treatment with Wnt signaling inhibitor IWR-1 after the pre-tip cell specification phase (29–72 hpf) resulted in severe vascular defects and hemorrhages in the brain, but not in the trunk vasculature (Supplementary Fig. 1a). Notably, there are further requirements for Wnt signaling during brain angiogenesis. In this study, we clarify the distinct requirements for Wnt signaling during brain angiogenesis. Whereas Wnt signaling is essential before sprouting to regulate yet to be defined early function of the future tip cells (pre-tip cells) within the parental vessels, it is surprisingly dispensable during sprout elongation and migration, although it remains continuously active in the invading sprout. We show that Wnt signaling is regulating brain capillary anastomosis and lumen formation. Interestingly, Wnt signaling is crucial for VE-cadherin localization at cell–cell junctions in a transcription-independent manner. During active brain capillary angiogenesis, Wnt signaling counteracts S1pr signaling, which enables VE-cadherin-dependent anastomosis and lumen formation. In contrast, when angiogenesis is completed at later stages, S1pr signaling regulates BBB formation. Our data therefore reveal an important functional link between the intertwined processes of brain capillary angiogenesis and BBB formation.

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Treatment with Wnt signaling inhibitor IWR-1 after the pre-tip cell specification phase (29–72 hpf) resulted in severe vascular defects and hemorrhages in the brain, but not in the trunk vasculature (Supplementary Fig. 1a), indicating that there are further requirements for Wnt signaling during brain angiogenesis. We therefore analyzed during which stages of brain capillary angiogenesis active Wnt signaling can be observed using confocal live imaging and two different transgenic Wnt signaling reporter lines, which express short-lived fluorophores controlled by Wnt-responsive promoters: $Tg(axin2BAC:Venus-Pesypm288)$ and $Tg(14TCF:loxP-STOP-loxP-dGFPMm22033)$. Both reporter lines showed robust fluorophore expression within the migrating CtA sprouts at 34 hpf (Supplementary Fig. 1b,c), which confirmed to be EC specific (Supplementary Fig. 1d). We next performed time-lapse analysis using cre mRNA-injected $Tg(14TCF:loxP-STOP-loxP-dGFPMm22032)$ embryos starting from 29 hpf (Fig. 1b, Supplementary movies 1, 2). We detected high Wnt reporter activity in the determined CtA pre-tip cells, which are forming within the PHBC. Despite a putative half-life of about 2 h, the destabilized GFP (dGFp) expression remained high in the emerging CtA sprouts during the following 5.5 h of development, indicating continuous Wnt signaling activity during CtA sprout migration and cell–cell contact formation (open arrow, Fig. 1b). With the onset of lumen formation about 2 h after cell–cell–contact formation, the dGFP signal decreased to a baseline level, which was maintained in the perfused CtAs, pointing to a post-determination role of Wnt signaling.

After pre-tip cell formation, CtA angiogenesis proceeds by sprouting and sprout invasion into the brain parenchyma. We asked whether endothelial Wnt signaling regulates these processes. We therefore generated a transgenic zebrafish line expressing a dominant-negative Tcf transcription factor fused to mCherry (mCherry-dnTcf) under the control of a heat shock inducible...
To restrict Wnt inhibition to ECs, we placed a loxP-flanked (floxed) STOP-cassette upstream of the mCherry-dnTcf coding sequence (Supplementary Fig. 2a, c). This STOP cassette was removed only in ECs by mating to fish with endothelial-specific Cre recombinase expression (Tg(kdrl:cre)s898). Validation of Wnt inhibition by heat shock controlled mCherry-dnTcf expression is documented in Supplementary Fig. 2.

To address whether Wnt signaling regulates sprout elongation or EC migration, we blocked Wnt signaling by EC-specific mCherry-dnTcf expression or IWR-1 treatment starting from 26 hpf and analyzed CTA sprouts at 32 hpf (Fig. 1c, d). Surprisingly, we did not observe differences in CTA sprout number or sprout length after Wnt signaling inhibition (Fig. 1c, d). Furthermore, sprout formation, behavior, and filopodia appearance were not affected by IWR-1 treatment (Fig. 1e, f, Supplementary movies 3,
normal CtA sprout formation in number of analyzed embryos; BA, basilar artery; CtAs, central arteries; ECs, endothelial cells; hpf, hours post fertilization; PHBC, primordial hindbrain images show dorsal (affected by IWR-1 treatment (Fig. 2d, e). After inhibition of Wnt signaling by IWR-1, we noted a drastic delay in lumen formation or even completely impaired lumenization within the observation window (Fig. 2e; Supplementary movie 5-8). Additionally, tip cells in CtA sprouts of IWR-1-treated embryos failed to establish stable cell–cell contacts, but instead continuously produced filopodia (>10 µm) was marginally decreased after IWR-1 treatment (Fig. 1f). Similarly, heat shock-induced EC-specific overexpression of mCherry-dnTcf (mCherry-dnTcfEC, c) or pharmacologically by IWR-1 treatment (d) resulted in normal CtA sprouting at 32 hpf. CtA sprout number or length of the sprouts was not affected by mCherry-dnTcfEC expression in Tg(kdrl:GFP)s843 embryos (c: mCherry-dnTcfEC; n = 11; mCherryEC; n = 12) or IWR-1 treatment (d: DMSO: number n = 22, length n = 17; IWR-1: number n = 24, length n = 18). e Still images from time-lapse movies starting at around 28 hpf displayed normal CtA sprout formation in Tg(kdrl:GFP)s843; (flh:ZGA(GFP)17) double transgenic embryos treated with IWR-1. f g CtA sprout morphology was not affected by IWR-1 treatment (f) or dnTcf expression (g) in Tg(flh:lifeact-GFP)mu240 embryos. IWR-1 treatment slightly reduced the formation of long filopodia (>10 µm) compared to DMSO control (f: IWR-1: n = 15; DMSO: n = 15). Images are displayed in inverted color for better visualization. Confocal images show dorsal (b) or lateral views (c–g), anterior to the left. Values represent mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. Student’s t-test; n, number of analyzed embryos; BA, basilar artery; CtAs, central arteries; ECs, endothelial cells; hpf, hours post fertilization; PHBC, primordial hindbrain channel; Scale bars: 30 µm (b), 50 µm (c–f), 6 µm (g).

4). Only the formation of long filopodia (>10 µm) was marginally decreased after IWR-1 treatment (Fig. 1f). Similarly, heat shock-induced EC-specific overexpression of mCherry-dnTcf (mCherry-dnTcfEC) did not result in differences in filopodia morphology, but instead in high filopodia motility, which resulted in slightly blurry images due to fast filopodia movement (Fig. 1g).

We conclude that after being required early within the parental vessel, Wnt signaling is continuously active during CtA angiogenesis, but dispensable for efficient sprout elongation and invasion of CtA tip cells into the brain parenchyma.

Wnt regulates brain capillary anastomosis and lumenization.

To address the role of the active Wnt signaling in the developing CtAs, we inhibited Wnt signaling by inducing mCherry-dnTcf expression in ECs at 26 hpf or by applying IWR at 29 hpf and analyzed CtA pattern and lumen formation at 48 hpf (Fig. 2a, b). We observed a reduced CtA number, reduced connections to the BA and ectopic connections between CtAs. CtA patterning appeared more disorganized, and CtAs had non-lumenized protrusions. To address whether the reduced number of CtAs (Fig. 2a, b) correlated to a reduced number of EC within CtAs, we blocked Wnt signaling as before, and quantified the number of cell nuclei in Tg(flh:GFP)17 embryos. Both, treatment with IWR-1 or heat shock-induced EC-specific mCherry-dnTcf expression, reduced the EC number within CtAs (Supplementary Fig. 3a, b). However, blocking cell proliferation via the administration of aphidicolin and hydroxyurea (AHU) did not result in any patterning defects in CtAs, and the combined inhibition of Wnt signaling and proliferation did not aggravate the phenotype of Wnt deficiency (Supplementary Fig. 3c). We therefore conclude that Wnt signaling is required for CtA patterning, but does not act via cell proliferation.

To understand the processes underlying the CtA patterning defects observed after Wnt signaling inhibition (Fig. 2a, b), we performed angiography following IWR-1 treatment (Fig. 2c). In control embryos, more than 80% of the CtAs were perfused at 48 hpf, whereas after IWR-1 treatment only about 25% of the CtAs carried the fluorescent tracer. We next performed time-lapse analysis following IWR-1 treatment or EC-specific mCherry-dnTcf expression using Tg(flh:lifeact-GFP)mu240 embryos to illustrate the actin cytoskeleton of ECs (Fig. 2d–f). We focused on laterally connecting CtAs for better visualization and quantified the time from cell–cell contact until completion of lumen formation until 48 hpf (Fig. 2d). In control embryos, the average time from cell–cell contact to lumen formation is less than 2 h (Fig. 2d, e). After inhibition of Wnt signaling by IWR-1, we noted a drastic delay in lumen formation or even completely impaired angiogenesis localizes to the hindbrain. Brain capillary (central artery, CtA) pre-tip cells can be detected within two bilateral primordial hindbrain channels (PHBCs, light gray) and sprout dorsally from PHBCs around 32 hpf. Between 32 and 36 hpf, CtA sprouts extend long toward the basilar artery (BA, dark gray). At 48 hpf CtAs have fused with either the BA or neighboring CtAs and carry blood flow. b Time-lapse analysis of a cre mRNA-injected Tg(14TCF:loxP-stor-lap-dGFP)mu202 embryo starting from around 29 hpf showed continuously active Wnt signaling in CtAs (arrowheads) before and during sprouting, during invasion, cell–cell contact (open arrowhead) and lumen formation. The bottom panel represents single channel images in inverted color for better visualization. c, d Inhibition of Wnt signaling by EC-specific dnTcf expression after heat shock at 26 hpf (mCherry-dnTcfEC, c) or pharmacologically by IWR-1 treatment (d) resulted in normal CtA sprouting at 32 hpf. CtA sprout number or length of the sprouts was not affected by mCherry-dnTcfEC expression in Tg(kdrl:GFP)s843 embryos (c: mCherry-dnTcfEC; n = 11; mCherryEC; n = 12) or IWR-1 treatment (d: DMSO: number n = 22, length n = 17; IWR-1: number n = 24, length n = 18). e Still images from time-lapse movies starting at around 28 hpf displayed normal CtA sprout formation in Tg(kdrl:GFP)s843; (flh:ZGA(GFP)17) double transgenic embryos treated with IWR-1. f g CtA sprout morphology was not affected by IWR-1 treatment (f) or dnTcf expression (g) in Tg(flh:lifeact-GFP)mu240 embryos. IWR-1 treatment slightly reduced the formation of long filopodia (>10 µm) compared to DMSO control (f: IWR-1: n = 15; DMSO: n = 15). Images are displayed in inverted color for better visualization. Confocal images show dorsal (b) or lateral views (c–g), anterior to the left. Values represent mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. Student’s t-test; n, number of analyzed embryos; BA, basilar artery; CtAs, central arteries; ECs, endothelial cells; hpf, hours post fertilization; PHBC, primordial hindbrain channel; Scale bars: 30 µm (b), 50 µm (c–f), 6 µm (g).

In summary, our analysis reveals that Wnt signaling is required for anastomosis of brain capillaries.

Block of Wnt signaling reduces VE-cadherin at cell junctions.

To elucidate how Wnt signaling regulates CtA angiogenesis, we investigated the abundance of the EC-specific adhesion molecules VE-cadherin and Esama at cell–cell junctions of the CtAs. VE-cadherin, a member of the cadherin protein family expressed in ECs, has been shown to be crucial for EC contact formation during blood vessel anastomosis. Esama belongs to the protein family of Junction adhesion molecules (Jam) and two paralogues exist in zebrafish, esama and esamb, of which esama is mainly expressed in the vasculature. To address, whether Wnt signaling inhibition affects protein localization of VE-cadherin or Esama, we performed immunostaining for VE-cadherin, Esama and ZO-1 in 42 hpf-old embryos (Fig. 3a–d). In control embryos (DMSO), VE-cadherin and Esama were expressed continuously along cell–cell junctions of ECs within CtAs and in anastomosis rings, which form at positions where two CtAs fuse (arrows in Fig. 3b). After IWR-1 treatment (Fig. 3b) or EC-specific mCherry-dnTcf expression (Fig. 3c, d), we found that the protein abundance of VE-cadherin, Esama, and ZO-1 at cell–cell junctions was dramatically reduced. Furthermore, we frequently observed clustered protein aggregates (stars in Fig. 3b) and the formation of many small adhesion spots (arrows in Fig. 3b) in Wnt-inhibited embryos, which is in line with our previous observations of multiple filopodia contacts (Fig. 2e, f). Hence, reduction and aberrant localization of VE-cadherin and Esama at the cell junctions could cause the anastomosis defects. To validate these findings in another model, we isolated primary brain endothelial cells (BECs) from microvascular fragments of either P3 or adult mice. We were able to block Wnt signaling using 10 µM IWR-1 for P3 and 20 µM for adult brain ECs (Fig. 3e, Supplementary Fig. 2e). To analyze the effect on VE-cadherin distribution we measured the length of the potential cell–cell contact area in form of opposing or touching membrane length and the actual
coverage of that area by VE-cadherin. We observed a marked reduction in the length of VE-cadherin junctions and the coverage of cell–cell contact sides by VE-cadherin in ECs isolated from P3 mice. Interestingly, Wnt signaling inhibition did not affect junctional VE-cadherin in adult brain ECs. We conclude that brain capillary ECs from P3 mice are capable of responding to Wnt signaling, presumably because these brain ECs are in a more angiogenic state, whereas ECs from adult mice gain mature BBB properties and are no longer sensitive to Wnt signaling inhibition.

We next analyzed how a complete loss of VE-cadherin affects CTA lumen formation using homozygous ve-cadherin<sup>ub8</sup> mutant embryos. In ve-cadherin<sup>ub8</sup>-deficient mutants, CTAs form and invade similarly to wild-type siblings (Supplementary movies 11, 12), but fail to establish stable cell–cell contacts, continuously produce filopodia toward the ECs in neighboring CTAs or BA and fail to form continuous lumens (Supplementary Fig. 4a, b, Supplementary movies 11, 12), a behavior, which mimics our observations after Wnt signaling inhibition (Fig. 2e, f). Interestingly, intracellular signaling via VE-cadherin and its
Fig. 2 Wnt signaling is crucial for brain capillary patterning and anastomosis. a, b Inhibition of Wnt signaling by IWR-1 treatment (a) or dnTcf expression (b) impaired CTA patterning, a IWR-1 treatment from 29 to 48 hpf reduced CTA number (n = 39) and the proportion of CTAa connecting to the BA (n = 39), but increased the proportion of interconnecting CTAa (n = 33), compared to DMSO treated embryos (CTAa number: n = 35; BA connection: n = 35; interconnected: n = 29). b Expression of mCherry-dnTcfEC through heat shock at 26 hpf reduced the number of CTAa (n = 40) and decreased the proportion of CTAa connecting to the BA (n = 32) or interconnecting (n = 32), compared to mCherryEC control at 48 hpf (CTAa number: n = 39; BA connection: n = 33; interconnected: n = 33). c Analysis of lumen formation by fluorescent tracer injection into the blood stream at 48 hpf. Treatment with IWR-1 decreased the proportion of lumenized CTAa (n = 16) compared to DMSO control (n = 18). d Schematic representation of CTAa anastomosis (dorsal view, -36 hpf). CTA sprouts (gray) migrate, extend filopodia and form cell-cell contacts to neighboring CTA sprouts (asterisk) or the BA. Cell-cell contact formation triggers anastomosis, cell rearrangements and lumen formation through the connected sprouts in a distinct time window (Δt). e, f Inhibition of Wnt signaling drastically extended the time window from cell-cell contact formation until lumen formation. Still images from time-lapse movies embryos treated with DMSO (n = 58, N = 9) or IWR-1 (n = 33, N = 9) or expressing mCherryEC (n = 36, N = 6) or mCherry-dnTcfEC (n = 39, N = 8) after heat shock treatment at 26 hpf. Note that for events, where lumen formation was not completed until the end of the time-lapse recording (~48 hpf), the last measured time point was used for quantification. Confocal images show dorsal views (anterior to the left) of Tg(flh:fla:Ga4)Uas:KaedeRK8 (a, b, e, f) or Tg(kdr:ga4)Uas:KaedeRK8 (c) embryos. Values represent mean ± SD (a–c) or ± SEM (e, f). *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t-test; n number of analyzed embryos (a–c) or n number of CTA fusion events analyzed; N, number of embryos analyzed (e, f). BA, basilary artery; CTAa, central arteries; ECs, endothelial cells; Scale bars: 50 μm

association with intracellular adaptor proteins seems to be required, as overexpression of VE-cadherin lacking the intracellular domain could not rescue the lumen formation defects (Supplementary Fig. 4d). Additionally, rescuing the ve-cadherinab58 variant by expression of endogenous levels of the VE-cadherin tension sensor (VE-cad-TS)36,37 did not completely restore wild-type VE-cadherin function, (Supplementary Fig. 4a), indicating that the presence of the fluorophores within the intracellular domain of VE-cadherin reduces its signaling somewhat. We also analyzed lumen formation of CTAa in esamaab1924 mutant embryos. Homozygous esamaab19variants displayed fewer lumenized CTAa compared to heterozygous controls (Supplementary Fig. 4c). We conclude that VE-cadherin is required for CTAa anastomosis and that Esama contributes to efficient CTAa lumen formation, which is in agreement with previous reports23,24. Therefore, we reason that the lumen formation defects caused by Wnt signaling inhibition were the result from decreased VE-cadherin and Esama protein levels at the cell–cell contact sites in CTAa.

Wnt does not regulate transcription of ve-cadherin and esama. We hypothesized that the reduced protein levels of VE-cadherin and Esama could result from reduced transcription in embryos lacking Wnt signaling. In order to analyze ve-cadherin and esama mRNA expression levels, we performed whole mount fluorescent in situ hybridization (FISH) after Wnt signaling inhibition by IWR-1 or EC-specific mCherry-dnTcf expression (Fig. 4a, b, Supplementary Fig. 4e). We quantified the fluorescence intensity within CTAa at 32 or 42 hpf and normalized the fluorescence intensity to the respective CTAa volume (generated from Tg(kdr:GFP)843 signal). Surprisingly, we did not find severe changes of the overall ve-cadherin or esama mRNA abundance in CTAa ECs at 32 or 42 hpf (Fig. 4a, b, Supplementary Fig. 4e).

At 32 hpf, ve-cadherin mRNA was slightly decreased following IWR-1 treatment and slightly increased following mCherry-dnTcfEC expression. At 42 hpf, at which we detected reduced VE-cadherin protein levels at the cell–cell junctions (Fig. 4b), ve-cadherin mRNA was increased using either of the Wnt signaling inhibition approaches. This indicates, that ve-cadherin mRNA levels in CTAa were not decreased (as expected from a putative Wnt target gene), but rather slightly increased over time, presumably due to a compensatory mechanism. The mRNA level of esama in CTAa ECs was slightly decreased after Wnt signaling inhibition by IWR-1 or mCherry-dnTcf expression both at 32 and 42 hpf (Fig. S4e).

Additionally, to gain insights into transcriptional changes in CTAa mediated by Wnt signaling inhibition, we isolated Kaede-photoconverted CTA ECs from 1.5 days-old Tg(flh:fla:Ga4)Uas:KaedeRK8, (UAS:Kaede)K8 embryos, which were treated with IWR-1 or DMSO (Fig. 4c, d) and performed gene expression profiling by RNA sequencing. We confirmed that ve-cadherin and esama expression levels were not regulated by Wnt signaling in sharp contrast to classical Wnt target genes (e.g., axin2 and lef1, Fig. 4d). We also analyzed the effect of Wnt inhibition on ve-cadherin mRNA levels by qPCR in BECs isolated from P3 and adult animals. In line with our zebrafish data, we observed no transcriptional regulation of mouse VE-cadherin by Wnt signaling (Fig. 4e).

We therefore conclude that post-transcriptional mechanisms account for the changes in protein abundance at brain capillary cell–cell junctions following Wnt signaling inhibition.

Wnt signaling counteracts S1pr1 signaling in angiogenesis. To address how VE-cadherin localization at cell–cell junctions can be regulated during brain angiogenesis, we focused on Sphingosine-1-phosphate (S1p) signaling, as it is known to regulate EC integrity and behavior by modulating junction protein localization and cell contractility27,28. In particular, S1p-induced signaling mainly via S1p receptor 1 (S1pr1) has been shown in vivo and in vitro to increase recruitment of Cadherin protein family members, including VE-cadherin and N-cadherin, to the plasma membrane31,38,39. Additionally, S1p receptor 3 (S1pr3) was found to function cooperatively with S1pr1 in promoting adherens junction formation in ECs38, whereas S1p receptor 2 (S1pr2) signaling disrupted EC junctions, thereby increasing vascular permeability40. We confirmed that similar to what is seen in mice30, blocking of S1prs using the pharmacological antagonist VPC23019 (VPC) during barriergenesis (48–72 hpf) resulted in extravasation of red blood cells into the surrounding brain tissue, indicating breakdown of the BBB (Supplementary Fig. 5a).

Our RNA sequencing data showed expression of s1pr1 and s1pr5a as well as very low level expression of s1pr2 and s1pr3b in zebrafish brain capillary ECs (Supplementary Fig. 5b). In order to functionally dissect the contribution of these different S1pr’s we validated various pharmacological antagonists by their potency to rescue phenotypes of S1pr overexpression (Supplementary Fig. 5c–g). In the zebrafish, VPC seems to antagonize the tested S1pr’s (S1pr1, S1pr2, S1pr3a, and S1pr5a), whereas TYS2156 (TY) acts specifically on S1pr1. In our hands the S1pr2 antagonist JTE013 (JTE) did not rescue S1pr2 overexpression (Supplementary Fig. 5c–g).
To test the effects of S1pr inhibition on lumen formation and VE-cadherin localization, we next applied the antagonists of S1pr signaling during CtA angiogenesis at 29 hpf and assessed patterning and lumen formation at 48 hpf. In contrast to barrierogenesis, treatment during brain angiogenesis with either VPC or TY did not affect CtA development and lumen formation in zebrafish (Fig. 5a). It is therefore likely that in wild-type embryos S1pr signaling is required only after the onset of blood circulation in CtAs, presumably through delivery of high amounts of its ligand S1p by the blood41. To our surprise, co-treatment of embryos with the Wnt signaling inhibitor IWR-1 and either VPC or TY increased the proportion of lumenized CtAs compared to single IWR-1 treatment and rescued CtA patterning and lumen formation (Fig. 5a). S1pr inhibition by VPC was also able to...
rescue EC-specific loss of Wnt signaling in embryos over-expressing mCherry-dnTcfE (Fig. 5b).

We further analyzed, whether not only lumen formation, but also VE-cadherin and Esama levels at cell–cell junctions were rescued by inhibiting S1pr signaling in Wnt-depleted embryos. We observed that the reduced VE-cadherin and Esama localization at cell–cell contact sides and in anastomosis rings following Wnt-1 treatment was restored by co-treatment with the S1pr1 antagonist TY and similar to control embryos when analyzed either by immunostaining for VE-cadherin and Esama or by live imaging of the Venus-fluorophore distribution of the VE-cadherin-TS (Supplementary Fig. 6). Hence, blocking of S1pr1 in parallel to IWR-1 treatment was sufficient to rescue VE-cadherin and Esama protein levels at cell–cell junctions in IWR-1 treated P3 BECs (Fig. 5c).

As an alternative approach to pharmacological S1pr1 inhibition in zebrafish, we used transient CRISPR-Cas9-mediated s1pr1 knockdown in combination with IWR-1 treatment. This manipulation restored lumen formation of CtAs (Fig. 5d) similar to TY or TY treatment (Fig. 5a). Furthermore, Wnt signaling inhibition was unable to affect VE-cadherin localization in S1pr1E/EC knockout BECs isolated from P3 mice (Fig. 5e).

Therefore, loss of S1pr1 signaling rescued Wnt depletion phenotypes, such as impaired lumen formation and VE-cadherin localization. Taken together, the studies reveal that Wnt signaling regulates brain capillary angiogenesis by counteracting S1pr1 signaling in zebrafish and mouse ECs, presumably to prevent premature barrier formation, which would impair brain angiogenesis (Fig. 5f).

Wnt signaling prevents S1pr1-mediated Rac1 activation. So far we have shown that Wnt signaling regulates brain capillary angiogenesis in an EC-specific manner by post-transcriptionally influencing VE-cadherin localization. Furthermore, the mislocalization of VE-cadherin caused by Wnt signaling inhibition was dependent on S1pr1 signaling (Fig. 5, Supplementary Fig. 6). In order to understand the regulatory interaction of these two signaling pathways, we addressed at which level Wnt signaling interferes with S1pr1 signaling activation. We therefore stimulated S1pr1 signaling using a combination of two agonists (CYM5541 and CYM5542; validation for agonist activity, see Supplementary Fig. 5h). Overstimulation of S1pr1 signaling activity did not induce lumen formation defects (Fig. 6a) and therefore did not overcome the intrinsic counteraction by Wnt signaling. However, block of S1pr signaling in ve-cadherin or esama-deficient embryos did not result in a rescue of lumen formation defects (Supplementary Fig. 7a).

Therefore, Wnt signaling antagonizes S1pr1 signaling downstream of receptor activation, but upstream of VE-cadherin and Esama function.

One potential mechanism that could account for the downregulation of VE-cadherin could be the regulation of VE-cadherin degradation or internalization. We therefore inhibited proteasomal degradation by MG132, dynamin-dependent internalization by Dynasore and lysosomal degradation by Chloroquine; however, none of these treatments rescued Wnt-deficiency (Supplementary Fig. 7b, c). Interestingly, we also observed a mislocalization of VE-cadherin to seemingly intracellular vesicular structures in Wnt signaling-deficient BECs (Fig. 6b), indicating changes in cellular architecture rather than VE-cadherin degradation following Wnt signaling inhibition.

S1pr-induced signaling in cultured ECs has been linked to activation of the Rho/Rac-pathway for stabilization of the cytoskeleton and recruitment of junction proteins to the cell membrane[8,12,43]. Similar to S1pr signaling, Rac1 activation as well as Rho inactivation is known to promote EC barrier function in mature blood vessels[44]; however, their contribution to brain capillary angiogenesis has not been addressed yet. We therefore applied the pharmacological Rac1 inhibitor (NSC23766, NSC) or Rho inhibitor (CT03) alone or together with IWR-1 as described. Single treatment with either Rac1 or Rho inhibitors, did not affect CtA development or lumen formation. Indeed, co-treatment of IWR-1 and Rac1 inhibitor, but not of IWR-1 and Rho inhibitor, robustly restored the lumen formation defects caused by IWR-1 (Fig. 6c, Supplementary Fig. 8a).

We hypothesized that if Rac1 was mediating premature S1pr1 signaling activation, overexpression of constitutive-active Rac1 (CA-Rac1) should phenocopy Wnt signaling inhibition. Therefore, we mated Tg(fliala:Gal4)B953 fish with Tg(UAS:CA-Rac1)ym217 and analyzed brain capillary lumen formation in 48 hpf-old embryos (Fig. 6d). Vascular-specific overexpression of CA-Rac1 caused strong lumen formation defects and was accompanied by multiple filopodia contact sides comparable to IWR-1-treated (Fig. 2a) or ve-cadherin-deficient (Supplementary Fig. 4a) embryos. Additionally, vascular-specific Gal4-UAS-mediated overexpression of dominant negative Rac1 (DN-Rac1, from Tg(UAS:DN-Rac1)ym212) could partially rescued lumen formation in IWR-1-treated embryos (Fig. 6e).

Hence, Rac1 activation downstream of S1pr1 signaling is required and sufficient to mediate lumen formation defects caused by Wnt signaling inhibition.

In summary, we identified a temporal-specific function of Wnt signaling for brain capillary angiogenesis by post-transcriptionally and EC-specifically regulating junction protein localization upstream or at the level of Rac1 activity, but downstream of S1pr1 signaling. We suggest that EC-specific Wnt signaling
Fig. 4 Wnt signaling does not regulate ve-cadherin mRNA expression. a Whole mount fluorescent in situ hybridization (FISH, red) in combination with anti-GFP immunostaining (green) of Tg(kdrl:GFP)6843 embryos. For quantification the FISH signal within CtA sprouts was normalized to the respective GFP volume (blue) of each sprout. b Inhibition of Wnt signaling either by incubation with IWR-1 or expression of mCherry-dnTcfEC did not severely affect ve-cadherin mRNA expression at 32 or 42 hpf. Treatment with IWR-1 resulted in slightly decreased ve-cadherin mRNA levels at 42 hpf (32 hpf: DMSO: n = 18, N = 6; IWR-1: n = 19, N = 7). After mCherry-dnTcfEC expression in ECs ve-cadherin mRNA expression was slightly increased (32 hpf: mCherryEC: n = 40, N = 7; mCherry-dnTcfEC: n = 16, N = 7). Quantifications were represented by Box-and-Whisker plots with median (center line), 25th and 75th percentiles (bounds of box) and Min-to-Max (whiskers). *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t-test; n, number of CtA volumes analyzed; N, number of embryos analyzed. Scale bars: 50 μm. c, d Expression levels of ve-cadherin or esama were not altered by inhibition of Wnt signaling, whereas classical Wnt-target genes (e.g., axin2, left) were downregulated. For RNA sequencing, CtA ECs of Tg(fli1a:Gal4);(UAS:Kaede)6, and IWR-1: n = 7, and IWR-1: n = 19, N = 7). Quantiﬁcations were represented by Box-and-Whisker plots with median (center line), 25th and 75th percentiles (bounds of box) and Min-to-Max (whiskers). *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t-test; n, number of biological replicates. BECs, brain ECs; CtAs, central arteries; ECs, endothelial cells; FISH, fluorescent in situ hybridization.
counteracts S1pr1 signaling during CtA angiogenesis, presumably to prevent premature barrier formation processes, which would impair brain angiogenesis (Fig. 6f,g).

Interestingly this mechanism seems to be specific to the role of Wnt signaling in regulating anastomosis and lumen formation, but not to the essential function of Wnt signaling during the earlier pre-tip cell phase. Treatment of embryos lacking Wnt signaling following IWR-1 treatment or of gpr124 mutants with S1pr antagonists did not restore CtA sprout formation (Supplementary Fig. 8b, c).
In conclusion, we describe a temporal control of different steps of brain angiogenesis and BBB formation, which are differentially regulated by Wnt signaling. We also discover an interplay between Wnt and S1pr signaling during brain angiogenesis.

Discussion

We addressed the role of β-catenin-dependent Wnt signaling during early brain capillary angiogenesis in vivo. We found that (1) Wnt signaling is highly active in brain capillary sprouts, but after pre-tip cell formation it is dispensable for brain capillary invasion into the brain parenchyma. Instead, (2) Wnt signaling is required for brain capillary anastomosis. During this process, (3) Wnt signaling influences the post-transcriptional regulation and cell–cell junction localization of the EC-specific adhesion molecules, VE-cadherin, and Esama, by counteracting S1pr1 signaling (Fig. 6f, g).

We and others have shown that Wnt signaling is active in brain capillaries during brain angiogenesis (Fig. 1, 11, 12, 14). Recent reports demonstrate that Gpr124-CKO mediated Wnt signaling is essential for brain vascularization, as homozygous gpr124 or rock mutants are completely devoid of brain capillaries12, 15–17. We hypothesized that Wnt signaling might be involved in the formation of pre-tip cells within the parental vessel, which most likely occurs at earlier developmental stages12. In line with this, a role of Wnt signaling for EC specification has already been suggested to drive cell fate from the Fli1a-positive precursor cell population into ECs at the expense of primitive erythrocytes32. At later developmental stages, Wnt signaling is required for specification of EC populations within the vasculature, which has been shown for hemogenic EC formation45, 46 and lymphatic cell fate specification47.

Here we demonstrate that Wnt signaling is continuously activated in CtAs during all stages of angiogenesis. Surprisingly, Wnt signaling is not involved in the process of CtA sprout invasion and filopodia formation (Fig. 1).

However, our data demonstrate that Wnt signaling is essential for brain capillary anastomosis (Fig. 2). During the anastomosis process in intersegmental vessels, VE-cadherin is required for cell–cell contact formation and together with Esama has been shown to be essential for efficient blood vessel fusion23, 24. We detected reduced protein levels of VE-cadherin, Esama, and ZO-1 at the cell–cell junctions following Wnt signaling inhibition (Fig. 3), which presumably impair anastomosis and lumen formation. In line with these findings, knockouts of VE-cadherin (by ve-cadherinub8/ub8) or Esama (by esamaub19/ub19) phenocopied CtA lumen formation defects (Supplementary Fig. 4). Surprisingly, neither ve-cadherin nor esama are transcriptional targets of Wnt signaling (Fig. 4). Similar to Hupe and colleagues, we detected mildly increased amounts ve-cadherin mRNA when Wnt signaling inhibition persists for longer time intervals (12 h), indicating a compensatory response to the reduced protein levels of VE-cadherin at cell–cell junctions48. We therefore conclude that VE-cadherin and Esama downregulation occurs via a post-transcriptional mechanism, which interferes with their membrane localization. Using a series of inhibitors, we excluded internalization as well as proteasomal and lysosomal degradation as potential mechanisms (Supplementary Fig. 7). Previously, Wnt signaling has been shown to be required for the junction localization of Claudin 1 and 3 in murine brain ECs during BBB formation, but no direct transcriptional regulation has been detected15, 14. As there have been no mechanisms proposed of how Wnt signaling could regulate these junctional molecules, we speculate that they might also be downregulated post-transcriptionally as a consequence of imbalanced S1pr signaling.

The concept that S1pr signaling regulates EC integrity by modulating translocation of junction proteins and actomyosin contractility is widely accepted27, 28. So far, S1pr signaling has not been linked to brain capillary angiogenesis and our data also indicate that the endogenous S1pr signaling is neither required for CtA tip cell specification (Supplementary Fig. 5) nor for other aspects of CtA angiogenesis (Fig. 5). However, as recently shown in mice30, we demonstrate that S1pr signaling indeed regulates BBB formation and integrity in zebrafish (Supplementary Fig. 5). Moreover, our data points to a novel regulatory mechanism of Wnt signaling during brain capillary angiogenesis, which is to counteract S1pr1-induced BBB formation in order to ensure efficient anastomosis (Fig. 5). We propose that Wnt signaling suppresses S1pr1-mediated signaling in CtAs during early stages of brain vascularization. After lumen formation, Wnt signaling activity within brain capillaries decreases to a low baseline level (Fig. 14). In lumenized vessels, activation of S1pr signaling would occur and be most likely enhanced by increased supply of the ligand S1p through the blood circulation41, which would enable functional S1pr signaling for BBB formation and maintenance (Supplementary Fig. 5).

It seems likely that the S1pr1-mediated pathway signals via Gα1i and activates Rac1 (Fig. 67, 49). We hypothesize that over-activation of Rac1 after Wnt signaling inhibition interferes with efficient cellular junction formation during brain capillary angiogenesis. To date, Rac1 activation is well described to promote EC barrier formation in mature blood vessel44, indicating
that Rac1 can act downstream of S1pr signaling in barrier genesis. However, we demonstrate a different regulation in Wnt-responsive brain capillary ECs during capillary angiogenesis, which is similar to our observation of S1pr signaling regulation (Fig. 5). Our data in zebrafish and mouse BECs clearly show altered responsiveness of the ECs to Wnt inhibition depending on their developmental state. This indicates that S1pr-Rac1-mediated regulation of VE-cadherin is differently affecting the modulation of established junctions in mature blood vessels compared to junctions newly forming during brain angiogenesis.

Additionally, over-activation of Rac1 could destabilize junction molecule complexes, as has been shown for initial stages of pharyngeal pouch formation in zebrafish. In this study, the authors suggested that Wnt signaling mediates activation of Rac1 by an unknown mechanism; however, a possible role of S1pr signaling has not been addressed. The authors also observed a...
reduced membrane localization of the adherens junction molecules E-Cadherin and Alcama after Wnt signaling inhibition by morpholino-mediated knockdown of Disheveled, which is in line with our findings during brain angiogenesis. We therefore speculate that similarly to brain capillary ECs, Wnt signaling regulates Rac1-mediated S1pr signaling and thereby modulate junction localization of adhesion molecules also during pharyngeal pouch formation.

During brain neo-vascularization, we and others report diverse functions of Wnt signaling, which regulate many steps of brain capillary angiogenesis and BBB formation and cross-communicate with other signaling cues. First, Wnt signaling is required for the formation of the brain capillary pre-tip cells within the parental vessel, which is dependent on a functional Gpr124-RecK co-receptor complex. Second, Wnt signaling limits S1pr signaling during brain capillary sprouting and promotes brain capillary anastomosis through efficient VE-cadherin and Esama localization to the membrane (as shown in this work). And third, Wnt signaling leads to the induction of BBB components, such as glucose transporter Glut1 and Claudin family members, such as glucose transporter Glut1 and Claudin family

**Methods**

**Zebrafish husbandry and strains.** Zebrafish (Danio rerio) husbandry and embryo maintenance were carried out under standard conditions at 28.5 °C. Embryonic developmental stages are determined according to Ref. 37.

Transgenic lines used in this study were Tg(14TCflox:STOP-loxp-S1pr2-EGFP)iu283, Tg(axin2BAC:Venus-Pest)mu288, Tg(14TCflox:CARlmu211), and Tg(hsp70l:loxP-STOP-BACE1)g11ap. During brain neo-vascularization, we and others report diverse functions of Wnt signaling, which regulate many steps of brain capillary angiogenesis and BBB formation and cross-communicate with other signaling cues. First, Wnt signaling is required for the formation of the brain capillary pre-tip cells within the parental vessel, which is dependent on a functional Gpr124-RecK co-receptor complex. Second, Wnt signaling limits S1pr signaling during brain capillary sprouting and promotes brain capillary anastomosis through efficient VE-cadherin and Esama localization to the membrane (as shown in this work). And third, Wnt signaling leads to the induction of BBB components, such as glucose transporter Glut1 and Claudin family members, such as glucose transporter Glut1 and Claudin family members.

**Microinjections.** For ubiquitous removal of the CRE-labeled STOP cassette by Cre-mediated recombination, 2 nl of cre mRNA (200 pg/nl) were injected at single cell stage. Therefore the cre coding sequence was cloned into pCS2+ and transcribed into mRNA by using the Sp6 mRNA machine kit (Ambion) following NotI-digest as previously described.

For overexpression of S1pr1, the coding sequences of s1pr1, s1pr2, s1pr3a, and s1pr5a were amplified from 24 hf-dDNA and cloned into pCS2+ using the following primers (see also Supplementary Table 1): s1pr1 fwd 5'-ATGGATGACGAGCTTGAACC-3', s1pr2 fwd 5'-ATGACATCAGACTGGCTGTG-3', and s1pr5a rev 5'-GGGATCTGCAAAC-3'. Transcription into mRNA following NotI-digest was carried out using the Sp6 mRNA machine kit (Ambion) as described. 2 nl of 75 ng/µl or 150 ng/µl s1pr1 mRNA, 10 ng/µl s1pr2 mRNA, 150 ng/µl s1pr3a mRNA, or 150 ng/µl s1pr5a mRNA were injected at single cell stage as indicated for each experiment. For agonist experiments, 75 ng/µl s1pr1 mRNA was additionally supplemented with 200 µM Sphingosine-1-phosphate (S1p, Tocris Bioscience, 1370), and for transient CRISPR-Cas9-mediated knockout of s1pr1, annealed template oligonucleotides were transcribed into gRNAs using MEGAscript T7 Kit (Ambion) s1pr1 5'-AAAGCCAGCGACTGGCTGACTTTCAGTATG

**Generation of transgenic Wnt-manipulating lines.** The Tg(hsp70:loxP-STOP-loxp-S1pr2-EGFP)iu283 downstream of a heat shock inducible promoter (hsp70°). A floxed STOP cassette (loxP-STOP-loxp, Hesselson 2009) was inserted upstream of mCherry by Alili-Spel digest. Using the gateway system, hsp70:loxP-STOP-loxp-mCherry-dntf was cloned into the destination vector pTol2Dest31. The Tg(hsp70:loxP-STOP-loxp-mCherry)iu283 line was generated by removing the dntf sequence from Tol2:hsp70:loxP-STOP-loxp-mCherry-dntf Tol2 using Afel-SnBAL digest followed by Klenow blunting and religation. For both lines, standard transgenesis was performed.
AAPCAGTACGCTTTTACCTGTATCTGCTATGCTAAATCCTGCTGAAACGCTCGTATATCGGATGATACCTACTG-3' (spt1 target sequence: 5'-TCTTCTGAAAACCTAGCTTTTG-3'). A total of 2 nL of 500 ng/µL gRNA and 300 ng/µL nls-cas9-nls mRNA were control as were injected at single cell stage. Efficacy of the CRISPR-targeting was accessed by PCR using spt1 fwd 5'-TCTTACGCTGCACTTGG-3' and rev 5'-CCTGATGACGATAGCGACC-3' (see Supplementary Table 1) and a subsequent restriction enzyme digest using Hpy188L. This leads to 136 and 69 bp fragments in control samples and additionally ~205 bp fragments in samples obtained from spt1 CRISPR injected embryos.

Vascular-specific Cre-mediated recombination. For vascular-specific removal of floxed STOP-cassettes, transgenic fish (e.g. Tg(hsp70:loxP-STOP-loxP-mCherry-dntf)β267) were mated with Tg(kdrl:cre)β267. The resulting embryos carrying both transgenes were used for analysis and in the case of heat shock inducible transgenes were labeled by iEC (m.CherryβC, m.Cherry-dTecβC).

Heat shock and pharmacological treatments. In order to induce gene expression by heat shock, 26 hpf-old dechorionated embryos were incubated 40 min at 39 °C in pre-warmed E3 medium and analyzed at indicated developmental stages. For validation of the Tg(hsp70:loxP-STOP-loxP-mCherry-dntf)β267 and Tg(hsp70:loxP-STOP-loxP-mCherry-dntf)β267 lines, embryos were incubated at 10 hpf and 15 hpf at 1 h for 39 °C and analyzed at 24 hpf. For pharmacological treatments, dechorionated embryos were incubated from 5.5 to 24 hpf (for validation of Spt1 antagonists), from 29 to 48 hpf or from 26 to 32 hpf in E3 medium supplemented with 150 µM Aplidicolin (A, Sigma-Aldrich)33, 100 µM Chloroquine (Sigma-Aldrich)27,28, 10 µM CMX5541 (Sigma-Aldrich), 10 µM CMY5541 (Tocris Bioscience), 80 µM Dynasore (Sigma-Aldrich)27,29, 20 mM Hydroxyurea (HU, Sigma-Aldrich)30, 20 µM IWR-1 (Sigma-Aldrich)30, 50 µM JTE013 (JTE, Cayman Chemical), 10 µM MG132 (Sigma-Aldrich)30,62, 100 µM NSC23766 (NSC, Sigma-Aldrich), 5 µg/mL Rho Activator II (Cytoskeleton)59, 2 µg/mL of proteinase K (Roth, 7528.4, 10 µg/mL) for 12 min and re

RNA sequencing of zebrafish brain capillary ECs. For RNA-seq, RNA was isolated from FACS sorted zebrafish CA3 ECs cells using the mirNeasy micro Kit (Qagen) combined with on-column DNA digestion (DNase-Free DNase Set, Qiagen) to avoid contamination by genomic DNA. RNA and library preparation integrity were verified with a Bioanalyzer 2100 (Agilent) or LabChip Gx Touch 24 (Perkin Elmer). RNA amount was adjusted on number of isolated cells by FACS and ~250–500 pg total RNA was used as input for SMART-Seq® v4 Ultra® Low Input RNA Kit (TakaRa Clontech) for cDNA pre-amplification. Obtained full-length cDNA was checked on LabChip and fragmented by UltraSonication by E220 machine (Covaris). Final Library Preparation was performed by Low Input Library Prep Kit 2 (TakaRa Clontech). Sequencing was performed on the NextSeq500 instrument (Illumina) using v2 chemistry, resulting in minimum of 30M reads per library with 1 x 75 bp single end setup. The resulting raw reads were assessed for quality, adapter content, and duplication rates with FastQC (available online at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc) and Trimmed reads were mapped to zebrafish genome (DanDar10) using STAR 2.4.0a with the parameter ‘‐‐outFilterMismatchNmax 0.1’ to increase the number of reads aligned to genes with featureCounts 1.4.5‐p1 tool from the Subread package84. Only reads mapping at least partially inside exons were admitted and aggregated per gene. Reads overlapping multiple genes or aligning to multiple regions were excluded. The Ensembl annotation was enriched with UniProt data (release 06.04.2014) based on Ensembl gene identifiers (activities at the Universal Protein Resource (UniProt)).

RNA isolation from zebrafish embryos and RT-qPCR. RNA was isolated from zebrafish embryos with Trizol reagent (Thermo Fisher Scientific), and CDNA was generated using the SuperScript II reverse transcriptase kit (Invitrogen). Real-time quantitative PCR (RT-qPCR) was conducted using Power SYBR Green (Applied Biosystems) and the following primers (see also Supplementary Table 1): 3′-CCACATGGGAGGCTGAGAGCT-5′ (axin2 fwd), 3′-GTTGGACAGATGACCCCTCC-5′ (β-catenin rev), 3′-TCTATACTGCCAACATCCTG-5′ (gata6 rev), 3′-GAAGAACCATTGCGTTATAGTGAGTCGTATTACGC-5′ (spt1 target), 3′-CTGTGCGACAGACGTCAACT-5′ (gata6 fwd), and 3′-CTGATGACGATAGCGACC-5′ (β-catenin fwd). Obtained full-length cDNA was checked on LabChip and fragmented by UltraSonication by E220 machine (Covaris). Final Library Preparation was performed by Low Input Library Prep Kit 2 (TakaRa Clontech). Sequencing was performed on the NextSeq500 instrument (Illumina) using v2 chemistry, resulting in minimum of 30M reads per library with 1 x 75 bp single end setup. The resulting raw reads were assessed for quality, adapter content, and replication rates with FastQC. admire input for SMART-Seq® v4 Ultra® Low Input RNA Kit (TakaRa Clontech) for cDNA pre-amplification. Obtained full-length cDNA was checked on LabChip and fragmented by UltraSonication by E220 machine (Covaris). Final Library Preparation was performed by Low Input Library Prep Kit 2 (TakaRa Clontech). Sequencing was performed on the NextSeq500 instrument (Illumina) using v2 chemistry, resulting in minimum of 30M reads per library with 1 x 75 bp single end setup. The resulting raw reads were assessed for quality, adapter content, and replication rates with FastQC. admirable input for SMART-Seq® v4 Ultra® Low Input RNA Kit (TakaRa Clontech) for cDNA pre-amplification. Obtained full-length cDNA was checked on LabChip and fragmented by UltraSonication by E220 machine (Covaris). Final Library Preparation was performed by Low Input Library Prep Kit 2 (TakaRa Clontech). Sequencing was performed on the NextSeq500 instrument (Illumina) using v2 chemistry, resulting in minimum of 30M reads per library with 1 x 75 bp single end setup. The resulting raw reads were assessed for quality, adapter content, and duplication rates with FastQC. admirable input for SMART-Seq® v4 Ultra® Low Input RNA Kit (TakaRa Clontech) for cDNA pre-amplification. Obtained full-length cDNA was checked on LabChip and fragmented by UltraSonication by E220 machine (Covaris).
centrifugation (10 min, 3000g) the微vascular fragments were resuspended in EGM2–EBM media supplemented with puromycin (Sigma-Aldrich, P9620, 10 μg/mL) and plated on collagen-1-coated μ-Slide 8-well ibiTreat cell culture chambers (Ibidi, 80826) or 24-well cell culture plates. Hereafter, cells were kept in culture at 37 °C and 5% CO2. Twenty hours later, wells were washed three times with PBS to remove dead and unattached cells and fresh EGM2–EBM2 media supplemented with puromycin (5 μg/mL) was added. The next day, pharmacologic treatment was started with drugs diluted in EGM2–EBM2 media.

IWR-1 (Sigma-Aldrich) and VPC23019 (VPC, Cayman Chemical) were dissolved in DMSO to a 10 mM concentration. Inhibition of Wnt signaling was achieved after 48 h treatment with 10 or 20 μM IWR-1 for endothelial cells from young pups or adult animals, respectively. Alternatively, 10 μM IWR-1-treated cells from young pups were exposed in parallel to 2 μM VPC to assess the effect of St1pr1 signaling blocking upon Wnt inhibition. Vehicle (DMSO) treated was used in all experiments as control.

**Immunohistochemistry of mouse brain primary EC.** After inhibitor treatment, cells growing in 8-well ibiTreat cell culture chambers were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich, P6148) for 15 min at room temperature, washed three times with PBS and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, T8787) in PBS for 15 min. After washing three times with PBS, cells were blocked by incubation with 2% normal donkey serum (Abcam, ab74759) in PBS in a humidified chamber for 30 min. Primary antibodies (goat polyclonal anti-CDS1, R&D Systems, AF5862, 1:100; rat monoclonal anti-VE-cadherin clone 11D4, BD Biosciences, 555289, 1:100; and rabbit polyclonal anti-ZO-1, Invitrogen, 402200; 1:100) were diluted in blocking buffer and incubated with the cells in a humidified chamber overnight at 4 °C. Wells were then washed three times with PBS and secondary antibodies (donkey anti goat Alexa Fluor 488, Invitrogen, A10055, 1:400; donkey anti rat Alexa Fluor Cy3, Jackson Immuno Research, 712-165-153, 1:400; and donkey anti rabbit Alexa Fluor 647, Invitrogen, A31573, 1:400) together with DAPI (Sigma-Aldrich, D9542, 1 μg/mL) were diluted in blocking buffer and incubated at room temperature for 2 h. After secondary antibody incubation, cells were washed as described above and covered with PBS until imaging.

**RT-qPCR of mouse brain primary ECs.** After inhibitor treatment, cells growing in 24-well cell culture plates were collected in 350 μL RTL Plus Buffer (Qiagen, 1053393) supplemented with 1% b-mercaptoethanol (Sigma, M6250) and total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen, 74034) following the manufacturer’s instructions. Whole RNA was quantified in terms of quantity and quality using Bioanalyzer 2000 (Agilent), reverse transcribed and converted to complementary DNA (cDNA) using the Script cDNA synthesis kit (BioRad, 170-8890). The following FAM-conjugated TaqMan gene expression probes (all from Thermo Fisher Scientific) were used: ve-cadherin (Cdh5, Mm00469398_m1), Actb (Mm00465651_m1), Ccm2 (Mm01265780_m1), Lef1 (Mm01310389_m1), and Cdh12 (Mm04336001_m1). VIC-conjugated Actb (4352341E) TaqMan probe was used to normalize gene expression. Quantitative PCR (qPCR) reactions were performed on a CFX96 Touch Real-Time PCR Detection System (BioRad) using the Sure Advanced Universal Probes Supermix (BioRad, 1725281). All relative gene expression analyses were performed using the 2^(-ΔΔCt) method in a minimum of four animals per group with triplicate reactions for each gene evaluated.

**Confocal microscopy.** Confocal microscopy was performed using LSM780 and LSM880 microscopes (Carl Zeiss Microscopy GmbH; objective lenses: Plan-Apochromat ×20/0.8; LD C-Apochromat ×40/1.1 W Korr M27). For confocal microscopy, the Online Focus and Airyscan module. For life imaging of VE-cadherinTS embryos, the Online Focus and Airyscan module. For life imaging of VE-cadherinTS embryos, the Online Focus and Airyscan module.

**Statistical analysis.** For all quantifications, statistical analysis was performed using Prism6 software (GraphPad). Graphs show mean ± standard deviation (SD) or standard error of the mean (SEM) and p-values were calculated using unpaired two-tailed Student’s t-test or one-way ANOVA for single and multiple comparisons as indicated for each experiment.

**Animal models.** All animal experiments were performed in compliance with the relevant laws and institutional guidelines, were approved by local animal ethics committees and were conducted at the University of Münster and the MPI for Molecular Biomedicine with permissions granted by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) of North Rhine-Westphalia.

**Data availability.** The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. The RNA sequencing data of this study have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE121041. The rest of the data are available from the authors upon reasonable request.

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Author contributions
K.H. performed experiments and together with W.H., analyzed the data, created the figures and wrote the manuscript. P.C., S.G., and B.V. performed and analyzed the RNA sequencing experiment, C.W., H.G.B., and M.A. analyzed ve-cadherin and esama mutants, R.D.-H. and R.A. performed and discussed primary mouse brain E.C. experiments, Y.W. and M.H. performed S1pr overexpression and agonist experiments, and K.G. generated dnTcf zebrafish lines. All authors provided experimental suggestions, critical comments, and reviewed the manuscript.

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