Endothelial Notch signalling limits angiogenesis via control of artery formation

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Angiogenic sprouting needs to be tightly controlled. It has been suggested that the Notch ligand dll4 expressed in leading tip cells restricts angiogenesis by activating Notch signalling in trailing stalk cells. Here, we show using live imaging in zebrafish that activation of Notch signalling is rather required in tip cells. Notch activation initially triggers expression of the chemokine receptor cxcr4a. This allows for proper tip cell migration and connection to the pre-existing arterial circulation, ultimately establishing functional arterial–venous blood flow patterns. Subsequently, Notch signalling reduces cxcr4a expression, thereby preventing excessive blood vessel growth. Finally, we find that Notch signalling is dispensable for limiting blood vessel growth during venous plexus formation that does not generate arteries. Together, these findings link the role of Notch signalling in limiting angiogenesis to its role during artery formation and provide a framework for our understanding of the mechanisms underlying blood vessel network expansion and maturation.

The formation of a functional vasculature entails the initial sprouting of new blood vessels and the subsequent development of properly connected arteries and veins. Despite the importance of these processes, we still lack an understanding of the mechanisms balancing blood vessel expansion with artery–vein maturation and the establishment of efficient blood flow patterns. Previous studies have shown that Notch signalling controls angiogenic growth by determining the positions of endothelial cells (ECs) in growing blood vessel sprouts. In this setting, vascular endothelial growth factor (VEGF) triggers expression of the Notch ligand dll4 in leading tip cells, which in turn activates Notch signalling in trailing stalk cells, thereby preventing the formation of supernumerary tip cells. Studies using embryoid bodies suggested that ECs compete for the tip cell position during blood vessel sprouting. Furthermore, Notch signalling is essential for arterial differentiation in zebrafish and mouse embryos prior to the onset of blood flow. Intriguingly, several tip cell–enriched genes, such asDll4(ref. 7), Cxcr4 (refs 16,17) or Apelin18,19, display an arterial restricted expression pattern, raising the possibility that tip cell and arterial fate specification might be coupled. Time-lapse imaging of blood vessel sprouting in zebrafish and genetic lineage tracing in mice showing that vein-derived tip cells contribute to newly forming arteries further supported this notion. However, to date, it is not clear whether Notch signalling can simultaneously control tip cell specification and artery formation. In addition, we have only an incomplete understanding of the Notch downstream signalling molecules in ECs that might mediate these processes.

One key molecule influencing EC migration during angiogenesis is the chemokine receptor cxcr4a. In the early zebrafish embryo and during tissue regeneration, cxcr4a is important for arterial morphogenesis. It also has a role in guiding the formation of the coronary arteries and controls artery–nerve alignment in the mouse skin. Despite the importance of CXCR4 function in these different vascular beds, it is still not clear which signalling pathways control CXCR4 expression in sprouting ECs.

Here we show, using time-lapse imaging in zebrafish embryos, that endothelial tip cells activate the Notch signalling pathway during blood vessel sprouting. We identify the chemokine receptor cxcr4a as an important Notch target during this process. Initially, Notch signalling induces cxcr4a expression, allowing proper tip cell migration towards the arterial circulation, thereby establishing optimal blood flow. At later stages, we observe downregulation of cxcr4a expression via Notch signalling and blood flow, which is important to prevent blood vessel hypersprouting. Together, our results link the role of Notch signalling during artery formation to its role in restricting angiogenesis, and elucidate a complex regulatory interplay between Notch and cxcr4a signalling.

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RESULTS

Live imaging reveals activation of Notch signalling in endothelial tip cells

To investigate Notch signalling during angiogenic sprouting in real time, we chose to study the development of the ocular vasculature in zebrafish embryos (Fig. 1a). In this setting, ECs sprout from the venous primordial midbrain channel (PMBC) and connect to the cranial division of the carotid artery (CrDI), ultimately forming the nasal ciliary artery (NCA)\(^{30,32}\). The main Notch ligand in the vasculature is dll4 (ref. 1). We developed a dll4 reporter transgenic line Tg(dll4:gal4)\(^{mu106}\), Tg(UAS:GFP)\(^{muasgfp14}\). We detected a good correlation between dll4 messenger RNA, GFP mRNA and protein in ECs (Supplementary Fig. 1a,b). We then combined this line with Tg(kdrl-NLS-mcherry)\(^{44}\), which labels all EC nuclei, and performed time-lapse imaging for 12 h (Supplementary Videos 1–3, see also Supplementary Fig. 1c–f). dll4 expression initiated in the leading tip cell (Fig. 1a, cell 1, 2:36 h time point, Fig. 1c) followed by activation of the reporter line in stalk cells (cells 2 and 3, starting at the 5:12 h time point). By contrast, we did not find dll4 expression in cells that kept the connection to the PMBC (Fig. 1a, cell 4, 9:58 h time point, Fig. 1c). Expression of dll4 was also detected in the CrDI, the blood vessel to which the NCA connected (Fig. 1a, arrows) and was still detected in both vessels 12 h after their fusion (50 hours post fertilization (hpf) time point, Supplementary Fig. 2a). NCA sprouting and dll4 expression required VEGF signalling (Supplementary Fig. 2c–f). Therefore, in agreement with previous results in the mouse retina\(^{5,7,10,33,34}\), our analysis revealed that dll4 became activated in tip cells of growing blood vessel sprouts in the zebrafish eye.

We next analysed activation of Notch signalling during the same time frame, using an established Notch indicator line, Tg(TP1:GFP)\(^{jun14}\) (ref. 35) (Fig. 1b,d, Supplementary Videos 4–6 and Supplementary Fig. 2g–j). We expected to observe Notch activation in trailing stalk cells. Surprisingly, Notch activation was first initiated in the leading tip cell (Fig. 1b, 6:15 h time point, d) and increased over time (Fig. 1b, 9:10 h time point, d). Subsequently, trailing cells were also marked by EGF in the Notch indicator line (Fig. 1b, 9:10 h time point, cells 2 and 3, 10 h Fig. 1d). We observed Notch pathway activation several hours after the onset of dll4 expression (compare Fig. 1c,d). We further noted that ECs within the NCA showing Notch pathway activation fused to the Notch-positive CrDI (Fig. 1b, arrows). Both vessels remained Notch positive at 12 h after fusion (50 hpf time point, Supplementary Fig. 2b). To detect possible fluctuations in Notch pathway activation, we analysed Tg(TP1::glob:VenusPEST)\(^{840}\) embryos\(^{86}\), in which the PEST domain reduces fluorescent protein half life\(^{87}\). These embryos also displayed stable activation of the Notch pathway within tip cells (Supplementary Fig. 3 and Supplementary Videos 7–9). Finally, we generated Tg(dll4:gal4)\(^{mu106}\); Tg(UAS:GFP)\(^{muasgfp12}\); Tg(TP1::H2B-mcherry)\(^{939}\) triple transgenic embryos to image dll4 expression and Notch pathway activation simultaneously. These embryos comparably showed activation of dll4 expression followed by Notch pathway activation in leading NCA tip cells (Supplementary Fig. 4 and Supplementary Videos 10–12). Thus, our results show that expression of dll4 precedes Notch activation and that Notch signalling initially occurs in tip cells prior to pathway activation in stalk cells (Fig. 1c).

Notch signalling occurs in trans

Knockdown of dll4 led to an almost complete absence of Tg(TP1::GFP)\(^{jun14}\) activation (Fig. 2a,b and Supplementary Fig. 5a–d and Supplementary Videos 13–15), showing that dll4 is the relevant endothelial Notch ligand. Notch signalling can occur in trans or in cis\(^{38,39}\). To distinguish between these possibilities, we transplanted dll4 knockdown cells that were also transgenic for the Notch indicator line into wild-type (WT) embryos (Fig. 2c). In the case of cis-activation, we would expect to observe absence of Notch activation within dll4-deficient tip cells, while activation in trans would allow for Notch activation from neighbouring, dll4-positive host ECs (Fig. 2d).

We observed Notch pathway activation in dll4 knockdown tip cells when they were in association with WT host cells (Fig. 2e–k and Supplementary Fig. 5e–v). Thus, our results suggest that Notch pathway activation in tip cells occurs in trans.

Notch-ligand-deficient cells can maintain tip cell positions

These transplantation experiments furthermore revealed an unexpected ability of ECs lacking dll4 to occupy tip cell positions. Previous studies proposed that ECs shuffle between tip and stalk positions during angiogenic sprouting and that these behaviours depend on Notch activity in trailing stalk cells\(^{7}\). According to this model, dll4-deficient cells would be excluded from the tip position (Fig. 3a). To corroborate our findings in mosaic NCA s, we examined the distribution of either control- or dll4-morpholino-injected Tg(kdrl::EGFP)\(^{943}\) donor cells within sprouting intersegmental blood vessels (ISVs) (Fig. 3b). This analysis revealed that control-morpholino-injected (Fig. 3c, arrowhead, Fig. 3d) and dll4-morpholino-injected (Fig. 3c, arrow, Fig. 3d) donor cells were equally distributed between tip and stalk cell positions. To investigate whether cell shuffling took place during ISV sprouting, we performed time-lapse analysis of mosaic embryos. These videos showed that dll4-deficient cells could remain in tip cell positions throughout the sprouting process (Fig. 3e, arrowheads, see also Supplementary Fig. 6a–d). Of note, and in agreement with a recent publication\(^{80}\), we also did not detect EC shuffling during ISV sprouting in WT embryos (Supplementary Fig. 6h,i).

Several Notch ligands are expressed in zebrafish ECs, which could potentially activate Notch receptors in the absence of dll4 function. We therefore analysed the distribution of mib (mib) mutant donor cells. Mib mutants lack an essential ubiquitin ligase necessary for Notch ligand processing\(^{41}\). We observed that mib mutant cells were also equally distributed between tip and stalk cell positions during ISV sprouting (Fig. 3f,g and Supplementary Fig. 6e–g). Together, these results indicate that Notch-ligand-deficient cells can occupy tip cell positions during blood vessel sprouting at a similar frequency as WT cells.

Loss of Notch signalling impairs the connection of new blood vessel sprouts to the existing arterial vasculature

On the basis of our observations that Notch signalling is activated within tip cells, we hypothesized that interfering with Notch signalling might compromise tip cell biology. Previous studies showed that Notch signalling controls EC proliferation and vascular expansion\(^{57,18,42}\). In agreement, we found that NCA area and cell numbers were increased in embryos lacking dll4 function (Fig. 4a–c). However, we also detected that NCA-forming cells displayed aberrant migratory behaviours and...
Figure 1 Time-lapse imaging of dll4 and Notch reporter lines during blood vessel development. (a) Still images at the indicated time points of NCA sprouting from the venous PMBC and connecting to the arterial CrDi in Tg(dll4:gal4)\textsuperscript{mu106}; Tg(UAS:GFP)\textsuperscript{nkuasgfp1a}, a dll4 expression reporter (green), and Tg(kdrl:NLS-mcherry)\textsuperscript{is4}, which marks all EC nuclei in red, triple-transgenic embryos (n = 3 embryos). White arrowheads with numbers mark individual ECs. Note the onset of GFP expression in cell number 1 at the 2:36 h time point, followed by GFP expression in cells 2 and 3. The arrows point to CrDi. (b) Still images at the indicated time points of NCA sprouting in Tg(TP1:GFP)\textsuperscript{um14}, a reporter for Notch signalling pathway activation, and Tg(kdrl:NLS-mcherry)\textsuperscript{is4} double-transgenic embryos (n = 3 embryos). The white arrowheads with numbers mark individual ECs. Note the onset of GFP expression in cell number 1 at the 6:15 h time point, followed by GFP expression in cells 2 and 3. Arrows point to CrDi. (c) Quantification of fluorescence intensity of the green Tg(dll4:gal4)\textsuperscript{mu106}; Tg(UAS:GFP)\textsuperscript{nkuasgfp1a} signal normalized to red Tg(kdrl:NLS-mcherry)\textsuperscript{is4} signal over time. (d) Quantification of fluorescence intensity of the green Tg(TP1:GFP)\textsuperscript{um14} signal normalized to red Tg(kdrl:NLS-mcherry)\textsuperscript{is4} signal over time. (e) Comparison of onset of dll4 expression and Notch pathway activation in tip and stalk cells in Tg(dll4:gal4)\textsuperscript{mu106}; Tg(UAS:GFP)\textsuperscript{nkuasgfp1a}; Tg(TP1:H2B-mcherry)\textsuperscript{s939} triple-transgenic embryos (see Supplementary Fig. 4) (n = 3 embryos, 3 independent experiments). Wilcoxon matched pairs-signed rank test. Error bars, mean ± s.e.m. Videos are representative of 3 independent experiments. t\textsubscript{0} = 26 hpf. Scale bars, 50 μm. a.u., arbitrary units; CrDi, cranial division of the internal carotid artery; EC, endothelial cell; h, hour; hpf, hours post fertilization; NCA, nasal ciliary artery; PMBC, primordial midbrain channel.
Figure 2 Dll4 trans-activates Notch signalling in tip cells. (a) Representative images from time-lapse analysis of Notch reporter Tg(TP1:GFP)um14, marking cells with active Notch signalling in green, and endothelial nuclear marker Tg(kdrl:NLS-mcherry)um14, marking EC nuclei in red, double-transgenic embryos injected with dll4 morpholino. (b) Quantification of fluorescent intensity of the green Tg(TP1:GFP)um14 signal normalized to red Tg(kdrl:NLS-mcherry)um14 signal over time. t0 = 26 hpf (n = 3 embryos, 3 independent experiments). (c) Transplantation scheme. Tg(kdrl:Hsa.HRAS-mcherry)u16 marks EC membranes of donor cells in red, while Tg(TP1:GFP)um14 marks donor cells with Notch signalling pathway activation in green. (d) Schematic detailing different outcomes of cell transplantations. Transferring dll4-knockdown cells from Tg(kdrl:Hsa.HRAS-mcherry)u16; Tg(TP1:GFP)um14 into WT hosts allows for distinguishing trans- from cis-activation of Notch signalling in ECs. (e) NCA cells stained with a Fli1b antibody detecting endothelial cell nuclei (white) (n = 3 mosaic embryos with single donor cell at tip position, 3 independent experiments). (f) Transplanted dll4-knockdown ECs (red). (g) Tip cell with activated Notch signalling identified via GFP expression (green arrow). (h) Overlay of transplanted NCA cells with host NCA cells, showing that cells 1 and 4 are donor-derived, while cells 2, 3 and 5 are host-derived. (i) Overlay of transplanted cells showing Notch pathway activation (green arrow) in host cells. Only the tip cell (number 1) shows GFP expression. (j) Overlay image, identifying a transplanted dll4-knockdown tip cell being GFP positive. (k) Zoom-in of the boxed area in j. Note the tip cell (number 1) being next to a WT host cell. Scale bars, 50 μm. a.u., arbitrary units; CrDI, cranial division of the internal carotid artery; EC, endothelial cell; h, hour; hpf, hours post fertilization; MO, morpholino; NCA, nasal ciliary artery; PMBC, primordial midbrain channel. WT, wild type.

Notch regulation of cxcr4a expression is responsible for establishing arterial connections

We speculated that Notch signalling might regulate the expression of the chemokine receptor cxcr4a due to the reported roles of CXCR4 in cell migration and arterial morphogenesis21,22. We detected
Figure 3: Notch signalling does not mediate competition between endothelial cells during intersegmental blood vessel sprouting. (a) Schematic drawing illustrating expected positions of ECs with indicated genotypes within blood vessel sprouts if dll4 would be required to activate Notch signalling in stalk cells (shuffling) or in tip cells (no shuffling). (b) Schematic drawing of transplantation procedure. (c) Representative image of transplanted morpholino-injected donor ECs in ISVs at 28 hpf. The arrowhead marks a transplanted control-morpholino-injected cell, while the arrow marks a dll4-morpholino-injected cell. DA and PCV are indicated. Tg(kdrl:EGFP) marks donor cells by virtue of cytoplasmic EGFP expression in green, while Tg(kdrl:Hsa.HRAS-mcherry) marks host cells by virtue of membrane mcherry expression in red. (d) Quantification of tip cell contribution of control-morpholino-injected (n=21 mosaic ISVs from 17 embryos) or dll4-morpholino-injected (n=19 mosaic ISVs from 15 embryos) donor cells (4 independent experiments). (e) Time-lapse imaging of mosaic ISV sprouts showing a dll4-morpholino-injected cell maintaining the tip cell position (arrowheads; n=3 embryos, 3 independent experiments). (f) Still images showing ECs with the indicated mib genotype maintaining tip cell positions (arrowheads). (g) Quantification of tip cell contribution of WT (n=9 mosaic ISVs from 7 embryos), mib heterozygous (n=26 mosaic ISVs from 19 embryos) or homozygous (n=13 mosaic ISVs from 9 embryos) mutant donor cells (4 independent experiments). Scale bars, 50 μm. Ctr, control; DA, dorsal aorta; EC, endothelial cell; hpf, hours post fertilization; ISV, intersegmental blood vessel; MO, morpholino; PCV, posterior cardinal vein; WT, wild type.

cxcr4a expression in sprouting NCA cells (Fig. 5a, 28 and 32 hpf time points, arrows). In agreement with the regulation of cxcr4a expression by haemodynamic forces, we observed downregulation of cxcr4a expression at the 38 h post fertilization (hpf) time point (Fig. 5a), when the NCA displayed blood flow. The expression of cxcr4a was almost completely absent in newly sprouting ECs in...
Figure 4 Loss of Notch signalling in sprouting ECs causes vascular hyperplasia and aberrant arterial–venous connections. (a) NCA phenotypes in dll4-morpholino-injected Tg(kdr:H2B-GFP)\(^{\text{mu122}}\), marking all EC nuclei in green, and Tg(kdr:Hsa.HRAS-mcherry)\(^{\text{mu14}}\), marking EC membranes in red, double-transgenic embryos. The white dots indicate EC nuclei, and the white dotted lines outline vessel areas. The arrowheads mark connected and perfused NCA in the control-morpholino condition (2 arrowheads), while in dll4-morpholino-injected embryos, NCA connections were dysmorphic (one arrowhead). (b–d) Quantification of NCA EC numbers (b), area (c) and NCA–CrDI connections (d) in control-morpholino-injected (\(n = 20\)) and dll4-morpholino-injected (\(n = 20\)) embryos. (e) NCA blood flow at the 38 hpf time point in control- and dll4-morpholino-injected embryos visualized using Tg(gata1a:dsRed)\(^{\text{sd}}\) embryos. Note blood flow from artery to vein. Tg(kdr:EGFP)\(^{\text{ms14}}\) marks ECs in green. Tg(gata1a:dsRed)\(^{\text{sd}}\) marks erythrocytes in red. Lack of NCA–CrDI connection leads to absence of blood flow in dll4-morpholino-injected embryos. (f) NCA blood flow at the 48 hpf time point in control- and dll4-morpholino-injected embryos. Note blood flow from artery into two veins. Lack of NCA–CrDI connection in dll4-morpholino-injected embryos causes aberrant blood flow patterns, including flow reversal due to direct connection of two veins. (g) Quantification of NCA flow defects in control-morpholino-injected (\(n = 30\)) and dll4-morpholino-injected (\(n = 30\)) embryos at 38 hpf. (h) Quantification of NCA flow defects in control-morpholino-injected (\(n = 30\)) and dll4-morpholino-injected (\(n = 30\)) embryos at 48 hpf. (i) Schematic drawing of NCA–CrDI connection in control-morpholino-injected embryos. The black arrows denote the blood flow direction, and the black arrowheads indicate the direction of EC migration. (j) Schematic drawing of NCA–CrDI connection in dll4-MO-injected embryos. The black arrows denote the blood flow direction, and the black arrowheads indicate the direction of EC migration. Images are representative of 3 independent experiments. Error bars, mean ± s.e.m. For b,c, \(* P < 0.001,\) unpaired Student’s t-test with Welch’s correction. For d,g,h, ***\(P < 0.0001,\) Fischer’s exact test. Scale bars, 50 μm. CrDI, cranial division of the internal carotid artery; Ctr, control; EC, endothelial cell; hpf, hours post fertilization; MO, morpholino; NCA, nasal ciliary artery; PMBC, primordial midbrain channel.
**Figure 5** Notch induction of cxcr4a expression mediates arterial–venous connections. (a) FISH to reveal expression of cxcr4a in NCA ECs in control-morpholino-injected embryos between 28 and 38 hpf (arrows). Tg(kdrl:EGFP)y^843 marks all ECs in green. Expression of cxcr4a becomes downregulated in NCA cells at 38 hpf. (b) Expression of cxcr4a in dll4-morpholino-injected embryos is largely absent (arrows). (n/N reports the number of embryos with staining pattern in image/total embryos from 3 independent experiments, N=10 embryos for each time point.) (c) Loss of cxcr4a function causes NCA-CrDI connection defects starting at the 36 hpf time point (arrows). At the 48 hpf time point, NCA formation is still impaired in cxcr4a-deficient embryos (brackets). (d) Quantification of NCA-CrDI connections in sibling (n=9) and cxcr4a mutant (n=7) embryos at 36 hpf. (e) Loss of cxcr4a function does not lead to changes in NCA EC numbers, as analysed in Tg(fli1a:nEGFP)y^7 fish, marking EC nuclei in green. Brackets indicate location of NCA. (f) Quantification of EC numbers in sibling (n=9) and cxcr4a mutant (n=7) embryos at 36 hpf. (g) Schematic drawing of cxcr4a expression during NCA-CrDI connection. The black arrows denote the blood flow direction; the black arrowheads indicate the direction of EC migration. (h) Schematic drawing of NCA-CrDI connection defects in cxcr4a-deficient embryos. The black arrows denote the blood flow direction; the black arrowheads indicate the direction of EC migration. Images are representative of 3 independent experiments. Error bars, mean ± s.e.m. ***P < 0.0001. For d, Fischer’s exact test. For f, unpaired Student’s t-test with Welch’s correction. Scale bars, 50 μm in b and 30 μm in c,e. FISH, fluorescence in situ hybridization; NS, not significant; Ctr, control; EC, endothelial cell; hpf, hours post fertilization; MO, morpholino; NCA, nasal ciliary artery.

We reasoned that if lack of cxcr4a expression was responsible for the observed failure of NCA cells to connect to the CrDI in embryos lacking proper Notch signalling, then cxcr4a mutants should...
recapitulate this phenotype. Analysis of cxcr4a mutant zebrafish showed an absence of NCA–GrDi connection at the 36 hpf time point (Fig. 5c, arrows). By 48 hpf, the NCA in WT embryos carried blood flow (Fig. 5c, 48 hpf time point, bracket, Supplementary Fig. 7a,b and Supplementary Videos 16 and 17), while cxcr4a mutants displayed a dysmorphic NCA still lacking CrDI connection (Fig. 5c, 48 hpf time point, bracket, 5d, Supplementary Videos 18 and 19). Analysis of EC numbers in cxcr4a mutants revealed no significant difference between siblings and mutants (Fig. 5e,f). Therefore, loss of cxcr4a function recapitulated the migratory phenotype of NCA cells in embryos lacking dll4–Notch signalling without affecting EC (Fig. 5g,h and Supplementary Fig. 7c,d). Together, our findings suggest that Notch signalling induces cxcr4a expression during angiogenic sprouting.

**Loss of cxcr4a function rescues hypersprouting phenotype in Notch-deficient blood vessels**

We next wanted to examine whether the observed regulation of cxcr4a expression via Notch signalling was a general phenomenon. We therefore analysed cxcr4a expression in newly forming intersegmental blood vessel sprouts (ISVs). We detected cxcr4a expression in the dorsal aorta and in sprouting ISVs (Fig. 6a, 28 and 32 hpf time points, arrows). However, at later stages and after the onset of blood flow (2.5 days post fertilization (dpf) time point)44, we observed downregulation of cxcr4a expression in ISVs and in the dorsal longitudinal anastomotic vessel (DLAV; Fig. 6a, 2.5 dpf time point, arrow). By contrast, dll4-morpholino-injected embryos showed an initial reduction of cxcr4a expression (Fig. 6b, arrows, 28 and 32 hpf time points), as we had observed in the eye vasculature. However, at later stages, cxcr4a expression was upregulated (Fig. 6b, 2.5 dpf time point, arrows).

Motivated by these observations, we explored possible genetic interactions between Notch and cxcr4a signalling. To do so, we knocked down dll4 in homozygous cxcr4a mutants. Of interest, while the amount of ectopic sprouts (Fig. 6c, arrows, Fig. 6e) was strongly reduced in dll4-knockdown embryos lacking cxcr4a function, EC numbers were still increased (Fig. 6f). cxcr4a/dll4 double-mutant embryos showed a similar rescue of hypersprouting (Fig. 6d,g,h). Thus, our results show that the hyperangiogenesis phenotype observed in dll4-knockdown embryos is partly due to an increase of cxcr4a expression.

To investigate the mechanism by which loss of Notch signalling might influence cxcr4a expression, we examined ISV blood flow patterns in dll4 knockout embryos. As previously reported8, we observed a reduction of ISVs carrying blood flow, both at the 54 hpf and at the 72 hpf time points (Supplementary Fig. 7e–h). More than 90% of ISVs showing ectopic blood vessel sprouts at 72 hpf did not carry circulation at the 54 hpf time point, while of the ISVs showing no ectopic sprouts, only 60% did not carry blood flow at the 54 hpf time point (Supplementary Fig. 7g). At the 72 hpf time point, almost 80% of ISVs with ectopic sprouts still did not carry blood flow, while only 60% of ISVs without ectopic sprouts did not carry blood flow. We also detected a marked increase in cxcr4a mRNA in the DA and in the DLAV when we blocked blood flow between 48 and 52 hpf (Supplementary Fig. 7h). Together, our findings indicate that Notch signalling differentially regulates cxcr4a expression and that there is a genetic interaction, which may be direct or indirect, between both pathways. They furthermore suggest that aberrant blood flow patterns contribute to increases in cxcr4a expression in dll4-deficient embryos.

**Notch signalling differentially regulates cxcr4 expression**

Using a human umbilical artery endothelial cell (HUVEC) culture system, in which Notch signalling was activated by plating cells on DLL4, we found CXCR4 upregulation between 4 and 8 h of incubation (Fig. 7a). This was unexpected, as previous studies showed that Notch signalling negatively regulates CXCR4 expression in ECs45,46. However, when we exposed HUAECs to DLL4 for longer times (24 h), we observed a comparable downregulation of CXCR4 expression (Fig. 7a). Thus, Notch signalling can differentially regulate CXCR4 expression in cultured ECs, depending on the duration of Notch pathway activation.

To further explore this aspect of Notch signalling, we analysed CXCR4 regulatory regions. This analysis identified three RBPJ-binding sites, the downstream transcription factor of the Notch signalling pathway48 (Fig. 7b), of which site 2 is being conserved among mammals (see Methods). We then performed chromatin immunoprecipitation (ChIP) in basal conditions and 4 h after DLL4-mediated Notch pathway activation. As a control, we used a previously described RBPJ-binding site in the HES4 promoter (Fig. 7c). Surprisingly, all RBPJ-binding sites within CXCR4 regulatory regions showed strong enrichment already in basal conditions (Fig. 7d). Subsequent to Notch pathway activation, there was a trend towards reduction in enrichment, although this was not statistically significant (Fig. 7d). Control regions 5’ and 3’ of the CXCR4 promoter did not show enrichment (Fig. 7d). Thus, our analysis uncovers three RBPJ-binding sites that might control CXCR4 expression. It furthermore suggests that these sites are differentially occupied by RBPJ depending on Notch signalling status.

To determine the significance of each site for CXCR4 expression, we generated a luciferase construct containing 2.5 kilobases upstream of the CXCR4 start codon, as well as the first intron. In keeping with our qPCR results, Notch pathway activation led to an increase in luciferase expression (Fig. 7e). Mutating RBPJ-binding site 1 caused a small reduction of luciferase expression, while mutating the conserved RBPJ-binding site 2 increased luciferase expression already in basal conditions. These results suggest that RBPJ acts as a transcriptional repressor and that Notch pathway activation might release this repressive activity. However, we noted that Notch pathway activation still increased luciferase expression irrespective of functional RBPJ-binding sites. This might argue for the existence of other indirect mechanisms downstream of Notch signalling controlling CXCR4 expression.

To investigate whether Notch signalling can directly influence cxcr4a expression during angiogenesis, we generated triple transgenic embryos Tg(cdh5:gal4)mut1; (UAS:GFP)knuagfla2; (UAS:myc-NICD)lac3. In these embryos, myc-tagged Notch intracellular domain (NICD) is specifically expressed in the vasculature, albeit in a mosaic fashion. We also injected these embryos with dll4 morpholino. Eighty-five per cent of myc+ ECs in ISV sprouts showed cxcr4a expression (Fig. 7f, arrows, Fig. 7g) compared with only 15% of myc- ECs (Fig. 7f, asterisk, Fig. 7g), thus suggesting a cell autonomous role of Notch signalling in inducing cxcr4a expression. Together, our findings argue for an initially repressive function of RBPJ on the CXCR4 promoter that might be relieved in a cell autonomous manner following Notch pathway activation.
**Figure 6** Aberrant cxcr4a expression contributes to excessive blood vessel sprouting in embryos lacking dll4–Notch signalling. (a) FISH to detect cxcr4a expression in sprouting ISVs. Expression starts in newly forming sprouts (arrows, 28 hpf). Expression of cxcr4a is visible in the newly forming DLAV (arrows, 32 hpf). Subsequently, cxcr4a expression is downregulated (arrow, 2.5 dpf). Tg(kdrl:EGFP)s843 marks all ECs in green. (b) FISH to detect cxcr4a expression in sprouting ISVs in dll4-morpholino-injected embryos. Expression is reduced in ISVs (arrow, 28 hpf) and in the newly forming DLAV (arrow, 32 hpf). Subsequently, cxcr4a expression is upregulated in DLAV cells (arrows, 2.5 dpf). (n/N reports the number of embryos with staining pattern in image/total embryos from 3 independent experiments, N = 10 embryos for each time point, except for b, where N = 9 embryos.) (c,d) Loss of cxcr4a function can rescue excessive blood vessel sprouting in dll4-morpholino-injected embryos (c) and dll4<sup>++</sup> mutants (d). Representative pictures of endothelial hypersprouting (arrows) in embryos with the indicated genotypes at 3 dpf. Tg(fli1a:nEGFP)<sup>y7</sup> marks EC nuclei in green, while Tg(-0.8 flt1:RFP)<sup>hu</sup>5333 marks arterial ECs in red. (e) Quantification of ectopic sprouts in embryos (n = 10) with the indicated genotypes. (f) Quantification of EC numbers in embryos (n = 10) with the indicated genotypes. (g) Quantification of ectopic sprouts in embryos (n = 10) with the indicated genotypes. Images are representative of 3 independent experiments. Error bars, mean ± s.e.m.; ***P < 0.0001, Mann–Whitney U-test. Scale bars, 50 µm. NS, not significant; aISV, arterial intersegmental blood vessel; Ctr, control; DA, dorsal aorta; DLAV, dorsal longitudinal anastomotic vessel; EC, endothelial cell; FISH, fluorescence in situ hybridization; hpf, hours post fertilization; dpf, days post fertilization. ISV, intersegmental blood vessel; MO, morpholino; vISV, venous intersegmental blood vessel.

**Notch signalling does not control venous blood vessel sprouting**

Our results suggest that Notch signalling restricts angiogenesis in settings of coordinated artery formation. We therefore wanted to analyse whether Notch signalling also had a role during the expansion of a venous vascular plexus (Fig. 8a–c). Analysis of caudal vein plexus (CVP) formation in dll4-morpholino-injected embryos in comparison
Figure 7 Regulation of CXCR4 expression by Notch. (a) Time course of CXCR4 expression in HUAECs grown on DLL4-coated dishes to activate Notch signalling (n = 4 independent experiments). (b) Schematic representation of the CXCR4 genomic locus. The rectangles represent CXCR4 exons and the arrow represents the transcription start site. The positions of identified RBPJ-binding sites in CXCR4 intron (1 and 2) and promoter (3) are shown and their positions relative to the ATG are indicated above. (c,d) ChIP qPCR to detect RBPJ binding in the regulatory regions of HES4 (c) and CXCR4 (d) (n = 3 independent experiments). The cells were seeded on DLL4 for 4 h. The data are represented as fold enrichment relative to a negative region (alpha Satellite repeat), which was set as 1. The positions of the primers amplifying RBPJ-binding sites (1–3) in CXCR4 regulatory regions, as well as the regions with no RBPJ sites (4–5) are shown in b as red bars. (e) Luciferase assay showing the response of the plasmid containing the WT CXCR4 regulatory region (2.5-kilobase (kb) promoter and intron), as well as the constructs with one or two mutated RBPJ-binding sites (corresponding to the numbers in b), to Notch activation 24 h after seeding the cells on DLL4 (n = 6 independent experiments). (f) EC-specific NICD overexpression in dll4-morpholino-injected Tg(cdh5:gal4ff); (UAS:GFP); (UAS:myc-NICD) triple-transgenic embryos (n = 10), followed by FISH and myc staining to detect cxcr4a and NICD expression, respectively. Myc-NICD-positive (+) ECs express cxcr4a (arrows) whereas myc-NICD negative (−) ECs do not (asterisk). (g) Quantification of cxcr4a expression in myc-NICD-positive (+) and myc-NICD-negative (−) ECs. Images are representative of 3 independent experiments. Error bars, mean ± s.e.m. *P < 0.05, **P < 0.001, ***P < 0.0001, statistical analysis in a,c,d was performed using a paired two-tailed Student’s t-test and in g using Fisher’s exact test. In a the Student’s t-test was performed at each time point by comparing control and DLL4 conditions. In e the data were analysed using repeated measures one-way ANOVA and Tukey’s multiple comparison test. MO, morpholino; hpf, hours post fertilization; reg, regulatory region; mut, mutated; NS, not significant. Scale bar, 50 μm.

to control-morpholino-injected embryos (Fig. 8b and Supplementary Video 20) revealed normal migratory behaviours of ECs (Fig. 8c, 3:10 h time point, arrowheads, Supplementary Video 21), sprout fusion events (Fig. 8c, 5:17 h time point, arrowhead) and lumen formation (Fig. 8c, 7:08 h time point, arrowhead). In addition, we did not observe an increase in EC numbers or CVP area in dll4-mutant embryos.
VENOUS PLEXUS BLOOD VESSEL SPROUTING OCCURS NORMALLY IN THE ABSENCE OF NOTCH SIGNALLING. (A) SCHEMATIC DRAWING INDICATING THE LOCATION OF THE CVP. (B) TIME-LAPSE IMAGING OF CVP SPROUTING (3:10 H, ARROWHEADS), CONNECTION BETWEEN TWO SPROUTS (4:29 H, ARROWHEAD) AND LUMEN FORMATION (7:08 H, ARROWHEAD) IN CONTROL-MORPHOLINO-INJECTED EMBRYO (N=3 EMBRYOS). Tg(fli1a:nEGFP)y7 MARKS EC NUCLEI IN GREEN, WHILE Tg(kdrl:Hsa.HRAS-mcherry)s916 MARKS EC MEMBRANES IN RED. (C) TIME-LAPSE IMAGING OF CVP SPROUTING (3:10 H, ARROWHEADS), CONNECTION BETWEEN TWO SPROUTS (4:29 H, ARROWHEAD) AND LUMEN FORMATION (7:08 H, ARROWHEAD) IN dll4-morpholino-injected embryo (n=3 embryos). (D) COMPARISON OF CVP IN WT AND dll4-MUTANT EMBRYOS. Tg(fli1a:nEGFP)y7 MARKS EC NUCLEI IN GREEN, WHILE Tg(-0.8fltl1:RFP)hu5333 MARKS ARTERIAL BLOOD VESSELS IN RED. (E) QUANTIFICATION OF EC NUMBERS IN WT (N=10) AND dll4 MUTANT (N=10) EMBRYOS. (F) QUANTIFICATION OF CVP AREA IN WT (N=10) AND dll4 MUTANT (N=10) EMBRYOS. (G) ANALYSIS OF NOTCH ACTIVATION IN ECs OF THE CVP IN Tg(TP1:GFP)um14; Tg(kdrl:Hsa.HRAS-mcherry)s916 DOUBLE-TRANSGENIC ZEBRAFISH (N=10 EMBRYOS). NO GFP EXPRESSION CAN BE DETECTED IN ECs AT ANY GIVEN TIME POINT (ARROWHEADS). VIDEOS AND IMAGES ARE REPRESENTATIVE OF 3 INDEPENDENT EXPERIMENTS. ERROR BARS, MEAN ± S.E.M. UNPAIRED T-TEST WITH WELCH’S CORRECTION. SCALE BARS, 50 µM. CTR, CONTROL; CVP, CAUDAL VEIN PLEXUS; DA, DORSAL AORTA; DV, DORSAL VEIN; EC, ENDOTHELIAL CELL; HPF, HOURS POST FERTILIZATION; MO, MORPHOLINO; PCV, POSTERIOR CARDINAL VEIN; VV, VENTRAL VEIN; NS, NOT SIGNIFICANT.

DISCUSSION

In this report we used time-lapse imaging to examine the spatio-temporal sequence of Notch pathway activation within angiogenic blood vessel sprouts. Previous reports investigated activation of Notch signalling at fixed time points. We have now found that dll4 expression preceded Notch pathway activation both in leading tip and subsequently in trailing stalk cells. These cells ultimately connected to a Notch-positive arterial blood vessel, thereby terminating the angiogenesis program. Our results therefore show how Notch pathway activation and artery formation are integrated during angiogenesis (Supplementary Fig. 8c,d). They help to explain how growing tissues can generate the appropriate amount of arteries, as these are continuously being generated from venous-derived tip cells that activate Notch signalling. These will subsequently be exposed to arterial blood flow patterns, which can induce the expression of arterial genes.
These discoveries redefine the role of Notch signalling during angiogenesis in comparison with current models of blood vessel sprouting, where DLL4 from tip cells would activate Notch signalling in trailing stalk cells and prevent the formation of excessive tip cells (Supplementary Fig. 8c,d). Our work suggests important differences concerning Notch signalling pathway activation during blood vessel sprouting in distinct settings. We previously suggested that tip cells in ISV sprouts lack Notch signalling. However, this might be a special scenario, since ISVs do not sprout from a vein, but rather from an artery.

An outstanding unresolved question is why we do not observe activation of Notch signalling in stalk cells first, as tip cells express DLL4 prior to stalk cells. One potential explanation is that stalk cells are less competent to receive Notch signalling compared with tip cells, a possibility we are currently investigating. Alternatively, cis-inhibition\textsuperscript{18}, where interaction between the ligand and the receptor present on the same cell leads to endocytosis and further degradation of the receptor–ligand complex, might influence Notch pathway activation in tip and stalk cells.

Loss of Notch signalling leads to overproliferation of ECs and the formation of a hyperbranched vasculature. Previous studies showed that PTEN mediates the Notch-induced proliferation arrest in ECs\textsuperscript{48}. We now identify the chemokine receptor CXCR4 as another critical downstream target of Notch signalling, which specifically regulates cell migration. Thus, our work suggests that distinct target genes are responsible for the different phenotypes observed in angiogenic blood vessels lacking proper Notch signalling. We furthermore uncover temporal differences in CXCR4 regulation by Notch. After an initial increase in expression, Notch signalling subsequently negatively regulates CXCR4 expression. Our work reveals that the duality of this regulation is important, since early loss of CXCR4 causes migratory defects in tip cells, while ectopic expression at later stages contributes to excessive blood vessel sprouting. A better understanding of the regulatory modules that mediate the observed temporal differences in CXCR4 expression downstream of Notch signalling will therefore be of great future interest.

A report analysing artery formation during angiogenic blood vessel sprouting in the mouse retina similarly found a role for Notch signalling in limiting CXCR4 expression\textsuperscript{49}. In addition, the authors provide evidence that also in this setting, Notch pathway activation in endothelial tip cells is necessary for these cells to incorporate into nascent arteries. Thus, the function of Notch signalling in regulating angiogenesis via artery formation appears to be conserved between mice and zebrafish. However, Pitulescu et al. did not observe an initial induction of CXCR4 expression via Notch signalling. They propose that Notch signalling is rather required to limit endothelial VEGF mRNA levels, thereby secondarily affecting CXCR4 expression. Future work will be necessary to better understand to which extent these observations reflect temporal or species-specific differences in the control of CXCR4 expression downstream of Notch pathway activation.

Ectopic CXCR4 expression can be detected on tumour ECs\textsuperscript{46}, which strongly respond to inhibition of Notch signalling\textsuperscript{29}. Our findings will therefore also be of importance for improving treatments aiming at controlling blood vessel formation in pathological settings, when achieving correctly patterned vascular networks is a therapeutic aim.\underline{50}

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

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

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**AUTHOR CONTRIBUTIONS**

S.H. and A.F.S. designed the experiments, performed experiments, analysed the data and wrote the manuscript. M.L. performed in situ hybridization and analysed data. R.T. performed HUAEC experiments, qPCR analysis and ChIP. L.W. analysed data. K.W. analysed data. J.C.M. and N.D.L. provided the fli1b antibody. H.S. provided HUAECs.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Zebrafish strains. Zebrafish were maintained as described previously 27. Embryos were staged by hours post fertilization (hpf) at 28.5°C (ref. 52). The following transgenic lines were used: Tg(kdrb1:NLS-mcherry) 30, Tg (dll4:gal4) 9 (this study), Tg(UAS:GFP) 77, Tg(Tp1:GFP) 31 (ref. 55), Tg(gdr:HA2B-EGFP) 22, Tg(kdrb1:EGFP) 22 (ref. 56), Tg(flxEGFP) 78 (ref. 57), Tg(kdrt:Hras-mCherry) 19 (ref. 58), Tg(k1a:EGFP) 31 (ref. 59), Tg(0.8fl1:RFP) 22 (ref. 60), Tg(gata1:DsRed) 61, cxcr4a 22 (ref. 26), dll1kca (ref. 62), Tg(Tp1:Venus-Pest) 39 (ref. 36), Tg(Tp1:H2B-mcherry) 64,65 (ref. 36), Tg(ci/8s20^gal4) 22 (ref. 16) abbreviated as Tg(ci/8s20^gal4) 64,65, Tg(UASmyc-Notch1a-intra) 66 (ref. 63) abbreviated as Tg(UASmyc-Notch1a-intra) 66.

Zebraline used in this study were between 1 and 2 years of age and were not selected for gender. All animal experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by local animal ethics committees of the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen.

Generation of Tg(dll4:gal4) 86 86/86 line. We applied BAC recombineering as described previously 86/86 using BAC CH211-19M2 and modified with a PCR-clamp fragment amplified from pCS2 86/86 86/86 with primers dll4_gal4FF_fw: 3'-ccttctgggg aataatactggtgagctgctgagaagtaacccATCATAGAATCAGTCCCTATTC GaAC-5' and dll4_kan_rev: 3'-tgcgtcaaaacggtcgtgaaaaatgcgatgagaaaggtgagccaagc tgctcAGAACTGCTCAGAAGGGCC-5'.

Live imaging, confocal microscopy and image processing. For in vivo imaging, live embryos were mounted in 1% low-melting-point agarose in E3 embryo medium with 168 mg l−1 tricine (1xtimes) for anaesthetization and 0.03% phenolthiourea to inhibit pigmentation. Imaging was carried out on SP8 or SP8 (Inverted) confocal microscopes using a 20 x 40 dry objective (Leica Microsystems). A heated microscope chamber at 28.5°C was used for recording time-lapse videos. Stacks were taken every 15–25 min with a step size of 2.0 μm. Confocal stacks and time-lapse videos were analysed using IMARIS Software (Bitplane) and ImageJ (NIH). Vessels in close proximity to the GDI and NCA were cropped for better visualization.

Quantifications. To quantify the fluorescence intensity of the reporter lines for eye vessel live imaging experiments, surface rendering was performed around eye vessel live imaging experiments, surface rendering was performed around eye vessel live imaging experiments, surface rendering was performed around eye vessel live imaging experiments, surface rendering was performed around

Chromatin immunoprecipitation (ChIP). Zebrafish were starved overnight in 0.1% FCS-containing medium and seeded on DLL4-coated or uncoated dishes in the same medium. After 4 h the cells were fixed with 1% formaldehyde for 5 min at room temperature. The crosslinking was stopped with 0.125 M glycine and after washing with cold PBS, cells were collected by scraping in PBS containing protease inhibitors. The cells were then resuspended in the nuclease buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) and sonicated with Covaris S1 to obtain DNA fragments of 250 bp on average. Chromatin was diluted (final buffer composition: 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% NLaCl, 1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate) and an aliquot was used to confirm sonication efficiency on Bioanalyzer (Agilent) and to quantify chromatin amount. Five micrograms of chromatin was incubated with 2 μg RBPJ antibody (Abcam, ab25949) overnight and then 50 μl of pre-washed Dynabeads Protein G (Life Technologies) was added for 4 h to each reaction to pull down DNA/protein complexes. The beads were then washed 5 times with the buffers with increasing ionic strength and resuspended in the buffer containing 10 mM Tris-HCl pH 8.0, 0.3 M NaCl, 5 mM EDTA pH 8.0, 0.5% SDS. The beads were then incubated with RNase A at 65°C for 6 h and with Proteinase K at 55°C overnight. DNA was then isolated by phenol/chloroform extraction and ethanol precipitation and resuspended in TE buffer. An aliquot of chromatin and DNA was isolated in the same way. Input and ChIP DNA was used for qPCR using SYBR Green as described above. HES4 primers (7273, Cell Signaling) were used as positive and alpha Satellite repeat primers (Abcam, ab85782) as negative control. The primer sequences of ChIP were used for qPCR analysis with FAM-labeled probes and quencher labeled probes.

Cell culture experiments. Human umbilical artery endothelial cells (HUAEcs) were isolated from umbilical cords of anonymized donors as described previously 79 and according to the principles outlined in the Declaration of Helsinki; this was approved by the ethics board of the WW-University of Münster (2009-537-F-S). HUAECs were cultured in M200 medium with LSGS supplements (Invitrogen) and used for experiments until passage 5. For stimulation with DLL4, cell culture dishes (ibidi) were coated overnight at 4°C with 10 μg ml−1 DLL4 (R&D) diluted in PBS with 0.2% gelatin (Sigma). The cells were then starved overnight in M200 medium supplemented with 0.1% FCS (Sigma) and reseeded on the dishes coated with DLL4 or only gelatin in the starvation medium. No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample. The cell lines were not authenticated. The cell lines were not tested for mycoplasma contamination.

Quantitative polymerase chain reaction (qPCR). RNA was isolated with RNasy Plus Micro kit (Qiagen) from HUAECs and reverse transcribed with Script cDNA Synthesis Kit (BioRad). For qPCR, cDNA produced from 5 ng RNA and 8 pmol of each forward and reverse primer per reaction and Power SYBR Green PCR Master Mix (Applied Biosystems) were used. Relative expression was quantified by the ΔΔCt method using RPL13A as an endogenous control. At each time point, the expression in the control sample (cells grown on gelatin alone) was set as 1 (0 on log, scale). ΔCt values were used for statistical analysis. The following primers were used:

- CXCR4-3-fwd: 5'-GCCCTCTCTGCTGACTTCC-3';
- CXCR4-rev: 5'-GGCAGGATAAGGGCAAACATC-3';
- RPL13A-fwd: 5'-TCGTAACGCTGTGAAGGGCATC-3';
- RPL13A-rev: 5'-CAGCATACCTCAGCAACGTC-3'.

Chromatin immunoprecipitation (ChIP). HUAECs were starved overnight in 0.1% FCS-containing medium and seeded on DLL4-coated or uncoated dishes in the same medium. After 4 h the cells were fixed with 1% formaldehyde for 5 min at room temperature. The crosslinking was stopped with 0.125 M glycine and after washing with cold PBS, cells were collected by scraping in PBS containing protease inhibitors. The cells were then resuspended in the nuclease buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) and sonicated with Covaris S1 to obtain DNA fragments of 250 bp on average. Chromatin was diluted (final buffer composition: 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% NaCl, 1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate) and an aliquot was used to confirm sonication efficiency on Bioanalyzer (Agilent) and to quantify chromatin amount. Five micrograms of chromatin was incubated with 2 μg RBPJ antibody (Abcam, ab25949) overnight and then 50 μl of pre-washed Dynabeads Protein G (Life Technologies) was added for 4 h to each reaction to pull down DNA/protein complexes. The beads were then washed 5 times with the buffers with increasing ionic strength and resuspended in the buffer containing 10 mM Tris-HCl pH 8.0, 0.3 M NaCl, 5 mM EDTA pH 8.0, 0.5% SDS. The beads were then incubated with RNase A at 65°C for 6 h and with Proteinase K at 55°C overnight. DNA was then isolated by phenol/chloroform extraction and ethanol precipitation and resuspended in TE buffer. An aliquot of chromatin and DNA was isolated in the same way. Input and ChIP DNA was used for qPCR using SYBR Green as described above. HES4 primers (7273, Cell Signaling) were used as positive and alpha Satellite repeat primers (Abcam, ab85782) as negative control. To identify potential RBPJ-binding sites, we analysed CXCR4 regulatory sequences with the FIMO algorithm of the MEME Suite using the SUH_HUMAN.H10MO.C matrix from the HOCOMOCO database. In addition we investigated conservation of RBPJ-binding sites between human and mouse using Molan and multiTF algorithms and VSRBPX_01 matrix. The primers amplifying the regions containing RBPJ-binding sites predicted by FIMO were:

- CXCR4-1-fwd: 5'-TGAGAATCCTCAACAATCTCCTCCTCCATCT-3';
- CXCR4-1-rev: 5'-ACCCTCAGCCTGCTAGTCCGTCT-3';
- CXCR4-2-fwd: 5'-GGTTGCGAACAGACATCTCCTCATAA-3';
- CXCR4-2-rev: 5'-GCAATACGCTCAAAACCCAGGAA-3';
- CXCR4-3-fwd: 5'-TGCTTGCTGAGAGACCAGTTG-3';
- CXCR4-3-rev: 5'-AAGAGATACGTGATGACGCGCT-3'.

In addition the primers to amplify the regions upstream and downstream of CXCR4 containing no predicted RBPJ-binding sites were:

- CXCR4-4-fwd: 5'-CTGCTACCCGCTAATCGTCTGAG-3';
- CXCR4-4-rev: 5'-TCTATATCTGGAGCCACACAAA-3';
- CXCR4-5-fwd: 5'-GGTTAGATTAGGCTTCTCCAGACTGAA-3';
- CXCR4-5-rev: 5'-ACAGCAGGATAGGGCGAAAGGG-3'.

The data were calculated as percentage of input and then normalized to a negative region (alpha Satellite repeat) to obtain fold enrichment.
Luciferase assay. For luciferase assays, we cloned a 2.5-kilobase upstream piece of the human CXCR4 promoter including the first intron via PCR and restriction digest into pGL4.10 (Promega). The promoter piece was amplified with PfuU polymerase from a BAC containing the human CXCR4 locus (IMAGE873781221860; Source Bioscience) using primers Hs cxcr4 Pr 2.5 P (GGATGAACCCAAAAATCTTGTTCAAC- TATTCCACTAACAGT) and Intron R (GTGGTCTGAGTGGCCAGCAGG) and subsequently cloned into pSCE Blunt (Stratagene). The intron piece was amplified with primers Intron F (TGAGGCGAGGGCCCTGAGTGCCT) and Hs cxcr4 Bsp R (TTATCATGATTCCTCGAGCCCATTTCTCGGTAGCAG) and cloned into pSCE Blunt. The promoter piece was then digested using SpeI and Acc65I, while the intron piece was digested using Acc65I and BspHI and pGL 4.10 using Nhel and Ncol. The three pieces were subsequently ligated together and confirmed via sequencing.

The predicted intronic RBPI-binding sites were mutated as follows: 5' site gcgtgggaaaa into gcgtCCgaC, the 3' site catgggaaaa into ctaCCggCTAa. We also mutated both sites simultaneously. Mutated nucleotides are shown in capital letters.

HUAECs were starved in the medium with no FCS for 4 h and transfected for 45 min with CXCR4 constructs using Lipofectamine 2000 and Plus Reagent (Invitrogen). Immediately after transfection, the cells were reseeded in 0.1% FCS-containing medium on the dishes, pre-coated with DLL4 as described above. Twenty-four hours after seeding, the cells were lysed and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega). For normalization the cells were co-transfected with pRL-CMV vector (Promega) and the values of firefly luciferase activity were divided by the values of Renilla luciferase.

Statistics and reproducibility. Sample sizes were not predetermined, the experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment. Column statistics were performed on data sets to check for normal distribution and appropriate tests were performed. Each experiment was performed at least three times with the exception of RNA data sets to check for normal distribution and appropriate tests were performed.

Data availability. All data supporting the findings of this study are available from the corresponding author on reasonable request.

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Supplementary Figure 1 Validation and activation of dll4 transgenic reporter line. (a) Double FISH of dll4 mRNA together with GFP mRNA and an antibody staining to detect GFP protein at different time points in Tg(dll4:gal4)mu106; Tg(UAS:GFP)nkuasgfp1a double transgenic embryos to mark ECs with dll4 expression. Inset indicates zoomed in NCA region below (n=8 embryos for each time point, 2 independent experiments). (b) Double FISH of dll4 mRNA together with GFP mRNA and an antibody staining to detect GFP protein in the trunk region of a zebrafish embryo at 24 hpf. Insets mark zoomed in region. Arrowheads mark dll4 mRNA (red), GFP mRNA (green), GFP protein (white) or overlay of channels (n=8 embryos, 2 independent experiments). (c) Still images of video 2 at indicated time points of NCA sprouting from the PMBC and connecting to the CdI in Tg(dll4:gal4)mu106; Tg(UAS:GFP)nkuasgfp1a; Tg(kdrl:NLS-mcherry)iso4 triple transgenic embryos. White arrowheads with numbers mark individual ECs. Note onset of GFP expression in cell number 1 at the 2:36 h time point, followed by GFP expression in cells 2 and 3. (d) Still images of video 3 at indicated time points of NCA sprouting from the PMBC and connecting to the CdI in Tg(dll4:gal4)mu106; Tg(UAS:GFP)nkuasgfp1a; Tg(kdrl:NLS-mcherry)iso4 triple transgenic embryos. White arrowheads with numbers mark individual ECs. Note onset of GFP expression in cell number 1 and 2 at the 5:00 h time point, followed by GFP expression in cell number 3. (e) Quantification of fluorescence intensity of the green Tg(dll4:gal4)mu106; Tg(UAS:GFP)nkuasgfp1a signal normalized to red Tg(kdrl:NLS-mcherry)iso4 signal over time in video 2. (f) Quantification of fluorescence intensity of the green Tg(dll4:gal4)mu106; Tg(UAS:GFP)nkuasgfp1a signal normalized to red Tg(kdrl:NLS-mcherry)iso4 signal over time in video 3. (n=3 embryos, 3 independent experiments). t₀= 26 hpf. Scale bars, 50 μm. A.U.-arbitrary units; CdI-cranial division of the internal carotid artery; EC-endothelial cell; h-hour; hpf-hours post fertilization; ISV-intersegmental blood vessel; NCA-nasal ciliary artery; PMBC-primordial midbrain channel; FISH-Fluorescent in situ hybridization.
**Supplementary Figure 2** Notch signalling activity in newly forming arteries.

(a) Living triple transgenic Tg(dll4:gal4)um106; Tg(UAS:GFP)nkuasgfp1a; Tg(kdrl:HRAS-mcherry)s916 zebrafish embryo at 50 hpf. Tg(dll4:gal4)um106; Tg(UAS:GFP)nkuasgfp1a double transgenic embryos mark ECs with dll4 expression in green, while Tg(kdrl:HRAS-mcherry)s916 marks EC membranes in red. Note EGFP expression in NCA (n=10 embryos, 3 independent experiments).

(b) Living double transgenic Tg(TP1:GFP)um14; Tg(kdrl:HRAS-mcherry)s916 zebrafish embryo. Tg(TP1:GFP)um14 marks cells with Notch pathway activation in green (n=10 embryos, 3 independent experiments).

(c) Transplantation scheme. Kdrl knockdown cells (green) were transferred into wt hosts (red).

(d) Representative embryo, showing sprouting NCA cells at 26 hpf time point. Note absence of green fluorescent donor ECs in sprouting NCA (n=9 embryos, 3 independent experiments).

(e) dll4 expression in sprouting NCA cells in control embryo at 30 hpf after DMSO treatment for 4 h and experimental embryo at 30 hpf after SU5416 (VEGF inhibitor) treatment for 4 h (n=10 embryos, 2 independent experiments). Arrowheads indicate dll4 expressing ECs, marked by Tg(kdrl:EGFP)um14. In experimental embryos, while ECs are present in the position of the NCA, these do not express dll4 (n=10 embryos).

(f) dll4 expression in sprouting NCA cells in control embryo at 32 hpf after DMSO treatment for 4 h and experimental embryo at 30 hpf after SU5416 (VEGF inhibitor) treatment for 4 h (n=9 embryos, 3 independent experiments). Arrowheads indicate dll4 expressing ECs, marked by Tg(kdrl:EGFP)um14. In experimental embryos, while ECs are present in the position of the NCA, these do not express dll4 (n=10 embryos).

(g, h) Still images of g video 2 and h video 3 at indicated time points of NCA sprouting in Tg(TP1:GFP)um14; Tg(kdrl:NLS-mcherry)is4 double transgenic embryos. White arrowheads with numbers indicate individual ECs. Note onset of GFP expression in cell number 1 at the 4:35 h time point, followed by GFP expression in cells 3 and 4 in video 2. Note onset of GFP expression in cell number 1 at the 3:28 h time point, followed by division of this cell, in video 3. GFP expression can be subsequently detected in cell 2.

(i, j) Quantification of fluorescence intensity of green Tg(TP1:GFP)um14 signal normalized to red Tg(kdrl:NLS-mcherry)is4 signal over time in i video 2 and j video 3. (n=3 embryos, 3 independent experiments). Scale bars, 50 μm. A.U.-arbitrary units; CrDI-Cranial division of the internal carotid artery; PMBC-primordial midbrain channel; NCA-nasal ciliary artery; h-hour; hpf-hours post fertilization.
Supplementary Figure 3: Activation of Notch signalling in transgenic reporter zebrafish line expressing destabilized Venus protein in sprouting eye blood vessels. (a) Still images of video 1 at indicated time points of NCA sprouting in Tg(TP1:Venus-PEST)^s940; Tg(kdrl:NLS-mcherry)^s34 double transgenic embryos. Tg(TP1:Venus-PEST)^s940 marks cells with Notch pathway activation in green. Tg(kdrl:NLS-mcherry)^s34 marks EC nuclei in red. White arrowheads with numbers mark individual ECs. Note onset of Venus-PEST expression in cell number 1.1 at the 3:28 h time point, followed by Venus-PEST expression in cells 1.2 and 3. (b) Still images of video 2 at indicated time points of NCA sprouting in Tg(TP1:Venus-PEST)^s940; Tg(kdrl:NLS-mcherry)^s34 double transgenic embryos. White arrowheads with numbers mark individual ECs. Note onset of GFP expression in cell number 1.1 and 1.2 at the 7:37 h time point. (c) Still images of video 3 at indicated time points of NCA sprouting in Tg(TP1:Venus-PEST)^s940; Tg(kdrl:NLS-mcherry)^s34 double transgenic embryos. White arrowheads with numbers mark individual ECs. Note onset of Venus-PEST expression in cell number 1 at the 6:36 h time point. (d) Quantification of fluorescence intensity of green Tg(TP1:Venus-PEST)^s940 signal normalized to red Tg(kdrl:NLS-mcherry)^s34 signal over time in video 1. (e) Quantification of fluorescence intensity of green Tg(TP1:Venus-PEST)^s940 signal normalized to red Tg(kdrl:NLS-mcherry)^s34 signal over time in video 2. (f) Quantification of fluorescence intensity of green Tg(TP1:Venus-PEST)^s940 signal normalized to red Tg(kdrl:NLS-mcherry)^s34 signal over time in video 3. (n=3 embryos, 3 independent experiments). t₀ = 26 hpf. Scale bars, 50 μm. A.U.-arbitrary units; CrDI-Cranial division of the internal carotid artery; PMBC-primordial midbrain channel; NCA-nasal ciliary artery; hpf-hours post fertilization.
Supplementary Figure 4 Hasan et al. Simultaneous detection of dll4 expression and Notch pathway activation during NCA formation. Still images at indicated time points of NCA sprouting in Tg(dll4:gal4)mut106; Tg(UAS:GFP)nkuasgfp1a; Tg(TP1:H2B-mcherry)s939 triple transgenic embryos to detect dll4 expression and Notch pathway activation simultaneously. Tg(dll4:gal4)mut106; Tg(UAS:GFP)nkuasgfp1a marks cells with dll4 expression in green, while Tg(TP1:H2B-mcherry)s939 marks cells with Notch pathway activation in red. White arrowheads with numbers mark individual ECs. (a) Video 1: Note onset of dll4 expression in cell number 1 at the 2:15 h time point, followed by Notch activation at 5:10 h time point. (b) Video 2: Note onset of dll4 expression in cell number 1 at the 1:16 h time point, followed by Notch activation at 5:34 h time point. (c) Video 3: Note onset of dll4 expression in cell number 1 at the 2:16 h time point, followed by Notch activation at 7:21 h time point. (n=3 embryos, 3 independent experiments). t₀ = 26 hpf. Scale bars, 50 μm. CrDI-Cranial division of the internal carotid artery; EC-endothelial cell; NCA-nasal ciliary artery; h-hour; hpf-hours post fertilization.
Supplementary Figure 5 Absence of Notch activation in dll4 knockdown embryos during NCA formation. (a, b) Still images of a video 2 and b video 3 at indicated time points of NCA sprouting in Tg(TP1:GFP)um14; Tg(kdrl:NLS-mcherry)id4 double transgenic embryo injected with dll4 MO. Tg(TP1:GFP)um14 marks cells with Notch pathway activation in green, while Tg(kdrl:NLS-mcherry)id4 marks EC nuclei in red. White arrowheads with numbers indicate individual ECs. (c, d) Quantification of fluorescence intensity of green Tg(TP1:GFP)um14 signal normalized to red Tg(kdrl:NLS-mcherry)id4 signal over time in c video 2 and d video 3. t0 = 26 hpf. (n=3 embryos, 3 independent experiments). (e) Transplantation scheme. Transferring dll4 knockdown cells from Tg(kdrl:Hsa.HRAS-mcherry) id4, Tg(TP1:GFP)um14 into wt hosts allows to distinguish trans- from cis- activation of Notch signalling in ECs. Tg(kdrl:Hsa.HRAS-mcherry) id4 marks donor EC membranes in red, while Tg(TP1:GFP)um14 marks donor cells with Notch signalling pathway activation in green. (f-h) Schematic detailing different outcomes of cell transplantsations. (i-o) Embryo 2. (p-v) Embryo 3

(i, p) NCA cells stained with a Fli1b antibody detecting EC nuclei (white). (j, q) Transplanted dll4 knockdown ECs. Arrows mark tip cells. (k, r) Cells with activated Notch signalling identified via GFP expression (green arrows). (l, s) Overlay of transplanted NCA cells with host NCA cells, showing that only cell 1 is donor-derived, while cells 2, 3, 4 and 5 are host-derived. (m, t) Overlay of transplanted cells showing Notch pathway activation (green arrows) with host cells. Only the tip cell (number 1) shows GFP expression. (n, u) Overlay of all three channels, identifying transplanted dll4 knockdown tip cell being GFP positive. (o) Zoom in of boxed area in (n). Note tip cell (number 1) being next to a wt host cell (number 2). (v) Zoom in of boxed area in (o). Note tip cell (number 1) being next to a wt host cell (number 2). Green arrows denote Notch pathway activation (n=3 mosaic embryos with single donor cell at tip position, 3 independent experiments). Scale bars, 50 μm. Ab-antibody; EC-endothelial cell; hpf-hours post fertilization; CrDI-Cranial division of the internal carotid artery; PMBC-primordial midbrain channel; NCA-nasal ciliary artery.
Supplementary Figure 6  Notch signalling does not mediate competition between endothelial cells during intersegmental blood vessel sprouting. (a) Transplantation scheme. Tg(kdrl:EGFP)y1 marks donor cells that were either injected with a ctr or dll4 MO in green. Tg(kdrl:Hsa.HRAS-mcherry)s916 marks host vasculature in red. (b) Representative image of mosaic embryos at 32 hpf. Arrowheads mark donor cells at tip positions. (c) Quantification of contribution of ctr MO (n=2 embryos, 2 independent experiments) or homozygous dll4 mutant (n=12 mosaic embryos in 3 independent experiments) ECs to different positions within the trunk vasculature. Images are representative of three independent experiments. (b) Still images of video 1 at indicated time points of ISV sprouting. Tg(fli1a:nEGFP)y7 marks EC nuclei in green. Numbers delineate individual ECs or their progeny. Images are representative of three independent experiments.

(c) Quantification of contribution of mib sibling (n=17 mosaic embryos in 3 independent experiments) or homozygous mib mutant (n=12 mosaic embryos in 3 independent experiments) ECs to different positions within the trunk vasculature. Images are representative of three independent experiments. (d) Still images of video 1 at indicated time points of ISV sprouting. Tg(fli1a:nEGFP)y7 marks EC nuclei in green. Numbers delineate individual ECs or their progeny. Out of 10 ISVs observed, we only detected one shuffling event (white arrowhead in panel g). (f) Representative image of mosaic embryos at 32 hpf. Arrowheads mark donor cells at tip positions. (g) Quantification of contribution of mib sibling (n=17 mosaic embryos in 3 independent experiments) or homozygous mib mutant (n=12 mosaic embryos in 3 independent experiments) ECs to different positions within the trunk vasculature. Images are representative of three independent experiments. (h) Still images of video 2 at indicated time points of ISV sprouting. Tg(fli1a:nEGFP)y7 marks EC nuclei in green. Numbers delineate individual ECs or their progeny. Out of 10 ISVs observed, we did not detect EC shuffling. Scale bars, 50 μm. aISV-arterial intersegmental vessel; ctr-control; DA-dorsal aorta; DLAV-dorsal longitudinal anastomotic vessel; hpf-hours post fertilization; MO-morpholino; PCV-posterior cardinal vein.
Supplementary Figure 7 The chemokine receptor cxcr4a affects tip cell migration, while dll4 knockdown causes aberrant blood flow patterns. (a) Wt ctr video 1 showing sprouting of the NCA from the PMBC at indicated time points. Note migratory behaviours of cells 1 and 2 towards the CrDI during the 3:30 h time course. Tg(kdrl:EGFP)1842 marks ECs in green. (b) Wt ctr video 2 showing sprouting of the NCA from the PMBC at indicated time points. Note migratory behaviours of cells 1 and 2 towards the CrDI during the 3:30 h time course. (c) NCA sprouting in cxcr4a mutant embryo 1. Note normal initiation of NCA sprouting, but subsequent stalling of EC 1. Even at the 8:14 h time point, no connection between CrDI and NCA can be observed. At 16:47 h time point, a small sprout extends from the NCA towards the CrDI (arrowhead). (d) NCA sprouting in cxcr4a mutant embryo 2. Note stalling of ECs 1 and 2. At the 6:46 h time point, a sprout emanates from the CrDI (arrow) and subsequently connects to the NCA, but without generating a proper lumen. (n=3 embryos, 3 independent experiments). (e) Quantification of blood flow patterns at 54 hpf and 72 hpf in ctl MO injected embryos (n=19 embryos, 2 independent experiments). Note regular flow patterns and absence of ectopic sprouts. (f) Quantification of blood flow patterns at 54 hpf and 72 hpf in dll4 MO injected embryos (n=19 embryos, 2 independent experiments). Note lack of blood flow, increase in venous ISVs and ectopic sprouts (green bars). (g) Aberrant blood flow patterns correlate with increased ectopic blood vessel sprouting in dll4 knockout embryos (n=19, 2 independent experiments). White arrows indicate ectopic blood vessel sprouts. These are more frequently found in areas containing ISVs without blood flow. (h) Blocking blood flow from 48 to 52 hpf leads to upregulation of cxcr4a expression in the DA and DLAV (arrows). ‘n/N’ reports the ‘number of embryos with staining pattern in image/’total embryos from three experiments’ (N=20 embryos for DMSO and N=15 embryos for Nifedipine treatment). ***p<0.0001, Fischer’s exact test. Scale bars, 50 μm. CrDI-Cranial division of the internal carotid artery; EC-endothelial cell; PMBC-posterior cardinal vein; NCA-nasal ciliary artery; h-hour; DA-dorsal aorta; PCV-posterior cardinal vein.
**Supplementary Figure 8** dll4 and cxcr4a are not expressed in endothelial cells during venous plexus blood vessel sprouting. (a) Expression of the dll4 reporter Tg(dll4:gal4)mu106; Tg(UAS:GFP)nkuasgfp1a (green) cannot be detected in sprouting venous plexus ECs (arrowheads) marked with Tg(kdrl:Hsa.HRAS-mcherry)s916 (red) at 28 hpf, 32 hpf or 38 hpf (n=10 for each time point). (b) Expression of cxcr4a (red) cannot be detected in sprouting venous plexus ECs marked with an anti-GFP antibody staining in Tg(kdrl:EGFP)s843 (green) embryos (n=10 for each time point). Visible red staining comes from overlying fin fold tissue. Images are representative of three independent experiments. (c) Current model of tip/stalk cell specification through lateral inhibition. VEGF signalling induces the Notch ligand dll4 in tip cells. High dll4 expression in tip cells activates Notch signalling in neighbouring stalk cells thereby preventing them from acquiring a tip cell phenotype. (d) Model integrating artery formation and Notch pathway activation during angiogenesis. Initially, Notch negative tip cells sprout from veins. These tip cells subsequently express dll4. Stalk cells also start expressing dll4. This leads to Notch activation via trans-activation first in tip and then also in stalk cells. Notch activation induces expression of the chemokine receptor cxcr4a. This allows proper tip cell migration towards the existing artery, ultimately allowing blood flow from arteries to veins. Scale bars, 50 μm. CVP-caudal vein plexus; DA-dorsal aorta; EC-endothelial cell; hpf-hours post fertilization.
Supplementary Video Legends

Supplementary Video 1 Time lapse imaging of \textit{dll4} reporter line during eye vessel development- Embryo 1. Time lapse confocal imaging of NCA development in \textit{Tg(dll4-gal4 \textit{myo106}: Tg(UAS:GFP)\textit{ykaug612}; Tg(kdrl:NLS-mcherry)\textit{is4}} triple transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes:seconds.

Supplementary Video 2 Time lapse imaging of \textit{dll4} reporter line during eye vessel development- Embryo 2. Time lapse confocal imaging of NCA development in \textit{Tg(dll4-gal4 \textit{myo106}: Tg(UAS:GFP)\textit{ykaug612}; Tg(kdrl:NLS-mcherry)\textit{is4}} triple transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes:seconds.

Supplementary Video 3 Time lapse imaging of \textit{dll4} reporter line during eye vessel development- Embryo 3. Time lapse confocal imaging of NCA development in \textit{Tg(dll4-gal4 \textit{myo106}: Tg(UAS:GFP)\textit{ykaug612}; Tg(kdrl:NLS-mcherry)\textit{is4}} triple transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes:seconds.

Supplementary Video 4 Time lapse imaging of Notch reporter line during eye vessel development- Embryo 1. Time lapse confocal imaging of NCA development in \textit{Tg(\textit{TP1}:GFP)\textit{um114}; Tg(kdrl:NLS-mcherry)}\textit{is4} double transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes:seconds.

Supplementary Video 5 Time lapse imaging of destabilized Notch reporter line during eye vessel development- Embryo 2. Time lapse confocal imaging of NCA development in \textit{Tg(\textit{TP1}:GFP)\textit{um114}; Tg(kdrl:NLS-mcherry)}\textit{is4} double transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes:seconds.

Supplementary Video 6 Time lapse imaging of destabilized Notch reporter line during eye vessel development- Embryo 3. Time lapse confocal imaging of NCA development in \textit{Tg(\textit{TP1}:GFP)\textit{um114}; Tg(kdrl:NLS-mcherry)}\textit{is4} double transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes:seconds.

Supplementary Video 7 Time lapse imaging of destabilized Notch reporter line during eye vessel development- Embryo 1. Time lapse confocal imaging of NCA development in \textit{Tg(\textit{TP1}:Venus-\textit{PEST})\textit{is40}; Tg(kdrl:NLS-mcherry)}\textit{is4} double transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 8 Time lapse imaging of destabilized Notch reporter line during eye vessel development- Embryo 2. Time lapse confocal imaging of NCA development in \textit{Tg(\textit{TP1}:Venus-\textit{PEST})\textit{is40}; Tg(kdrl:NLS-mcherry)}\textit{is4} double transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 9 Time lapse imaging of destabilized Notch reporter line during eye vessel development- Embryo 3. Time lapse confocal imaging of NCA development in \textit{Tg(\textit{TP1}:Venus-\textit{PEST})\textit{is40}; Tg(kdrl:NLS-mcherry)}\textit{is4} double transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 10 Simultaneous imaging of \textit{dll4} expression and Notch activation during eye vessel development- Embryo 1. Time lapse confocal imaging of NCA development in \textit{Tg(dll4-gal4 \textit{myo106}: Tg(UAS:GFP)\textit{ykaug612}; Tg(\textit{TP1}:H2B-mcherry)}\textit{is439}} triple transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 11 Simultaneous imaging of \textit{dll4} expression and Notch activation during eye vessel development- Embryo 2. Time lapse confocal imaging of NCA development in \textit{Tg(dll4-gal4 \textit{myo106}: Tg(UAS:GFP)\textit{ykaug612}; Tg(\textit{TP1}:H2B-mcherry)}\textit{is439}} triple transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 12 Simultaneous imaging of \textit{dll4} expression and Notch activation during eye vessel development- Embryo 3. Time lapse confocal imaging of NCA development in \textit{Tg(dll4-gal4 \textit{myo106}: Tg(UAS:GFP)\textit{ykaug612}; Tg(\textit{TP1}:H2B-mcherry)}\textit{is439}} triple transgenic embryo. Image acquisition started at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 13 Time lapse imaging of Notch reporter line during eye vessel development with \textit{dll4} knockdown- Embryo 1. Time lapse confocal imaging of NCA development in \textit{Tg(\textit{TP1}:GFP)\textit{um114}; Tg(kdrl:NLS-mcherry)}\textit{is4} double transgenic embryo injected with \textit{dll4} morpholino. Image acquisition started at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 14 Time lapse imaging of Notch reporter line during eye vessel development with \textit{dll4} knockdown- Embryo 2. Time lapse confocal imaging of NCA development in \textit{Tg(\textit{TP1}:GFP)\textit{um114}; Tg(kdrl:NLS-mcherry)}\textit{is4} double transgenic embryo injected with \textit{dll4} morpholino. Image acquisition started at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 15 Time lapse imaging of Notch reporter line during eye vessel development with \textit{dll4} knockdown- Embryo 3. Time lapse confocal imaging of NCA development in \textit{Tg(\textit{TP1}:GFP)\textit{um114}; Tg(kdrl:NLS-mcherry)}\textit{is4} double transgenic embryo injected with \textit{dll4} morpholino. Image acquisition started at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 16 Time lapse imaging of \textit{Tg(kdrl:EGFP)\textit{s843}} during eye vessel development- Embryo 1. Time lapse confocal imaging of NCA development in \textit{Tg(kdrl:EGFP)\textit{s843}} wt embryo starting at 30 hpf. Elapsed time in hours:minutes.

Supplementary Video 17 Time lapse imaging of \textit{Tg(kdrl:EGFP)\textit{s843}} during eye vessel development- Embryo 2. Time lapse confocal imaging of NCA development in \textit{Tg(kdrl:EGFP)\textit{s843}} wt embryo starting at 30 hpf. Elapsed time in hours:minutes.
Supplementary Video 18  Time lapse imaging of cxcr4a<sup>um20</sup> mutant during eye vessel development- Embryo 1. Time lapse confocal imaging of NCA development in cxcr4a<sup>um20</sup> Tg(kdrl:EGFP)<sup>s843</sup> embryo starting at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 19  Time lapse imaging of cxcr4a<sup>um20</sup> mutant during eye vessel development- Embryo 2. Time lapse confocal imaging of NCA development in cxcr4a<sup>um20</sup> Tg(kdrl:EGFP)<sup>s843</sup> embryo starting at 26 hpf. Elapsed time in hours:minutes.

Supplementary Video 20  Time lapse imaging of caudal vein plexus development in control morpholino injected embryo. Time lapse confocal imaging of caudal vein plexus development in Tg(fli1a:nEGFP)<sup>y7</sup>; Tg(kdrl:Hsa.HRAS-mcherry)<sup>s916</sup> double transgenic embryo injected with control morpholino. Image acquisition started at 24 hpf. Elapsed time in hours:minutes:seconds.

Supplementary Video 21  Time-lapse imaging of caudal vein plexus development in dll4 morpholino injected embryo. Time lapse confocal imaging of caudal vein plexus development in Tg(fli1a:nEGFP)<sup>y7</sup>; Tg(kdrl:Hsa.HRAS-mcherry)<sup>s916</sup> double transgenic embryo injected with dll4 morpholino. Image acquisition started at 24 hpf. Elapsed time in hours:minutes:seconds.
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### Experimental design

1. **Sample size**
   
   Describe how sample size was determined.
   
   No sample size calculation was performed. Sample sizes were chosen based on previous experience.

2. **Data exclusions**
   
   Describe any data exclusions.
   
   No data were excluded from analysis.

3. **Replication**
   
   Describe whether the experimental findings were reliably reproduced.
   
   The experimental findings were reliably reproduced several times.

4. **Randomization**
   
   Describe how samples/organisms/participants were allocated into experimental groups.
   
   Not relevant to our study. Embryos were allocated into experimental groups based on which morpholino they were injected with or whether they were genotypically identified as mutants or wildtypes.

5. **Blinding**
   
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   
   No blinding was used during data acquisition or analysis. Blinding was not possible since due to the experimental setups.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**

   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

   | n/a | Confirmed |
   |-----|-----------|
   | x   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
   | x   | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly. |
   | x   | A statement indicating how many times each experiment was replicated |
   | x   | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
   | x   | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
   | x   | The test results (e.g. p values) given as exact values whenever possible and with confidence intervals noted |
   | x   | A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
   | x   | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

### Software

Policy information about availability of computer code

7. **Software**

   Describe the software used to analyze the data in this study.

   N/A
For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The *Nature Methods* guidance for providing algorithms and software for publication may be useful for any submission.

### Materials and reagents

**Policy information about availability of materials**

**8. Materials availability**

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

Fish line generated during the course of study is available from the author. Fli1b antibody can be obtained from the laboratory of Nathan Lawson (reference see below).

**9. Antibodies**

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Rabbit anti-Fli1b: From Nathan Lawson's lab (Moore, J.C. et al. Post-transcriptional mechanisms contribute to Etv2 repression during vascular development. Dev Biol 384, 128-140 (2013).)

Rabbit anti-GFP: Torres-Pines (ABIN1574093)
Mouse anti c-myc: Sigma-Aldrich (M5546)
Goat anti-mouse Alexa Fluor 647: Invitrogen (A21236)
Goat anti-rabbit Alexa Fluor 647: Invitrogen (A32733)
Goat anti-rabbit Alexa Fluor 488: Invitrogen (A11034)

**10. Eukaryotic cell lines**

a. State the source of each eukaryotic cell line used.

b. Describe the method of cell line authentication used.

c. Report whether the cell lines were tested for mycoplasma contamination.

d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

We used primary HUAECs obtained from Prof. Hans Schnittler. They were cultured until maximally passage #5.

Cell lines were not authenticated.

Cell lines were not tested for mycoplasma contamination.

No commonly misidentified cell lines were used.

### Animals and human research participants

**Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines**

**11. Description of research animals**

Provide details on animals and/or animal-derived materials used in the study.

Zebrafish used in this study were between 1 and 2 years of age and were not selected for gender. The following transgenic lines were used: Tg(kdrl:NLS-mcherry)s4, Tg(dll4:gal4)mu106 (this study), Tg(UAS:GFPInuasgfp1a, Tg(TP1:GFPUm14, Tg(kdrl:H2B-EGFP)mu122, Tg(kdrl:EGFP)s843, Tg(fli1a:EGFP)y1, Tg(0.8flt1:RFP)hu5333, Tg(gata1:DsRed)sd2, cxcr4a um20, dll4 j16e1, mib ta52b, Tg(TP1:Venus-PEST)s940, Tg(TP1:H2B-mcherry)s939, Tg(cdh5BAC:gal4ff)mu101, abbreviated as Tg(cdh5:Gal4)mu101, Tg(UAS:myc-Notch1a-intra)kca3, abbreviated as Tg(UAS:NICD)kca3. The fli1b antibody was obtained from the laboratory of Dr. Nathan Lawson, UMass Medical School, Worcester, USA.

**12. Description of human research participants**

Describe the covariate-relevant population characteristics of the human research participants.

Study did not involve human research participants.