Neuronal sFlt1 and Vegfaa determine venous sprouting and spinal cord vascularization

Raphael Wild¹,², Alina Klems¹,², Masanari Takamiya², Yuya Hayashi²,³, Uwe Strähle², Koji Ando⁴, Naoki Mochizuki⁴, Andreas van Impel⁵,⁶, Stefan Schulte-Merker⁵,⁶, Janna Krueger⁷, Laetitia Preau¹, & Ferdinand le Noble¹,²

Formation of organ-specific vasculatures requires cross-talk between developing tissue and specialized endothelial cells. Here we show how developing zebrafish spinal cord neurons coordinate vessel growth through balancing of neuron-derived Vegfaa, with neuronal sFlt1 restricting Vegfaa-Kdrl mediated angiogenesis at the neurovascular interface. Neuron-specific loss of flt1 or increased neuronal vegfaa expression promotes angiogenesis and peri-neural tube vascular network formation. Combining loss of neuronal flt1 with gain of vegfaa promotes sprout invasion into the neural tube. On loss of neuronal flt1, ectopic sprouts emanate from veins involving special angiogenic cell behaviours including nuclear positioning and a molecular signature distinct from primary arterial or secondary venous sprouting. Manipulation of arteriovenous identity or Notch signalling established that ectopic sprouting in flt1 mutants requires venous endothelium. Conceptually, our data suggest that spinal cord vascularization proceeds from veins involving two-tiered regulation of neuronal sFlt1 and Vegfaa via a novel sprouting mode.
he vascular network closely associates with the neuronal network throughout embryonic development, in adulthood and during tissue regeneration\textsuperscript{1,2}. Close association of vessels and nerves allows reciprocal cross-talk involving diffusible molecules, which is important for physiological functions in both domains\textsuperscript{3,4}. Arteries secrete factors that attract sympathetic axons, and adrenergic innervation of arteries allows the autonomic nervous system to control arterial tone and tissue perfusion\textsuperscript{5}. The nervous system, on the other hand, requires a specialized network of blood vessels for its development and survival. Metabolically active nerves rely on blood vessels to provide oxygen necessary for sustaining neuronal activity\textsuperscript{6}, and disturbances herein result in neuronal dysfunction\textsuperscript{7,8}.

How nerves attract blood vessels is debated, but several studies addressing vascularization of the mouse and chicken embryo nervous system suggest that the angiogenic cytokine VEGF-A is involved\textsuperscript{9,10}. In the mouse peripheral nervous system axons of sensory nerves innervating the embryonic skin trigger angiogenesis involving VEGF-A–Neuropilin-1 (NRP1) dependent signalling\textsuperscript{11,12}. While these studies provide evidence for the physical proximity and cooperative patterning of the developing nerves and vasculature, relatively little is known about mechanisms controlling VEGF-A dosage at the neurovascular interface. This is of great importance considering that blood vessels are very sensitive to changes in VEGF-A protein dosage and even moderate deviations from its exquisitely controlled physiological levels result in dramatic perturbations of vascular development\textsuperscript{13,14}. VEGF-A levels must therefore be well titrated, and several strategies have evolved to achieve this.

Mouse retinal neurons for example can reduce extracellular VEGF-A protein via selective endocytosis of VEGF-A–VEGF receptor-2 (KDR/FLK) complexes. Inactivation of this uptake causes non-productive angiogenesis\textsuperscript{15}. In the vascular system, spatio-temporal control of VEGF-A protein dosage is thought to be achieved by soluble VEGF receptor-1 (sFLT1), an alternatively spliced, secreted isoform of the cell-surface receptor membrane-bound FLT1 (mFLT1)\textsuperscript{16,17}. Soluble FLT1 binds VEGF-A with substantially higher affinity than KDR, thereby reducing VEGF-A bioavailability and attenuating KDR signalling\textsuperscript{18}. While originally discovered as a vascular-specific receptor, evidence is emerging showing neuronal FLT1 expression\textsuperscript{18}. To what extent endogenous neuronal Flt1 has a physiological role in titrating neuronal VEGF levels controlling angiogenesis at the neurovascular interface independent of vascular Flt1 remains to be determined.

Angiogenesis involves complex and dynamic changes in endothelial cell behaviour\textsuperscript{19}. In the zebrafish embryo these events can be studied in detail at the single cell level in vivo through the use of vascular-specific reporter lines\textsuperscript{20,21}. The stereotyped patterning of arteries and veins in the trunk of the zebrafish embryo prior to 48 hpf is mediated by cues derived from developing somites and the hypochord, controlling angiogenic sprout differentiation and guidance\textsuperscript{22,23}. Sprouting of intersegmental arterioles (aISV) requires Vegfaa-Kdr/fkdr, kdr, flt4 and the ligands vegfaa, vegfab, and plgf (Supplementary Fig. 1b,e)\textsuperscript{24}. Flt1 was expressed in a comparable range as neuronal guidance molecules (Supplementary Fig. 1c,f). Real-time qPCR analysis for the onset and the extent of the vascular network that supplies the spinal cord. We demonstrate that on genetic ablation of neuronal sFlt1 this brain is relieved resulting in the formation of a vascular network supplying the spinal cord in a Vegfaa-Kdrl dependent manner. Using inducible neuron-specific vegfaa gain-of-function approaches and analysis of several mutants with vegfaa gain-of-function scenarios, we furthermore show that the neuronal Vegfaa dosage determines the extent of the neovascularization supplying the spinal cord, as well as sprout invasion into the spinal cord. Interestingly, loss of flt1 or augmenting neuronal vegfaa promotes sprouting from intersegmental veins involving distinctive angiogenic cell behaviours including nuclear positioning and a molecular signature not observed in primary arterial or secondary venous sprouting. Cell transplantation experiments confirm the role of neuronal flt1 in venous sprouting and furthermore show that vascular flt1 is dispensable herein. Taken together, our data suggest that spinal cord vascularization proceeds from veins and is coordinated by two-tiered regulation of neuronal sFlt1 and Vegfaa determining the onset and the extent of the vascular network that supplies the spinal cord via a novel sprouting mode.

In this study we show that developing spinal cord neurons located in the trunk of the zebrafish embryo produce Vegfaa and sFlt1 affecting the angiogenic behaviour of intersegmental vessels at the neurovascular interface. We find that during early development neuronal sFlt1 restricts angiogenesis around the spinal cord. We demonstrate that on genetic ablation of neuronal sFlt1 this brain is relieved resulting in the formation of a vascular network supplying the spinal cord in a Vegfaa-Kdrl dependent manner. Using inducible neuron-specific vegfaa gain-of-function approaches and analysis of several mutants with vegfaa gain-of-function scenarios, we furthermore show that the neuronal Vegfaa dosage determines the extent of the neovascularization supplying the spinal cord, as well as sprout invasion into the spinal cord. Interestingly, loss of flt1 or augmenting neuronal vegfaa promotes sprouting from intersegmental veins involving distinctive angiogenic cell behaviours including nuclear positioning and a molecular signature not observed in primary arterial or secondary venous sprouting. Cell transplantation experiments confirm the role of neuronal flt1 in venous sprouting and furthermore show that vascular flt1 is dispensable herein. Taken together, our data suggest that spinal cord vascularization proceeds from veins and is coordinated by two-tiered regulation of neuronal sFlt1 and Vegfaa determining the onset and the extent of the vascular network that supplies the spinal cord via a novel sprouting mode.

**Results**

**Spinal cord neurons express sflt1, mflt1 and vegf ligands.** Analysis of TgBAC(fltl:YFP)aw624, Tg(kdr:hsa.HRAS-mCherry)K16 transgenic embryos showed flt1 expression in the aorta, arterial intersegmental vessels (aISVs), dorsal part of venous intersegmental vessels (vISVs) and spinal cord neurons located in the neural tube (Fig. 1a,b,d–g)\textsuperscript{18}. Spinal cord neurons were in close proximity to blood vessels (Fig. 1c–e) and 3D-rendering of confocal z-stacks obtained from Tg(kdr:EGFP)G24, Tg(Xla.Tubb:DsRed)G48 double transgenic embryos showed the dorsal aspect of ISVs ‘indenting’ the neural tube indicative of close contact (Fig. 1c; Supplementary Movie 1). Optical sections confirmed close contact between the outer neuronal layers of the neural tube and the dorsal part of ISVs, as well as the dorsal longitudinal anastomotic vessel (DLAV) (Fig. 1d–g). Such anatomical juxtapositioning of trunk vessels and neurons may provide a template for molecular cross-talk (Fig. 1d,c; pink box).

**TaqMan analysis using FAC-sorted neuronal cells from two different neuronal reporter lines (Supplementary Fig. 1a–k) showed expression of mflt1, sflt1, kdr, kdr, flt4 and the ligands vegfaa, vegfab, and plgf (Supplementary Fig. 1b,e)\textsuperscript{24}. Flt1 was expressed in a comparable range as neuronal guidance molecules (Supplementary Fig. 1c,f). Real-time qPCR analysis for vegfaa and vegfab in the trunk of developing zebrafish embryos confirmed expression of both isoforms (Supplementary Fig. 1l,m).

**Loss of flt1 induces ectopic vascular networks.** In zebrafish flt1 consists of 34 exons encoding membrane-bound mflt1 and soluble sflt1, which is formed by alternative splicing at the exon 10—intron 10 boundary (Supplementary Fig. 2a)\textsuperscript{18}. To obtain loss of both mflt1 and sflt1 (flt1 full mutants) we targeted flt1-exon 3 using a CRISPR/Cas approach (Supplementary Fig. 2a–d) and analysed in detail the vascular phenotypes of three mutant alleles, flt1\textsuperscript{KAb01} (~1 nt), flt1\textsuperscript{KAb02} (~5 nt) and flt1\textsuperscript{KAb03} (~5 nt) (Fig. 2a–f; Supplementary Fig. 2a–d). To obtain mflt1-specific mutants we targeted exon 11b, the alternative exon essential for mflt1 transcription (Fig. 2g; Supplementary Fig. 2a,e)\textsuperscript{18}. Both the flt1\textsuperscript{KAb01} and flt1\textsuperscript{KAb05} mutant showed no signs of non-sense mediated decay (Supplementary Fig. 3a,b).

**Loss of flt1 induces ectopic vascular networks.** In zebrafish flt1 consists of 34 exons encoding membrane-bound mflt1 and soluble sflt1, which is formed by alternative splicing at the exon 10—intron 10 boundary (Supplementary Fig. 2a)\textsuperscript{18}. To obtain loss of both mflt1 and sflt1 (flt1 full mutants) we targeted flt1-exon 3 using a CRISPR/Cas approach (Supplementary Fig. 2a–d) and analysed in detail the vascular phenotypes of three mutant alleles, flt1\textsuperscript{KAb01} (~1 nt), flt1\textsuperscript{KAb02} (~5 nt) and flt1\textsuperscript{KAb03} (~5 nt) (Fig. 2a–f; Supplementary Fig. 2a–d). To obtain mflt1-specific mutants we targeted exon 11b, the alternative exon essential for mflt1 transcription (Fig. 2g; Supplementary Fig. 2a,e)\textsuperscript{18}. Both the flt1\textsuperscript{KAb01} and flt1\textsuperscript{KAb05} mutant showed no signs of non-sense mediated decay (Supplementary Fig. 3a,b).
Zebrafish homozygous for the \textit{flt1} -1 nt allele (\textit{flt1}\textsuperscript{ka601}) displayed severe hyper-branching of the trunk vasculature at 3–4 dpf (Fig. 2a,b). Supernumerous amounts of branches developed in the dorsal aspect of the trunk at the level of the neural tube (Fig. 2b,c). Comparable observations were made in embryos homozygous for the \textit{flt1}/C0 5 nt allele (\textit{flt1}\textsuperscript{ka602}) and the \textit{flt1}\textsuperscript{+} 5 nt allele (\textit{flt1}\textsuperscript{ka603}) (Fig. 2d,e,f). Analysis of four \textit{mflt1} mutant alleles (\textit{flt1}\textsuperscript{ka605-608}, Supplementary Fig. 2e) did not reveal any obvious vascular malformations or alterations in vascular branching morphogenesis (Fig. 2g). These observations are compatible with absence of angiogenic defects in mouse Flt1TK/C0/C0 embryos lacking mFlt1 signalling\textsuperscript{28,29}. The vascular phenotype observed in the \textit{flt1}\textsuperscript{ka601} mutants thus most likely involved soluble Flt1.

Since the vascular phenotypes of the \textit{flt1}\textsuperscript{ka601}, \textit{flt1}\textsuperscript{ka602} and \textit{flt1}\textsuperscript{ka603} mutant alleles (\textit{flt1} full mutants) were indistinguishable, we focused on analysing \textit{flt1}\textsuperscript{ka601} embryos (Fig. 2p–s). \textit{flt1}\textsuperscript{ka601} mutants showed normal arterial-venous remodelling (Fig. 2b,p–s) and adequate perfusion of both aISVs and vISVs. No significant changes in heart frequency were noted (Supplementary Fig. 4a). The vascular phenotype of \textit{flt1}\textsuperscript{ka601} mutants emerged around day 2.5 (Fig. 2q,r) with sprouts emanating exclusively from the dorsal aspect of the venous ISVs at the level of the neural tube (Supplementary Movie 2); ectopic arterial ISV sprouting was not observed (Fig. 2r). In \textit{flt1}\textsuperscript{ka601}/+ heterozygotes (Fig. 2l–o) ectopic sprouting was rarely observed (Fig. 2n,o; Supplementary Movie 3). In wild-type (WT) embryos such endothelial cell behaviours were not observed (Fig. 2h–k, Supplementary Movie 4).

We furthermore examined whether \textit{flt1} targeting morpholino could recapitulate the \textit{flt1}\textsuperscript{ka601} mutant phenotype (Supplementary Fig. 4b–h). We evaluated two dosages of a published \textit{flt1} ATG targeting morpholino (MO) and found that 1 ng \textit{flt1} MO induced hyper-branching in WT at levels comparable to \textit{flt1}\textsuperscript{ka601} (Supplementary Fig. 4g,h)\textsuperscript{18,30}. Injection of 1 ng MO into \textit{flt1}\textsuperscript{ka601} mutant background did not induce additional sprouting defects (Supplementary Fig. 4d), suggesting that the 1ng dosage targets \textit{flt1} specifically. In contrast, 3 ng MO introduced
additional branches at 2 dpf that were not observed in the flt1^ka601 mutant at this stage (Supplementary Fig. 4e). Since we did not observe maternal contribution of flt1 these observations suggest that 3 ng MO introduced non-specific effects^31.

**Sprouts in flt1^ka601 display distinctive cell behaviours.** Compatible with ectopic sprouting we identified hyperactive endothelial cells extending filopodia in the dorsal aspect of vISVs of flt1^ka601 mutants (Fig. 3a; Supplementary Movie 2). About 55% of hyperactive endothelial cells investigated generated a patent sprout (Fig. 3a); in the remaining 45%, filopodia and sprouts retracted (Fig. 3b). From the population of patent ectopic venous sprouts 95% formed an anastomosis with an aISV, whereas only 5% made a connection with a vISV (Fig. 3c). The preference for arterial anastomosis may be physiologically

---

**Figure 2 | Flt1 mutants develop hyper-branched vascular networks at the level of the neural tube.** (a) Trunk vasculature in 4 dpf WT sibling. (b) trunk vasculature in 4 dpf flt1^ka601 mutant, in Tg(kdrl:EGFP)s843 background. Perfused aISVs with red arrow, veins with blue arrow. Note the extensive amount of hyper-branching (dotted box) at the level of the neural tube. (c) Schematic representation of hyper-branching phenotype along the neural tube; ectopic vessels make anastomosis between vISV (blue) with aISVs (red). (d) Hyper-branching (dotted box) is also observed in flt1^ka602 and (e) flt1^ka603 mutants. (f) Quantification of hyper-branching for indicated mutant alleles. Mean ± s.e.m., n = 10 per group, ANOVA. (g) Membrane-bound flt1 mutant (flt1^ka605) without vascular phenotype (compare dotted box in g, with control in a). (h–k) Trunk vascular network in WT embryos at indicated time points. (l–o) Trunk vasculature in flt1^ka601 embryos at indicated time points. Arrowheads indicate ectopic branches. DA, dorsal aorta; PCV, posterior cardinal vein; DLAV, dorsal longitudinal anastomotic vessel; NT, neural tube; hpf, hours post fertilization; dpf, days post fertilization. Scale bar, 50 μm in a,b,d,e,g,i,m,q,k,o,s; 25 μm in h,l,p,j,n,r.
Figure 3 | Imaging and quantification of sprouting kinetics in flt1ka601 mutants. (a) Time lapse imaging of sprout initiation and anastomosis formation in flt1ka601 mutant. Sprout initiation (60.0 hpf), elongation (65.2 hpf) and connection-anastomoses (72.5 hpf) with adjacent aISV. (b) Time lapse imaging of sprout initiation and retraction in flt1ka601 mutant. Endothelial cells produce filopodia (68.8 hpf), extend a sprout (71.6 hpf), which subsequently retracts (80.8 hpf). (c) Quantification of data in (a,b) showing % of sprouts retracting (top part, 45%) or connecting (bottom part, 55%) to adjacent ISVs. In the latter scenario sprouts in 95% of cases connected to aISV (red) and in 5% of cases to vISV (blue). Angiogenic behaviour was analysed in time-lapse confocal movies, n = 20 embryos. (d-f) Filopodia directionality and length in flt1ka601 mutants (n = 10 embryos, n = 920 filopodia). (g-j) Time lapse imaging of endothelial nuclei in Tg(fli1a:nGFP)², Tg(kdr:hsa.HRAS-mcherry)³ showing association between nuclear position and spraying initiation point (SIP). Note that sprouts arise in close proximity to the position of the nucleus. Arrowheads indicate sprouts; nuclei at indicated time points (sprout initiation with actively migrating nucleus towards SIP I, II, III, IV and nucleus already located at SIP 1,2,3,4). (k) Schematic representation of nuclear position with respect to SIP. (l,m) Quantification of observations in g-j. Red dot indicates sprout initiation time point. Note that sprouting preferentially occurs when endothelial nuclei are within less than 5 µm from SIP (SIP below dotted line in (l)). n = 5 (l) and n = 13 (m). (n-q) Quantification of EC nuclei in aISV, vISVs, DLAV, DA and PCV of WT and flt1ka601 embryos at 4 dpf; mean ± s.e.m., t-test, n = 21 embryos per genotype. (r) Quantification of DLAV width in WT and flt1ka601 mutant, n = 9 embryos per genotype. A, artery; aISV, arterial intersegmental vessel; DA, dorsal aorta; DLAV, dorsal longitudinal anastomotic vessel; EC, endothelial cell; PCV, posterior cardinal vein; SIP, sprout initiation point; V, vein; vISV, venous intersegmental vessel. Scale bar, 30 µm in a,b; 10 µm in de and 50 µm in op.

### Raw Text

**Cardinal Vein; SIP, Sprout Initiation Point; V, Vein; vISV, Venous Intersegmental Vessel. Scale Bar, 30 µm.**

**Figure 3** Imaging and quantification of sprouting kinetics in flt1ka601 mutants. (a) Time lapse imaging of sprout initiation and anastomosis formation in flt1ka601 mutant. Sprout initiation (60.0 hpf), elongation (65.2 hpf) and connection-anastomoses (72.5 hpf) with adjacent aISV. (b) Time lapse imaging of sprout initiation and retraction in flt1ka601 mutant. Endothelial cells produce filopodia (68.8 hpf), extend a sprout (71.6 hpf), which subsequently retracts (80.8 hpf). (c) Quantification of data in (a,b) showing % of sprouts retracting (top part, 45%) or connecting (bottom part, 55%) to adjacent ISVs. In the latter scenario sprouts in 95% of cases connected to aISV (red) and in 5% of cases to vISV (blue). Angiogenic behaviour was analysed in time-lapse confocal movies, n = 20 embryos. (d-f) Filopodia directionality and length in flt1ka601 mutants (n = 10 embryos, n = 920 filopodia). (g-j) Time lapse imaging of endothelial nuclei in Tg(fli1a:nGFP)², Tg(kdr:hsa.HRAS-mcherry)³ showing association between nuclear position and spraying initiation point (SIP). Note that sprouts arise in close proximity to the position of the nucleus. Arrowheads indicate sprouts; nuclei at indicated time points (sprout initiation with actively migrating nucleus towards SIP I, II, III, IV and nucleus already located at SIP 1,2,3,4). (k) Schematic representation of nuclear position with respect to SIP. (l,m) Quantification of observations in g-j. Red dot indicates sprout initiation time point. Note that sprouting preferentially occurs when endothelial nuclei are within less than 5 µm from SIP (SIP below dotted line in (l)). n = 5 (l) and n = 13 (m). (n-q) Quantification of EC nuclei in aISV, vISVs, DLAV, DA and PCV of WT and flt1ka601 embryos at 4 dpf; mean ± s.e.m., t-test, n = 21 embryos per genotype. (r) Quantification of DLAV width in WT and flt1ka601 mutant, n = 9 embryos per genotype. A, artery; aISV, arterial intersegmental vessel; DA, dorsal aorta; DLAV, dorsal longitudinal anastomotic vessel; EC, endothelial cell; PCV, posterior cardinal vein; SIP, sprout initiation point; V, vein; vISV, venous intersegmental vessel. Scale bar, 30 µm in a,b; 10 µm in de and 50 µm in op.
relevant as it creates a pressure gradient promoting blood flow perfusion. Sprout filopodia length ranged from 1 to 20 μm, and filopodia projected at an angle between 90 and 120° with respect to the vISV compatible with arterial anastomosis formation (Fig. 3d–f). Current models posit that Flt1 produced in angiogenic sprouts mainly prevents back-branched of nascent sprouts. We find that in the absence of Flt1 sprouts retain their directionality and migrate away from the parent vessel. Within ISVs endothelial nuclei migrated at velocities of up to 1 μm min⁻¹ (Fig. 3g–j). Careful analysis of nuclear positioning within endothelial cells revealed an association between nuclear position and sprout initiation (Fig. 3k–m, Supplementary Movie 2). Nuclei migrated actively into the direction of future sprout initiation points (SIP), and in more than 80% of the studied sprout initiations nuclear positioning was directly linked with sprout initiation (linkage was defined as nucleus-SIP distance of <5 μm at sprout initiation) (Fig. 3l,m). This nuclear movement is in contrast to rearward nuclear positioning in migrating angiogenic endothelial cells in vitro and is not described in vivo for primary artery or secondary venous sprouting events in zebrafish. Analysis of endothelial cell numbers at 4 dpf showed increased endothelial cell numbers in aISVs, vISVs, DLAV, DA and PCV of flt1ka601 compared with WT (Fig. 3n–q); DLAV size was not statistically different (Fig. 3r). At earlier stages (17 hpf) we found no difference of vISV cell numbers in aISVs, vISVs, DLAV, DA and PCV of flt1ka601 compared with WT (Supplementary Fig. 4i–l). The cell numbers in aISVs, vISVs, DLAV, DA and PCV of flt1ka601 mutants showed significantly increased levels of other genes implied in sprouting cell behaviour. RNA-seq of flt1ka601 mutants revealed upregulated expression of other genes implied in sprouting cell behaviour. RNA-seq and qPCR of flt1 mutants showed significantly increased levels of apelin receptor-a (aprla), angiopoietin-2a (angpt2a), and endothelial cell specific molecule-1 (esm1) (Supplementary Fig. 5b,c), genes previously shown to be enriched in angiogenic vessels. In addition, we observed a significant upregulation of plgf, which encodes the Flt1-specific pro-angiogenic ligand PIGF, and lyve1, a gene expressed in veins and implied in lymphangiogenesis, in line with the venous expansion phenotype in flt1ka601 mutants (Supplementary Fig. 5c).

**flt1ka601 display upregulation of angiogenic sprout markers.** We next performed RNA sequencing of flt1ka601 and analysed genes implicated in sprouting angiogenesis (Supplementary Fig. 5a–c). Expression of the classical tip-stalk cell markers including notch1a, notch1b, dll4, nrarp, nrarpb, hey1, hey2, her6 and flt4 were not altered (Supplementary Fig. 5b). This result may not be surprising since ectopic venous sprouts emanated from venous ISVs, andDll4-Notch signalling is absent in this domain. Instead we found upregulated expression of other genes implied in sprouting cell behaviour. RNA-seq and qPCR of flt1 mutants showed significantly increased levels of apelin receptor-a (aprla), angiopoietin-2a (angpt2a), and endothelial cell specific molecule-1 (esm1) (Supplementary Fig. 5b,c), genes previously shown to be enriched in angiogenic vessels. In addition, we observed a significant upregulation of plgf, which encodes the Flt1-specific pro-angiogenic ligand PIGF, and lyve1, a gene expressed in veins and implied in lymphangiogenesis, in line with the venous expansion phenotype in flt1ka601 mutants (Supplementary Fig. 5c).

**Origin of endothelial cells in ectopic venous sprouts.** It is established that artery-derived ECs, on arteriovenous (AV) remodelling, contribute to the dorsal aspect of vISVs (ref. 39). Besides these remodelled artery-derived cells, another source may be PCV-derived venous endothelial cells as they can migrate over long distances. However, a specific contribution of these venous ECs in populating the dorsal aspect of vISVs has not been shown thus far. To determine whether PCV-derived venous cells can colonize the dorsal aspect of vISVs, we performed cell tracking experiments using the Tg(kdrl:nlskikGR)hsc transgenic line (Fig. 4a–i). A small part of the PCV was photo-converted at 30 hpf and individual venous endothelial cells were tracked in the period 30–60 hpf by time-lapse imaging (Fig. 4a–f, Supplementary Movie 5). We observed three scenarios (Fig. 4i). In scenario (I): PCV-derived venous endothelial cells migrated into the vISV and reached the most dorsal aspect of the vISV (Fig. 4c–e). In the dorsal aspect of vISVs, PCV-derived endothelial cells were observed together with the remodelled artery-derived endothelial cells (Fig. 4f; artery-derived cells in green). Scenario (I), which we refer to as ‘mixed’ (both artery and vein-derived ECs), accounted for 43.2% of cases (Fig. 4h,i). Of the mixed population 67.9% of endothelial cells were of venous origin and 32.1% of arterial origin (Fig. 4h, right panel). In scenario (II), the dorsal part of vISV only contained PCV-derived venous endothelial cells; artery-derived endothelial cells were absent. Scenario (II) accounted for 48.6% of cases (Fig. 4h,i). In scenario (III) we find that the dorsal part of vISV only contained artery-derived ECs; in this scenario the dorsal aspect of vISVs was not colonized by migrating PCV-derived venous endothelial cells (Fig. 4h,i). This scenario was observed in 8.2% of cases.

The flt1enh promoter marks ISV-ECs of arterial origin. Loss of flt1 in Tg(flt1enh::Tdtomato; flt4:mCitrine) showed ectopic venous sprouts containing flt1enh-expressing ECs (Fig. 4k,l,n–p). In the same embryo, we furthermore noted ectopic venous sprouts devoid of flt1enh-expressing ECs (Fig. 4j,m,p), suggesting that these sprouts were only made of vein-derived ECs (Fig. 4m). To confirm a contribution of PCV-derived venous endothelium we performed cell tracking experiments in Tg(kdrl:nlskikGR)hsc on loss of flt1 and indeed we found that PCV-derived venous ECs were capable of contributing to ectopic sprouting (Supplementary Fig. 6a). Interestingly, besides sprouts exclusively containing artery-derived ECs, or venous-derived endothelium (Fig. 4m,o), we observed composite sprouts with artery and venous-derived endothelial cells juxtapositioned (Fig. 4k,n).

**Vegfaa gain-of-function promotes venous sprouting.** Before 48 hpf trunk arterial sprouting is driven by Vegfaa and venous sprouting by Vegfc (refs 24,26,27). Since loss of flt1 mimics vegfaa gain-of-function, we expected changes in arterial branching in flt1ka601. Rather surprisingly, we observed ectopic venous sprouting after 2.5 dpf (Fig. 3a,b; Fig. 5a,b,e). Primary artery development was not affected in flt1 mutants (Supplementary Fig. 6b,c,f,g), although primary arterial sprouts developed in close proximity to the neural tube (Supplementary Fig. 6h–k).

Ectopic venous sprouting was conserved in several other vegfaa gain-of-function scenarios, including vhlhu2114 mutants and ptena/−/−;ptenb/−/− double mutants (Fig. 5c–e). Von Hippel-Lindau protein (pVHL) is essential for the proteolytic degradation of Hif-1α, an evolutionarily conserved transcription factor important for regulating vegfaa transcription. Loss of vhl prevents Hif-1α degradation and augments vegfaa expression. Accordingly, vhlhu2114 mutants developed ectopic sprouts emanating from vISVs but not from aISVs (Fig. 5c,e). Changes in primary aISV sprouting were not observed (Supplementary Fig. 6d,f,g).

PTEN is a tumour suppressor gene acting as a PI3K/Akt signalling attenuator and linked to the progression of many tumours involving VEGF-A (refs 43,44). In zebrafish, two orthologues of pten exist, and ptena/−/−;ptenb/−/− double mutant zebrafish show increased vegfaa levels. Detailed analysis of ptena/−/−;ptenb/−/− double mutants identified pronounced ectopic venous sprouting at the level of the neural tube (Fig. 5d,e). In pten double mutants ectopic venous sprout numbers were higher when compared with flt1ka601 single mutant or vhlhu2114 single mutant (Fig. 5e). In addition, in a small percentage of ISVs, ptena/−/−;ptenb/−/− double mutants displayed very few ectopic arterial sprouts (Fig. 5e).

Mechanistically, loss of vhl and flt1 augments Vegfaa function at different levels, through increased vegfaa transcription and higher Vegfaa protein bioavailability, respectively. We reasoned that combining both mutants should increase Vegfaa.
Figure 4 | Arterial and venous-derived endothelial cells populate the dorsal part of venous ISV and contribute to sprouting upon loss of flt1.

(a) Endothelial cell tracking in Tg(kdrl:nlsKikGR)hsc7 embryos. Photo-converted PCV-derived venous endothelial cells express red-kikGR. (b–f) Endothelial cell tracing during 30–60 hpf, showed that PCV-derived endothelium, indicated in red & labelled a,b,c,d,e, migrated along the ISV, from ventral to dorsal up to the most dorsal part of vISVs (cell labelled b). Pre-existing arterial endothelial cells in ISV, in green and labelled 1,2,3,4, shows artery-derived ECs in the dorsal part of vISVs (cell labelled 2) adjacent of PCV-derived EC (cell labelled a,b). (g) Schematic representation of the scenario imaged in b–f. (h) Left panel: Identity analysis of endothelial cells in the dorsal part of vISV revealed three different scenarios: (I) mixed, both arterial and venous-derived endothelium were present, (II) only vein-derived endothelium, (III) only artery-derived endothelium. (n = 10 experiments & 6 ISVs/embryo). Right panel: % of artery and vein-derived endothelium in the mixed population scenario. (i) Schematic representation of the three identity scenarios in dorsal part of vISV. Arterial derived EC in red, venous-derived EC in blue. (j–l) Ectopic sprouting scenarios in flt1 morphants in Tg(flt1enh:Tdtomato; flt4:mCitrine), n = 6 embryos. (j) Ectopic venous sprout devoid of fltrenh expressing artery-derived EC (arrowhead). (k) Ectopic venous sprout (arrowhead) containing both arterial and venous-derived ECs; the fltrenh expressing artery-derived EC is juxtaposed to the venous-derived cell at the tip (arrowhead). (l) Ectopic venous sprout only containing fltrenh expressing artery-derived ECs; fltrenh (red) and flt4 (green) were expressed by the same cell which appears in yellow (arrowhead). (m–o) Schematic representation of the three ectopic venous sprouting scenarios. (p) Ectopic sprouting upon loss of flt1 in Tg(flt1enh:Tdtomato; flt4:mCitrine) (representative of 5 embryos). flt1enh positive sprouts (pink arrowhead) and sprouts devoid of flt1enh (white arrowhead). MO, flt1 morpholino, 1ng, aISV, arterial intersegmental vessel; DA, dorsal aorta; DLAV, dorsal longitudinal anastomotic vessel; PCV, posterior cardinal vein; vISV, venous intersegmental vessel. Scale bar, 20 μm in j–l, 10 μm in b–f, p.
and activate downstream Kdr signalling even further. Indeed, flt1<sup>ka601</sup>;vhlhu<sup>2114</sup> double mutants showed more severe hyper-branching of the trunk vasculature when compared with single mutants (Fig. 5e,h). Accordingly, flt1<sup>ka601</sup>;vhlhu<sup>2114</sup> double mutants developed more ectopic venous sprouts when compared with either single flt1<sup>ka601</sup> or single vhlhu<sup>2114</sup> mutants (Fig. 5e). The flt1<sup>ka601</sup>;vhlhu<sup>2114</sup> double mutants also developed a small number of ectopic arterial sprouts after 2.5 dpf (Fig. 5e).

However, venous sprout numbers were three times higher (P<0.001) than arterial sprout numbers at this stage (Fig. 5e). Changes in primary aISV (24 hpf) sprouting were not observed (Supplementary Fig. 6e,f,g). Endoxifen-induced neuronal-specific overexpression of vegfa<sup>a165</sup> at 52 hpf in WT embryos also promoted ectopic venous sprouting (Fig. 5e; Supplementary Fig. 8e,f). In addition, a smaller number of ectopic arterial sprouts was noted, similar to flt1<sup>ka601</sup>;vhlhu<sup>2114</sup> double mutants (Fig. 5f).
double mutants (Fig. 5e). Taken together, ectopic venous sprouting was conserved in five vegfaa gain-of-function scenarios.

Ectopic sprouting in fli1ka601 mutants requires veins. To prove that in fli1ka601 mutant sprouts indeed emanated from veins, we interfered with early arterial-venous remodelling by blocking flt4 (ref. 26) (Fig. 5f,g; Supplementary Fig. 6l). Loss of flt4 in fli1ka601 mutants interfered with arterial-venous remodelling; as a consequence almost all trunk ISVs remained arterial28 (Supplementary Fig. 6l). In line with the requirement for veins, the fli1ka601 hyper-branching phenotype was rescued (Fig. 5f,g). Furthermore, flt4 loss-of-function in the fli1ka601;vhl2114 double mutants (denoted as double in Fig. 5h,i) also significantly reduced branching complexity (Fig. 5h-j; method quantification of branch points in Supplementary Fig. 1n). As ectopic sprouting requires venous endothelium, we next reasoned that promoting vISV formation in fli1ka601 mutants should augment branching. Vessel identity and Notch signalling are linked. In zebrafish, it is established that loss of the Notch ligand Dil4 promotes venous cell fate and dll4 loss-of-function embryos display a trunk vasculature consisting almost exclusively of vISVs57. Accordingly, loss of dll4 in fli1ka601 mutants significantly augmented ectopic branching when compared with control fli1ka601 mutants (Fig. 5k,l).

Notch, pericytes and ectopic venous sprouting in fli1ka601. One explanation for the low arterial responsiveness in vegfaa gain-of-function scenarios may involve high arterial Notch activity since Notch acts as a repressor of sprouting in arteries, downstream of Vegfaa signalling25,35,46. To inhibit endogenous Notch signalling in arterial ISVs of fli1ka601 mutants, we expressed a dominant negative form of the Notch co-activator MAML (DN-MAML-EGFP) in an endodoxin inducible manner (Fig. 6a,b)55. We used the fli1enh promoter construct which is mainly active in aISVs (ref. 39) to drive gal4-E2T2;UAS:DN-MAML-EGFP (notchΔE5C) in fli1ka601 mutants. Transgene expression was initiated at 52 hpf by adding endoxifen. Endothelial-specific DN-MAML gain-of-function in fli1ka601 mutants induced ectopic aISV sprouting at the level of the neural tube (Fig. 6a,b,f). Even more pronounced ectopic arterial sprouting was observed with the γ-secretase inhibitor LY-411575 that blocks Notch activation; adding LY-411575 at 2 dpf activated ectopic arterial sprouting in fli1ka601 mutants (Fig. 6c-f). Venous sprout numbers were not significantly changed upon DN-MAML (16.1 ± 3.45 versus 17.1 ± 2.88) or LY-411575 treatment (15.9 ± 2.89 versus 14.2 ± 1.69). Addition of LY-411575 to WT at 2 dpf had no effect. To explain differential AV responsiveness, we also considered differences in pericyte cell coverage (Fig. 6g–j). Overall, pericytes were scarce with 88% of all ISVs investigated not being covered by pericytes. In the remaining 12% of cases, pericytes were found in both aISVs (9.94%) and vISVs (1.91%) along the ISV ventral-dorsal axis. In the most dorsal aspect of aISV and vISV, the region where ectopic sprouting occurs in fli1ka601, pericytes were comparable between aISV and vISV (2.48% and 1.91% respectively, Fig. 6k).

Vegf and Flt1 determine extent of spinal cord vascularization. Neurons expressed vegfaa (Supplementary Fig. 1), and neuronal cells of both 3 dpf WT and vhl loss-of-function embryos had significantly higher vegfaa levels than non-neuronal cells (Fig. 7a,b; FACS settings in Supplementary Fig. 7a–d). Furthermore, neuronal vegfaa expression was significantly increased in vhl loss-of-function when compared with WT (Fig. 7a,b). Thus, at this stage of development neurons are the major source of vegfaa, and not other tissues like developing muscle48. We next examined whether neurons can direct sprouts into the neural tube (Fig. 7c–i). We compared the fli1ka601;vhl2114 double mutant (Fig. 7c,d) with fli1ka601 mutant and WT and found striking changes in optical sections of the neurovascular interface (Fig. 7e–h). In fli1ka601, sprouts occasionally projected into the neural tube (Fig. 7g), whereas in fli1ka601;vhl2114 double mutants many branches invaded the neural tube (Fig. 7h,i).

In the mutants with vegfaa gain-of-function, the spinal cord becomes vascularized relatively early, between 3 and 4 dpf. In WT, the spinal cord is vascularized much later in development starting in the period between 12 and 14 dpf (Fig. 7j–l). In those older WT embryos, sprouts preferentially emanated from venous ISVs, displayed nuclear positioning as described for the fli1 mutant (Supplementary Fig. 7e,f) and the onset of vascularization of the WT spinal cord coincides with decreased sflt1 expression during this stage of development (Supplementary Fig. 7g).

Neuronal sflt1 and Vegfaa regulate sprouting from veins. We next generated tissue-specific and inducible fli1 and vegfaa gain-of-function models. Loss of neuronal sflt1 in fli1ka601 mutants may augment neuron-derived Vegfaa availability and promote ISV sprouting. Hence, restoring neuronal sflt1 in fli1ka601 mutants should provide a rescue, whereas neuronal-specific fli1 loss-of-function should induce hypersprouting. To test the first scenario we expressed -3.2elav3Xgal4-ERT2;UAS:GFP-p2A-sflt1 (sflt1TNC) in fli1ka601 mutants (Fig. 8a–d, branch quantification method in Supplementary Fig. 1n). This construct allows precise time-controlled expression of sflt1 specifically in neurons. We found that transgene activation in neurons at 52 hpf, just before the emergence of the ectopic sprouts in fli1ka601 mutants, rescued the vascular hyper-branching phenotype (Fig. 8b–d).

We next explored whether neuron-specific loss of fli1 is sufficient to induce ISV hyper-branching (Fig. 8e–i). To accomplish neuron-specific loss of fli1 we expressed the fli1 targeting sgRNAfli1E3 (U6:sgRNAfli1E3), the same sgRNA as used to generate fli1ka601 mutants; expressed in all cells) together with the Cas9 construct employing the Gal4-UAS system under the control of the pan-neuronal promoter Xla.Tubb (3.8Xla.Tubb:gal4-VP16/UAS:Cas9-12A-eGFP (fli1ANC); (Fig. 8e))99. To optimize the biallelic knockout efficacy, we injected the construct into embryos heterozygous for the fli1–1 nt allele (fli1ka601/). GFP signal was detected in spinal cord neurons indicating efficient Xla.Tubb-driven neuron-specific expression of Cas9 (Fig. 8h). Neuronal loss of fli1 significantly induced ectopic venous sprout formation when compared with WT and fli1ka601 heterozygous mutants (Fig. 8f–i). In contrast, sprouting was not observed when Cas9 was expressed under a vascular promoter (Supplementary Fig. 8a) or in embryos only carrying the sgRNA without Cas9.

To substantiate the contribution of neuronal sflt1 we next employed multiplexed custom designed miRNAs directed against sflt1 3’UTR arranged with a common miR-155 backbone50 (Supplementary Fig. 8b). The constructs were expressed under control of vascular (fli1TNC) and neuronal (Xla.Tubb) specific promoters. Targeting neuronal sflt1 resulted in ectopic sprouting (Supplementary Fig. 8c), but targeting vascular sflt1 failed to induce sprouts (Supplementary Fig. 8d).

Next we performed cell transplantation experiments, which demonstrated that neuronal fli1 and not vascular fli1 is the physiologically relevant mediator of sprouting at the level of the neural tube (Fig. 8j–l). Transplantation of fli1 mutant neurons into WT hosts induced ectopic sprouting (Fig. 8k).
In contrast, transplantation of flt1 mutant endothelial cells into WT hosts failed to induce sprouting (Fig. 8i).

To prove that neuron-derived Vegfaa promotes hyper-branching, we generated neuronal tissue-specific and inducible vegfaa165 gain-of-function zebrafish (Supplementary Fig. 8e,f; quantification in Fig. 5e). Transgenic expression was initiated by adding endoxifen after completion of AV remodelling at 52 hpf. In this scenario hyper-branched neovascular networks formed at the level of the neural tube, similar to flt1ka601 mutants (Supplementary Fig. 8e,f). Neuronal vegfaa121 was also capable of inducing sprouting (Supplementary Fig. 8g). In contrast, neuron-specific and inducible vegfc gain-of-function, induced at 54 hpf, did not induce ectopic sprouts (Supplementary Fig. 8h). Timing of transgene expression was relevant as inducible neuron-specific vegfaa165 overexpression prior to completion of AV remodelling resulted in thickened abnormal vascular structures (Supplementary Fig. 8i,j). In the same line, neuron-specific constitutive overexpression of sflt1 completely annihilated ISV formation (Supplementary Fig. 8k).

To confirm that the flt1ka601 phenotype involved gain of Vegfa, we titrated vegfaa levels using a low dose vegfaa targeting morpholin51. Reducing vegfaa in flt1ka601 mutants rescued the hyper-branching phenotype (Fig. 8m–o). Vegfaa signals via Kdrl and application of ki8751, an established Kdrl tyrosine kinase inhibitor in zebrafish 52 to flt1ka601 mutants rescued the hyper-branching phenotype (Fig. 8m–o).

In this scenario hyper-branched neovascular networks formed at the level of the neural tube, similar to flt1ka601 mutants (Supplementary Fig. 8e,f). Neuronal vegfaa121 was also capable of inducing sprouting (Supplementary Fig. 8g). In contrast, neuron-specific and inducible vegfc gain-of-function, induced at 54 hpf, did not induce ectopic sprouts (Supplementary Fig. 8h). Timing of transgene expression was relevant as inducible neuron-specific vegfaa165 overexpression prior to completion of AV remodelling resulted in thickened abnormal vascular structures (Supplementary Fig. 8i,j). In the same line, neuron-specific constitutive overexpression of sflt1 completely annihilated ISV formation (Supplementary Fig. 8k).

Figure 6 | Notch inhibits ectopic arterial sprouting in flt1ka601 mutants. (a) flt1ka601 mutants show ectopic venous sprouts (blue circles), but no arterial sprouts. (b) Inhibiting arterial Notch by endoxifen-induced arterial ISV-specific expression of dominant negative MAML-eGFP (NotchΔEC) at 52 hpf under control of the flt1 promoter in flt1ka601 mutants results in the emergence of ectopic arterial sprouts (red circles); representative image from 7 experiments. (c–e) Trunk vasculature of WT (c), flt1ka601 (d) and flt1ka601 treated with Notch inhibitor LY-411575 (e). LY-411575 was added at 2 dpf. Note the emergence of ectopic arterial sprouts upon LY-411575 treatment. (red arrowhead: arterial sprout; blue arrowhead: venous sprout). (f) Quantification of experiments in (a–e), mean ± s.e.m., n = 7 for NotchΔEC, n = 10 for LY-411575 treatment, n = 10 for flt1ka601. t-test. (g–i) Imaging of pericytes in TgBAC(pdgfrb:EGFP);Tg(fli1a:myr-mcherry) double transgenic at 54 hpf. (j) Schematic representation of pericyte number counting in ISVs as performed in (k). (k) Quantification of pericyte recruitment in aISVs and vISVs at 54 hpf. (n = 246 ISVs from 14 embryos). ISV, intersegmental artery; NotchΔEC, inducible ISV-specific loss of Notch; vISV, intersegmental veina. Scale bar, 25 μm in a–e,g–i.
Figure 7 | Neurons are a major source of Vegfaa and attract sprouting vessels. (a) FACS procedure for obtaining neuronal cells in control and vhl morphants using Tg(Xla.Tubb:DsRed)zf148 neuronal reporter embryos. (b) Quantification of vegfaa expression using real-time qPCR in FAC-sorted cell populations at 3 dpf. Note that neuronal cells expressed significantly more vegfaa than non-neuronal cells. Loss of vhl promoted neuronal vegfaa expression. Mean ± s.e.m., n = 3 separate experiments in triplicate (two-way ANOVA). (c) Schematic representation: loss of vhl augments vegfaa transcription, loss of flt1 augments Vegfaa bioavailability; combining both mutants augments Vegfaa bioavailability above single mutant level. (d) Trunk vasculature in flt1ka601;vhlhu2114 double mutants at 4 dpf. Note the severe hyper-branching at the level of the neural tube, red-dotted box. (e) Schematic representation of optical section (shown in f–h) through the neural tube and associated trunk vasculature. (f–h) Dorsal view on optical section through WT (f), flt1ka601 (g) and flt1ka601;vhlhu2114 double mutants (h). Note invasion of sprouts into the neural tube in double mutants (arrowheads in h). Red circle indicates position of ISVs, dotted line neural tube boundary. (i) Transverse 3D-rendered view of vasculature (green) through the trunk in WT (left panel) and flt1ka601;vhlhu2114 double mutants (right panel); note vessels penetrating the neural tube in mutant (compare vessel in dotted circle right panel, arrowhead; such vessels are absent in WT left panel; representative image from 3 separate experiments). (j) Representative image of spinal cord vascular network in Tg(Xla.Tubb:DsRed)zf148 x Tg(kdrl:EGFP)s843 double transgenic at 13 dpf. (k,l) Comparison of trunk vasculature in WT at 4 dpf (k) and at 13 dpf (l); note the emergence of ectopic branches (pink arrowheads) at level of the spinal cord. DA, dorsal aorta; f.c. fold change; KD, knockdown; NT, neural tube; NC, neuronal cell; PCV, posterior cardinal vein. Mutants are in Tg(kdrl:EGFP)s843 background. Scale bar, 50 μm in d, 25 μm in f–l.
**Figure 8 | Neuronal Flt1 regulates vascular branching by titrating neuronal Vegfaa.** (a) Schematic representation of endoxifen inducible gain-of-function approach in zebrafish. In the present situation Gal4 is under the control of neuron-specific promoters elavl or Xla.Tubb. Expression can be observed within 1.5 h upon endoxifen application. (b) Hyper-branching in fit1/fit1 mutants (dotted box). (c) Endoxifen inducible neuron-specific sflt1 gain-of-function rescues hyper-branching in fit1/fit1 mutants; compare dotted box in c and b. Purple arrowheads indicate vISVs; endoxifen was applied at 52 hpf. (d) Quantification of rescue in (b,c), mean ± s.e.m, n = 15-19 embryos per group. (e) Approach for generating a neuron-specific fit1 mutant. Cas9 was expressed under control of neuronal promoter Xla.Tubb; sgRNA was expressed ubiquitously, resulting in Cas9 activity in neuronal cells only (domain marked by orange border). Heterozygous fit1/fit1 mutants were used to facilitate biallelic knockout. (f-h) Neuron-specific loss of fit1 gain-of-function (iNC) induces ectopic sprouting (h), sprouts in yellow dotted ellipse, arrowheads indicate neuronal cells with Cas9 expression. (i) Quantification of ectopic sprouting for indicated genotypes. Note that neuron-specific loss of fit1 significantly augments ectopic sprouting (green bar) mean ± s.e.m, n = 16 embryos per group, t-test. (j-l) Transplantation of fit1 mutant neuronal cells (k) and endothelial cells (l) into WT. Note: transplantation of fit1 mutant neuronal cells induced sprouting (k, arrowheads); 9 out of 12 neuronal cell transplants resulted in sprout formation. In all 10 endothelial cell transplants, sprouts were absent (l). (m,n) Low dose morpholino-mediated reduction of vegfaa expression in fit1/fit1 mutants rescues sprouting defects; compare dotted box in (m,n). (o) Quantification of rescue in (m,n), mean ± s.e.m, n > 5 per group, t-test. DA, dorsal aorta; PCV, posterior cardinal vein; DLAV, dorsal longitudinal anastomotic vessel; NT, neural tube. GOI, gene of interest; POI, protein of interest; iNC, inducible, neuronal cell specific gain-of-function; ΔNC, neuron-specific loss of fit1; MO, morpholino. Scale bar, 50 μm in b-h,m,n, 25 μm in k,l.

**Discussion**

Intimate cross-talk between vessels and the nervous system is important for tissue homeostasis. During embryonic development, neuronal stem cells differentiate into mature neurons, a process that associates with a change in cellular metabolism. Concomitantly with developmental neurogenesis, changes occur in the vascular network feeding the spinal cord. We show in the zebrafish embryo that neurons in the developing spinal cord express the pro-angiogenic ligand Vegfaa and anti-angiogenic soluble Vgf receptor-1, sFlt1, which acts as a Vegfaa scavenger (Fig. 9). Spinal cord neurons are in close contact to the developing trunk vasculature, and we show that these vessels are responsive to changes in neuronal sFlt1 and Vegfaa. Using a combination of global and tissue-specific...
**Figure 9 | Schematic representation of neurovascular communication involving neuronal sFlt1-Vegfaa and sprouting from intersegmental veins.**

(a) Spinal cord neurons produce both sFlt1 and Vegfaa in close proximity to the dorsal aspect of intersegmental arteries and veins. (b) Schematic representation of vascularization around the neural tube in WT (top left), flt1ka601 single mutant or vhlhu2114 single mutant (top right), and flt1ka601;vhlhu2114 double mutant (bottom). Loss of flt1 or vhl induces the formation of a peri-neural tube network, and combining both mutants in addition promotes sprouting into the neural tube. NT, neural tube; ISV, intersegmental vessel (a-arterial, v-venous); DA, dorsal aorta; PCV, posterior cardinal vein; DLAV, dorsal longitudinal anastomotic vessel; hpf, hours post fertilization.

**Flt1ka601** mutants display ectopic sprouting in vISVs but not in aISVs, indicating that AV vessel identity or compartment-specific cues may be involved in the novel sprouting type described here. Notch is tightly linked to both AV vessel specification and sprouting, as Notch programs arterial identity and Notch signalling represses sprouting of arteries.

Loss of flt1 mutants, and further substantiated by vegfaa loss- and gain-of-function experiments as well as cell transplantsations, we demonstrate that neuronal sFlt1 restricts neuronal Vegfaa and vessel branching morphogenesis at the neurovascular interface. Differential regulation of vegfaa and sflt1 allows orchestration of the onset and extent of spinal cord vascularization (Fig. 9). We propose that neurons may use sflt1-Vegfaa to adjust vascularization according to their developmental needs.

Flt1ka601 mutants develop ectopic sprouts emanating from venous ISVs around embryonic day 2.5. Neural-specific targeting of flt1 or sflt1 using CRISPR/Cas9- and miRNA-based approaches respectively, result in flt1ka601, vegfaa gain-of-function phenotypes. Transplantation of flt1 mutant neurons into WT hosts induces ectopic sprouting which is not observed after transplantation of flt1 mutant endothelial cells, suggesting that neuronal flt1 is the physiologically relevant mediator in our mutant. Neuronal-specific gain of sflt1, reducing vegfaa levels, or inhibition of Kdrl signalling provides an important cost of ectopic venous sprouting is mediated by the Vegfaa-Kdrl signalling axis. Accordingly, ectopic venous sprouting from the dorsal aspect of vISVs is conserved in five independent vegfaa gain-of-function scenarios.

Previous studies have shown that during AV remodelling, aISV-derived endothelial cells remain integrated in the dorsal aspect of vISVs. We confirm that remodelled artery-derived endothelial cells indeed contribute to this domain although they are not the sole or most important endothelial source. Using in vivo cell tracking we find posterior cardinal vein-derived endothelial cells migrating against the direction of blood flow to populate venous ISVs including the dorsal aspect where sprouts are formed. Here, venous-derived endothelium can co-exist with the artery-derived endothelium. With respect to the endothelial cells populating the dorsal part of vISVs, our data now reveal three different scenarios. The dorsal aspect can contain a mix of both artery and venous-derived endothelium (43%), only vein-derived endothelium (48%) or only artery-derived endothelium (8%). On loss of flt1, both artery- and vein-derived endothelium give rise to ectopic vISV sprouts. This prompts toward the concept that integration into the local venous ISV environment constitutes a permissive factor for sprouting, regardless of the endothelial origin.
Using sFlt1 as a rheostat to control Vegfaa bioavailability, constitutes a means to regulate Vegfa independent of vegfaa promoter activity, vegfaa mRNA or protein stability. We propose that this enables neurons to dynamically fine-tune the extent and onset of peri-neuronal vascular network formation and sprouting into the spinal cord. While the peri-neural network may serve to sustain growth of the developing nervous system, vessel sprouting into the spinal cord and relief of hypoxia has been associated with changes in neuronal stem cell metabolism, triggering differentiation events33. Therefore, untimely or excessive vascularization of the spinal cord is potentially harmful as it may promote premature stem cell differentiation and disrupt the correctly orchestrated neuronal specification process. We propose a two-tiered checkpoint mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13991

Methods

has been completed.

sufficient vessels into the spinal cord after stem cell differentiation and disrupt the carefully orchestrated neuronal mechanism involving sFlt1 and Vegfaa, requiring two decisions to guide vascularization, namely Vegfaa up- and sFlt1 downregulation, to protect neurons from harmful angiogenesis and oxygen variations during early stages on the one hand, and more mature neurons to attract sufficient vessels into the spinal cord after stem cell differentiation has been completed.

Methods

Ethics statement
and p3E_polyA were recombined into pDestToZGC2 (pCG2_Xla.Tubb-3.8_gEFPl, pME_gal4ERT2 and p3E_polyA were recombined into pDestToZGC2 (pCG2_gal4ERT2, p3E_flt1enh, pME_gal4ERT2 and p3E_polyA were recombined into pDestToZGC2 (pCG2_gal4ERT2).

Generation of tissue-specific KO constructs. pME-Cas9-T2A-GFP and pDestToZGC2-U6-egRNA were a gift from Leonard Zen (Addgene plasmid # 63163 and # 63155). pDestToZGC2-U6-egRNA was generated by annealed oligo cloning. Oligos U6_B131E_1 and U6_B131E_2 were cloned into pDestToZGC2-U6-egRNA following BseRI restriction digest. To drive Cas9 expression specifically in neurons, the Gal4 driver construct pCG2_Xla.Tubb-3.8_gal4ERT2 was recombined by generating recombinant p3E_flt1enh, pME_gal4ERT2, p3E_polyA and pDestToZGC2. To drive Cas9 expression specifically in endothelial cells, the Gal4 driver construct pCG2_flt1enh_gal4ERT2 was generated by recombining p3E_flt1enh, pME_gal4ERT2, p3E_polyA and pDestToZGC2. For the Gal4 efector construct, p3E_UAS, pME_cas9T2a-egFP and p3E_polyA were recombined into pDestToZGC2 (pCG2_UAS_Cas9-T2a-egFP_U6_gRNA[15]).

Tissue-specific miR155-flt1-1-2-3 knockdown constructs. sflt1 3’UTR-specific miRNAs were designed using the BLOCK-IT RNAi Designer website (https://rnaidesigner.thermoscientific.com/miarexpress/). To enhance miRNA effectiveness three sflt1 3’UTR-specific target sites with miRNA backbone were cloned in series. A fragment containing the three multiplexed miRNAs were synthesized by Eurofins Genomics and cloned into 641-pMER-GFP-miR155empty and 641-pMER-DsRed-miR155empty using restriction enzymes BamHI and XhoI. The target sites are listed in Supplementary Table 7. The expression construct of Xla.Tubb-3.8_gal4ERT2 transgene was generated by using expression cloning. p3E_flt1enh, 641-pMER-GFP-miR155-flt1-1-2-3 and p3E_polyA were recombined into pDestToZGC2 (pCG2_Xla.Tubb_gfpRNA[15], p5E_flt1enh, 641-pMER-DsRed-miR155-sflt1-1-2-3 and p3E_polyA were recombined into pDestToZGC2 (pCG2_flt1enh_DsRed-miR155-sflt1-1-2-3).

FACS. Approximately 500 embryos Tg(mnx1:GFP)ml2, Tg(Huc:EGFP)as8 or vhl MO injected Tg(Xla.Tubb:DsRed)zf148 embryos were dechorionated at 24 hpf using pronase (0.5 mg/ml). Cells were dissociated using btsmx as recommended by the manufacturer. Tg(mx1:GFP)ml2, Tg(Huc:EGFP)as8 embryos were dissociated and sorted at 24 hpf, control and vhl MO injected Tg(Xla.Tubb:DsRed)zf148 embryos were dissociated and sorted at 3 dpf. Dissociated cells were FACS sorted using BD-FACS-Aria I and Aria II. The sorted cells (~0.5 x 106 cells per experiment) were spun down at 310 g for 5 min and resuspended in lysis buffer contained in the RNeasy mini kit (Qiagen). RNA was extracted as described in the manual. Because of limited amounts of cells, the Gal4 driver construct pCG2_Xla.Tubb-3.8_gal4ERT2 was generated by recombining p5E_flt1enh, pME_gal4ERT2, p3E_polyA and pDestToZGC2. To drive Cas9 expression specifically in endothelial cells, the Gal4 driver construct pCG2_flt1enh_gal4ERT2 was generated by recombining p5E_flt1enh, pME_gal4ERT2, p3E_polyA and pDestToZGC2. For the Gal4 efector construct, p3E_UAS, pME_cas9T2a-egFP and p3E_polyA were recombined into pDestToZGC2 (pCG2_UAS_Cas9-T2a-egFP_U6_gRNA[15]).

Gene expression analysis by real-time qPCR and TaqMan. Total RNA of zebrafish embryos was isolated with TRIzol, purified with RNeasy mini kit (Qiagen) and quantity and quality were measured using an Agilent 2,100 bioanalyzer (Agilent Technologies) according to the manufacturer’s instructions. We performed DNase on-column digestion using RNase-free DNase Set (Qiagen) according to the manufacturer, followed by cDNA synthesis using the ThermoScript First-Strand Synthesis System (Thermo Fisher Scientific). Primer probe sets (FAM and TAMRA labels) were obtained from Thermo Fisher Scientific. Amplification was carried out using an ABI Prism 7,000 thermocycler (Applied Biosystems). qPCR was conducted with SYBR Green PCR Master Mix (Thermo Scientific) in a StepOnePlus real-time qPCR system (Applied Biosystems). Primers for real-time qPCR were ordered from Eurofins Genomics. Gene expression data were normalized against zebrafish elongation factor 1-alpha. Primers and probes are listed in Supplementary Table 1-3.

RNA-seq library preparation and sequencing. Zebrafish RNA was isolated and purified from 4 dpf zebrafish larvae using total RNA and RNeasy mini kit (Qiagen) as recommended by the manufacturers. A cDNA library was generated using the TrueSeq RNAI library sample prepv2 kit according to the manufacturer’s protocol (Illumina).

Identification of differentially expressed genes. Raw sequencing reads were mapped to the transcriptome and the zebrasoma reference genome (GRCz10 dianerix10) using Bowtie2.0.2 and TopHat 2.0 (ref. 67). On average 44,490,573 reads (81.6% of total reads) were assigned to genes with Cufflinks and the HTSeq software package. Differentially expressed genes (control vs. mutant) were identified using DESeq and Cuffdiff.78,68 Genes were defined as differentially expressed if ≥2 fold significantly regulated (P<0.05) with two independent methods (DESeq and Cuffdiff).

Zebrafish histological sectioning. Dechorionated larvae were fixed in 4% PFA for 2h and subsequently transferred to 20% DMSO/80% Methanol and incubated overnight at 20 °C. Larvae were then washed in 100 mM NaCl, 100 mMTris-HCl, pH 7.4, for 40 min at room temperature. Washed larvae were embedded in gelatin from cold water fish skin/sucrose (Sigma). Larvae were sectioned (20 μm) in a cryocutomere.

Inhibitor treatments. All stock solutions were prepared in DMSO. Embryos were dechorionated at 24 hpf using Pronase (Roche, Basel, Switzerland). For Notch signalling inhibition embryos were incubated from 2 dpf with 10 μM of LY-411575 (Sigma, St Louis, MO, USA) and imaged at 3 dpf. For VEGER2 and VEGFR3 inhibition embryos were treated with 23.5 μM MAZ51 (Merck Millipore, Billerica, Massachusetts, USA) from 2.5 dpf or from 3 dpf with 0.125 μM ku8751 (Sigma, St Louis, MO, USA) and imaged at 4 dpf. To inhibit PI3K/Akt signalling embryos were incubated with 1.25 μM wortmannin from 3 dpf and imaged at analysed at 4 dpf. Heartbeat was blocked using 15 mM 2,3-Butanedione 2-monoxide (BDM) dissolved in E3 media. Control embryos were mock treated with DMSO (Sigma, St Louis, MO, USA). Embryos were randomized assigned to experimental groups. Investigators were blinded to inhibitor treatment.

Photocconversion of kikGR and migration tracking. Dechorionated embryos were embedded in 0.7% low-melting agarose at 30 hpf and a small part of the posterior cardinal vein of Tg(kdr:tdkikGR)doi7 transgenics was converted for several seconds using UV-light with the smallest available field diaphragm of the Leica SP8 confocal microscope. Subsequently embryos removed from the agarose and allowed to develop in E3 medium until imaging or were immediately used for time-lapse imaging.

Gal4ERT2 endoxifen activation. Endoxifen (Sigma) was solved in DMSO. Zebrafish embryos expressing Gal4ERT2 were incubated from 52 hpf onwards in 0.5 μM endoxifen in E3 medium in the dark. GFP positive cells could be observed approximately 1.5 h after induction.

Vascular network analysis. To assess sprout number and length, we developed a semi-automated analysis of the DLAV-INS vessel network using ImageJ (Supplementary Fig. 1n). Image-stacks of ISVs were acquired using the Leica SP8 confocal microscope. Stack projections of one side of the trunk were generated. Dorsal region of the ISVs was used for analysis. ImageJ a Gaussian blur filter was applied followed by a black/white threshold and subsequent skeletonization to generate a skeleton of the vasculature. Segment number, branch point number and total branch length were calculated using the ‘analyse skeleton’ plugin. The semi-automated pipeline was applied for analysis of 4 dpf vascular networks, while sprout numbers in 2–3 dpf zebrafish embryos were counted manually.

Imaging. Zebrafish larvae were embedded in 0.7% low-melting agarose with 0.112 mg ml−1 Tricaine (E10521, Sigma) and 0.003% PTU (P7629, Sigma) in glass bottom dishes (MatTek, P35G-0.170-14-C). Images presented in this study were acquired using a Leica SP8 confocal microscope with ×20 multi-immersion and ×4 water immersion objectives and LAS X software. Images were processed using ImageJ. Vascular branching was quantified using a semi-automated ImageJ pipeline (Supplementary Fig. 1n). Animal numbers used are indicated in figure legends. For zebrafish mutants more than 100 embryos per genotype were analysed. In morpholin experiments morphologically malformed embryos were excluded from analysis.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6. Each dataset was tested for normal distribution (D’Agostino and Pearson test). Parametric method (unpaired Students t-test) was only applied if the data were normally distributed. For non-normal distributed data sets, a non-parametric test (Mann Whitney U test) was applied. When appropriate in case of multiple comparisons, ANOVA plus Bonferroni correction was applied. Values <0.05 were considered significant. Data are represented as mean ± s.e.m., unless otherwise indicated. “*P<0.05,” **P<0.01 and ***P<0.001.

Data availability. The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding author on reasonable request. The RNA-seq data generated in this study have been deposited into the NCBI Gene Expression Omnibus database with the accession code http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89350.
Acknowledgements
We thank the colleagues of the KIT–European Zebrafish Resource Center (EZRC) for handling and maintenance of the zebrafish lines. We are very grateful to Dr Caroline Burns, Cardiovascular Research Center, Charlestown for sharing the pME_DN-MAML-GFP entry clone. We thank Dr Thomas Becker and Dr Jean Giacomotto, Brain and Mind Research Institute, University of Sydney for the 641-pMER-GFP/DrRed-miR155empty plasmids. We thank Leonard I. Zon M.D., Boston Children’s Hospital and Dana Farber Cancer Institute, Boston for the pDestTol2CG2-U6gRNA and the pME-Cas9-T2A-GFP constructs and Dr Keith Joung, Massachusetts General Hospital, Charlestown for DR274 and MLM3613 plasmids. We are also very grateful to Dr Jeroen den Hertog, Hubrecht Institute, Utrecht for sharing the zebrafish ptena/C0/C0;ptenb/C0/C0 double mutants. S.S.-M., U.S. and F.L.N. are members of the EuFishBioMed zebrafish initiative. S.S.-M. and F.L.N. are supported by grants from the Deutsche Forschungsgemeinschaft (DFG)–FOR2325 ‘Interactions at the Neurovascular Interface’. Y.H. is supported by an individual grant from the Danish Council for Independent Research. We acknowledge support by Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Karlsruhe Institute of Technology.

Author contributions
R.W. designed and performed experiments and interpreted experimental data. A.K. and R.W. performed and analysed FACS experiments. K.A. participated in manuscript preparation. L.P. performed and analysed inhibitor experiments. J.K. performed and analysed Taqman experiments. M.T. & U.S. contributed the Gal4ERT2 construct, Y.H. performed FACS analyses. K.A. & N.M. performed the analysis on pericyte contribution. A.van I. and S.S.-M. contributed transgenic fish, constructs, interpreted data and discussed the conceptual framework. F.L.N. conceived and designed the project, analysed the data and supervised the overall project. F.L.N. and R.W. wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Wild, R. et al. Neuronal sFlt1 and Vegfaa determine venous sprouting and spinal cord vascularization. Nat. Commun. 8, 13991 doi: 10.1038/ncomms13991 (2017).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/