Venous identity requires BMP signalling through ALK3

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Venous endothelial cells are molecularly and functionally distinct from their arterial counterparts. Although veins are often considered the default endothelial state, genetic manipulations can modulate both acquisition and loss of venous fate, suggesting that venous identity is the result of active transcriptional regulation. However, little is known about this process. Here we show that BMP signalling controls venous identity via the ALK3/BMPR1A receptor and SMAD1/SMAD5. Perturbations to TGF-β and BMP signalling in mice and zebrafish result in aberrant vein formation and loss of expression of the venous-specific gene Ephb4, with no effect on arterial identity. Analysis of a venous endothelium-specific enhancer for Ephb4 shows enriched binding of SMAD1/5 and a requirement for SMAD binding motifs. Further, our results demonstrate that BMP/SMAD-mediated Ephb4 expression requires the venous-enriched BMP type I receptor ALK3/BMPR1A. Together, our analysis demonstrates a requirement for BMP signalling in the establishment of Ephb4 expression and the venous vasculature.

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Arteriovenous differentiation begins prior to the onset of blood flow, indicating an important role for genetic fate determination. Mammalian arterial–venous fate is acquired in a stepwise manner: arterial identity is established first, while the initial venous structures express both arterial and venous markers prior to embryonic day (E) 9.0, when full venous differentiation occurs concurrently with the expression of Eph receptor B4 (Ephb4). Ephb4, encoding a transmembrane tyrosine kinase, is highly expressed in definitive venous endothelium but is not found in arterial endothelium. Consequently, it is often treated as the definitive venous endothelial identity marker, although some expression is also detected in capillaries and in lymphatic valves. Loss of Ephb4 expression results in embryonic lethality by E10.5, with significant defects in the formation of the cardinal vein while the dorsal aorta is relatively unaffected.

It has been hypothesized that endothelial cells (ECs) are venous by default while arterial identity is acquired; however, growing evidence suggests that venous EC identity is dependent upon dynamic gene regulation. For example, the phosphoinositide-kinase-AKT pathway downstream of vascular endothelial growth factor (VEGF-A) actively promotes venous differentiation through inhibition of extracellular signal–regulated kinase/mitogen-activated protein kinase (ERK/MAPK), whereas the venous-specific orphan nuclear receptor Coup-TFII (Nr2f2) actively represses arterial gene expression. However, relatively little is known about the regulatory mechanisms that control venous differentiation: no venous specific enhancers or promoters have been described in the literature, and it is unclear whether venous gene expression is actively stimulated in veins or repressed in arteries.

While the roles of the Notch and VEGF-A signalling pathways in arteriovenous differentiation have been thoroughly investigated, the involvement of other vascular signalling networks in this process is less well understood. In particular, the precise role played by transforming growth factor (TGF)-β signalling in arteriovenous differentiation has been challenging to determine. The TGF-β superfamily of pleiotropic cytokines, including three TGF-β and multiple bone morphogenetic protein (BMP) ligands, are widely found in blood vessels, as are their cognate type II and type I transmembrane receptor kinases. Ligand binding results in the intracellular phosphorylation of the receptor-regulated SMADs (R-SMADs), which form heteromeric complexes with the common mediator protein SMAD4 and translocate to the nucleus where they directly bind DNA.

Mutations in the BMP receptor ALK1 (ACVR1L) and SMAD4 and the accessory type III receptor ENG are associated with the human condition Hereditary Hemorrhagic Telangiectasia (HHT), characterized by arteriovenous malformations and mucocutaneous telangiectasias. However, although gene ablation studies in mice support a crucial role for TGF-β and BMP signalling in arteriovenous differentiation, research in these mutants has made conclusive analysis of the role of these pathways in early arterial and venous identity challenging. Furthermore, while studies in zebrafish demonstrate a role for BMP signalling through the receptor BMPR2 in venous-specific angiogenic sprouting, the requirement for BMP signalling in dorsal–ventral axis specification prior to vascular specification has thus far prevented analysis at stages relevant to arterial or venous identity.

In this paper, we investigate arteriovenous differentiation after EC-specific deletion of SMAD4 in both mice and fish, demonstrating a requirement for SMAD4 in the acquisition of venous but not arterial identity. Further, we conduct a comprehensive analysis of the transcriptional regulation of the essential venous identity gene Ephb4, identifying a venous endothelial-specific enhancer containing essential SMAD-binding motifs and binding SMAD1/5 in ECs. Analysis of SMAD1/5 chromatin immunoprecipitation–sequencing (ChIP-seq) data finds similar SMAD1/5–binding peaks around other venous-associated genes, including Coup-TFII/Nr2f2. Lastly, we demonstrate that the BMP type I receptor ALK3/BMPRIA1 is specifically expressed in early venous ECs and is required for venous identity.

**Results**

**Absence of endothelial Smad4 results in the loss of Ephb4.** To investigate the role of the canonical TGF-β and BMP pathways in arteriovenous identity, we re-examined the consequences of loss of the common mediator protein SMAD4 in ECs. As reported by Lan et al., mouse embryos with endothelial-specific deletion of Smad4 (Tie2:Cre;Smad4fl/fl, referred to here as Smad4EC/EC) die between E9.5 and E10.5 and exhibit growth retardation and gross vascular defects. Analyses of wild-type (WT) and mutant mouse embryos at E10.5 suggest that the early arterial identity is unperturbed after Smad4 deletion: the dorsal aorta could be clearly detected by morphological analysis, arterial markers DLL4 and NRP1 were detected in all Smad4EC/EC embryos and expression of the arterial Dll4in3:LacZ enhancer transgene was clearly detected in the apparent dorsal aorta in even severely growth retarded Smad4ECEC, Dll4in3:LacZ embryos (Fig. 1a, b and Supplementary Fig. 1a–d).

To analyse venous formation in the absence of SMAD4, knock-in Ephb4lacZ+ mice (which express LacZ specifically in Ephb4+ venous ECs) were crossed into the Smad4EC/EC line. Strikingly, very little Ephb4lacZ expression was detected in Smad4ECEC embryos by E10.5 (Fig. 1c, d and Supplementary Fig. 1e). Transverse sections through E10.5 Smad4ECEC;Ephb4lacZ+ embryos confirmed the lack of Ephb4lacZ expression and revealed a morphological absence of a discernible cardinal vein (Fig. 1d), a phenotype shared with Ephb4 null embryos. Loss of differentiated venous endothelium was further confirmed by immunohistochemical analysis of endogenous EphB4 and Coup-TFII (a venous-specific orphan nuclear receptor) expression relative to the pan-endothelial CD31 marker, demonstrating a requirement for Ephb4 expression: some venous-positioned vessels were detected in some E9.5 Smad4EC/EC embryos (Supplementary Fig. 1g), potentially reflecting Smad4 expression in early vessels prior to Tie2:Cre activity or indicating that SMAD4 is not required for initial Ephb4 expression. However, this result clearly demonstrates a requirement for SMAD4 in Ephb4 expression as the early veins develop and differentiate and for the proper formation of the venous vasculature.

While these results strongly support a role for the TGF-β or BMP pathways in venous identity, these pathways have also been implicated in other endothelial functions, including angiogenic sprouting, smooth muscle recruitment, vascular stability and proliferation. Therefore, it is possible that the venous phenotype seen in Smad4EC/EC embryos is secondary to a more general vascular defect that more severely affects veins comparative to the earlier fated arteries. However, Ephb4lacZ expression was also consistently reduced in the heterozygous Smad4EC+/EC; Ephb4lacZ embryos (Fig. 1c), which were normal sized and exhibited no embryonic lethality. Similar sized (but younger) Smad4EC+/EC;Ephb4lacZ+ embryos also displayed clear venous LacZ expression (Supplementary Fig. 2a), suggesting growth retardation was unlikely to be the principle driver of reduced Ephb4lacZ.
cev = these mice three days after Cre induction, they exhibited defective aorta, ica endothelial cells, COUP-TFII is expressed by arterial smooth muscle cells and other mesenchymal cells (as reported by You et al.7). White scale bars are μ picture shown. Grey scale bars are 500 endothelial cell markers EPHB4 (mouse (Dll4in3:Cre). Like endogenous front19,21. Consequently, Dll4in3:Cre is speci is active in arterial but not in venous ECs and at the angiogenic isv c knockout of Smad4EC/EC n=9), heterozygous Smad4EC/+: (n = 9) and homozygous Smad4EC/EC (n = 8) embryos also transgenic for the venous marker Ephb4LacZ (four litters total). Robust X-gal activity is detected in the veins of Smad4+/+ embryos but is reduced in Smad4EC/+ embryos and absent in Smad4EC/EC. Red box denotes zoomed region, grey numbers on bottom right denote the number of embryos similar to picture shown. Grey scale bars are 500 μ, black scale bars are 100 μ. Outliers are shown in Supplementary Figure 1a. e, f Expression of the venous endothelial cell markers EphB4 (e) and COUP-TFIi (f) in transverse sections from E10.5 Smad4+/+ and Smad4EC/EC embryos. In addition to venous endothelial cells, COUP-TFIi is expressed by arterial muscle cells and other mesenchymal cells (as reported by You et al.7). White scale bars are 100 μ. Smad4+/+ indicates Tie2:Cre-mediated deletion, /+ indicates Cre−, /EC+ indicates Cre+, Smad4+/+ and /EC+ indicates Cre+. da = dorsal aorta, ica = internal carotid artery, isa = intersomitic arteries, isv = intersomitic vessel, baa = branchial arch arteries, nt = neural tube, cv = cardinal vein, cev = branches of cerebral venous plexus. See also Supplementary Figure 1.

Additionally, induced endothelial-specific deletion of Smad4 (referred to here as indEC) after the initial vasculature was formed (using induction of CDH5(PAC);CreERT223 at either E9.5 or E11.5) also resulted in significant loss of Ephb4LacZ activity and eventual embryonic death (Fig. 2 and Supplementary Fig. 2b−c). While some venous structures could be detected in these mice three days after Cre induction, they exhibited defective morphology and fewer Ephb4LacZ+ cells (Fig. 2c).

To further rule out the possibility that either a general vascular or arterial defect underlies the lack of venous identity in Smad4EC/EC, we examined the consequences of deleting Smad4 specifically in a subset of ECs using a Dll4in3 enhancer-driven Cre transgenic mouse (Dll4in3:Cre). Like endogenous Dll4, the Dll4in3 enhancer is active in arterial but not in venous ECs and at the angiogenic front19,21. Consequently, Dll4in3:Cre is specifically active in these cell types (Supplementary Fig. 3a). This transgene is also active within the endocardium and valves of the heart in similar patterns to Tie2:Cre (Supplementary Fig. 3b−c). While analysis of the activity of the Cre reporter Rosa26R:LacZ (R26R:LacZ) in E10.5 Tie2-Cre; Smad4+/fl embryos recapitulated the venous defects reported in Figs. 1 and 2 (Fig. 3a, b), analysis of R26R:LacZ activity in Dll4in3:Cre;Smad4+/fl embryos (referred to here as Smad4ART/ART) found no vascular defects (Fig. 3c, d). Further, the observed frequency of Smad4ART/ART embryos corresponded to expected Mendelian ratios until E13.5 (Fig. 3e). This further indicates that the early venous defects seen in Smad4EC/EC embryos are not downstream of a general vascular phenotype or from arterial-specific defects and that SMAD4 is not required for arterial identity and differentiation in the early embryo.

A venous endothelium-specific enhancer for the Ephb4 gene. Our results clearly demonstrate that vascular ablation of SMAD4 results in the loss of Ephb4 expression and venous development. This suggests a crucial role for TGF-β or BMP
signalling in Ephb4 expression and consequently in venous identity. However, analysis of Smad4 mutant embryos cannot differentiate between direct and indirect targets of the TGF-β and BMP pathways. Therefore, to investigate whether SMAD4 (in combination with R-SMADs) was involved in Ephb4 expression, we investigated the transcriptional regulation of Ephb4 in the early vasculature. Complex spatial and temporal patterns of gene expression often involve the use of one or more gene enhancers, and most endothelial-specific genes are known to be primarily regulated by distal enhancer elements. Enhancers are cis-acting DNA sequences enriched for transcription factor-binding sites and are associated with regions of open chromatin with specific histone marks (e.g. H3K4Me1 and H3K27Ac). Analysis of the Ephb4 locus for sequences rich in enhancer-associated histone modifications and DNaseI hypersensitivity sites identified only two regions containing these marks specifically in ECs (Fig. 4a and Supplementary Fig. 4a). We termed these putative regulatory elements the Ephb4-2 and Ephb4-10 enhancers, reflecting their distance in kb from the transcriptional start site (TSS). Sequence analysis after ClustalW alignment of the human and mouse sequences of Ephb4-2 and Ephb4-10 showed that both enhancers contained a number of conserved ETS-binding elements (EBE), which are known to be essential for endothelial enhancer activity (Supplementary Fig. 4b–d). ETS transcription factor binding at these two sites was also indicated by analysis of a human genome-wide map of the Ets family member ERG binding in human umbilical vein endothelial cells (HUVECs) generated by Fish et al. (Supplementary Figure 4e).

Fig. 2 Induced endothelial-specific deletion of Smad4 after E9.5 results in reduced Ephb4 expression and dysfunctional venous structures.

**a–c** Representative whole-mount images (**a**), zoomed head region images (**b**) and transverse sections through head and torso regions (**c**) from Smad4+/+ and Smad4indEC/indEC embryos also transgenic for the venous marker Ephb4lacZ. Embryos investigated three and four days after tamoxifen induction at E9.5 and E10.5; Smad4+/+ control for E13.5 embryos is shown in Supplementary Fig 2b. Ephb4lacZ expression was greatly decreased in Smad4indEC/indEC embryos compared to wild-type (WT) control at E12.5 (WT n = 5, null n = 4 from two litters) and near-ablated in Smad4indEC/indEC embryos by E13.5 (n = 2 from one litter). Grey scale bars are all 1000 μm, black scale bars are all 100 μm. **d** Schematic detailing tamoxifen regime and timing. Blue and red boxes denote zoomed region, numbers on bottom right-hand corner of each whole-mount image denote the number of embryos similar to picture shown. indEC indicates CDH5(PAC):Cre/ERT2-mediated deletion, +/+ indicates Cre−. cev = branches of cerebral venous plexus, ca = cerebral artery, ccv = common cardinal vein, ao = aortal, nt = neural tube. See also Supplementary Figure 2.
To establish whether the putative Ephb4-2 and Ephb4-10 enhancers functioned in vivo, we first cloned the mouse sequences upstream of the E1b minimal promoter and the green fluorescent protein (GFP) reporter gene (Fig. 4b) and examined their activity in transgenic zebrafish. The Ephb4-10 putative enhancer did not drive GFP expression in ECs (Supplementary Fig. 4f). The Ephb4-2 putative enhancer was able to direct robust GFP expression in transgenic fish specifically in the axial veins but not in the axial arteries from early venous specification through later stages of differentiation (Fig. 4c and Supplementary Fig. 4f–j). To determine whether this enhancer was also active in mice, we next cloned the same murine Ephb4-2 enhancer upstream of the hsp68 minimal promoter and LacZ reporter gene and generated transgenic mice. As expected, the Ephb4-2 enhancer was able to drive reporter gene activity specifically to venous ECs during embryonic development (Fig. 4d, e). Tg(Ephb4-2:LacZ) mouse embryos showed patterns of LacZ expression strikingly similar to those seen in Ephb4LacZ/+ embryos (Fig. 4e), although the intensity of expression was greater in the enhancer line, reflecting the multiple copies of the Ephb4-2: LacZ transgene comparative to the single copy in Ephb4LacZ/+ knock-in. Of note, Ephb4-2: LacZ expression exactly correlated with the specific expression of endogenous Ephb4 in the venous intersomitic vessels. This contrasts with the activity of Ephb4-2: GFP in zebrafish, where expression was seen in the orthologous intersegmental arteries and veins (Fig. 4). This discrepancy may be due to differences in the pathways driving the formation of these vessels between mammals and fish (as the Ephb4-2:GFP transgene contains the mouse sequence) or reflect the requirement for flow in the specification of these vessels in

**Fig. 3** Deletion of Smad4 specifically in arterial endothelial cells does not affect vascular patterning or early embryonic development. a, b Representative pan-endothelial Tie2:Cre;Smad4fl/+ and Tie2:Cre;Smad4fl/fl whole-mount images (a) and transverse sections (b) from E10.5 embryos also transgenic for the Cre-reporter Rosa26R:LacZ. The cardinal vein (cv) cannot be identified in the Smad4fl/fl embryo, although the dorsal aorta (da) can be seen in both. Grey scale bars are 500 μm, black scale bars are 100 μm. c, d Representative arterial-specific Dll4in3:Cre;Smad4fl/+ and Dll4in3:Cre;Smad4fl/fl whole-mount images (c) and transverse sections (d) from E10.5 embryos also transgenic for the Cre-reporter Rosa26R:LacZ. Arterial-specific deletion of Smad4 had no effect on vasculature development at E10.5. Grey scale bars are 500 μm, black scale bars are 100 μm. e Observed frequency of Smad4fl/fl;Dll4in3:Cre embryos from E9.5 to P5. Only two sick (*) P5 Smad4fl/fl;Dll4in3:Cre animals were recovered. EC indicates Tie2:Cre-mediated deletion, ART indicates Dll4in3:Cre-mediated deletion, da = dorsal aorta, nt = neural tube, cv = cardinal vein, ica = inner cerebral artery, isa = intersomitic arteries. See also Supplementary Figure 3.

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**Table 1** Observed frequency of Smad4fl/fl;Dll4in3:Cre embryos from E9.5 to P5. Only two sick (*) P5 Smad4fl/fl;Dll4in3:Cre animals were recovered. EC indicates Tie2:Cre-mediated deletion, ART indicates Dll4in3:Cre-mediated deletion, da = dorsal aorta, nt = neural tube, cv = cardinal vein, ica = inner cerebral artery, isa = intersomitic arteries. See also Supplementary Figure 3.
zebrafish or relate to the high levels of proliferation seen in these zebrafish vessels\(^3\). In conclusion, this enhancer analysis clearly demonstrates that the mouse Ephb4-2 sequence represents a venous-specific enhancer for Ephb4 that closely mimics the expression of the endogenous gene in mice, providing us with a powerful tool to elucidate the regulators of Ephb4 in the venous vasculature.

The Ephb4-2 enhancer requires SMAD-binding motifs. We directly tested whether the Ephb4-2 enhancer was active in the absence of endothelial SMAD4 by generating \textit{Smad4EC/EC;Tg} (Ephb4-2::LacZ) mouse embryos. Ephb4-2::LacZ expression was largely absent in E10.5 \textit{Smad4EC/EC;Tg(Ephb4-2::LacZ)} embryos, similar to that seen with \textit{Ephb4::LacZ} (Fig. 5a and Supplementary Fig. 5a–b). These results demonstrate that endothelial SMAD4 is required for both endogenous \textit{Ephb4} expression and activity of the Ephb4-2 enhancer.

The R-SMAD/SMAD4 complexes recognize a variety of DNA sequences. These include the SMAD-binding element (SBE; Supplementary Fig. 4b), and a variety of GC-rich sequences primarily associated with the BMP-driven R-SMADS 1, 5 and 8\(^3\). The SBE was initially identified as a motif bound by human SMAD3 and SMAD4. However, SBE motifs are highly enriched within ChIP-seq and ChIP-chip peaks after immunoprecipitation with SMAD4\(^3\), with TGF-\(\beta\)-associated R-SMAD SMAD3\(^3\), and with BMP-associated R-SMADs SMAD1/5\(^3\) emphasizing the commonality of this motif in DNA regions bound by different types of R-SMAD/SMAD4 complexes. We therefore investigated whether the Ephb4-2 enhancer sequence contained any consensus or near-consensus SBEs (as defined by JASPAR\(^3\)). This analysis identified nine potential binding motifs conserved between human and mouse sequences (Supplementary Fig. 4c–d). Mutation of all nine SBEs within the Ephb4-2 enhancer (Ephb4-2::mutSBEall) resulted in a massive reduction in vascular expression in transgenic mouse and zebrafish (Fig. 5c and Supplementary Fig. 5c–e). Similar mutations to other conserved regions away from the SBEs and EBEs did not result in any alteration of

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\( \text{Fig. 4} \) The vein-specific Ephb4-2 enhancer recapitulates endogenous \textit{Ephb4} expression in fish and mouse. \( \textbf{a} \) Region around the human \textit{EPHB4} gene as seen on UCSC Browser (http://genome.ucsc.edu). Within histone marks, the three tracks show the enhancer-associated H3K4Me1 and H3K27Ac and promoter-associated H3K4Me3 marks found in human umbilical vein endothelial cells as light blue peaks. Within DNAse hypersensitivity, the heat maps show DNAse hypersensitive (HS) regions found in different cell lines, with endothelial cells labelled in red and non-endothelial cells labelled in orange. The endothelial histone marks and endothelial-specific DNAse I hypersensitivity indicate two potential endothelial enhancer regions (named -2 and -10, marked as red horizontal lines). \( \textbf{b} \), \( \textbf{c} \) The Tol2 Ephb4-2: \( \text{E}1\beta\text{GFP} \) reporter gene in transgenic zebrafish (\( \textbf{b} \)) directs venous expression of the green fluorescent protein (GFP) reporter gene in a transgenic zebrafish line expressing the pan-endothelial \textit{kdr};HRAS-mCherry (\( \textbf{c} \)). No enhancer activity (as detected by GFP expression) was seen in the dorsal aorta, whereas robust activity was detected in the cardinal and ventral veins. Expression is detected in all intersegmental vessels. White scale bars are 100 \( \mu\text{m} \). Red bracket = dorsal artery; white bracket = axial veins; iv = intersegmental vein. \( \textbf{d} \), \( \textbf{e} \) The Ephb4-2::hsp68::LacZ transgene (\( \textbf{d} \)) directs vein-specific expression of the \textit{LacZ} reporter gene in transgenic mice (\( \textbf{e} \)) as compared to \textit{Ephb4::LacZ} (\( \text{E}1\beta\text{GFP} \)). Black scale bars are 100 \( \mu\text{m} \), grey scale bars are 500 \( \mu\text{m} \), red scale bars are 1000 \( \mu\text{m} \). iv = intersomitic vessel, cv = cardinal vein, da = dorsal aorta, jv = jugular vein, ca = carotid artery, ccv = common cardinal vein, cvv = branches of the cerebral venous plexus, lv = left ventricle. See also Supplementary Figure 4.
expression (Supplementary Fig. 4c and 5c–d). In conclusion, these analyses demonstrate that both endogenous Ephb4 and Ephb4-2 enhancer activity requires SMAD4 and indicate an essential role for SMAD4/R-SMAD-binding motifs for venous expression of the Ephb4-2 enhancer.

**SMAD1/5 binds Ephb4-2 to influence Ephb4 expression.** SMAD4 forms heteromeric transcriptional complexes with multiple different phosphorylated R-SMADS prior to nuclear translocation and direct DNA binding. In mice, the combined endothelial-specific deletion of the R-SMADS Smad1 and Smad5, which are activated by BMP ligands, closely phenocopies the vascular defects and early lethality seen in Smad4EC/EC embryos, although this study ascribed the defective vasculature to an angiogenic defect, not an arteriovenous one. In contrast, combined endothelial-specific deletion of Smad2/Smad3, activated by TGF-β ligands, has no vascular effects at E10.5 and does not affect survival until after E12.5. This suggests that SMAD4-mediated venous identity involves the BMP-activated R-SMADs SMAD1 and SMAD5 (commonly written as SMAD1/5). Although SMAD1 and 5 have high affinity for SBE motifs, they prefer alternative GC-rich binding motifs. This was confirmed by Morikawa et al., who performed ChIP-seq analysis on HUVECs stimulated with high levels of BMP9 to phosphorylate SMAD1/5. This identified four over-represented motifs within SMAD1/5-bound regions (reproduced in Supplementary Fig. 6a), only one of which was localized around peak summits and therefore assumed to directly bind SMAD1/5. This motif, summarized as GGA/CGCC, was similar to the GCCG and GGCGCC SMAD1/5-binding motifs described elsewhere. In total, these GC-rich elements (referred to as GC-SBE) were found in nearly half of all SMAD1/5-bound motifs. This analysis also found significant enrichment of the SBE motif within SMAD1/5-bound regions, and demonstrated that, when present, both SBE and GC-SBE motifs were required for BMP responsiveness. While the spacer length between SBE and GC-SBE motifs varies between different SMAD1/5-bound sequences, a 5 bp spacer between the two motifs was over-represented in SMAD1/5-bound regions.

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**Fig. 5** Activity of the Ephb4-2 enhancer requires SMAD4. **a** Two sets of E10.5 Smad4+/+; Ephb4-2:LacZ and Smad4EC/EC; Ephb4-2:LacZ littersmates demonstrates loss of Ephb4-2 enhancer activity after loss of SMAD4 regardless of extent of growth retardation (n = 4 from 4 litters). Although some embryos exhibited severe growth retardation, similar sized younger embryos had robust Ephb4-2:LacZ expression (see Supplementary Fig. 5). Grey scale bars are 1000 μm. **b, c** Mutation of two composite SMAD-binding elements (SBE2/3 and SBE6/7) within the Ephb4-2 enhancer resulted in near-total loss of enhancer activity compared to wild-type enhancer in F0 transgenic zebrafish (b, mosaic due to nature of Tol2-mediated F0 transgenesis) and mice (c). Transverse section taken through the strongest lacZ-expressing F0 Ephb4-2mutSBE(2/3,6/7) embryo confirmed lack of vascular expression. Grey numbers in bottom right corner indicate the number of F0 mouse with similar expression patterns to the image shown. White and black scale bars both represent 100 μm, grey scale bars are 1000 μm. da = dorsal aorta, cv = cardinal vein, nt = neural tube; red bracket = dorsal artery; white bracket = axial veins. See also Supplementary Figure 5.
To determine whether SMAD1/5 directly binds the Ephb4-2 enhancer, we examined the Ephb4-2 enhancer sequence for GC-SBE SMAD1/5-binding motifs adjacent to the previously identified SBE motifs. This analysis identified two conserved SBE/GC-SBE composite motif elements and one additional non-conserved SBE/GC-SBE motif element (Fig. 6a and Supplementary Fig. 6a). Strikingly, the 3' conserved SBE/GC-SBE composite element contains the SBE6/7 motif that we previously demonstrated was required for Ephb4-2 enhancer activity (Fig. 5c). This SBE/GC-SBE composite element contains the optimal 5 bp spacer sequence separating the SBE and GC-SBE (Fig. 6a). The Ephb4-2 enhancer also contained numerous sequences correlating to the other three SMAD1/5-bound region-associated motifs (Fig. 6a and Supplementary Fig. 6a).

Fig. 6 The Ephb4-2 enhancer is bound by SMAD1/5. a Mouse (m) and orthologous human (h) DNA sequence of the Ephb4-2 enhancer. Red nt on human sequence correspond to regions bound by SMAD1/5 as determined from SMAD1/5 human umbilical vein endothelial cell (HUVEC) chromatin immunoprecipitation-sequencing (ChIP-seq) data32 (see also Supplementary Fig. 6c). Green boxes denote GC-SBE motifs associated with SMAD1/5 binding, blue boxes denote SBE motifs associated with all SMAD binding and grey boxes denote other over-represented motifs (MEME1, 3 and 532). Black outlined boxes denote composite SBE/GC-SBE elements, italic nt indicate linker sequences. Grey text under sequences indicates motifs previously identified as the EBEs and SBEs in earlier analysis (Supplementary Fig. 4). For sequence logos of motifs, see Supplementary Fig. 6b. b SMAD1/5 HUVEC and PASMC ChIP-seq data from Morikawa et al.32 (red peaks = statistically significant peaks after BMP9 stimulation in HUVEC). Significant binding peaks are not observed in PASMC after BMP4 stimulation (green). See also Supplementary Fig. 6c). c Box and whiskers plot of ChIP-qPCR data shows significant enrichment of SMAD1 binding at the Ephb4-2 enhancer in BMP9-stimulated HUVECs (red) compared to a control intergenic region on the same chromosome (p = 0.00015, paired two-tailed t test). SMAD1 binding at the Ephb4-2 enhancer region is not enriched over control in the absence of BMP stimulation (green) (p = 0.19212). No enrichment is observed between IgG control regions (p = 0.3154 and p = 0.19212, paired two-tailed t test). Horizontal lines = medians, boxes = interquartile range (IQR); vertical lines = minimal/maximal values (up to 1.5x IQR) and black dots = data points outside of 3x IQR. Data represents three biological replicates each with three technical replicates performed in triplicate. All data points were included in statistical analysis. d, e Morpholino (MO)-mediated partial knockdown of smad1/5 on GFP expression in arterial tg(Dll4in3:GFP) (d) and venous tg(Ephb4-2:GFP) (e) transgenic zebrafish lines, using 0.5 ng smad1 MO and 0.25 ng smad5 MO. Graphs depict expression in all embryos, Dll4in3:GFP WT n = 58, MO n = 48; Ephb4-2:GFP WT n = 104, MO n = 84. High expression = solid colour, weak = pattern, absent = solid white. Zebrafish embryos shown are representative of the predominant phenotype, red bracket = dorsal aorta, white bracket = posterior cardinal and ventral vein(s). White scale bars represent 100 μm.
To further determine whether phosphorylated SMAD1/5 directly binds the Ephb4-2 sequence, we re-examined the HUVEC SMAD1/5-biding data generated by Morikawa et al.32. In agreement with our motif analysis, significant EC-specific binding peaks for SMAD1/5 were found directly over the two conserved regions of the orthologous human EphB4-2 enhancer sequence (Fig. 6b and Supplementary 6b–c). The ability of SMAD1/5 to bind these enhancers was further confirmed by ChIP quantitative polymerase chain reaction (qPCR) using the same conditions as in Morikawa et al.32 (Fig. 6c). No other SMAD1/5-biding peaks were found elsewhere in the Ephb4 gene locus (Supplementary Fig. 6b).

To determine whether reduction in SMAD1/5 levels affected Ephb4-2 activity, we examined the consequences of morpholinino (MO)-mediated smad1/5 knocking on Ephb4-2-GFP in transgenic zebrafish. While the effects of complete deletion of smad1/5 on Ephb4-2 could not be investigated owing to severe patterning defects40, we were able to knockdown smad1/5 in tg(Ephb4 2:GFP) zebrafish using lower doses of smad1 and smad5 MO40. These MOs adhere to current guidelines41, in that they were not signifi cantly affected in mouse cardinal vein ECs (E11.5) and postnatal retinal veins (P4) (Supplementary Fig. 8). Although it is unwise to make definitive conclusions based on MO-based knockdown analysis alone, these results were strikingly similar to those seen in Smad4EC/EC mice. Consequently, when combined with enhancer sequence analysis, ChIP-seq and ChIP-qPCR results, our data strongly support a direct role for SMAD1/5–SMAD4 in the regulation of venous Ephb4 expression. SMAD1/5 binding is not strongly associated with pan-EC or arterial gene expression, even though HUVECs express many arterial-associated genes47, including the BMP type I receptor ALK1 (ACVRL1) through which BMP9 can signal42,48. This result therefore indicates that, unlike ETS factors, SMAD1/5 binding is not an essential component of all endothelial enhancers.

The loss of venous structures in Smad4EC/EC embryos suggests the possibility that SMAD4–SMAD1/5 binding may be a shared feature of venous-expressed genes. Supporting this hypothesis, our re-examination of the HUVEC SMAD1/5 binding43 found SMAD1/5-bound peaks within the loci of the venous-associated Coup-TFII (Nr2f2), Nrp2 and Einch genes (Fig. 7a and Supplementary Fig. 10a). In each case, SMAD1/5 binding coincided with enhancer-associated histone marks, DNasel hypersensitivity and sequence motifs for both ETS (EBE) and SMAD (SBE and GC-SBE) (Fig. 7a and Supplementary Fig. 10a–b), suggesting that these sequences may represent endothelial enhancers. Supporting this hypothesis, the Coup-TFII–Nr2f2–26 and EMCN-22 enhancers were all able to direct endothelial-specific LacZ activity in transgenic mice (Fig. 7b and Supplementary Fig. 10c–d).

Coup-TFII, like Ephb4, is specifically expressed in venous but not arterial ECs, where it is essential for vein acquisition and identity in early embryonic development7. Similar to the Ephb4-2 enhancer, the Coup-TFII-965 enhancer was able to drive venous endothelial-specific reporter gene expression in both transgenic mice and transgenic zebrafish lines (Fig. 7b, c and Supplementary Fig. 11a–c). Unlike Ephb4-2, Coup-TFII-965 expression was also seen in the early dorsal aorta (E8.5 and 24 hpf) and paraxial mesoderm (E9.5 and 24 hpf) in both mouse and zebrafish transgenic models (Fig. 7b and Supplementary Fig. 11a), suggesting that Coup-TFII-965 may bind additional factors not shared with Ephb4-2 that regulate non-venous endothelial activity. Although we could not independently verify SMAD1/5 binding to the Coup-TFII-965 enhancer region, analysis of the Coup-TFII-965 enhancer sequence identifi ed three conserved consensus SBE-binding motifs, the second of which was located within a core SMAD1/5-binding peak (Supplementary Fig. 10b and 11d). Unlike Ephb4-2, no conserved GC-SBE motifs were found surrounding these SBE sites, although non-conserved GC-SBE were separately identifi ed in both human and mouse sequences (Supplementary Fig. 11d). To determine whether the core SBE (SBE-peak) was required for Coup-TFII-965 activity, we tested enhancers in which this motif was mutated. Expression of the Coup-TFII-965 enhancer was entirely ablated after mutation of SBE-peak in both mouse and zebrafish transient transgenic models (Fig. 7d). As with Ephb4-2, alternative mutations within the Coup-TFII-965 enhancer away from the EBEs and SBEs did not signifi cantly influence activity (Fig. 7d). Therefore, through these results cannot prove SMAD1/5 binding to the COUP-TFII enhancer, they suggest that SMAD-mediated activation may be a shared feature of both Ephb4 and Coup-TFII gene expression in veins.

SMAD1/5 regulates transcription of venous-specific genes. Although phosphorylated SMAD1/5 (pSMAD1/5) can be detected in both venous and arterial ECs42 (Supplementary Fig. 9a), the Id1 promoter-based BMP response element (BRE, containing SBE and GC-SBE motifs)43 has been reported to be preferentially expressed in the zebrafish caulif vein comparative to the dorsal aorta44,45 and is more strongly and consistently expressed in mouse cardinal vein ECs (E11.5) and postnatal retinal veins (P4) comparative to arterial ECs46. Further, analysis of the HUVEC SMAD1/5 ChIP-seq data by Morikawa et al.32 found significant SMAD1/5 binding within only two of the 11 in vivo-characterized pan-endothelial enhancers (Supplementary Fig. 9b), and no SMAD1/5-binding peaks within any of the six known arterial gene enhancers (Supplementary Fig. 9c). This suggests that SMAD1/5 binds to the Ephb4-2-GFP expression (Supplementary Fig. 12a). Similarly,
Notch signalling can repress BMP responsiveness through the activation of SMAD6 in the angiogenic sprout, yet inhibition of Notch had no effect on the intensity and vein specificity of Ephb4-2:GFP and CoupTFII-965:GFP expression, although the expected hyper-sprouting phenotype was observed (Fig. 8a and Supplementary Fig. 12d), suggesting a lack of BMP responsiveness or venous identity. Since the other BMP type II receptors, Acrv2a and Acrv2b, are ubiquitously expressed during arteriovenous differentiation, it is therefore unlikely that spatial restriction of type II receptors alone is responsible for vein identity during embryonic development.

To investigate a potential role of BMP type I receptors in Ephb4 expression, we first investigated Ephb4-2:GFP expression after addition of the chemical inhibitor DMH1. DMH1 specifically targets the BMP type I receptors ALK1 (ACVRL1), ALK2 (ACVR1), ALK3 (BMPR1A) and ALK6 (BMPR1B). Treatment of tg(Ephb4-2:GFP) embryos with DMH1 (at a dose that did not cause severe patterning defects) resulted in significantly reduced Ephb4-2:GFP activity (Supplementary Fig. 12d), suggesting a requirement for one or more of these receptors in venous identity. Unlike the early vascular defects and lethality seen in Smad4EC/EC and Smad1/5EC/EC mice, Alk6 null mice are born healthy at...
Fig. 8 Notch-independent Alk3a/b signalling is involved in venous identity in zebrafish. a Loss of Notch signalling had no effect on the expression of the venous Ephb4-2:GFP transgene in tg(Ephb4-2:GFP) transgenic zebrafish. Representative 48 hpf embryos demonstrate similar intensities of vein-specific green fluorescent protein (GFP) expression in both control and DAPM-treated embryos. Red bracket = dorsal aorta, white bracket = posterior cardinal and ventral vein. Graph depicts observed expression pattern of GFP in tg(Ephb4-2:GFP) embryos for control (n = 51) and 100 µM DAPM-treated embryos (n = 57); black denotes high expression, grey denotes weak. See Supplementary Fig. 12 for Coup-TFII-965:GFP results and controls. b Morpholino (MO)-induced reduction of alk1 and alk2 had little effect on Ephb4-2:GFP expression in 48hpf tg(Ephb4-2:GFP) transgenic zebrafish, whereas reduction of alk3a/b resulted in significantly decreased transgene expression. Representative 48 hpf tg(Ephb4-2:GFP) embryos demonstrate reduced GFP expression after alk3a/b MO injection. Red bracket = dorsal aorta, white bracket = posterior cardinal and ventral vein. Graph depicts observed expression patterns of GFP for control (n = 51), alk1 MO (n = 45), alk2 MO (n = 45) and alk3a/b MO (n = 48); black denotes high expression, grey denotes weak expression and white denotes no detectable GFP expression. c Whole-mount in situ hybridization for bone morphogenetic protein (BMP) receptors alk3a and alk3b compared to pan-endothelial kdrl in wild-type zebrafish embryos at 20, 24, 28 and 36 hpf. Earlier time points can be seen in Supplementary Fig. 13. Both alk3a and alk3b were detected in the axial vein but not in the artery, with greater early expression seen for alk3b. Red bracket = axial artery, blue bracket = axial vein. d Whole-mount in situ hybridization for BMP ligands bmp2b and bmp4 compared to pan-endothelial kdrl in wild-type zebrafish embryos at 28 hpf. Both bmp2b and bmp4 showed stronger expression around the axial veins comparative to the dorsal artery. Red bracket = axial artery, blue bracket = axial vein. All scale bars represent 100 µm. See also Supplementary Figures 12–13.
Mendelian ratios with no reported vessel defects. Consequently, we ruled out a potential essential role for ALK6 in embryonic vein development. However, individual loss of Alk1, Alk2 and Alk3 in mice each results in embryonic lethality with some degree of vascular defects, warranting further investigation.

To determine which ALK receptor(s) are required for venous identity, we first conducted a zebrafish screen, looking at the effects of reducing levels of the zebrafish orthologues of ALK1, 2 and 3 individually in tg(Ephb4-2:GFP) fish using MOs known to recapitulate mutant phenotypes. These experiments used reduced, sub-lethal concentrations of MOs where necessary to avoid previously reported gastrulation defects, effectively creating a knockdown but not full depletion. The alkl and alk2 morphants maintained reporter gene activity in the posterior cardinal vein despite considerable morphological defects (Fig. 8b). In contrast, Ephb4-2:GFP expression was severely diminished in combined alk3a/b morphants (Fig. 8b and Supplementary Fig. 13a). The alk3a and alk3b MO used here recapitulate the zygotic mutant phenotype and can be rescued by RNA injection. As with smad1/5 MO, a dose–response curve was also included in the analysis (Supplementary Fig. 13a–b).

The loss of Ephb4-2:GFP expression in alk3a/b morphants suggested a potential role in venous gene expression. We therefore investigated the expression pattern of alk3a/b in early zebrafish embryos. Although not previously reported to be strongly expressed in the zebrafish vasculature, we detected expression of alk3b in venous positioned angioblasts from 18 to 20 s (approximately 18 hpf), and both alk3a and alk3b were highly expressed in venous regions of the axial vasculature by 28 hpf (Fig. 8c and Supplementary Fig. 13c). In comparison, we detected little expression of endogenous ephb4a until 20 s
(approximately 19–20 hpf; Supplementary Fig. 13d), suggesting alk3a/b expression begins concurrently or just before that of ephb4a. The expression of known ALK3 ligands bmp2b and bmp4 were also highest around the developing trunk vein18 (Fig. 8d). Further, MO-induced depletion of alk3a/b resulted in significant reduction in endogenous ephb4a expression (Supplementary Fig. 14a–c), while morphological analysis of alk3a/b morphants demonstrated the presence of only one axial vessel and a lack of proper blood circulation (Supplementary Fig. 14d). These results therefore suggest that the spatially restricted ALK3 receptors may play a fundamental role in ephb4 expression and vein morphogenesis in zebrafish.

Endothelial-specific loss of Alk3 (Bmpr1a) in mice results in early vascular defects similar to Smad4EC/EC embryos12, supporting a specific role for ALK3 in venous identity in mammals as well as zebrafish. Comparatively, endothelial deletion of the vascular type I receptors Alk1(Acvrl1) and Alk2 (Acrv1) caused later lethality55,59. Analysis of murine ALK3 expression also found that it was preferentially found in venous ECs relative to arterial endothelium at E8.5 and E9.5 (Fig. 9a and Supplementary Fig. 15a). Further, expression of the ALK3 ligand Bmp4 was also preferentially found around venous vessels (Fig. 9b and Supplementary Fig. 15b). Although the vascular defects seen after endothelial-specific Alk3 deletion were previously attributed to defective vessel maturation and problems in atrioventricular endocardial cushion formation12, arteriovenous differentiation was not investigated in these embryos. Therefore, we re-investigated the consequences of endothelial-specific ablation of Alk3 in mice. As with Smad4EC/EC embryos, Tie2-Cre-mediated deletion of Alk3 (Alk3EC/EC) resulted in severe defects by E10.5 but did not ablate expression of the arterial-associated Dll4in3: LacZ transgene (Fig. 9c, e). However, no expression of Ephb4LacZ was detected in any Alk3EC/EC embryos by E10.5 (Fig. 9d, e). Morphological analysis of Alk3EC/EC/Dll4in3:LacZ and Alk3EC/EC; Ephb4LacZ embryos confirmed that these embryos contain Dll4in3:LacZ+ dorsal aortas but lack both Ephb4LacZ-positive vessels (Fig. 9e). Although many of these embryos were substantially defective by E10.5, all heterozygous Alk3EC/+ embryos also showed consistently reduced levels of Ephb4LacZ expression, although they exhibited no clear morphological defects (Fig. 9d). Strikingly, deletion of Alk3 using the arterial expressed Dll4in3:Cre (Alk3ART/ART) had little effect on embryos at E10.5 (Fig. 9f–h), demonstrating that, similar to Smad4EC/EC, the vascular defects seen in Alk3EC/EC embryos are not caused by general vascular maturation or cardiac defects. Previous analysis also found no defects in haematopoiesis in Alk3EC/EC embryos12. Because the ALK3 receptor is specific for the BMP pathway and signals only through SMAD1/5/860, these mutant mouse experiments also provide direct in vivo murine evidence supporting our zebrafish smad1/5 MO-mediated observations linking SMAD1/5 with the regulation of Ephb4 and venous identity. In conclusion, these results support a model of arteriovenous development in which the venous-restricted ALK3 receptor is indispensable for vein morphogenesis downstream of BMP ligands and upstream of SMAD4–SMAD1/5-dependent transcriptional activation of venous genes (Fig. 10).

Discussion

The crucial roles for VEGF-A and NOTCH in activating arterial EC identity has often led to the supposition that venous specification is either default or actively repressed in arteries. However, our results clearly demonstrate that venous genes are directly transcriptionally activated via a BMP/ALK3/SMAD1/5 signalling cascade. This therefore supports a model for arteriovenous differentiation in which endothelial progenitors positively acquire either arterial or venous fate downstream of independent signalling pathways early in development.

The specific expression of the Alk3 type I receptor and Bmp2/4 ligands in venous ECs suggests that venous ECs may be innately sensitive to BMP signalling65. Supporting this, the type II BMP receptors Bmpr2a and Bmpr2b and cargo-specific adaptor Dab2 are also enriched in zebrafish venous ECs18,44. Although neither Bmpr2a/b nor Dab2 appeared to be absolutely required for Ephb4-2 activity or venous specification, the combined expression of these BMP signalling components in addition to Alk3 may well contribute to an increased venous endothelial response to BMP ligands. It is also possible that the type I receptor ALK2 (ACVR1L) contributes to venous differentiation at later stages of embryogenesis and after birth: while endothelial-specific loss of Alk2 did not phenocopy the early vascular defects seen in Smad4EC/EC or Alk3EC/EC embryos, ALK2 is implicated in venous angiogenesis alongside Alk3 in zebrafish18 and is strongly expressed in venous ECs in the postnatal retina, where Alk2 and Alk3 are required for correct retinal vessel morphogenesis61.

The results in this paper strongly indicate that the establishment of venous identity is the principle function for BMP signalling in the early vasculature. In particular, the absence of detectable vascular defects in E10.5 Smad4ART/ART embryos suggests that many of the previous roles attributed to BMP signalling via SMAD4 in ECs, including vascular integrity, remodelling and smooth muscle recruitment12,14, may be secondary to the loss of correct arteriovenous specification and resultant breakdown of vascular patency. While we did not directly investigate cardiac valve defects, both Alk3ART/ART and Smad4ART/ART embryos survived until late gestation despite endocardial expression of Dll4in3:Cre, indicating that the requirement for BMP signalling in cardiac valve formation may manifest in death at later embryonic stages. This does not necessarily suggest that BMP signalling is unimportant for valve morphogenesis: endocardial nuclear factor of activated T cell (NFAT) signalling is absolutely required for heart valve morphogenesis, yet endothelial NFAT-deficient embryos display aberrant valves and gestational lethality only after E13.562. Similarly, our results do not directly contradict a role for BMP-SMAD1/5 in angiogenic sprouting15,22. While the Dll4in3:Cre used to generate Smad4ART/AKT embryos is also active in ECs at the angiogenic front, Dll4in3 is preferably...
active in tip cells. Conversely, the angiogenic role of BMP signalling is thought to occur primarily in stalk cells, where it co-operates with the Notch pathway. Agreeing with this role, endothelial SMAD1/SMAD5 binding was also enriched around Notch pathway genes, although not over the three characterized arterial Notch-pathway enhancers (this paper and ref.32). However, recent reports in zebrafish demonstrate that venous ECs are the primary source of cells at the angiogenic front, suggesting that it may be impossible to entirely separate the requirement of SMAD1/5 in venous identity with any potential role in embryonic angiogenic sprouting.

SMAD1/5 is involved in many other BMP-driven processes beyond the vasculature, suggesting that any recruitment of SMAD4–SMAD1/5 to venous enhancers would require additional cofactors33,64. The presence of ETS-binding motifs (EBEs) alongside SMAD motifs in each venous enhancer suggests a role for ETS transcription factors such as ERG, which are already established as central regulators of endothelial gene expression32, in conferring endothelial specificity to SMAD1/5-bound vein enhancers. These may potentially assist SMAD complexes in binding DNA, as alone they have only low affinity for DNA33. However, ETS factor binding is also a feature of pan-endothelial, arterial and angiogenic enhancers19–21, making it unlikely that ETS factors contribute directly to venous specificity.

Although ligand and receptor density or specificity may result in increased venous pSMAD1/5 during early arteriovenous development18, some BMP receptors are clearly expressed in both mouse and zebrafish arteries (e.g. ALK1)65,66, and HUVEC SMAD1/5 ChIP analyses demonstrate that BMP9, more commonly associated with ALK1-mediated angiogenesis, can stimulate SMAD1/5 to bind to venous enhancers in vitro. Nuclear pSMAD1/5 is also found in both arterial and venous ECs, yet both ErbB-2 and CoupTfII-965 enhancers were predominantly specific to venous endothelium. It is therefore highly probable that additional co-factors cooperate with ETS and SMAD1/5 to activate specific genes in veins and/or to repress these genes elsewhere. There is already some evidence supporting a role for direct transcriptional activation and repression in SMAD1/5-dependent vein-specific gene expression: the Id1-based BRE, effectively a group of SBE and GC-SBE motifs, is virtually silent in conferring endothelial specificity to SMAD1/5-bound vein enhancers19, as alone they have only low affinity for DNA33. Further, activity of the BRE-CMV is enriched but not specific to the venous endothelium. These observations therefore suggest that DNA motifs binding both activating and repressive transcription factors may be needed in addition to SBE and GC-SBE elements to achieve vein-specific gene activation.

Our results also present a compelling case for ALK3 to be considered as a target for antiangiogenic therapy. Current anti-angiogenic drugs, which aim to prevent the rapid vessel growth seen during tumourigenesis, primarily target the VEGF signalling pathway. However, the response is often limited and additional therapeutic targets are needed68. Combined with the emerging understanding of the importance of the venous endothelium at the angiogenic front63, the essential and independent role for ALK3 in venous growth demonstrated here suggests that targeting of ALK3 may effectively inhibit tumour angiogenesis.

Methods

Cloning. For venous enhancers, all enhancer sequences were initially generated as custom-made, double-stranded linear DNA fragments (GeneArt Strips™, Life Technologies) with the exception of Coup-TfII-965 and Nrp2+/-26, which were generated by PCR from genomic DNA. The sequences of all enhancers are provided in Supplementary Methods. DNA fragments/PCR products were cloned into pCR8/GW/enhancer entry vector to a suitable destination vector using Gateway LR Clonase II Enzyme mix (Life Technologies, 11791–100) following the manufacturer’s instructions. For mouse transgenic enhancer was cloned into the hp68 LaCZ Gateway vector (provided by N. Ahituv). For zebrafish transgenesis, the enhancer was cloned into the E1b-GFP-Tol2 vector (provided by N. Ahituv).

To drive the Dll4in3Cre transgene, the Dll4in3 enhancer19 was amplified by PCR to include engineered 5’ SacII and 3’ NotI restriction sites (sequences in Supplementary table 1) and cloned into the promoterless p-AUG-pGAL construct69. The Dll4in3 promoter was also generated by PCR with engineered 5’ SpeI and 3’ BamHI sites (sequences in Supplementary Methods) and cloned into the Dll4in3 enhancer–p-AUG-pGAL construct. Transgenic mice were generated to ensure enhancer/promoter context, after which the Zsgal marker was replaced by Cre-ORT/polyA amplified from the pCAG-Cre vector. The full sequence of the Dll4in3 enhancer/promoter is provided in Supplementary Methods.

Mice. All animal procedures complied with all relevant ethical regulations, were approved by the Clinical Medicine Local Ethical Review Committee, University of Oxford and licensed by the UK Home Office. Smad1fl/fl mice70–72 were provided by Ralf Adams and ALK1fl/fl mice73 were provided by Yuji Mishina. Dll4in3fl/fl mice were obtained from The Jackson Laboratory (stock number 004128), Ephb4fl/fl mice were purchased from JAX (stock number 004128), Ephb4fl/fl mice were provided by David Anderson, Cdh5(PAC)-CreERT2 mice74 were provided by Elizabeth Robertson, Ephb4fl/fl mice were purchased from JAX (stock number 004128), Gfpfl/fl mice were purchased from Life Technologies, 11791-100) following the manufacturer’s instructions. Once cloning was confirmed, the enhancer sequence was transferred from the pCR8/GW/enhancer entry vector to a

ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-08315-w
NATURE COMMUNICATIONS  | (2019) 10:453 | https://doi.org/10.1038/s41467-019-08315-w | www.nature.com/naturecommunications
command and combined with Z-stack collection under a confocal microscope Zeiss LSM 710 MP (Carl Zeiss) at 488 nm excitation and 590 nm emission (EGFP) and 587 nm and 610 nm (mCherry), respectively. For imaging of stable zebrafish transgenic lines (in Figs. 4 and 8), zebrafish embryos were treated with 0.03 mg/ml PTU (N-phenylthiourea, P7629, Sigma) at 24 hpf to prevent melanogenesis. Embryos were embedded in 0.4% TopVision low melting point agaroose (R8001, Thermofisher) in 0.14 mg/ml Tricaine (ethyl 3-aminobenzoate methanesulfonat, A5040, Sigma) in glass bottom multi-well culture plates (MatTek).

Analysis of transgene expression was qualitative not quantitative, therefore no statistical analysis was applied to the observations of reporter gene intensity and pattern. Numbers of transgenic zebrafish embryos used followed precedent set by similar published papers, and was no <40/transgene. Where variations in pattern. Numbers of transgenic zebrafish embryos were detected within a single experimental set, this is represented in the relevant figure by a graph summarizing variance with n numbers provided in the legend. No experimental randomization or blinding was used as we did not consider this necessary.

MO and chemical inhibition in zebrafish. MOs were dissolved in ultrapure water and injected into 1–2-cell stage zebrafish embryos as previously described23.

Sequential MO treatments were complemented with 100 µM DAPM (Calbiochem) added at 10 hpf49, 40 µM LY294002 (MedChem Express) and BMP, embryos were manually dechorionated and incubated with 100 µM DAPM (Calbiochem) added at 10 hpf49. Control embryos were treated with identical concentrations of dimethyl sulfoxide without inhibitor.

In situ hybridization. All in situ hybridization experiments were conducted at least three separate times. Analysis was qualitative not quantitative, therefore no statistical analysis was applied to the observations of staining intensity and pattern. Numbers of transgenic zebrafish embryos were no <40/g (transgene) line. Where variations in expression were detected within a single experimental set, this is represented in the relevant figure by a graph summarizing variance with n numbers provided in the legend. No experimental randomization was used as we did not consider this necessary. Experimental blinding was not used as notophytes of control and treated embryos were easily detectable due to dorsalization and sprouting defects.

In situ hybridization. For zebrafish whole-mount in situ hybridization, ephb4, efnb2, alk2, alk3a and alk3b probes were generated as custom-made, double-stranded linear DNA fragments (GeneArt Strings®, Life Technologies), cloned into the pT7-blue vector using the TOPO-TA Cloning Kit (Invitrogen) and transcribed using SP6 and T7. The sequences are provided below. dlv1, dlv1, bmp4 and bmp2 probes were used as previously described72,73. Whole-mount in situ hybridization was conducted as previously described4. Briefly, embryos were collected at 28 and 36 hpf, fixed overnight at 4 °C in 4% PFA, dehydrated and stored in 100% ethanol. Before ephb4, 1% PBS with 0.1% Tween-20 (PBST), blocked in 3% H2O2/0.5% KOH and then embryos were re-fixed for 4% PFA for 20 min. The embryos were made permeable by digestion with 15 µg/ml proteinase K (Sigma-Aldrich) for 10 min (28 hpf embryos) or 30 min (36 hpf embryos) followed by two PBST washes, fixed in 4% PFA for 5 min, washed five times with PBST, then transferred into hybridization solution (50% formamide, 5× Saline-Sodium Citrate (SSC), 0.1% Tween 20, 50 µg/ml heparin, 500 µg/ml yeast RNA, 10 mM citric acid) for 2 h at 65 °C, transferred into diluted antisense riboprobe/hybridization solution and incubated overnight at 65 °C. Probes were removed and embryos relocated to a Bioline HT1 in situ machine (Intavis). Embryos were washed through a dilution series of 2× SSC and incubated and followed by 0.2× SSC at 65 °C and thereafter taken through room temperature dilution washes of 100% MABT (0.1 M maleic acid, 0.15 M NaCl, pH 7.5). Non-specific sites were blocked with MAB block (MABT with 2% Böhringer block reagent), and the embryos were incubated for 1 h with 1:2000 antiDIG antibody (Roche) at 4 °C, before washing in PBST. Prior to staining, embryos were rehydrated in alkaline phosphatase (AP) buffer and the in situ signal was developed at room temperature for two washes in MABT buffer (0.1 M maleic acid, 0.15 M NaCl, 0.01% Tween-20, 2 mM Levamisole (Sigma-Aldrich), pH 7.5) before blocking for 1 h in 2% Böhringer Blocking Reagent (Roche)/10% sheep serum/MABT. They were then incubated overnight at 4 °C. Sections were then DAPI stained, mounted in Anti-dig-2-Anti-Telopeptide Antibody (Roche) diluted 1:2000. Finally slides were washed three times in MABT and then two times in AP buffer (100 mM Tris, pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween-20, 2 mM Levamisole), before AP activity was detected using BM Purple (Roche).

Immunostaining. For whole-mount DLL4 and CD31 staining, embryos were dissected and fixed in 4% PFA on ice for 1 h (DLL4) or overnight (CD31), rinsed in PBST (0.1% TritonX-100 in PBS), incubated in 1 h in blocking solution (10% Normal Donkey Serum in DLL4 (R&D Systems, AF1389, 1 in 50 dilution) or rat monoclonal to CD31 to 1:50 dilution) or rabbit monoclonal to DLL4 (Dia-310, Dianova, 1 in 250 dilution). Samples were washed in PBST and subsequently incubated overnight with suitable species-specific rabbit or mouse monoclonal or polyclonal anti-DIG antibody (1:300, Thermo Fisher Scientific) in 0.1% PBST at 4 °C.

For immunofluorescence staining on paraffin sections, E8.5–E10.5 mouse embryos were harvested and fixed overnight in 4% PFA/PBS at 4 °C, embedded, and sectioned as described above. Sections were dewaxed by heat, stained in Hematoxylin and counterstained in 5% Hydrogen Peroxide (Sigma Aldrich) and rehydrated through an ethanol series. Antigen retrieval was carried out by boiling slides in 10 mM sodium citrate buffer, pH 6.0, in a commercial pressure cooker for 3 min, followed by two washes in PBS. Sections were then incubated at room temperature in blocking solution (1% bovine serum albumin (BSA), 2% donkey serum in PBS or 10% Mouse donkey serum incubated in 3% hydrogen peroxide (Sigma Aldrich) for 5 min and rinsed in PBS. The Avidin-Biotin Blocking Kit (SP-2001, Vector Laboratories) and Mouse on Mouse (M.O.M.®) Basic Kit (BMB-2202, Vector Laboratories) were then used according to the manufacturer’s instructions, with a mouse monoclonal antibody to COUP-TFII (PP-H7147) in 1:100 dilution. For whole-mount DLL4 and CD31 staining, embryos were dissected and fixed in 4% PFA/PBS for 10 min and then incubated in 30% Sucrose/PBS at 4 °C, thawed at room temperature and washed in PBS to remove the OCT embedding medium. All in situ analyses were conducted at least two separate times. Analysis was qualitative not quantitative, therefore no statistical analysis was applied to the observations of staining intensity and pattern. Numbers of transgenic zebrafish embryos were no <40/g (transgene line). Where variations in expression were detected within a single experimental set, this is represented in the relevant figure by a graph summarizing variance with n numbers provided in the legend. No experimental randomization or blinding was used as we did not consider this necessary. Experimental blinding was not used as notophytes of control and treated embryos were easily detectable due to dorsi-
Received: 11 April 2018 Accepted: 20 December 2018
Published online: 28 January 2019

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Acknowledgements

We thank E. Robertson for providing the Smad4<sup>fl/fl</sup> mice, D. Anderson for providing the Ephb4<sup>KO</sup>/mice, Y. Mishina for providing the Alk5<sup>fl/fl</sup> mice, R. Adams for providing the Cib5<sup>fras2</sup>/Cib5<sup>fras2</sup> mice, N. Ahituv for providing GW vectors, and M. Shipman for help with layout. This work was supported by the Ludwig Institute for Cancer Research, the BBSRC (BB/I02038/1; to A.N. and S.D.V.), the BHF Centre of Research Excellence, Oxford (RE/08/004; to S.D.V.), and the BHF (PG/16/34/2135; to S.P.) and (FS/17/3532929; to S.D.V. and A.N.).

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Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-08315-w.

Competing interests: The authors declare no competing interests.

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Journal peer review information: Nature Communications thanks the anonymous reviewers for their contributions to the peer review of this work.

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