Vein Identity Requires BMP Signalling Through ALK3

Alice Neal1,2, Svanhild Nornes1,2, Sophie Payne1, Marsha D. Wallace1, Martin Fritzsche1, Pakavarin Louphrasitthiphol1, Robert N. Wilkinson3, Kira M. Chouliaras1, Ke Liu4, Karen Plant3; Radhika Sholapurkar1, Indrika Ratnayaka1, Wiebke Herzog5,6, Gareth Bond1, Tim Chico3, George Bou-Gharios4, Sarah De Val1,2

1 Ludwig Institute for Cancer Research Ltd, Nuffield Department of Medicine, University of Oxford, Oxford OX3 7DQ, United Kingdom.

2 Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford OX1 3PT, United Kingdom.

3 Department of Infection, Immunity and Cardiovascular Disease and Bateson Centre, University of Sheffield,

Sheffield S10 2TN, United Kingdom.

4 Institute of Aging and Chronic Disease, University of Liverpool, Liverpool L69 3GA, United Kingdom.

5 University of Muenster, Muenster, Germany.

6 Cells-in Motion Cluster of Excellence EXC1003-CiM, University of Muenster, Germany.

**Corresponding author:**

Sarah De Val

Ludwig Institute for Cancer Research Ltd

University of Oxford

Nuffield Department of Medicine

Old Road Campus Research Building (off Roosevelt Drive)

Headington

Oxford OX3 7DQ

01865 617435

sarah.deval@ludwig.ox.ac.uk

**Abstract**

Vein endothelial cells are molecularly and functionally distinct from their arterial counterparts. Although venous cells are often considered the default endothelial state, genetic manipulations can modulate both acquisition and loss of venous fate, indicating that vein 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 venous formation and loss of the venous-specific gene *Ephb4,* but do not affect arterial identity. Analysis of a novel venous endothelium-specific enhancer for *Ephb4* found enriched binding of SMAD1/5 and a direct requirement for SMAD binding motifs. Further, our data demonstrates that BMP/SMAD-mediated *Ephb4* activation requires the venous-enriched BMP type I receptor ALK3 (BMPR1a). This work therefore demonstrates an indispensable role for BMP signalling in the establishment of the venous vasculature.

**Introduction**

Arteriovenous differentiation begins prior to the onset of blood flow, indicating an important role for genetic fate determination1. 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 concurrent with the expression of *Ephrin b4 (Ephb4)*2. *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 valves3,4. Loss of *Ephb4* expression results in embryonic lethality by E10.5, with significant defects in the formation of the cardinal vein whilst the dorsal aorta is relatively unaffected5.

It has been hypothesized that endothelial cells are venous by default while arterial identity is acquired, however growing evidence suggests that venous endothelial cell identity is dependent upon dynamic gene regulation. For example, the PI3K-AKT pathway downstream of Vascular Endothelial Growth Factor (VEGF-A) actively promotes venous differentiation through inhibition of ERK/MAPK6, whereas the venous-specific orphan nuclear receptor Coup-TFII (*Nr2f2*) actively represses arterial gene expression7. 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 (BMPs) ligands, are widely found in blood vessels, as are their cognate type II and type I transmembrane receptor kinases8. Ligand binding results in the intracellular phosphorylation of the receptor-regulated SMADs (R-SMADs), which form heteromeric complexes with the common-mediator SMAD4 and translocate to the nucleus where they directly bind DNA

9.

Mutations in the BMP receptor *ALK1(ACVRL1)*, *SMAD4* and the accessory type III receptor *ENG* are associated with the human condition Hereditary Hemorrhagic Telangiectasia (HHT), characterised by arteriovenous malformations (AVMs) and mucocutaneous telangiectasias10. However, although gene ablation studies in mice support a crucial role for TGF-β and BMP signalling in the vasculature

11-16, the use of different Cre lines, confounding effects of cardiac valve defects and inconsistent analysis of arteriovenous differentiation 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 sprouting1,17,18, 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 have combined a detailed analysis of arteriovenous differentiation after TGF-β/BMP disruption in both mice and fish with a comprehensive delineation of the transcriptional regulation of the essential venous identity gene *Ephb4*, identifying a previously unsuspected role for BMP signalling in venous differentiation via transcriptional activation.

**Results**

**Endothelial cell-specific ablation of *Smad4* results in the loss of the definitive venous marker *Ephb4* but does not affect arterial identity**

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 endothelial cells. As reported by Lan et al.,14 mouse embryos with endothelial-specific deletion of *Smad4* (*Tie2:Cre;Smad4fl/fl,* referred to here as *Smad4EC/EC*) die between E9.5 and 10.5 and exhibit growth retardation and gross vascular defects. Analyses of wildtype and mutantmouseembryos at E10.5 suggest that 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 transgene19-21 was clearly detected in the apparent dorsal aorta in even severely growth retarded *Smad4EC/EC; Dll4in3:LacZ* embryos (Fig. 1a-b and Supplementary Fig. 1a-d).

To analyse venous formation in the absence of TGF-β and BMP signalling, knock-in *Ephb4LacZ/+*mice5 (which express *LacZ* specifically in *Ephb4*+ venous endothelial cells) were crossed into the *Smad4EC/EC* line. Strikingly, very little *Ephb4LacZ* expression was detected in *Smad4EC/EC* embryos by E10.5 (Fig. 1c-d and Supplementary Fig. 1e). Transverse sections through E10.5 *Smad4EC/EC;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 embryos5. 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 in *Smad4+/+*and *Smad4EC/EC*embryos (Fig. 1e-f). The requirement to use the endothelial-specific Tie2:Cre driver (which is not consistently expressed in early embryo litter-mates, Supplementary Fig. 1f) makes it challenging to establish from these results whether SMAD4 is required for initiation or maintenance of *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 data 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 novel role for the TGF-β or BMP pathways in venous identity, they have also been implicated in other endothelial functions including angiogenic sprouting, smooth muscle recruitment, vascular stability and proliferation 14,15,18,22. Therefore it is possible that the venous phenotype seen in *Smad4EC/EC* embryos is secondary to a more general vascular defect which more severely affects veins comparative to the earlier fated arteries. However, *Ephb4LacZ* expression was also consistently reduced in the heterozygous *Smad4EC/+; Ephb4LacZ* embryos (Figure 1c), which were normal sized and exhibited no embryonic lethality. Similar sized (but younger) *Smad4+/+;Ephb4LacZ/+* embryosalsodisplayed clear venous *LacZ* expression (Supplementary Fig. 2a), suggesting growth retardation was unlikely to be the principle driver of reduced *Ephb4LacZ*. 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 sub-set of endothelial cells using a novel Dll4in3 enhancer-driven Cre transgenic mouse (Dll4in3:Cre). Like endogenous *Dll4,* the Dll4in3 enhancer is active in arterial but not venous endothelial cells, and in tip cells at the angiogenic front 19,21. 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; Smad4fl/fl* embryos recapitulated the venous defects reported in Fig. 1-2 (Fig. 3a-b), analysis of *R26R:LacZ* activity in *Dll4in3:Cre;Smad4fl/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.

**Identification of 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*.* Thissuggests 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 enhancers24, and most endothelial-specific genes are known to be primarily regulated by distal enhancer elements25. 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) 26. Analysis of the *Ephb4* locus for sequences rich in enhancer associated histone modifications and DNaseI hypersensitivity sites 26-28 identified only two regions containing these marks specifically in endothelial cells (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 25 (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.,* 29 (Supplementary Figure 4e).

To establish if the putative Ephb4-2 and Ephb4-10 enhancers functioned *in vivo*, we first cloned the mouse sequences upstream of the E1b minimal promoter 30 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 endothelial cells (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 the axial arteries from early venous specification through later stages of differentiation (Fig. 4c and Supplementary Fig. 4f-j). To determine if 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 endothelial cells 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. These analyses therefore clearly demonstrate that the Ephb4-2 sequence represents a novel, venous-specific enhancer for *Ephb4* that closely mimics the expression of the endogenous gene, providing us with a powerful tool to elucidate the regulators of *Ephb4* in the venous vasculature.

**The Ephb4-2 venous-specific enhancer requires SMAD binding motifs**

We directly tested whether the Ephb4-2 enhancer was active in the absence of endothelial SMAD4 by generating *Smad4EC/EC;Tg(Ephb4-2:LacZ)* mouse embryos. *Ephb4-2:LacZ* expression was largely absent in E10.5 *Smad4EC/EC;Tg(Ephb4-2:LacZ)* embryos, similar to that seen with *Ephb4LacZ* (Fig. 5a and Supplementary Fig. 5a-b). These results demonstrate that endothelial SMAD4 is required for both endogenous *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 31,32. 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 (e.g. 33,34), with TGFß-associated R-SMAD SMAD3 (e.g 35,36), and with BMP-associated R-SMADs SMAD1/5 (e.g. 31) 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 JASPAR37). 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 (Ephb4-2mutSBEall) resulted in a massive reduction in vascular expression in transgenic fish (Supplementary Fig. 5c-d). Of these nine SBE, four occurred as part of palindromic repeat SBE sequences (SBE2/3 and 6/7) commonly associated with SMAD binding32. Mutations of these SBE2/3 and SBE6/7 sites separately also resulted in near-total loss of the Ephb4-2 activity in transient transgenic mice and zebrafish (Fig. 5 c and Supplementary Fig. 5c-e). Similar mutations to other conserved regions away from the SBEs and EBEs did not result in any alteration of expression (Supplementary Fig. 4c and 5 c-d). In conclusion, this analysis 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 the *Ephb4* enhancer region *in vitro* and can influence *Ephb4* expression**

SMAD4 forms heteromeric transcriptional complexes with multiple different phosphorylated R-SMADS prior to nuclear translocation and direct DNA binding32. 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 one15. 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 E12.516. 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 motifs38. This was confirmed by Morikawa et al., 31, who performed ChIP-seq analysis on human umbilical vein endothelial cells (HUVECs) stimulated with high levels of BMP9 to phosphorylate SMAD1/5. This identified four over-represented motifs within SMAD1/5-bound regions (reproduced in Supplemental 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 38. In total, these GC-rich elements (referred to as GC-SBE) were found in nearly half of all SMAD1/5-bound motifs. This work 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 responsiveness31. While the spacer length between SBE and GC-SBE motifs varies between different SMAD1/5-bound sequences, a 5bp spacer between the two motifs was over-represented in SMAD1/5-bound regions 31.

To determine if SMAD1/5 directly binds the Ephb4-2 enhancer, we therefore 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 5bp 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).

To further determine whether phosphorylated-SMAD1/5 directly bind the Ephb4-2 sequence, we re-examined the HUVEC SMAD1/5 binding data generated by Morikawa *et al*.31. In agreement with our motif analysis, significant endothelial cell-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 q-PCR using the same conditions as 31(Fig. 6c). No other SMAD1/5 binding peaks were found elsewhere in the *Ephb4* gene locus (Supplementary Fig. 6b).

To determine if reduction in SMAD1/5 levels affected Ephb4-2 activity, we examined the consequences of morpholino (MO)-mediated *smad1/5* knock-down on Ephb4-2:GFP in transgenic zebrafish. While the effects of complete deletion of *smad1/5* on Ephb4-2 could not be investigated due to severe patterning defects39, we were able to knock-down *smad1/5* in *tg(Ephb4-2:GFP)* zebrafish using lower doses of *smad1* and *smad5* MO (previously described by 39). These MOs adhere to current guidelines 40, in that they recapitulate the mutant phenotype, give similar results to other MOs targeting the same gene and can be rescued by RNA expression 39. Knock-down of *smad1/5* resulted in near-complete ablation of Ephb4-2:GFP activity in the trunk and segmental vessels (Fig. 6d and Supplementary Fig. 7a). Although all embryos also had a mild dorsalisation defect that significantly affected the tail region, expression of the arterial-specific Dll4in3:GFP transgene was still robust in the tail region of MO-treated embryos, suggesting that a pan-vascular defect was unlikely to explain the loss of Ephb4-2:GFP expression. A dose response curve (with total MO concentration ranging from 0.375ng-1.5ng), demonstrated both increased dorsalization and decreased Ephb4-2:GFP activity as MO concentrations increased (Supplementary Fig. 7b-c). Loss of Ephb4-2:GFP after *smad1/5* knock-down was also unlikely to be related to reduction of blood flow, as Ephb4-2:GFP expression was not significantly affected in *tnnt2* morphants, which have no heartbeat (Supplementary Fig. 7d). Wholemount *in situ* analysis in these morphants was used to also examine the expression patterns of endogenous *ephb4a*, *dll4* and the arterial marker *ephb2a*: arterial *dll4 and ephb2a* was detected in *smad1/5* morphants, while *ephb4a* expression was greatly reduced (Supplementary Fig. 8). Although it is unwise to make definitive conclusions based on morpholino-based knock-down analysis alone, these results were strikingly similar to those seen in the *Smad4EC/EC*mice. Consequently, when combined with enhancer sequence analysis, ChIP-seq and ChIP-qPCR results, our data strongly supports a direct role for SMAD1/5-SMAD4 in the regulation of venous *Ephb4* expression.

**SMAD1/5 directly regulates transcription of key venous differentiation genes**

Although phosphorylated SMAD1/5 (pSMAD1/5) can be detected in both venous and arterial ECs (Supplementary Fig. 9a and 41), the Id1 promoter-based BMP response element (BRE, containing SBE and GC-SBE motifs) 42 has been reported to be preferentially expressed in the zebrafish caudal vein comparative to the dorsal aorta 43,44, and is more strongly and consistently expressed in mouse cardinal vein ECs (E11.5) and post-natal retinal veins (P4) comparative to arterial ECs 45. Further, analysis of the Morikawa *et al*., 31 HUVEC SMAD1/5 ChIP-seq data found significant SMAD1/5 binding within only two of eleven *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 binding is not strongly associated with pan-EC or arterial gene expression, even though HUVECs express many arterial-associated genes (e.g 46), including the BMP type I receptor ALK1 (ACVRL1) through which BMP9 can signal 31,47. This data 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 binding 31 found SMAD1/5-bound peaks within the loci of the venous-associated *Coup-TFII (Nr2f2)*, *Nrp2* and *Emcn* genes (Fig. 7a and Supplementary Fig. 10a). In each case, SMAD1/5 binding coincided with enhancer-associated histone marks, DNaseI 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 novel endothelial enhancers. Supporting this hypothesis, the CoupTFII-965, Nrp2+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 endothelial cells, where it is essential for vein acquisition and identity in early embryonic development 7. Similar to the Ephb4-2 enhancer, the CoupTFII-965 enhancer was able to drive venous endothelial-specific reporter gene expression in both transgenicmice and transgeniczebrafish lines (Fig. 7b-c and Supplementary Fig. 11a-c). Unlike Ephb4-2, CoupTFII-965 expression was also seen in the early dorsal aorta (E8.5 and 24hpf) and paraxial mesoderm (E9.5 and 24hpf) in both mouse and zebrafish transgenic models (Fig. 7b and Supplementary Fig. 11a), suggesting that CoupTFII-965 may bind additional factors not shared with Ephb4-2 which regulate non-venous endothelial activity. Although we could not independently verify SMAD1/5 binding to the CoupTFII-965 enhancer region, analysis of the CoupTFII-965 enhancer sequence identified 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 identified in both human and mouse sequences (Supplementary Fig. 11d). To determine if the core SBE (SBE-peak) was required for CoupTFII-965 activity, we tested enhancers in which this motif was mutated. Expression of the CoupTFII-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 CoupTFII-965 enhancer away from the EBEs and SBEs did not significantly influence activity (Fig. 7d). Therefore, although these results cannot prove SMAD1/5 binding to the COUPTFII enhancer, they suggest that SMAD-mediated activation may be a shared feature of both *Ephb4* and *Coup-TFII* gene expression in veins.

**The BMP type I receptor ALK3 is required for vein formation**

Since multiple BMP ligands can be present in the bloodstream, we hypothesized that either repression of BMP-driven gene activation in early arterial endothelial cells or spatially selective expression of BMP receptors may contribute to venous-specific gene expression.We found no evidence of arterial repression of BMP-driven venous gene activation via known pathways. Specifically, chemical inhibition of ERK/MAPK signalling, which is active in arteries but repressed in veins6 and had previously been associated with inhibition of nuclear SMAD148, did not lead to arterial expansion of Ephb4-2:GFP expression (Supplementary Fig. 12a). Similarly, Notch signalling can repress BMP responsiveness through the activation of SMAD6 in the angiogenic sprout49, 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.12b)50. We also found little evidence that spatial restriction of BMP type II receptors played a crucial role in early venous identity. Wiley *et al*., demonstrated that zebrafish BMP type II receptors bmpr2a and bmpr2b (BMPR2 in mice) are important regulators of venous angiogenic sprouting18. However, when we repeated their MO-induced depletion of *bmpr2a* and *bmpr2b* in *tg(Ephb4-2:GFP)* zebrafish, we did not lose enhancer activity and could still see a developed axial vein although we recapitulated the venous sprouting defect (Supplementary Fig. 12c). Similarly, Kim et al., 43 reported an essential role for *dab2* as a mediator of BMP2 signalling in zebrafish venous sprouting. However, again when we repeated their MO-induced depletion of *dab2* in *tg(Ephb4-2:GFP)* zebrafish we saw little change in enhancer activity or axial vein formation although we again recapitulated the venous sprouting defect (Supplementary Fig. 12c). While the initial design of these MOs may not meet the current standards in all cases, mice lacking endothelial BMPR2 also exhibit no reported embryonic vascular defects 39, strongly suggesting that BMPR2 is unlikely to be absolutely required for early *Ephb4* expression or venous identity. Since the other BMP type II receptors, *Acvr2a* and *Acvr2b,* are ubiquitously expressed during arteriovenous differentiation

51, 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 activity 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 mendelian ratios with no reported vessel defects 52. 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

12,53,54, 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 morpholinos where necessary to avoid previously reported gastrulation defects

55,56, effectively creating a knock-down but not full depletion. The *alk1* 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 55,57. 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 geneexpression. We therefore investigated the expression pattern of *alk3a/b* in early zebrafish embryos. Although not previously reported to be strongly expressed in the zebrafish vasculature57, we detected expression of *alk3b* in venous positioned angioblasts from 18-20ss (approximately 18hpf), and both *alk3a* and *alk3b* were highly expressed in venous regions of the axial vasculature by 28hpf (Fig. 8c and Supplementary Figure 13c). In comparison, we detected little expression of endogenous *ephb4a* until 20ss (approximately 19-20hpf)(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 vein (Fig. 8d and 18). 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* embryos

12, supporting a specific role for ALK3 in venous identity in mammals as well as zebrafish. Compartively, endothelial deletion of the vascular type I receptors *Alk1(Acvrl1)* and *Alk2 (Acvr1)* caused later lethality54,58. Analysis of murine ALK3 expression also found that it was preferentially found in venous endothelial cells 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 formation

12, 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 and 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* embryosare not caused by general vascular maturation or cardiac defects. Previous analysis also found no defects in hematopoiesis in *Alk3EC/EC* embryos

12. Because the ALK3 receptor is specific for the BMP pathway and signals only through SMAD1/5/859, 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 dependant transcriptional activation of venous genes (Fig. 10).

**Discussion**

The crucial roles for VEGF-A and NOTCH in activating arterial endothelial cell identity has often led to the supposition that venous specification is either default or actively repressed in arteries. However, our data clearly demonstrates that venous genes are directly transcriptionally activated via a BMP/ALK3/SMAD1/5 signalling cascade. This therefore supports a new 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 ligandsin venous endothelial cells lends considerable weight to the concept that venous endothelial cells may be innately sensitive to BMP signalling 44. Supporting this, the type II BMP receptors Bmpr2a and Bmpr2bandcargo-specific adaptor Dab2are also enriched in zebrafish venous endothelial cells18,43. 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 (ACVRL1) 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 endothelial cells in the post-natal retina, where *Alk2* and *Alk3* are required for correct retinal vessel morphogenesis60.

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 endothelial cells, including vascular integrity, remodelling and smooth muscle recruitment

12,14, 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 *Smad4 ART/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 NFAT signalling is absolutely required for heart valve morphogenesis, yet endothelial NFAT-deficient embryos display aberrant valves and gestational lethality only after E13.5 61. 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/ART*embryos is also active in endothelial cells at the angiogenic front, Dll4in3 is preferentially active in tip cells21. Conversely, the angiogenic role of BMP signalling is thought to occur primarily in stalk cells, where it co-operates with the Notch pathway15,22. 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 31). However, recent reports in zebrafish demonstrate that venous endothelial cells are the primary source of cells at the angiogenic front62, 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 cofactors

32,63. 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 expression25, 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 DNA 32. 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 development 18, some BMP receptors are clearly expressed in both mouse and zebrafish arteries (e.g ALK1)64,65, and the others 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 endothelial cells, yet both Ephb4-2 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 BMP-response element (BRE), effectively a group of SBE and GC-SBE motifs, is virtually silent in the vasculature unless additional CMV-derived enhancer elements are added to the transgene66. 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 data also presents a compelling case for ALK3 to be considered as a novel target for anti-angiogenic 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 needed67. Combined with the emerging understanding of the importance of the venous endothelium at the angiogenic front 62, 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® Strings™, 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 the Supplementary Methods. DNA fragments/PCR products were cloned into the pCR8 vector using the pCR8/GW/TOPO TA Cloning Kit (Invitrogen, K2500-20) following manufacturer’s instructions. Once cloning was confirmed, the enhancer sequence was transferred from the pCR8/GW/enhancer entry vector to a suitable destination vector using Gateway LR Clonase II Enzyme mix (Life Technologies, 11791-100) following manufacturer’s instructions. For mouse transgenesis, the enhancer was cloned into the hsp68-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 generate the Dll4in3Cre transgene, the Dll4in3 enhancer 19 was amplified by PCR to include engineered 5’ SacII and 3’ Not1 restriction sites (F primer ATATCCGCGGCTGCTGATATCGCTATCTC R primer ATATGCGGCCGCAGAGTTTCCTGGCGAAGT) and cloned into the promoterless p-AUG-βGal construct 68. The Dll4in3 promoter was also generated by PCr with engineered 5’ SpeI and 3’ BamHI sites (F primer AGTAGACTAGTCTGGAAAGGAAAGGGAGATC R primer ATAATGGATCCCCCTTGGGGTGTCCTCTC), and cloned into the Dll4in3enhancer- p-AUG-βGal construct. Transgenic mice were generated to ensure activity of enhancer/promoter construct, after which the βGal sequence was replaced by Cre-ORT/polyA amplified from the pCAG-Cre vector. The full sequence of the Dll4in3 enhancer/promoter is provided in the Supplementary Methods.

**Mice**

All animal procedures comply with all relevant ethical regulations, were approved by local ethical review and licensed by the UK Home Office. *Smad4fl/fl* mice 69-72 were provided by Elizabeth Robertson, *Tg(Tie2:Cre)* mice, officially designated *Tg(Tek-cre)*12Flv/J 73,74 were purchased from JAX (stock number 004128), *Ephb4LacZ/+* mice 5,75 were provided by David Anderson, Cdh5(PAC)-CreERT2 mice23 were provided by Ralf Adams and *Alk3fl/fl* mice

12 were provided by Yuji Mishina. *Tg(Dll4in3:LacZ)* were as described in 19,31, while the *Tg(Ephb4-2:LacZ), Tg(Coup-TFII-965)* and *Tg(Dll4in3:Cre)* lines were generated for this paper. Transgenic mice were generated by oocyte microinjection of linearized DNA as described previously 76,77. Mouse embryos were collected along with yolk sac and fixed in 2% paraformaldehyde 0.2% glutaraldehyde 1X PBS. E8.5 embryos were fixed at 4ºC for 10 mins, E9.5 embryos fixed for 30 mins, E11.5 embryos for 60 mins and E13-E15 embryos for 120 mins. After fixation, embryos were rinsed in 0.1% sodium deoxycholate 0.2% Nonidet P-40 2 mM MgCl2 1 X PBS then stained for 2-24 hours in 1 mg/ml 5-bromo-4-chloro-3-indolyo-β-D-galactoside solution (X-gal) containing 5 mM potassium ferrocyanide, 5 mM ferricyanide, 0.1% sodium deoxycholate, 0.2% Nonidet P-40, 2 mM MgCl2 and 1 X PBS. After staining, embryos were rinsed through a series of 1 X PBS washes, then fixed overnight in 4% paraformaldehyde at 4°C. Embryos were imaged using a Leica M165C stereo microscope equipped with a ProGres CF Scan camera and CapturePro software (Jenoptik). In instances that images have been altered to improve quality and colour balance, each image within a set (e.g WT, het and null embryos) have been altered using the same parameters. This occasionally included to selective depletion of the yellow or red colour channel, in order to counteract issues from the X-gal stain solution (which is orange). All embryos are stored in 4% PFA indefinitely, and slowly become less yellow. Consequently, embryos stained more recently have a greater yellow/orange hue. An example of this alteration can be seen in Supplementary Figure 16.

The yolksac was used for genotyping. Tissue samples were incubated overnight at 55°C with 500 μL GNT buffer (50mmol/L KCl, 1.5mmol/L MgCl2, 10mmol/L Tris-pH8, 0.01% gelatin, 0.45% nonidet P40, 0.45% Tween) and proteinase K (10mg/ml), 0.5 μL of this supernatant was subsequently used in PCR reactions with the GoTag Green master mix (Promega, M7122) using relevant PCR primers. For *Smad4EC/EC*crosses with *Ephb4Lacz/+*and *Ephb4-2:LacZ*, in the rare cases where the LacZ genotyping results did not agree with the pattern of X-gal staining in WT embryos, error was presumed and the entire litter was excluded. For histological analysis of transgenic and mutant mouse sections, embryos were dehydrated through a series of ethanol washes, cleared by xylene and paraffin wax-embedded. 5 or 6-μm sections were prepared and de-waxed. For imaging of X-gal staining, slides were counterstained with nuclear fast red (Electron Microscopy Sciences). Analysis was qualitative not quantitative, therefore no statistical analysis was applied to the observations of staining intensity and pattern. Numbers of transgenic mice used followed precedent set by similar published papers. Where significant variations in expression were detected within a single experimental set this is represented in the relevant figure by representative pictures of each outcome in combination with the n number for each example. No experimental randomization or blinding was used as this was not considered necessary.

**Transgenic Zebrafish**

All animal procedures were approved by local ethical review and licensed by the UK Home Office. *tg(Dll4in3:GFP)* and *tg(kdrl:HRAS-mCherry*) fish lines were as previously described 19,31,78. The stable lines *tg(Ephb4-2:GFP*) and *tg(Coup-TFII-965)* were generated from an initial outcross of adult F0 carriers, and intercrossed with the *tg(kdrl:HRAS-mCherry)* to enable visualization of the entire vasculature. F0 transient mosaic transgenic zebrafish embryos were generated using Tol2 mediated integration79. Embryos were maintained in E3 medium (5 mM NaCl; 0.17 mM KCl; 0.33 mM CaCl2; 0.33 mM MgSO4) at 28.5 °C. To image, all embryos were dechorionated and anesthetized with 0.1% tricaine mesylate. For analysis of transient transgenic zebrafish, single embryos were transferred into a flat bottom 96-well plate, and GFP reporter gene expression screened with a Zeiss LSM 710 confocal microscope at 46-50 hpf. The total number of injected fish, fish with detectable GFP expression, and fish with GFP expression in the vasculature were noted. Whole fish were imaged using the “tile scan” command, combined with Z-stack collection under a confocal microscope Zeiss LSM 710 MP (Carl Zeiss) at 488nm excitation and 509nm emission (EGFP) and 587 nm and 610 nm (mCherry), respectively. For imaging of stable zebrafish transgenic lines (in Figure 4 and 8), zebrafish embryos were treated with 0.03mg/ml PTU (N-Phenylthiourea, P7629, Sigma) at 24hpf to prevent melanogenesis. Embryos were embedded in 0.4% TopVision low melting point agarose (R0801, Thermo) in 0.14mg/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 less than 40/transgene. 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 legend. No experimental randomization or blinding was used as we did not consider this necessary.

**Morpholino (MO) and chemical inhibition in zebrafish**

MOs were dissolved in ultrapure water and injected into 1 – to 2-cell stage zebrafish embryos as previously described 19. Sequences used were:

*smad1* MO (0.5ng) AGGAAAAGAGTGAGGTGACATTCAT 39;

*smad5* MO(0.25ng) ACATGGAGGTCATAGTGCTGGGCTG 39 ;

 alk1 MO ATCGGTTTCACTCACCAACACACTC 64;

alk2 MO (3ng) GATTCATGTTTGTGTTCAATTTCCG 80;

alk3a MO (5-7ng) GACGCATTGTCAAATTGTCTTGTCG

56;

alk3b MO (1.2-1.8ng) GTCGAGTTGTTGAACTGTATGGCTG

56;

bmpr2a MO (12ng) AGAGAAACGTATTTGCATACCTTGC 18;

bmpr2b MO (6ng) AGTTGATTCTGACCTTGTTTGACCA 18;

dab2 MO (2.5ng) TTCTGCTTCAGGTGACTGTGACATG 43,

tnnt2 MO (4ng) CATGTTTGCTCTGATCTGACACGCA81.

For pharmacological inhibition of pathways downstream of VEGF, Notch and BMP, embryos were manually dechorionated and incubated with either 100µM DAPM (Calbiochem) added at 10hpf 19, 40µM LY294002 (MedChem Express) added at 10hpf 82, 15-40µM SL327 (SellekChem) added at 10hpf 6, 5µM DMH1 (Calbiochem) added at 10hpf 18. Control embryos were treated with identical concentrations of DMSO without inhibitor.

All MO injections and use of chemical inhibitors 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 zebrafish embryos was no less than 40/tg(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 legend. No experimental randomization was used as we did not consider this necessary. Experimental blinding was not used as phenotypes 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 pCR2 vector using the TOPO/TA cloning kit (Invitrogen 450641) and transcribed using SP6 and T7. The sequences are provided below. *dll4*, *kdrl*, *bmp2b* and *bmp4* probes were used as previously described 69,71,72. Whole-mount *in situ* hybridizationwas conducted as previously described73. Briefly, embryos were collected at 28hpf and 36hpf, fixed overnight at 4 °C in 4% PFA, dehydrated and stored at -20°C in 100% ethanol. Before use, embryos were rehydrated in 1XPBS with 0.1% Tween-20 (PBST), bleached in 3% H2O2/0.5% KOH then embryos were re-fixed in 4% PFA for 20 min. The embryos were made permeable by digestion with15 μg/ml proteinase K (Sigma-Aldrich) for 10 min (28hpf embryos) or 30 min (36hpf embryos) followed by two PBST washes, fixed in 4% PFA for 20 min, washed five times with PBST, then transferred into hybridization solution (50% formamide, 5 ×SSC, 0.1% Tween 20, 50 μg/ml heparin, 500 μg/ml of tRNA adjusted, 10 mM citric acid) for 2 hours at 65 °C, transferred into diluted antisense riboprobe/hybridization solution and incubated overnight at 65 °C. Probes were removed and embryos relocated to a Biolane HT1 *in situ* machine (Intavis). Embryos were washed through a dilution series of 2 x SSC followed by 0.2 x SSC at 65 °C and thereafter taken through room temperature dilution washes of 100% MABT (0.1M Maleic Acid, 0.15 M NaCl, pH 7.5). Nonspecific sites were blocked with MAB block (MABT with 2% Boehringer block reagent) and the embryos incubated for 15 hours with 1:2000 antiDIG antibody (Roche) 4 °C, before washing in MABT. Prior to staining, embryos were washed in AP buffer and the *in situ* signal developed at room temperature with BM Purple (Sigma-Aldrich). Staining was stopped as appropriate by fixation in 4% PFA. Embryos were transferred to 80% glycerol for imaging and storage.

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 zebrafish embryos was no less than 20/in situ/condition. 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 legend, and by the numbers next to the representative pictures. No experimental randomization was used as we did not consider this necessary. Experimental blinding was not used as phenotypes of control and treated were easily detectable due to dorsalization and sprouting defects. Sequences of each probe are provided in the Supplementary Methods.

For mouse *in situ* hybridisation, *Bmp4* sense and antisense RNA probes (from Allen Brain Atlas Data Portal 72) were prepared as for the zebrafish. Wildtype E9.5 and E10.5 embryos were harvested, fixed overnight in 4% PFA/PBS at 4°C, and paraffin embedded as described above. 10µm sections were cut, de-waxed using Histoclear and rehydrated through an ethanol series. The sections were then digested in 20µg/ml Proteinase K (Sigma-Aldrich) for 8 min, followed by washes in 2mg/ml Glycine/PBS and then PBS. Sections were fixed for 20 min in 4% PFA/PBS, washed twice in PBS, and then incubated in a humidified chamber for 1 hour at 70°C in Hybridisation buffer (50% formamide, 5x Saline-Sodium Citrate (SSC) buffer pH 4.5, 50µg/ml yeast RNA, 1% SDS, 50µg/ml heparin). This was followed by an overnight incubation in Hybridisation buffer containing 1µg/ml sense or antisense RNA probe at 70°C. The following morning, slides were rinsed twice in 2x SSC buffer and then at 65°C underwent three 15 min washes in Solution I (50% formamide, 5x SSC pH4.5, 1% SDS) and two 15 min washes in Solution II (50% formamide, 2x SSC pH4.5). They were then returned to room temperature for two washes in MABT buffer (0.1M maleic acid, 0.15M NaCl, 0.01% Tween-20, 2mM Levamisole (Sigma-Aldrich), pH7.5) before blocking for 1 hour in 2% Boehringer Blocking Reagent (Roche)/10% sheep serum/MABT. They were then incubated overnight at 4°C in the blocking solution containing alkaline-phosphatase (AP) conjugated anti-DIG antibody (Roche) diluted 1:2000. Finally slides were washed in three times in MABT and then two times AP buffer (100mM Tris, pH 9.5, 50mM MgCl2, 100mM NaCl, 0.1% Tween-20, 2mM Levamisol), 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 hour (DLL4) or overnight (CD31), rinsed in PBST (0.1% TritonX-100 in PBS), incubated for 1 hour in blocking solution (10% Normal Donkey Serum in PBST), then overnight at 4°C with goat polyclonal to DLL4 (R&D systems, AF1389,1 in 50 dilution) or rat monoclonal to CD31 (DIA-310, Dianova, 1in 250 dilution). Samples were washed in PBS-T and subsequently incubated overnight with suitable species-specific Alexa Fluor® or HRP-conjugated secondary antibodies (1:300, Thermo Fisher Scientific) in 0.1% PBS-T 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 de-waxed by two washes in Histoclear and rehydrated through an ethanol series. Antigen retrieval was carried out by boiling slides in 10mM sodium citrate buffer, pH 6.0, in a commercial pressure cooker for 3 minutes, followed by two washes in PBS. Sections were then incubated at room temperature in blocking solution (1% BSA, 2% donkey serum in PBS or 10% donkey serum in PBS) for 1 hour in a humidified chamber, followed by overnight incubation at 4°C in primary antibodies diluted in blocking solution. Sections were rinsed in PBS and incubated in species-specific Alexa Fluor®-conjugated secondary antibodies diluted 1:1000 in blocking solution for 1 hour at room temperature. Finally, sections were rinsed further in PBS, stained with DAPI and mounted with glass coverslips using FluoromountTM Aqueous Mounting Medium (Sigma), before imaging on a Zeiss LSM 710 Confocal Laser Scanning Microscope. Primary antibodies used were rat monoclonal to CD31 (DIA-310, Dianova) diluted 1:300, and rabbit monoclonal to Neuropilin 1 (ab81321, Abcam) diluted 1:100, rabbit polyclonal to ALK3 (ab38560, Abcam) diluted 1:100, and goat polyclonal to EPHB4 (AF446, R&D Systems) diluted 1:50.

For COUP-TFII immunohistochemistry staining on paraffin sections, E10.5 mouse embryos were processed the same as for the immunofluorescence staining up to the antigen retrieval step. Following antigen retrieval, sections were washed in PBS and then incubated in 3% hydrogen peroxide (Sigma Aldrich) for 5 mins and rinsed again in PBS. The Avidin/Biotin Blocking Kit (SP-2001, Vector Laboratories) and Mouse on Mouse (M.O.M.™) Basic Kit (BMK-2202, Vector Laboratories) were then used according to manufacturer’s instructions, with a mouse monoclonal antibody to COUP-TFII (PP-H7147-00, Perseus Proteomics Inc) diluted 1:100 in the M.O.M.TM diluent. Following incubation in the M.O.M.™ Biotinylated Anti-Mouse IgG reagent, sections were incubated for 10 mins in VECTASTAIN® Elite® ABC Reagent (from PK-6102, Vector Laboratories), rinsed in PBS and then incubated in DAB substrate (SK-4100, Vector Laboratories), including Nickel solution, until colour developed. Slides were imaged using a NanoZoomer S210 slide scanner with NDP.view2 viewing software (Hamamatsu).

For immunofluorescence staining on cryosections, E9.5 mouse embryos were harvested and fixed in 4% PFA/PBS at 4°C for 1 hour, before incubation in 30% sucrose/PBS overnight at 4°C. They were then washed in a 50/50 mix of 30% sucrose/OCT Embedding Medium (Thermo Scientific), two washes in OCT and then mounted over dry ice and stored at -80°C. Cryosections were cut at a thickness of 12μm, thawed at room temperature and washed in PBS to remove the OCT embedding medium. They were fixed in 4% PFA/PBS for 10 mins, washed three times in PBS and permeabilised in 0.5% Triton X-100/PBS (Merck) for 12 mins. Following two further PBS washes, sections were blocked in 10% donkey serum/0.1% Triton X-100/PBS for 1 hour in a humidified chamber at room temperature, before overnight incubation at 4°C in primary antibodies diluted in the blocking solution. Following further PBS washes, sections were incubated at room temperature in Alexa Fluor®-conjugated secondary antibodies diluted 1:1000 in blocking solution for 1 hour. Sections were then DAPI-stained, mounted and imaged as described above. Primary antibodies used were rabbit monoclonal to Phospho-SMAD1 (Ser463/465)/ SMAD5 (Ser463/465)/ SMAD9 (Ser465/467) (D5B10, Cell Signaling Technology), goat polyclonal to EPHB4 (AF446, R&D Systems) diluted 1:50, and rat monoclonal to CD31 (DIA-310, Dianova) diluted 1:300.

**ClustalW and sequence motif analysis**

Mouse and human sequences of putative enhancers were aligned using ClustalW 75. Binding motifs for the vascular ETS factors ERG and ETV2, and SMAD4 were obtained from JASPAR and annotated by hand.

**Bioinformatic analysis of SMAD1/5-enriched binding sites**

SMAD1/5 binding information was obtained using publically available ChIP-seq data 31 via the NCBI GEO database 76, accession number GSE27661. Raw reads for HUVEC stimulated by BMP9 and PASMC stimulated by BMP4 sets were trimmed with Sickle. Reads were then aligned to human genome build hg19 using Bowtie2, duplicate PCR reads removed with rmdup, and peaks were called with MACS2. A bedgraph was generated of the significant peaks for visualization and comparison to other genomic datasets.

ERG binding information from HUVEC was obtained using publically available ChIP-seq data 29 via ArrayExpress73, accession number E-MTAB-5148. Raw reads were trimmed with Sickle, aligned to human genome build hg19 using Bowtie2, duplicate PCR reads removed with rmdup, and peaks were called with MACS2. A bedgraph was generated of the significant peaks for visualization and comparison to other genomic datasets.

**Chromatin immunoprecipitation (ChIP)**

Human umbilical vein pooled endothelial cells (PromoCell) were grown in Endothelial Cell Growth Medium 2 with BulletKit (PromoCell). Media was changed every 48 hours. Four 80% confluent 15cm dishes per condition were serum starved in 0.5% Foetal Bovine Serum (Gibco) overnight before being stimulated with BMP9 1ng/ml for 1.5 hours. Cells were then trypsinised and the cell pellet collected. Crosslinking was performed in 0.6% methanol-free formaldehyde (Pierce) under rotation at room temperature for 12’ before being quenched with glycine to a concentration of 0.2M. Lysis was achieved by passing cell suspension through a 25g needle (Terumo #NN-2525R) in 1ml of cell lysis buffer (50mM Tris-HCL(pH8.0), 10mM EDTA, 10mM Sodium butyrate, 1%SDS, 0.5mM PMSF and cOmplete, EDTA-free protease inhibitor cocktail (Roche)).

To obtain a mean chromatin fragment size of 650-850bp chromatin was sheared by sonication using a Covaris sonicator S220 in a Covaris AFA fiber tube at 160W peak incidence power, 5% duty cycle, 200 cycles, for 8’. Fragment size was checked by agarose gel. Sonicated chromatin was centrifuged at 14,000g, 10’ and supernatant diluted in 8ml ChIP dilution buffer (16.7mM Tris(pH8.0), 167mM NaCl, 1.2mM EDTA, 1%Triton X – 100, 0.01% SDS) and incubated overnight with 8ug of antibody, Smad1 (Iwai North America BMR00479), or IgG control (12-371 merckmillipore) and a no antibody control. IP was performed with Dynabeads-protien G (ThermoFischer), blocked overnight in 0.5mg/ml Bovine Serum Albumin (Sigma-Aldrich) under rotation for 1 hr. G-Dynabead immunocomplexes were washed with low-salt buffer (20mM Tris-HCL (pH8.0) 150mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), high-salt buffer (20mM Tris-HCL (pH8.0) 500mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS) and LiCl buffer (250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, Tris–HCL 10 mM, pH 8.0) . Beads were eluted in 0.2ml elution buffer and ChIPed-DNA was reverse crosslinked overnight at 55°C in elution buffer plus 0.3 M NaCL, 20ug RNase A (Invitrogen) and 20ug Proteinase K (Fermentas). DNA was column purified with QIAquick PCR purification Kit (Qiagen).

Immunoprecipitated DNA was analyzed by qPCR using TaqMan Custom Gene Expression Assay Probes (ThermoFischer) designed against 100 bp regions of the Ephb4-2 enhancer or a gene dessert region of Chromosome 7 as a negative control, sequences are detailed in the Supplementary Methods.

Each ChIP was performed on at least 3 biological replicates, with 3 technical replicates for each. Statistical analysis was performed in StepOne plus software, Microsoft Excel. Input was taken as the supernatant from the non-antibody control condition qPCR analysis of 1% input was run alongside SMAD1 antibody analysis for each region on each qPCR. Results are expressed as the mean of the % input defined as 100\*(2^(adjusted Input ct – ct IP)) across all replicates. Significance was calculated with a Paired 2 tailed t-test comparing % input of control region to % input of SMAD1 antibody conditions. Graphs were produced using R[] statistical package.

**Data Availability**

The authors declare that the main data supporting the findings of this study are available within the article, its Supplementary Figures and Methods. All ChIP-seq datasets used in this study were previously published and are publicly available, references and accession numbers are provided within the article. Extra data are available from the corresponding author upon request.

**Author Contributions**

Conceptualization, A.N and S.D.; Methodology, A.N., S.N. and S.D.; Formal Analysis, M.D.W and G.L.B.; Investigation, A.N., S.N. S.P. M.F., K.C., P.L., R.W., K.L., K.P, R.S., I.R.; Resources, M.D.W, K.L., W.H., G.B-G.; Data Curation, M.D.W. and G.L.B.; Writing – Original Draft, S.D.; Writing – Review and Editing, A.N., S.N., S.P., M.F., M.W., R.W., K.C., W.H., T.C., G.B-G., and S.D.; Visualization, A.N., S.N., S.D.; Supervision, A.N., G.B., T.C. and S.D.; Project Administration, S.D., Funding Acquisition, S.D.

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**FIGURES LEGENDS**

**Figure 1.**  **Endothelial specific knockout of *Smad4* does not affect arterial identity but results in the loss of *Ephb4* expression**.

**a-b.** Representative E10.5 whole-mount images (**a**) and transverse sections (**b**) from wildtype *Smad4+/+*(n=17), heterozygous *Smad4EC/+;* (n=12) and homozygous *Smad4EC/EC* (n=10) embryos all expressing the arterial Dll4in3:*LacZ* transgene. Five litters were investigated in total. Robust transgene expression, specific to arterial endothelial cells, was seen in all embryos regardless of *Smad4* genotype.

**c-d.** Representative E10.5 whole-mount images (**c**) and transverse sections (**d**) from wildtype *Smad4+/+*(n=9), heterozygous *Smad4EC/+;* (n=9) and homozygous *Smad4EC/EC* (n=8) embryos also transgenic for the venous marker *Ephb4LacZ.* 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-hand corner of each whole-mount image denote number of embryos similar to picture shown. Outliers are shown in Supplementary Figure 1e.

**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 smooth muscle cells and other mesenchymal cells (as reported by You et al7).

 EC indicates Tie2:Cre-mediated deletion, +/+ indicates Cre-, EC/+ indicates Cre+,Smad4fl/+ and EC/EC indicates Cre+;Smad4fl/fl. da=dorsal aorta; ica=internal carotid artery; isa=intersomitic arteries; baa=branchial arch arteries; nt=neural tube; cv=cardinal vein; cev=branches of cerebral venous plexus. Scale bars are all 100μm. See also Supplementary Figure 1.

**Figure 2. Induced endothelial-specific deletion of *Smad4* after the initial vasculature is established 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+/+ andSmad4indEC/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 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).

**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 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=aorta. Scale bars are all 100μm.

See also Supplementary Figure 2.

**Figure 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.

**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.

**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. Scale bars are all 100μm.

See also Supplementary Figure 3.

**Figure 4. The vein-specific Ephb4-2 enhancer recapitulates endogenous *Ephb4* expression in fish and mouse.**

**a**. Region around the human *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 HUVEC cells as light blue peaks. Within “DNaseI HS”, the heatmaps show DNaseI 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).

**b-c.** The Tol2 Ephb4-2:*E1b*:GFP transgene (**b**) directs venous expression of the GFP reporter gene in a transgenic zebrafish line also expressing the pan-endothelial kdrl:HRAS-mCherry (**c**). No enhancer activity (as detected by GFP expression) was seen in the dorsal aorta, while robust activity was detected in the cardinal and ventral veins. Expression is detected in all intersegmental vessels. Red bracket=dorsal artery, white bracket=axial veins, isv intersegmental vein, isv intersegmental vein.

**d-e**. The Ephb4-2:*hsp68:LacZ* transgene (**d**) directs vein-specific expression of the *LacZ* reporter gene in the transgenic mice (**e**) as compared to *Ephb4LacZ/+.* cv=cardinal vein, da=dorsal aorta, jv=jugular vein, ca=carotid artery, ccv=common cardinal vein, cev=branches of the cerebral venous plexus.

See also Supplementary Figure 4.

**Figure 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* littermates demonstrates loss ofEphb4-2 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).

**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 wildtype 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 number of F0 mouse or fish embryoswith similar expression patterns to the image shown. da=dorsal aorta, cv cardinal vein, nt= neural tube, red bracket=dorsal artery, white bracket=axial veins

See also Supplementary Figure 5.

**Figure 6. The Ephb4-2 enhancer is bound by SMAD1/5.**

**a.** Mouse (m) and orthologous human (h) DNA sequence corresponding to the entire Ephb4-2 enhancer. Red nt on human sequence correspond to regions bound by SMAD1/5 as determined from the SMAD1/5 HUVEC ChIP-seq data 31 (more detail shown in 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 5 31). Black outlined boxes denote composite SBE/GC-SBE elements, italic nt indicate linker sequences. Grey text under each line of sequence indicates the motifs previously identified as the EBEs and SBEs in earlier analysis in Supplementary Figure 4). Sequence logos for all motifs are in Supplementary Fig. 6b.

**b.** UCSC browser view (<http://genome.ucsc.edu)> of *EPHB4* locus incorporating SMAD1/5 HUVEC and PASMC ChIP-seq data from Morikawa et al31 (red peaks=statistically significant peaks after BMP9 stimulation in HUVEC). Significant binding peaks are not observed in PASMC after BMP4 stimulation (green). See Supplementary Fig. 6c for wider view of the *EPHB4* locus.

**c.** Box and whiskers plot shows ChIP- qPCR data demonstrating significant enrichment of SMAD1 binding at the Ephb4-2 enhancer region in BMP9 stimulated HUVECS (red) compared to a control intergenic region on the same chromosome (p=0.00015, paired 2 tailed ttest). 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 was observed between IgG control regions (p= 0.3154 and p=0.19212, paired 2 tailed ttest). Horizontal lines represent medians boxes represent the interquartile range (IQR); vertical lines show minimal/ maximal values (up to 1.5\* IQR) and the black dots are data points outside of 3\* IQR range. Data represents 3 biologocal replicates each with 3 technical replicates performed in triplicate. All data points were included in statistical analysis.

**d-e.** Effects of morpholino (MO)-mediated partial knock-down of *smad1/5* on GFP expression in arterial *tg(Dll4in3:GFP)* **(d)** and venous *tg(Ephb4-2:GFP)* **(e)** transgenic zebrafish lines, using 0.5ng *smad1* MO and 0.25ng *smad5* MO.Graphs depict observed expression in all embryos, Dll4in3:GFP WT n=58, MO n=48; Ephb4-2:GFP WT n=104, MO=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). A dose-response analysis looking at morphology and Ephb4-2:GFP expression in fish treated with lower and higher concentrations of MO can be seen in Supplementary Fig. 7b-c. Expression of endogenous *ephb4a* and *dll4a* can be seen in Supplementary Fig. 8.

**Figure 7. A SMAD1/5 binding peak identifies a novel vein endothelial enhancer for *Coup-TFII***

**a.** UCSC browser view (<http://genome.ucsc.edu)> of *COUP-TFII (NR2F2)* incorporating SMAD1/5 binding data from Morikawa *et al*.31. A statistically significant SMAD1/5 binding peak in HUVECs (red) -965kb upstream of Coup-TFII correlated with the H3K27Ac enhancer histone mark in HUVECs (blue peaks) and endothelial HMVEC DNaseI hypersensitivity (black heat map).

**b-c.** Stable transgenic mouse (**b**) and zebrafish (**c**) embryos expressing the *lacZ* and GFP reporter genes respectively under the control of the murine CoupTFII-965 enhancer. In both animal models, enhancer activity was primarily seen in the venous endothelium. cev=branches of cerebral venous plexus; cv=cardinal vein; pm=paraxial mesoderm, da=dorsal aorta, red bracket=dorsal aorta, white bracket=posterior cardinal and ventral vein(s).

**d.** Tables summarizing reporter gene expression in transient transgenic zebrafish and mouse embryos after mutation of the core SMAD-binding element (SBE-PEAK).

See also Supplementary Figure 10-11.

**Figure 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 48hpf embryos demonstrate similar intensities of vein-specific 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 100µM DAPM-treated embryos (n=57), black represents high expression, grey represents 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 48hpf *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 represents high expression, grey represents weak expression and white represents no detectable GFP expression.

**c.** Whole-mount *in situ* hybridization for BMP receptors *alk3a* and *alk3b* compared to pan-endothelial *kdrl* in wild type zebrafish embryos at 20, 24, 28 and 36hpf. Earlier time-points can be seen in Supplementary Fig. 13. Both *alk3a* and *alk3b* were detected in the axial vein but not 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.

See also Supplementary Figures 12-13.

**Figure 9.**  **Endothelial specific knockout of *Alk3* results in loss of venous identity in mice**.

**a-b**. Immunofluorescent analysis of CD31, EPHB4 and ALK3 (**a**) and *in situ* hybridization for *Bmp4* (**b**) in transverse sections from E9.5 mouse embryo, cv=cardinal vein, da=dorsal aorta.

**c.** Representative E10.5 whole-mount images from wildtype *Alk3+/+*(n=16), heterozygous *Alk3EC/+;* (n=7) and homozygous *Alk3EC/EC* (n=7) embryos all expressing the arterial Dll4in3:*LacZ* transgene. Nine litters were investigated in total. Robust transgene expression, specific to arterial endothelial cells, was seen in all embryos regardless of *Alk3* genotype although *Alk3EC/EC* embryos were often significantly growth retarded. The grey numbers on bottom right-hand corner of each whole-mount image denote number of embryos similar to picture shown, for *Alk3EC/EC* two different images are shown to indicate Dll4in3:LacZ expression in the range of morphological defects seen.

**d.** Representative E10.5 whole-mount images from wildtype *Alk3+/+*(n=16), heterozygous *Alk3EC/+;* (n=7) and homozygous *Alk3EC/EC* (n=7) embryos all expressing venous *Ephb4LacZ*. Twenty one litters were investigated in total, littermates are shown in Supplementary Fig. 16. Robust X-gal activity is detected in the veins of *Alk3+/+* embryos but is reduced in *Alk3EC/+*embryos and absent in *Alk3EC/EC* regardless of extend of growth retardation and morphological defects. The grey numbers on bottom right-hand corner of each whole-mount image denote number of embryos similar to picture shown, for *Alk3EC/EC* two different images are shown to indicate *Ephb4LacZ* expression in the range of morphological defects seen.

**e.** Representative transverse sections taken from E10.5 *Alk3EC/EC* embryos (representing the majority morphology) also transgenic for either arterial Dll4in3:LacZ or venous *Ephb4LacZ* at two different levels. In each some vessels were clearly seen, these expressed Dll4in3:lacZ but not *Ephb4LacZ* and were located in arterial positions, suggesting the presence of dorsal aorta but no visible cardinal vein.

**g-h.** Representative arterial endothelial specific *Alk3+/+* and *Alk3ART/ART* whole-mount E10.5 embryos (**g**) and transverse sections stained with CD31 (**h**), generated by crossing *Alk3fl/fl* with the arterial specific Dll4in3:Cre deleter line*.* Loss of *Alk3* in arterial endothelial cells had no effect on vascular development at E10.5

**i.** Observed frequency of *Alk3fl/fl;Dll4in3:Cre* embryos at E10.5 and P5. Medelian ratios were present at both time-points.

For all panels, EC isTie2:Cre-mediated deletion, ART is Dll4in3:Cre-mediated deletion. +/+ indicates Cre-, EC/+ indicates Cre+,Alk3fl/+ and EC/EC indicates Cre+;Alk3fl/fl. da=dorsal aorta; ica=internal carotid artery; isa=intersomitic arteries; baa=branchial arch arteries; nt=neural tube; cv=cardinal vein; cev=branches of cerebral venous plexus, mda=midline dorsal aorta. Scale bars are all 100μm.

See also Supplementary Figure 15-16.

**Figure 10. Proposed model**

Our data supports a model in which vein-enriched BMP ligands BMP2 and BMP4 signal through the vein-specific Alk3 type I receptor (in combination with multiple different BMP type II receptors) resulting in the phosphorylation of SMAD1/5, transcriptional activation of the Ephb4 and Coup-TFII genes and subsequent venous identity.