

Hedgehog signaling via a calcitonin receptor-like receptor can induce arterial differentiation independently of VEGF signaling in zebrafish

Robert N. Wilkinson,^{1,2} Marco J. Koudijs,³ Roger K. Patient,⁴ Philip W. Ingham,^{1,2} Stefan Schulte-Merker,^{3,5} and Fredericus J. M. van Eeden^{1,2}

¹MRC Centre for Developmental and Biomedical Genetics, University of Sheffield, Sheffield, United Kingdom; ²Department of Biomedical Sciences, University of Sheffield, Sheffield, United Kingdom; ³Hubrecht Institute–KNAW and University Medical Centre Utrecht, Utrecht, The Netherlands; ⁴MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom; and ⁵Experimental Zoology Department, Wageningen University, Wageningen, The Netherlands

Multiple signaling pathways control the specification of endothelial cells (ECs) to become arteries or veins during vertebrate embryogenesis. Current models propose that a cascade of Hedgehog (Hh), vascular endothelial growth factor (VEGF), and Notch signaling acts instructively on ECs to control the choice between arterial or venous fate. Differences in the phenotypes induced by Hh, VEGF, or Notch inhibition suggest that not all of the ef-

fects of Hh on arteriovenous specification are mediated by VEGF. We establish that full derepression of the Hh pathway in *ptc1;ptc2* mutants converts the posterior cardinal vein into a second arterial vessel that manifests intact arterial gene expression, intersegmental vessel sprouting, and HSC gene expression. Importantly, although VEGF was thought to be absolutely essential for arterial fates, we find that normal and ectopic arterial differ-

entiation can occur without VEGF signaling in *ptc1;ptc2* mutants. Furthermore, Hh is able to bypass VEGF to induce arterial differentiation in ECs via the calcitonin receptor-like receptor, thus revealing a surprising complexity in the interplay between Hh and VEGF signaling during arteriovenous specification. Finally, our experiments establish a dual function of Hh during induction of *runx1*⁺ HSCs. (*Blood*. 2012;120(2):477-488)

Introduction

Among the first fully differentiated cells to arise in the vertebrate embryo are the mesodermally derived hematopoietic and endothelial lineages, which compose the circulatory system, itself the first functional organ system to develop. Unraveling the signaling networks that govern the formation of the vasculature has taken on added significance with the recent demonstration of the emergence of HSCs from ventral endothelium of the dorsal aorta (DA).¹⁻³ In zebrafish, hematopoietic and endothelial lineages arise from a common bipotential progenitor, the hemangioblast.⁴ Hemangioblasts are specified within ventral mesoderm, alongside unipotent hematopoietic progenitors and angioblasts. These 3 cell types are transported via the cellular rearrangements and morphologic movements of gastrulation to reside as 2 bilateral cell populations within the lateral plate mesoderm. The first major vessels of vertebrate embryos form via vasculogenesis, whereby migratory angioblasts coalesce to generate lumenized vessels. In zebrafish, angioblasts within the posterior lateral mesoderm (PLM) migrate medially beneath the somites and coalesce ventral to the notochord and hypochord at the midline. These cells form the major trunk vessels, the DA and posterior cardinal vein (PCV).⁵

The exact regulation and timing of endothelial specification are an important developmental question, but published data are currently difficult to reconcile. A genetic basis for arterial and venous differentiation was established with the identification of Ephrin-B2 and EphB4 receptors as markers of arteries and veins, respectively.^{6,7} Using lineage tracking, clusters of endothelial cells (ECs) were identified within the zebrafish PLM, which either

contributed to the DA or PCV, but never both, leading to the suggestion that determination of arterial and venous fate within ECs occurs before angioblast migration.⁸ The molecular basis of this proposed fate restriction is unclear because differential expression of known arterial or venous markers (*ephrinb2a*, *kdr1*, and *flt4*) is observed after the migrating angioblasts arrive at the midline⁹⁻¹¹; however, the first angioblasts to reach the midline and contribute to the DA express activated ERK during their migration.¹² Importantly, recent studies show that some *flt1/kdr1*⁺ (arterial) angioblasts migrate ventrally and contribute to the PCV after their arrival at the midline.¹³ Taken together, these studies suggest that endothelial progenitors are specified as arterial or venous before circulation, but it is unclear whether they are fully determined before migration to the midline.

The Notch signaling pathway is a key determinant in the establishment of arterial identity.⁹ In mice, Notch receptors and ligands exhibit arterially restricted expression and targeted inactivation of Notch pathway components results in defective arterial specification.¹⁴ Zebrafish *mindbomb* (*mib*) mutants, which are defective for Notch signaling, exhibit arteriovenous shunts, defective PCV formation, and reduced arterial gene expression, whereas *gridlock* (*grl*) mutants, which contain a lesion in *hey2*, exhibit reduced arterial gene expression and DA maturation defects.^{9,15,16}

Although Notch signaling guides arterial fate, elegant studies in zebrafish and more recently in mouse revealed Hedgehog (Hh) and VEGF signaling to act upstream of Notch in arterial specification.^{17,18} Zebrafish *sonic-you* (*syu*) mutants, which lack midline

Submitted October 5, 2011; accepted May 21, 2012. Prepublished online as *Blood* First Edition paper, June 5, 2012; DOI 10.1182/blood-2011-10-383729.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2012 by The American Society of Hematology

Shha activity, exhibit a drastic reduction in arterial gene expression. This failure to specify the DA correctly was proposed to result from failure to express *vegfa* in the adjacent somites.¹⁷ How this transcriptional activation occurs has not been addressed. Recently, a study positioned *Crlra* downstream of Hh signaling and upstream of VEGF within this hierarchy, but the precise molecular details remain unclear.¹⁹ Whatever the exact mechanism of VEGF induction, *vegfa* morphants exhibit reduced arterial gene expression, whereas *vegfa* overexpression induces ectopic arterial differentiation and can rescue the loss of arterial differentiation in *syu* mutant embryos, indicating its indispensability.¹⁷ VEGF binds to Kdr1 or Kdr, a receptor tyrosine kinase, thereby activating downstream phospholipase C gamma1 (Plcg1).²⁰ Zebrafish *kdr1* and *plcg1* mutants are defective in arterial but not in venous specification.^{21,22} Less is known about venous determination; in mice, active repression of Notch signaling may occur in venous ECs.²³ In zebrafish, PI3K signaling downstream of VEGF may maintain venous identity via its ability to block MAPK signaling, which itself favors arterial differentiation via activation of ERK.¹²

Hh signaling inhibition in zebrafish embryos results in a failure to form 2 distinct trunk axial vessels, a phenotype more severe than in embryos lacking VEGF signaling, implying that additional components act downstream of Hh to regulate arterial differentiation.^{9,17,20} Consistent with this, ectopic lumenized vessels can be induced by exogenous Shh but not by VEGF.²⁴ Differences in the response of ECs to Hh and VEGF may account for this²⁵; however, recent studies in mammals have revealed VEGF-independent roles for Hh signaling in vascular lumenization, yet the underlying mechanism remains unclear.¹⁸ In zebrafish, activation of Hh signaling can induce arterial differentiation at the expense of venous identity²⁶; here we extend these findings through analysis of animals doubly mutant for the Hh receptors *Ptc1* and *Ptc2*, which exhibit constitutive activation of Hh signaling.^{27,28} We show that Hh induces arterial differentiation at the expense of venous fate, effectively converting the PCV into a second DA, which displays intact arterial expression in addition to arterial characteristics, such as precocious vessel sprouting and HSC gene expression. In addition, we show that, in *ptc1;ptc2* mutants, arterial differentiation can occur independently of VEGF signaling, via a parallel pathway whereby Hh signaling acts via *Crlra* to induce arterial differentiation. Given the high conservation of these genetic hierarchies between zebrafish and mammals, the identification of *Crlr* signaling in parallel with VEGF may account for the VEGF-independent effects of Hh signaling during mammalian arterial development.¹⁸

Methods

Zebrafish husbandry

Zebrafish (*Danio rerio*) embryos from wild-type, *ptch1*^{hu1602/+}; *ptch2*^{ij222}, *smo*^{hi1640Tg/+}, *plcg1*^{126480/+}, Tg(*flil1a:EGFP*)y1/+, and Tg(*gatal:dsred*)sd2/+ strains were raised at 28.5°C as described.²⁹ All animal studies were conducted with approval from the United Kingdom Home Office.

Drug treatments

Purmorphamine (Chemistry Research Laboratory; stock solution in DMSO: 2.5 mg/mL) was used as previously described.³⁰ SU5416 and Cyclopamine (Merck; stock solution 10mM in DMSO and 95% ethanol, respectively) were used in E3 medium at a concentration of 2μM from tailbud and 50μM from high stage, respectively.

Whole-mount RNA in situ hybridization

Experiments were performed as previously described.¹¹ Myc expression was detected using anti-c-Myc primary antibody (9E10; Abcam; 1:500) alongside anti-DIG antibody. Embryos were incubated with AlexaFluor-488 secondary antibody (Invitrogen; 1:200) and imaged, followed by developing of in situ staining.

Crlra overexpression constructs

Full-length *crlra* was amplified by RT-PCR with the SuperScript One-Step RT-PCR System (Invitrogen) using the primers 5'-ggatccggtccggtactctgacatc-3' and 5'-ctcgagcacattgcatgttgagg-3'. The resulting *Bam*HI/*Xho*I fragment was cloned into pCS2+. Capped mRNA was generated using mMessage Machine SP6 Kit (Ambion).

In situ probes

wnt16 probe was amplified using the primers 5'-cctttgtctctcagggaag-3' and 5'-gcgttgctctttatcctgc-3' and cloned into pGem-T-Easy (Promega). *crlra* probe was created via PCR generated from IMAGE:7141310 (Source Bioscience) using primers 5'-tggataaccgtattaccgcc-3' and 5'-cgcgcaattaac cctcactaaagcactagtcataccaggatc-3'.

Fli1a:Myc-dnPKA:IRES-EGFP construct

Construct was generated using the Tol2 kit via standard methods³¹ and the following components; Fli1a enhancer/promoter, P5efli1EP,³² *Bam*HI-*Xba*I fragment of pSP64T-PK1,³³ and p3E-IRES-EGFPpA.³¹ Embryos were injected at one cell stage with 25 ng/μL Tol2 mRNA and Fli1a:Myc-dnPKA:IRES-EGFP DNA.

Morpholino injections

Embryos were injected with 1nl *crlra* morpholino (Gene Tools; 0.67mM dissolved in distilled water) 5'-agctcgtctcattcttttggcat-3'¹⁹; 0.5 nl (0.5mM) *Su(H)* morpholino 5'-caaacctccctgtcacaacaggcgc-3'³⁴; 0.5 nl (10 μg/μL) *kdr* morpholino 5'-gtttttgatctcacctgaacct-3'²²; and 0.5 nl (10 μg/μL) *kdr1* morpholino 5'-ccgaatgatactccgtatgacatt-3'.³⁵

Confocal imaging

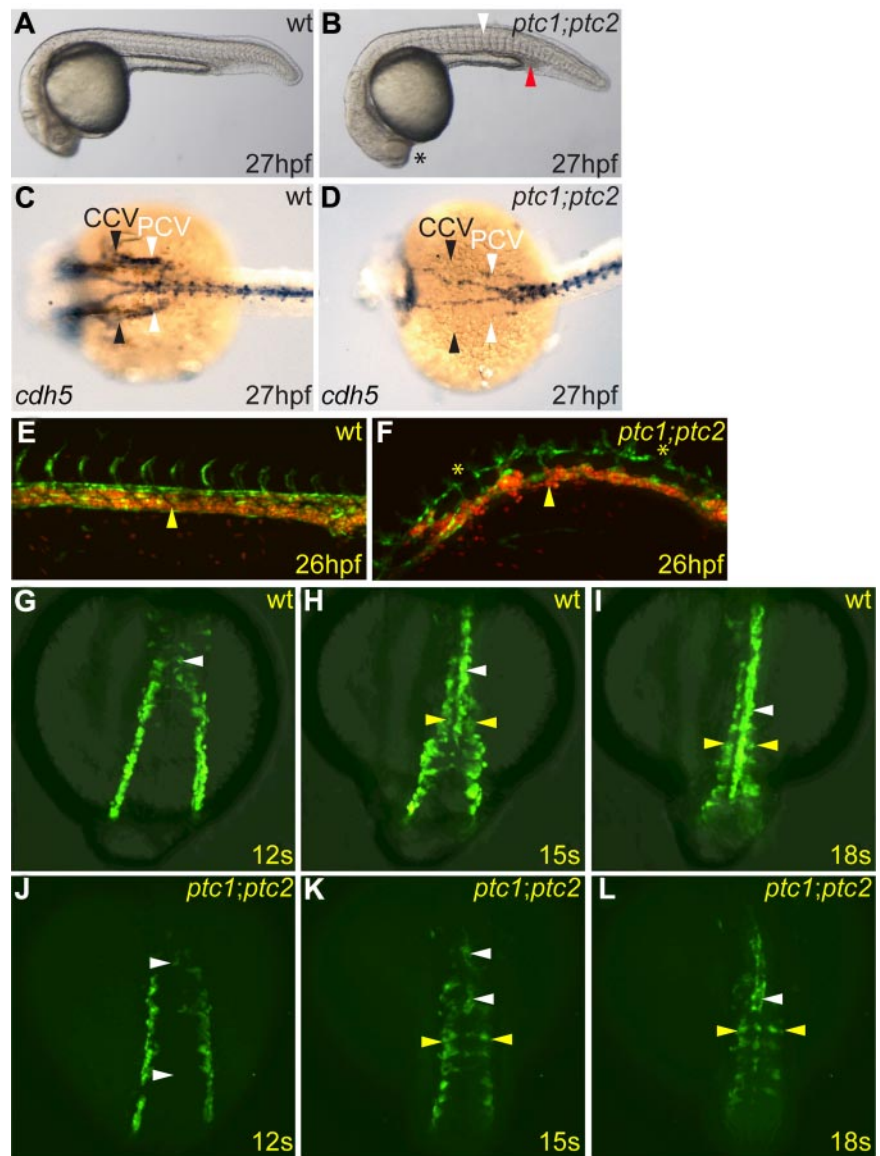
Confocal images were collected using a Leica DM IRE2 microscope at 20× magnification. Time-lapse confocal images were collected using a PerkinElmer Ultraview Vox microscope at 10× and 20× magnification. Images were analyzed using Volocity Version 6.0.1 (PerkinElmer) to reconstruct a 3-dimensional representation.

Results

Zebrafish *ptc1;ptc2* mutants show defective angioblast migration and fail to establish circulation

Although both *ptc1* heterozygous and homozygous embryos exhibit increased levels of Hh signaling, vascular development is not obviously affected in either, and the circulatory loop is established normally. Similarly, *ptc2* homozygotes have no detectable defects in the circulatory system.²⁸ However, double-mutant *ptc1;ptc2* embryos, which exhibit constitutive Hh signaling, by contrast, failed to establish circulation, resulting in pooling of primitive erythrocytes in the intermediate cell mass and posterior blood island (Figure 1A-B red arrowhead). This was further confirmed using the Tg(*flil1a:EGFP*)y1 and Tg(*gatal:dsred*)sd2 transgenes^{36,37} to visualize both developing vasculature and primitive erythrocytes at onset of circulation. Using time-lapse confocal microscopy, we observed that, in wild-type embryos, primitive erythrocytes entered circulation via the PCV, as previously described¹³ (Figure 1E yellow arrowhead; supplemental Video

Figure 1. Zebrafish *ptc1;ptc2* double mutants fail to establish circulation and exhibit multiple endothelial defects and defective angioblast migration. (A-B) *ptc1;ptc2* mutant (B) embryos at 27 hpf showed somitic flattening (white arrowhead) and loss of lens (asterisk). *ptc1;ptc2* embryos showed noncirculating primitive erythrocytes in the posterior intermediate cell mass (red arrowhead). (C-D) *cdh5* expression in *ptc1;ptc2* mutants (D) revealed defective formation of the common cardinal vein (CCV; black arrowheads) and PCV (white arrowheads) compared with wild-type (C). (E-F) Confocal image of trunk vasculature in Tg(*flil1a:EGFP*)y1;Tg(*gata1:dsred*)sd2/+ (E) and Tg(*gata1:dsred*)sd2/+;*ptc1;ptc2* (F) embryos; anterior to the left, posterior right. The primary vasculature formed normally in Tg(*flil1a:EGFP*)y1;Tg(*gata1:dsred*)sd2/+ embryos, and circulation commenced normally (yellow arrowhead; supplemental Video 1), whereas Tg(*flil1a:EGFP*)y1;Tg(*gata1:dsred*)sd2/+;*ptc1;ptc2* embryos had disorganized vasculature, axial vessels were noncontinuous along their anteroposterior axis (asterisks), and embryos lacked circulation (yellow arrowhead; supplemental Video 2). (G-L) Confocal images using a Tg(*flil1a:EGFP*)y1/+ background to visualize migrating angioblasts in indicated genetic backgrounds. Dorsal views are shown. (G-I) Normal angioblast migration in Tg(*flil1a:EGFP*)y1/+ embryos. (J-L) Fewer angioblasts migrated to the midline in Tg(*flil1a:EGFP*)y1/+;*ptc1;ptc2* embryos and formed a discontinuous endothelial cord by 18 somites (supplemental Video 3; white arrowheads). Angioblasts were present in more lateral positions (supplemental Video 4; L yellow arrowheads) than in controls at the corresponding stage (I yellow arrowheads).



1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article), whereas, in *ptc1;ptc2* embryos, they failed to enter the circulation, mainly residing within the PCV (Figure 1F yellow arrowhead; supplemental Video 2). Double-mutant embryos also exhibit disorganized vasculature. In wild-type embryos at 26 hpf, the DA and PCV were juxtaposed after the entry of primitive erythrocytes into circulation (Figure 1E), whereas in *ptc1;ptc2* mutants, the 2 axial vessels were clearly separated (Figure 1F). In more anterior regions, at 27 hpf, *ptc1;ptc2* embryos exhibited a drastic reduction of endothelial *cdh5* staining in the common cardinal vein (Figure 1C-D black arrowheads), and the adjoining PCV (Figure 1C-D white arrowheads), indicating these vessels were absent, which would explain the failure to establish a circulatory loop in *ptc1;ptc2* double mutants.

The medial migration of angioblasts is regulated by Hh signaling^{11,26}; thus, to investigate whether a migration defect could contribute to the failure to form proper vessels, we used the Tg(*flil1a:EGFP*)y1 transgene to follow medial migration of blood and endothelial progenitors in live *ptc1;ptc2* embryos (Figure 1G-L). In control Tg(*flil1a:EGFP*)y1/+ embryos, GFP⁺ angioblasts migrated, such that by 12 somites, the first GFP⁺ DA angioblasts

had reached the midline (supplemental Video 3; Figure 1G white arrowhead). By 15 somites, these cells had begun to coalesce into a vascular cord at the midline (Figure 1H white arrowhead), closely followed by angioblasts, which contribute to the PCV (Figure 1H yellow arrowheads). By 18 somites, the vascular cord extended posteriorly (Figure 1I white arrowhead), whereas some angioblasts remained more lateral and had not yet reached the midline (Figure 1I yellow arrowheads). In Tg(*flil1a:EGFP*)y1/+;*ptc1;ptc2* embryos, by contrast, quantification using ImageJ Version 1.44 software revealed a 38% decrease in GFP⁺ cells throughout the embryo at 12 somites and 55% fewer angioblasts migrated toward the midline at this stage (supplemental Video 4; Figure 1J white arrowheads), such that by 15 somites, 43% fewer angioblasts had reached the midline, forming a discontinuous vascular cord (Figure 1K white arrowheads) with many angioblasts present in more lateral positions (Figure 1K yellow arrowheads). By 18 somites, the vascular cord extended anteroposteriorly in Tg(*flil1a:EGFP*)y1/+;*ptc1;ptc2* embryos yet remained discontinuous and contained fewer GFP⁺ cells along the anteroposterior axis (Figure 1L, K white arrowheads). Many GFP⁺ cells also remained in more lateral positions at 18 somites in Tg(*flil1a:EGFP*)y1/+;*ptc1;ptc2* embryos (Figure

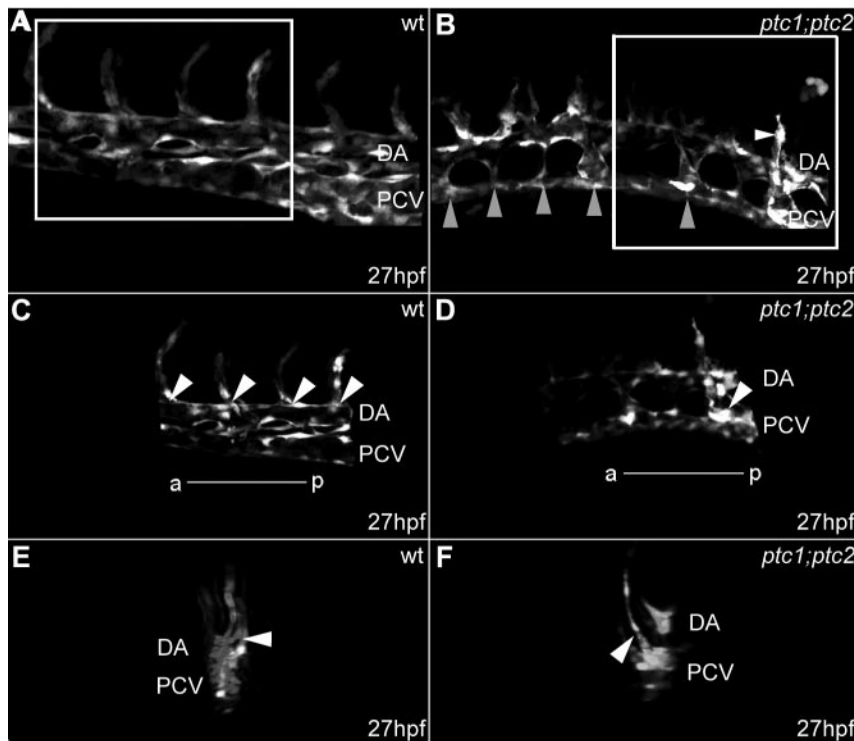


Figure 2. *ptc1;ptc2* mutants exhibit precocious vessel sprouting from the posterior cardinal vein. Confocal images of developing trunk vasculature in *Tg(fli1a:EGFP)y1/+* (A,C,E) and *Tg(fli1a:EGFP)y1/+;ptc1;ptc2* embryos at 27 hpf (B,D,F); anterior is left, posterior right. (A) Normal trunk vasculature. Highlighted area is shown in panel C. (B) Endothelial connections between the DA and PCV were present in *Tg(fli1a:EGFP)y1/+;ptc1;ptc2* embryos (gray arrowheads), and ectopic vessel sprouts arising from PCVs were present (B,D white arrowheads). Highlighted area is shown in panel D. 3-Dimensional reconstruction: transverse plane of embryo shown in panels A, C, and E and embryo shown in panels B, D, and F show the sprouting capacity of ECs in the PCV region in *ptc1;ptc2* mutants (white arrowhead).

1L yellow arrowheads). Thus, aberrant migration could contribute to the observed circulation defect. We also examined the effect of Hh signaling deficiency on angioblast migration using *Tg(fli1a:EGFP)y1/+;smo^{hi1640Tg}* embryos.³⁸ In contrast to recent studies,²⁶ we observed that angioblasts in *Tg(fli1a:EGFP);smo^{hi1640Tg}* embryos migrated in 2 waves yet failed to coalesce into an endothelial cord at the midline (supplemental Video 6; supplemental Figure 1D-F). Taken together, these results suggest that either elevated or absent Hh signaling results in impaired angioblast migration, implying that the levels of Hh signaling perceived by angioblasts are important in their successful migration to the midline.

More detailed analysis of angioblast migration for a period of 17 hours from 16 somite stage revealed that, whereas the vasculature formed normally in *Tg(fli1a:EGFP)y1/+* embryos (supplemental Video 7), *Tg(fli1a:EGFP)y1/+;ptc1;ptc2* embryos exhibited delayed formation of the DA, resulting from aberrant medial migration of angioblasts; however, the DA ultimately formed a continuous vessel in these embryos (supplemental Video 8). Importantly, no angioblasts were observed to migrate ventrally and contribute to the PCV, as described previously.¹³ Furthermore, in *Tg(fli1a:EGFP)y1/+;ptc1;ptc2* embryos, angioblasts in the position of the PCV in the posterior trunk were observed to migrate dorsally and contribute to the nascent DA (supplemental Video 8). VEGF has been shown to limit the ventral migration of angioblasts¹³; thus, in *ptc1;ptc2* mutants, where VEGF signaling is elevated (see Figure 3L green arrowhead), angioblasts migrated dorsally to contribute to the dorsal aorta, thereby underlining the importance of VEGF signaling as a directional cue in this process.

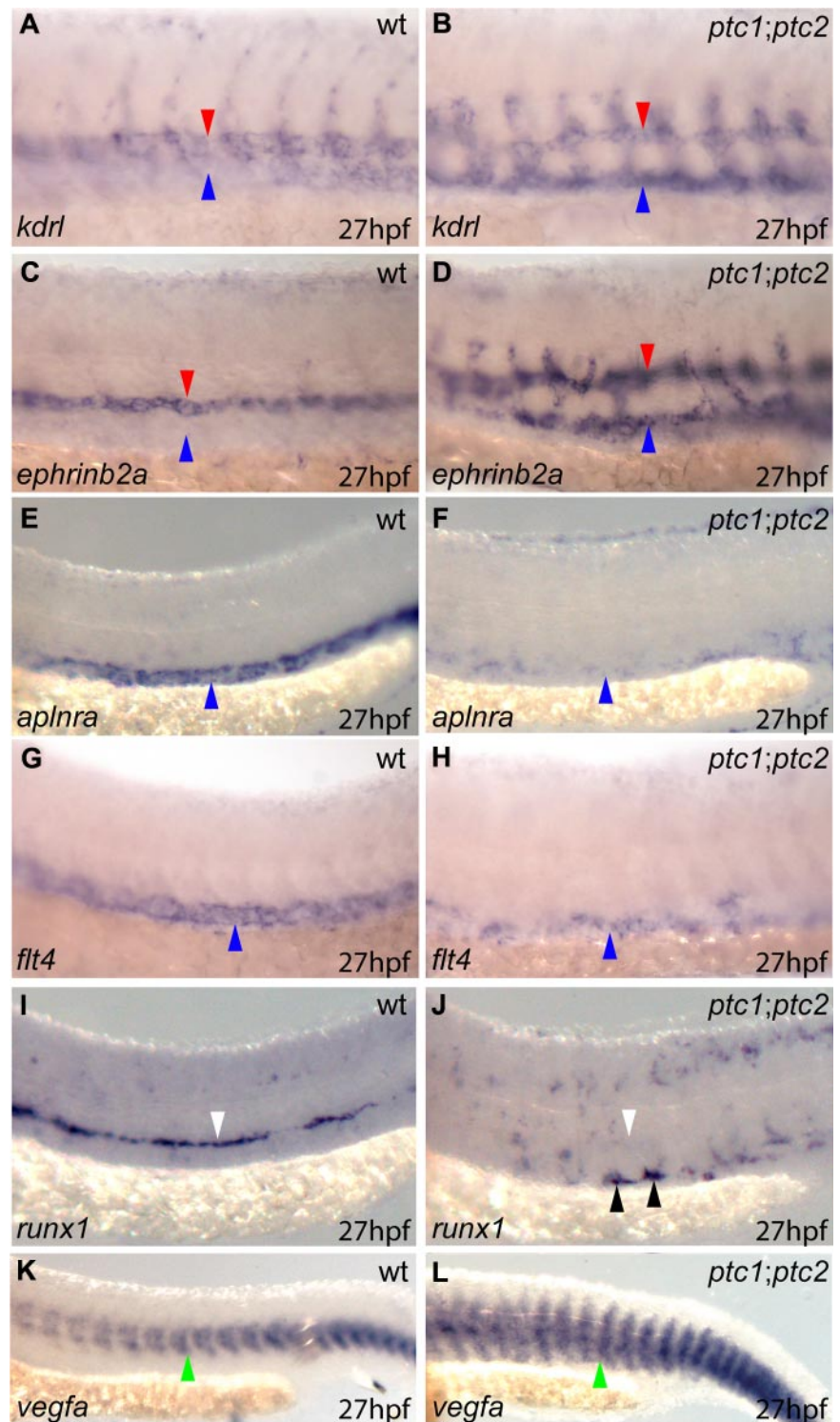
***ptc1;ptc2* mutants exhibit ectopic arterial differentiation at the expense of venous differentiation, resulting in conversion of the PCV into an arterial vessel**

During normal development, intersegmental vessels (ISVs) sprout from the DA from 24 hpf onwards, whereas venous ISVs sprout later between 1.5 dpf and 2 dpf.^{39,40} In *ptc1;ptc2* embryos, by

contrast, precocious ISV formation was clearly observed in the PCV at 27 hpf (Figure 2B-D white arrowheads), suggesting that the PCV had acquired arterial characteristics. Optical sectioning and 3-dimensional reconstruction confirmed that the sprouting ISVs originated from the PCV and not the DA (Figure 2F white arrowhead). In addition to the precocious PCV sprouts, *ptc1;ptc2* mutants displayed atypical endothelial connections between the DA and PCV (Figure 2B gray arrowheads), which were not present in control embryos (Figure 2A,C).

To evaluate the differentiation status of the vessels in *ptc1;ptc2* embryos, we assayed arterial and venous marker gene expression by in situ hybridization. Expression of the VEGF receptor *kdrl* normally exhibits an arterial bias, originally being present throughout the nascent vasculature, but becoming down-regulated in the PCV and restricted to the DA by 26 to 27 hpf (Figure 3A red arrowhead). In *ptc1;ptc2* mutants, by contrast, *kdrl* expression is maintained in the PCV during vascular development (Figure 3A-B blue arrowheads). Whereas the arterial marker *ephrinb2a* was ectopically expressed in the PCV (Figure 3C-D blue arrowhead), the expression of *aplrra* in venous endothelium⁴¹ was absent or substantially down-regulated in the PCV of *ptc1;ptc2* mutants (Figure 3E-F blue arrowheads). Furthermore, expression of the VEGF receptor *flt4*, which is restricted to the PCV at 27 hpf, was substantially down-regulated in *ptc1;ptc2* mutants (Figure 3G-H blue arrowheads). Ectopic arterial gene expression in the PCV of *ptc1;ptc2* mutants, combined with a loss of venous gene expression, indicates that the PCV had undergone a fate change and was converted into an artery. We also assessed the onset of arterial gene expression within the *ptc1;ptc2* mutants and did not observe any induction of arterial markers within premigratory angioblasts (data not shown); however, ectopic expression of the arterial marker *dll4* was observed as early as 18 somites (supplemental Figure 2A-B red arrowheads) and was detectable in angioblasts, which had not yet reached the midline in *ptc1;ptc2* mutants (supplemental Figure 2D asterisks). Similarly, precocious midline expression of *ephrinb2a*

Figure 3. *ptc1;ptc2* mutants exhibit ectopic arterial differentiation at the expense of venous differentiation, resulting in conversion of the PCV into a second artery. Lateral views of the trunk region are shown, oriented anterior to left, posterior right. (A) *kdr1* exhibited arterial preference in wild-type embryos (red arrowheads) but was present ectopically in the PCV in *ptc1;ptc2* embryos (B blue arrowheads). (C) *ephrinb2a* was restricted to the DA in wild-type embryos (red arrowhead) but present ectopically in the PCV in *ptc1;ptc2* embryos (D blue arrowheads). (E) *aplnra* and (G) *flt4* expression was restricted to the PCV in wild-type embryos (blue arrowheads) and down-regulated in *ptc1;ptc2* embryos (F,H blue arrowheads). (I) *runx1* was restricted to the DA in wild-type embryos (white arrowhead), down-regulated in the DA of *ptc1;ptc2* embryos (J white arrowhead), and ectopically expressed in the PCV of *ptc1;ptc2* embryos (J black arrowheads). (K) Increased trunk *vegfa* expression was present in *ptc1;ptc2* embryos (L green arrowhead) compared with wild-type (K green arrowhead).



was observed at 18 somites (supplemental Figure 2E-H red arrowheads) and 19 somites (supplemental Figure 2I-L red arrowheads) in *ptc1;ptc2* embryos. Thus, activation of Hh can change both spatial and temporal profile of arterial gene expression.

Analysis of *runx1*⁺ HSCs in *ptc1;ptc2* embryos confirms conversion of the PCV to an arterial vessel and indicates a dual function of Hh in their induction

Expression of the transcription factor *runx1* is normally restricted to cells in the ventral DA and also marks emerging definitive

hematopoietic progenitors and HSCs.^{11,42,43} In *ptc1;ptc2* mutants, *runx1* was expressed ectopically in the PCV, supporting the hypothesis that the PCV had acquired arterial character (Figure 3J black arrowheads). Quantification of the number of *runx1*⁺ cells in *ptc1;ptc2* mutants revealed an 89% reduction (supplemental Figure 3), and these cells were absent from the DA (Figure 3I,J white arrowheads). Recent studies have revealed a novel signaling pathway in the zebrafish somite,⁴⁴ whereby somitic *wnt16* signaling upstream of *deltaC/deltaD* was necessary for initiation of *runx1* expression within the DA. Because *ptc1;ptc2*

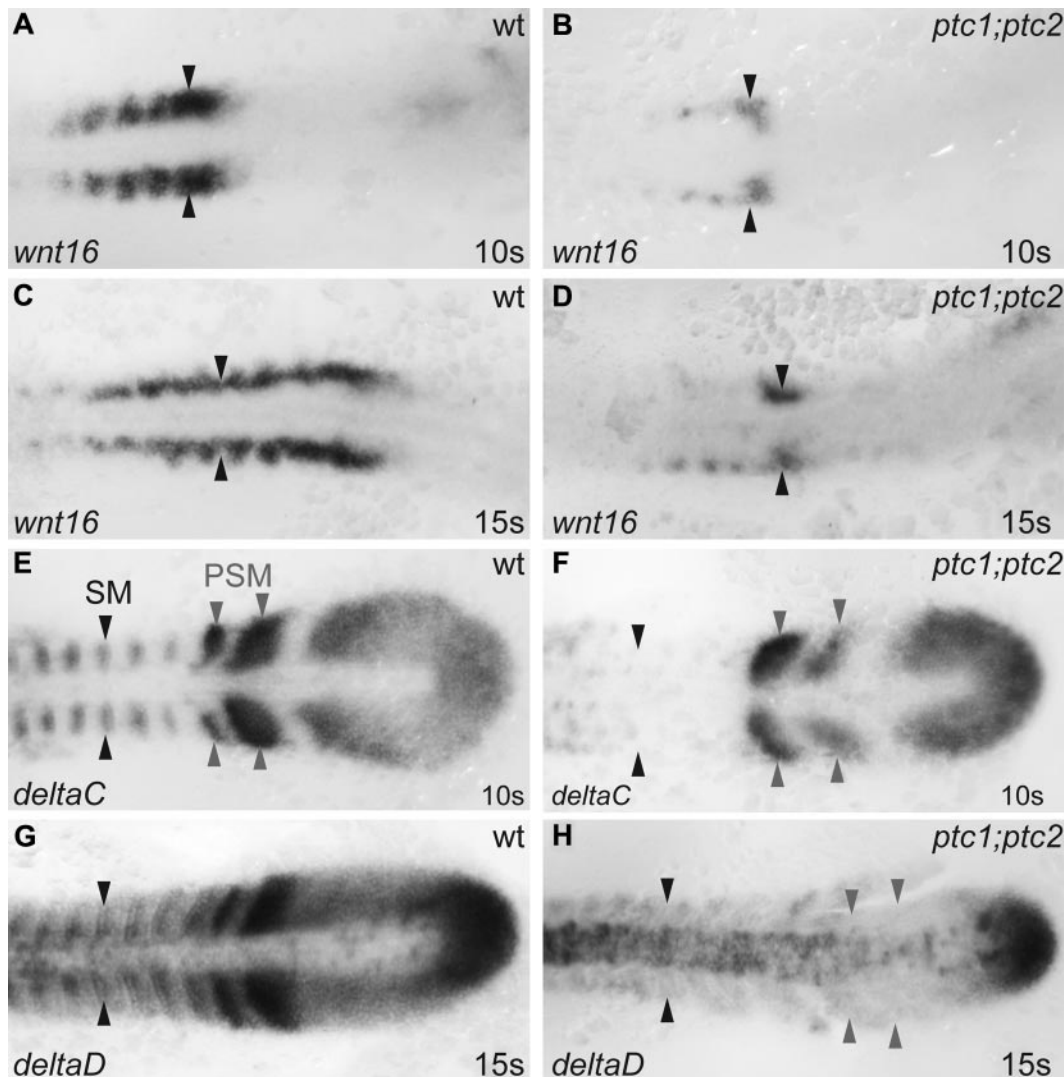


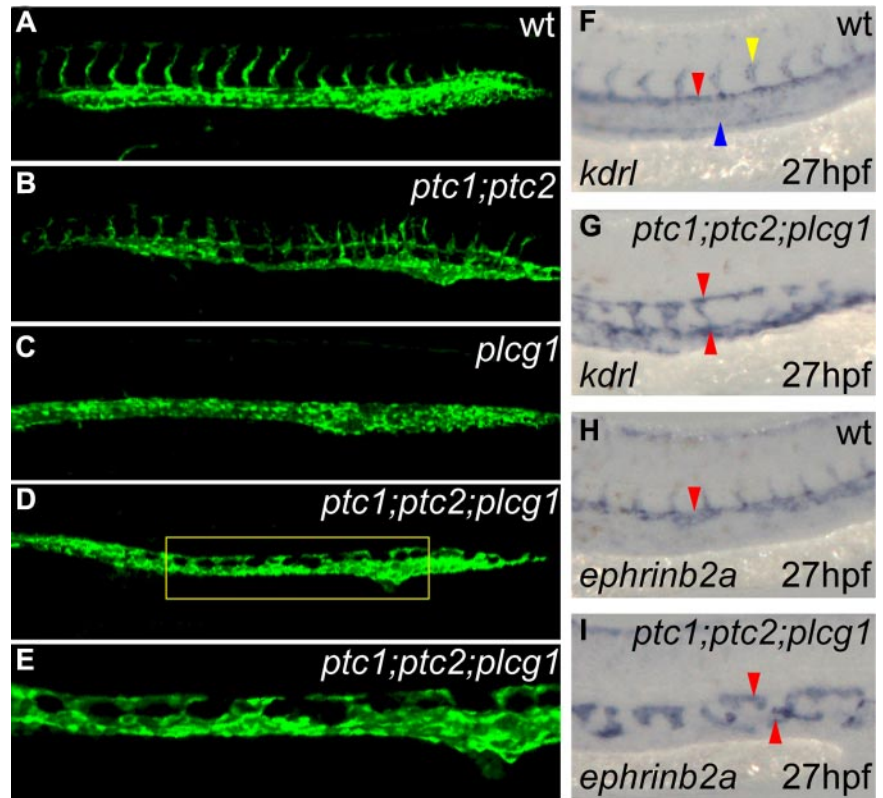
Figure 4. Somitic *wnt16*-*deltaC/deltaD* signaling required for HSC specification is abrogated in *ptc1;ptc2* mutants. Zebrafish flat mounts are shown with anterior to the left and posterior to the right. (A-B) Somitic *wnt16* expression is strongly down-regulated in *ptc1;ptc2* embryos at 10 somites (B black arrowheads), and this down-regulation persisted at the 15-second stage (C-D black arrowheads). (E-F) Expression of *deltaC* was absent from the somitic mesoderm (SM) of *ptc1;ptc2* embryos (F black arrowheads) but was present within the presomitic mesoderm (PSM; F gray arrowheads). (G-H) *deltaD* expression was strongly down-regulated within the SM (H black arrowheads) and PSM (H gray arrowheads) of *ptc1;ptc2* embryos.

embryos exhibit somite patterning defects, we analyzed *wnt16* expression and found that it was persistently reduced in the somites of *ptc1;ptc2* mutant embryos from 10 somites onwards (Figure 4A-D black arrowheads). In line with this, expression of *deltaD* was lost within the somitic mesoderm from 10 somites (Figure 4G-H black arrowheads), as was *deltaC* (Figure 4E-F).²⁷ That *runx1* is expressed at all is surprising given the early down-regulation of *wnt16* and *deltaC/deltaD*; however, we have previously demonstrated that *bmp4* expression is required for initiation of *runx1* expression in the DA.³⁰ Furthermore, *ptc1;ptc2* mutants exhibit strong up-regulation of *bmp4* within the pronephric ducts (data not shown). It is therefore possible that Bmp signaling is able to rescue the loss of somitic *wnt16*-*DeltaC/D* by inducing *runx1* expression within arterial ECs close to the source of *bmp4*. Taken together, these data indicate that ectopic Hh signaling affects *runx1*⁺ HSCs in 2 opposing ways: first by promoting arterial fate in the ventral vessel allowing ectopic HSCs to form, but at the same time antagonizing somitic *wnt16* and *deltaC/D*, leading to a reduction in overall HSC numbers.

The level of Hh and VEGF signaling determines the balance between venous and arterial fates

Because Hh sits atop a signaling hierarchy that activates VEGF,^{17,19} we analyzed *vegfa* expression in *ptc1;ptc2* mutants and found that it was significantly up-regulated throughout the trunk of mutant embryos (Figure 3K-L green arrowheads), consistent with previous studies.⁷ We also treated wild-type embryos with the Smoothed agonist Purmorphamine (PMA) from tailbud stage onwards⁴⁵ because PMA is capable of activating Hh signaling to a greater level than that observed in *ptc1* mutants but less than that of *ptc1;ptc2* mutants.³⁰ PMA-treated embryos demonstrated increased *kdrl* and *ephrinb2a* expression within the DA (supplemental Figure 4A-D red arrowheads). PMA-treated embryos also retained higher *kdrl* expression within the PCV (supplemental Figure 4A-B blue arrowheads), whereas *ephrinb2a* was not ectopically induced. In contrast to *ptc1;ptc2* mutants, PMA-treated embryos exhibited a moderate increase in *vegfa* expression (supplemental Figure 4G-H), and expression of the venous marker *aplnra*

Figure 5. Arterial specification occurs independently of VEGF signaling in *ptc1;ptc2;plcg1* triple mutants. (A-E) *Tg(fli1a:EGFP)y1/+;ptc1;ptc2* embryos formed 2 separate vessels, both with sprouting capacity (B), whereas *Tg(fli1a:EGFP)y1/+;plcg1²⁶⁴⁸⁰* embryos formed a single vessel without ISV sprouts and lacking arterial gene expression (data not shown; C). Two separate vessels were present in triple mutant embryos (D, zoomed view of panel E), but no ISVs formed. *kdrl* (F-G) and *ephrinb2a* (H-I) expression indicated that the DA remained duplicated in the triple mutants, albeit with fewer cells.



was considerably reduced or absent in the PCV of PMA-treated embryos (supplemental Figure 4E-F blue arrowheads). Taken together, these results indicate that the levels of Hh signaling and subsequent *vegfa* expression are critical for the balance between arterial versus venous differentiation.

Arterial specification occurs independently of VEGF signaling in *ptc1;ptc2* mutants

Up-regulated *vegfa* expression in *ptc1;ptc2* mutants (Figure 3K-L green arrowheads) implies that the aberrant PCV specification was the result of an increase in VEGF signaling. To address this, we crossed the *plcg1²⁶⁴⁸⁰* mutant,⁴⁰ which blocks VEGF signaling downstream of its receptor²⁰ into the *Tg(fli1a:EGFP)y1/+;ptc1;ptc2* background. Whereas the vasculature of *Tg(fli1a:EGFP)y1/+* siblings exhibited normal ISV sprouting (Figure 5A), ectopic ISVs were observed in *Tg(fli1a:EGFP)y1/+;ptc1;ptc2* embryos (Figure 5B). Furthermore, whereas the axial vessels were juxtaposed in *Tg(fli1a:EGFP)y1/+* embryos (Figure 5A), the 2 vessels could be observed discretely in *Tg(fli1a:EGFP)y1/+;ptc1;ptc2* embryos (Figure 5B). In contrast, no ISVs were present in *Tg(fli1a:EGFP)y1/+;plcg1* embryos, indicating defective angiogenesis arising from inhibition of VEGF signaling (Figure 5C).^{20,46,47} Whereas ISVs were absent in *Tg(fli1a:EGFP)y1/+;ptc1;ptc2;plcg1* embryos (Figure 5D-E), the developing vasculature consisted of a noncontinuous dorsal vessel, which was not closely juxtaposed to the ventral vessel. Expression of *kdrl* at 27 hpf was reduced in the PCV (Figure 5F blue arrowhead) of *Tg(fli1a:EGFP)y1/+* siblings and retained in the DA (Figure 5F red arrowhead) and ISVs (Figure 5F yellow arrowhead). In contrast, ectopic *kdrl* expression was retained in both vessels in *Tg(fli1a:EGFP)y1/+;ptc1;ptc2;plcg1* mutant embryos (Figure 5G red arrowheads). Consistent with this, arterial *ephrinb2a* was retained in all *Tg(fli1a:EGFP)y1/+;ptc1;ptc2;plcg1* embryos and demonstrated punctate expression through-

out the trunk in the position of the dorsal and ventral vessels (Figure 5I red arrowheads). Both *kdrl* and *ephrinb2a* are strongly down-regulated in *plcg1* mutants.³⁶

Although the *ptc1;ptc2;plcg1* triple mutant is expected to have no VEGF signaling, bifurcations in the downstream signaling cascade imply that the use of alternative effectors cannot be excluded. To control for this possibility, we used an inhibitor of VEGF signaling, SU5416, which acts at the level of the VEGF receptor.^{48,49} We treated the progeny of *Tg(fli1a:EGFP)y1/+;ptc1/+;ptc2/+* x *ptc1/+;ptc2/+* crosses, with high concentrations of SU5416 (2 μ M) from tailbud stage to ensure maximal inhibition of VEGF signaling (supplemental Figure 5). In treated *ptc1;ptc2* mutants, expression of arterial markers, such as *dll4* and *ephrinb2a*, were retained, albeit with reductions in the number of arterial ECs (supplemental Figure 5H,L red arrowheads) relative to DMSO-treated *ptc1;ptc2* mutants (supplemental Figure 5F,J red arrowheads). However, in SU5416-treated siblings, all arterial differentiation was absent (supplemental Figure 5E,G,I,K red arrowheads). Moreover, ectopic arterial differentiation was retained in SU5416-treated *ptc1;ptc2* mutants (supplemental Figure 5H,L red arrowhead). These results indicate that activation of Hh signaling can induce arterial differentiation independently of VEGF signaling.

This result was surprising given previously demonstrated requirements for VEGF in arterial differentiation. However, it remains formally possible that the levels of SU5416 used, which were determined by its nonspecific toxicity at higher doses, were insufficient to inhibit elevated VEGF signaling in *ptc1;ptc2* embryos. To address this issue, we asked whether the same dose of SU5416 was sufficient to inhibit the effects of exogenous *vegfa* mRNA. Embryos were injected with 50 pg *vegfa* mRNA, more than 2 times higher concentration than that sufficient to induce substantial ectopic arterial differentiation.¹⁷ As expected, embryos

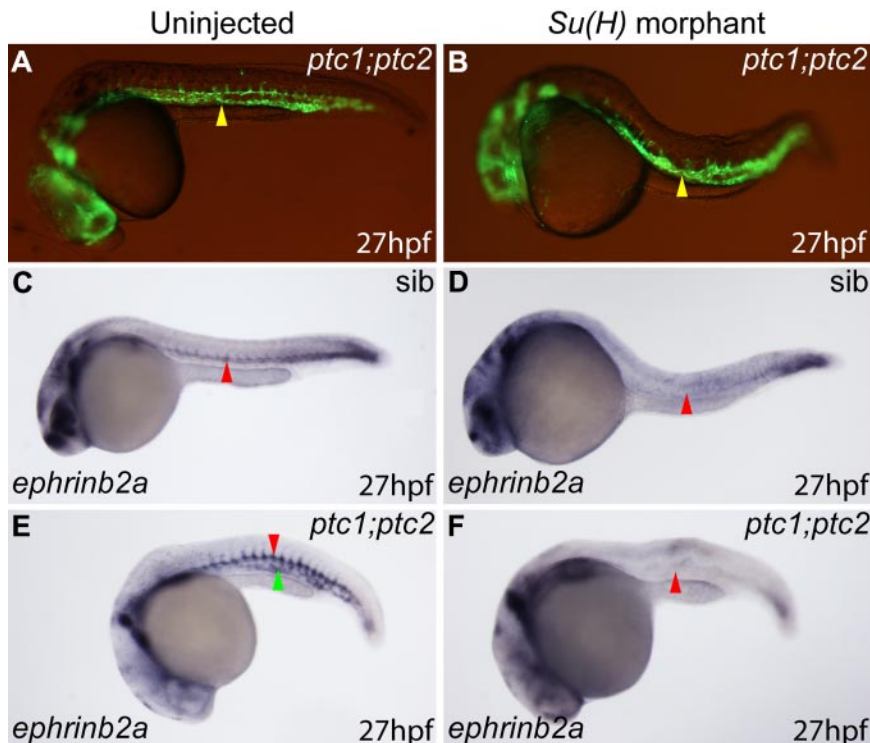


Figure 6. Arterial specification in *ptc1;ptc2* mutants is dependent on Notch signaling. (A) *Tg(fli1a:EGFP)y1/+; ptc1;ptc2* embryos exhibit blood vessel formation (B, yellow arrowhead; *n* = 15 of 230), even after *Su(H)* morpholino injection (B yellow arrowhead; *n* = 5 of 95). (C) Normal DA *ephrinb2a* expression in uninjected non-double mutant embryos (C red arrowhead; *n* = 157 of 167). (D) Loss of *ephrinb2a* in DA of non-double mutant *Su(H)* morphants (red arrowhead; *n* = 159 of 180). (E) Increased (red arrowhead), ectopic (green arrowhead) *ephrinb2a* expression in uninjected *ptc1;ptc2* embryos (*n* = 10 of 167). (F) Loss of *ephrinb2a* in DA of *ptc1;ptc2 Su(H)* morphants (*n* = 16 of 180).

injected with *vegfl21* mRNA and treated with DMSO exhibited ectopic *ephrinb2a* expression throughout the trunk (supplemental Figure 6B red arrowhead). By contrast, 63% of injected embryos treated with SU5416 exhibited total loss of *ephrinb2a* in the trunk (supplemental Figure 6D red arrowhead), whereas the remaining 37% exhibited weak expression and no ectopic *ephrinb2a* expression was observed (data not shown). These data clearly indicate that the concentration of SU5416 used is sufficient to inhibit very high levels of VEGF signaling.

As a further control, we coinjected *ptc1;ptc2* mutant embryos with morpholinos targeting the VEGF receptors *kdr* and *kdr1*,^{22,35} which together are required to transduce the VEGF signal. Combinatorial knockdown abolished arterial *ephrinb2a* expression in non-double mutant siblings (supplemental Figure 7A,C red arrowhead). By contrast, *ptc1;ptc2* mutant embryos retained ectopic *ephrinb2a* expression (supplemental Figure 7D red arrowhead), although no vessel sprouting was observed and fewer *ephrinb2a*⁺ cells were present (supplemental Figure 7B red arrowhead).

Finally, to demonstrate endothelial cell autonomy of Hh signaling, we mosaically activated Hh signaling within ECs by coinjecting a *Fli1a:Myc-dnPKA:IRES-EGFP* DNA construct and *Tol2* mRNA into embryos, which were treated with cyclopamine, to inhibit Hh signaling and downstream VEGF (supplemental Figure 8). Murine dnPKA is a well-established activator of Hh signaling.³³ Uninjected embryos treated with cyclopamine displayed a total absence of the early and late arterial markers *dll4* (supplemental Figure 8I-J black arrowheads) and *ephrinb2a* within the vasculature (supplemental Figure 8D,F black arrowheads).¹¹ By contrast, injected embryos treated with cyclopamine exhibited ECs coexpressing *Myc-dnPKA* and *dll4* (supplemental Figure 8G-H arrowheads) and *ephrinb2a* (supplemental Figure 8K-L arrowheads). This rescue of arterial differentiation indicates that Hh signaling is able to act cell autonomously in ECs. Taken together, our triple-mutant analysis, SU5416 exposure, VEGF receptor knockdown, and

demonstration of cell autonomy all show that high levels of Hh signaling are able to induce arterial differentiation in a VEGF-independent manner.

Arterial specification in *ptc1;ptc2* mutants is dependent on Notch signaling

To determine whether loss of Notch, which acts downstream of VEGF, could prevent ectopic arterial differentiation in *ptc1;ptc2* embryos, we injected a morpholino targeting *Su(H)*,³⁴ a protein essential for Notch signal transduction.⁵⁰ Knockdown of *Su(H)* in non-double mutant sibling embryos resulted in blood vessels (Figure 6A-B yellow arrowheads), which failed to express arterial *ephrinb2a* (Figure 6C-D red arrowheads). Interestingly, knockdown of *Su(H)* in *ptc1;ptc2* double-mutant embryos also resulted in a total loss of vascular *ephrinb2a* expression (Figure 6E-F red arrowheads). These data indicate that the VEGF-independent signaling in *ptc1;ptc2* embryos occurs upstream of Notch.

Knockdown of *crlra* in combination with VEGF inhibition prevents arterial differentiation in *ptc1;ptc2* mutants

Our finding that arterial differentiation can occur independently of VEGF signaling but is dependent on Notch raises the important question of how Hh is able to bypass VEGF to activate downstream target genes. We evaluated *crlra*, a downstream target of Hh with a role in arterial induction.¹⁹ In wild-type embryos, *crlra* was diffusely and weakly expressed throughout the somites (Figure 7A green arrowhead) but also weakly expressed in the DA (Figure 7A red arrowhead). Conversely, in *ptc1;ptc2* embryos, *crlra* was considerably up-regulated both in the somites (Figure 7B green arrowhead) and in the DA (Figure 7B red arrowhead). Thus, increased *Crlra* might account for the retention of arterial differentiation in VEGF inhibited *ptc1;ptc2* mutants.

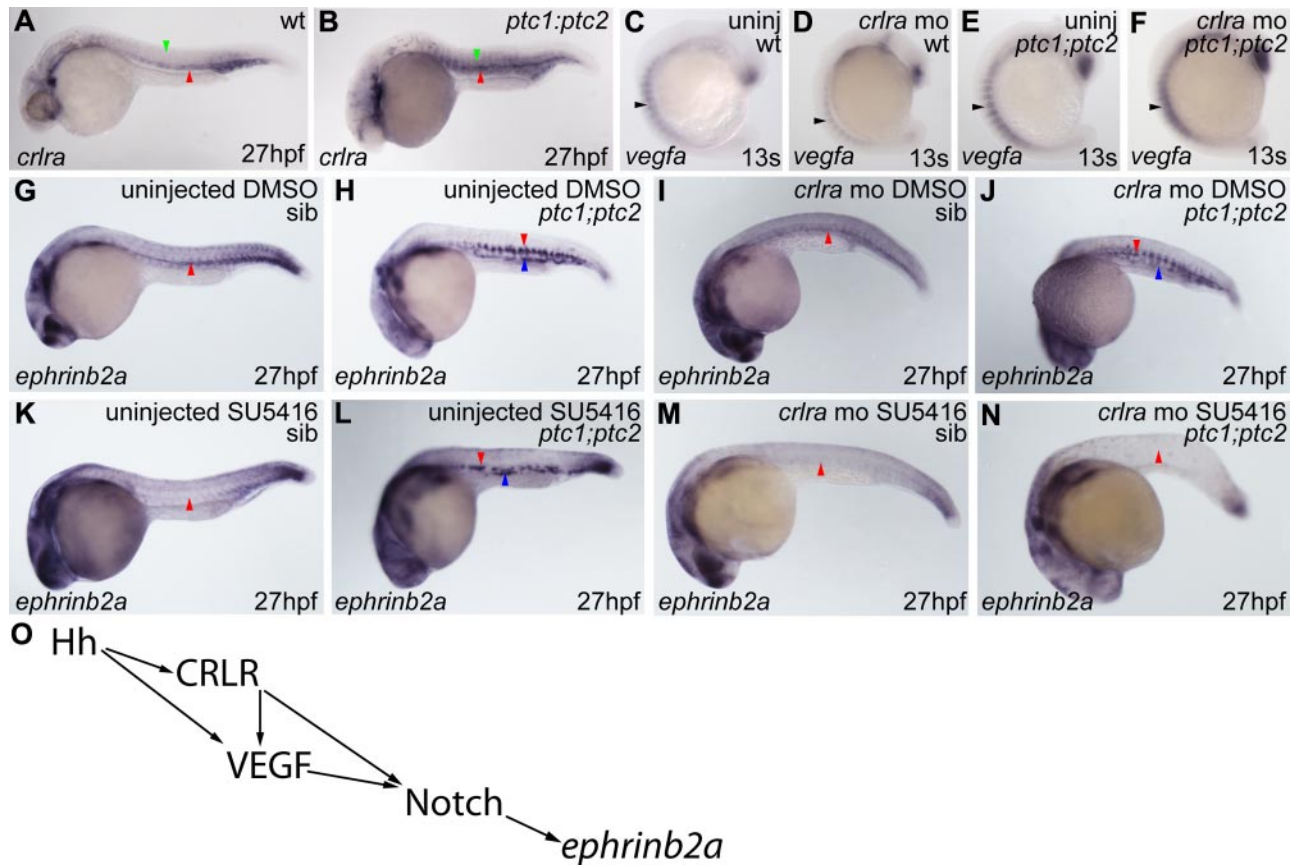


Figure 7. Knockdown of *crlra* in combination with VEGF inhibition prevents arterial differentiation in *ptc1;ptc2* mutants. (A) Wild-type somitic (green arrowhead) and DA *crlra* expression (red arrowhead). (B) Increased somitic (green arrowhead) and DA (red arrowhead) *crlra* expression in *ptc1;ptc2* embryos. (C) Normal somitic *crlra* expression in uninjected wild-type non-double mutant embryos (arrowhead; n = 58 of 66). (D) Decreased somitic *crlra* expression in non-double mutant *crlra* morphants (n = 58 of 71). (E-F) No difference in *crlra* expression (arrowheads) was detected between uninjected (n = 5 of 66) and *crlra* morphant (n = 4 of 71) *ptc1;ptc2* embryos. Uninjected wild-type siblings treated with SU5416 from tailbud until collection exhibited total absence of vascular *ephrinb2a* expression (K red arrowhead; n = 40 of 50) compared with controls (G red arrowhead; n = 46 of 49). SU5416-treated *ptc1;ptc2* mutants showed increased *ephrinb2a* (L red arrowhead) and ectopic expression in PCV region (L blue arrowhead; n = 3 of 49). Uninjected SU5416-treated *ptc1;ptc2* embryos exhibited no vessel sprouting compared with DMSO-treated *ptc1;ptc2* embryos. *ephrinb2a* expression was down-regulated in the DA of non-double mutant *crlra* morphants treated with DMSO (I red arrowhead; n = 78 of 96), whereas non-double mutant *crlra* morphants treated with SU5416 exhibited a loss of vascular *ephrinb2a* expression (M red arrowhead; n = 165 of 183). *ptc1;ptc2* *crlra* morphants treated with DMSO showed strong *ephrinb2a* expression (J red arrowhead), which was present ectopically in the region of the PCV (J blue arrowhead; n = 8 of 96), whereas vascular *ephrinb2a* was absent in *ptc1;ptc2* *crlra* morphants treated with SU5416 (N red arrowhead; n = 10 of 186). (O) Proposed model for arterial differentiation.

Embryos were injected with *crlra* morpholino¹⁹ and treated with SU5416. As previously described, SU5416 treated wild-type embryos revealed a total absence of arterial differentiation within the trunk vasculature (Figure 7G,K red arrowhead). In *ptc1;ptc2* mutants, by contrast, treatment with SU5416 was unable to block arterial *ephrinb2a* expression (Figure 7H,L blue arrowhead). Wild-type embryos injected with *crlra* morpholino exhibited considerable down-regulation of *ephrinb2a* (Figure 7G,I red arrowhead), whereas those injected with *crlra* morpholino and treated with SU5416 displayed a total absence of *ephrinb2a* throughout the trunk vasculature (Figure 7M red arrowhead). However, knockdown of *crlra* in *ptc1;ptc2* embryos failed to prevent induction of *ephrinb2a*, which was expressed ectopically (Figure 7J blue arrowhead). In *ptc1;ptc2* embryos, only combined knockdown of *crlra* and VEGF inhibition by SU5416 resulted in a total loss of *ephrinb2a* within the trunk vasculature (Figure 7N red arrowhead).

A confounding aspect of the latter experiment is that *crlra* normally activates somitic VEGF expression,¹⁹ so morpholino knockdown of *crlra* would be expected to lead to down-regulation both of Crlr and VEGF. To address whether activation of Hh can bypass this function of Crlr, we assayed *vegfa* expression in

ptc1;ptc2 mutant embryos and found that, in non-double mutant siblings, knockdown of *crlra* resulted in a decrease in somitic *vegfa* expression (Figure 7C-D arrowhead) as previously reported.¹⁹ However, in *ptc1;ptc2* mutants, *crlra* knockdown produced no observable difference in *vegfa* expression (Figure 7F arrowhead) relative to uninjected *ptc1;ptc2* mutants (Figure 7E arrowhead). We conclude that Hh is indeed able to signal in parallel to Crlr to induce expression of *vegfa*. Taken together with our observations that Notch signaling is required for arterial differentiation in *ptc1;ptc2* mutant embryos, these data indicate that Hh signaling is capable of inducing arterial differentiation via Crlr independently of VEGF signaling, and, conversely, via VEGF independently of Crlr, but that these signals converge again at the level of Notch (Figure 7O).

Discussion

Hh signaling plays a crucial role in inducing arterial fate in the zebrafish embryo. Elegant experiments by Lawson et al revealed a hierarchy of signaling, initiated by Hh and leading, via the

induction of VEGF, to activation of *hey2/gridlock* and Notch signaling to provide the final transcriptional output.¹⁷ More recently, an additional layer in this control has been suggested involving the Crlr receptor.¹⁹ We have exploited the constitutive activation of the Hh pathway in double-mutant *ptc1;ptc2* zebrafish embryos to explore further the relationships between Hh and VEGF in early vessel formation. In keeping with previous findings,¹⁷ we showed that activation of Hh leads to ectopic arterial gene expression, ultimately resulting in the absence of a functional circulatory loop. However, this endogenous hyperactivation of Hh signaling results in such strong repression of venous development that a correctly specified PCV fails to develop: rather, a second arterial vessel forms with an intact arterial marker signature, vessel sprouting, and initiation of HSC gene expression.

An earlier role has been postulated for Hh signaling in guiding endothelial migration.^{11,26} We have demonstrated that the levels of Hh signaling received by angioblasts are critical for their successful migration and that activation of the Hh pathway results in more severe defects in initial angioblast migration toward the midline than loss of Hh signaling. Previous studies have demonstrated that *smo* embryos exhibit a loss of arterial ECs and concomitant gain in venous ECs, and it was proposed that Hh is required cell autonomously already within the PLM to regulate arteriovenous specificity.²⁶ Importantly, we find that, in *smo*^{hi1640} mutants, the first wave of angioblast migration, which largely contributes to DA formation, is not lost as has previously been reported.²⁶ The discrepancy between our findings and those of Williams et al²⁶ may be explained by the higher resolution of the confocal imaging of angioblast migration used in our study. However, *smo* embryos lack arterial ECs, arguing that these 2 migratory waves are not differentially specified before migration. In line with this, the Hh receptors *ptc1* and *ptc2* are not expressed in the PLM,^{26,51} implying that cells of the PLM are unlikely to receive the Hh signal directly. From our high-resolution time-lapse movies, we suggest that Hh signaling is most important in the final stages of medial migration because *smo*^{-/-} angioblasts begin their medial migration normally yet fail to form a continuous endothelial cord as they reach the midline.

Interestingly, the arterial transformation of the ventral vessel leads to the generation of ISVs from this location, in addition to formation of atypical connections between the 2 axial vessels. Such connections have been observed in mutants with defects in arteriovenous specification, and it is probable that separation of the 2 vessels requires them to have different molecular identities.¹⁴ Considering the body of data implicating VEGF in arterial induction, we were initially surprised to find that mutation of *plcg1*, chemical ablation of VEGF signaling, or knockdown of the VEGF receptors failed to block normal and ectopic induction of arterial fates in double mutants. However, results from mice and also differences in phenotypic strength of VEGF and Hh pathway mutants^{18,25} indicated that VEGF-independent functions of Hh signaling in these processes were likely. It is important to stress, however, that under normal circumstances VEGF is essential for arterial induction.

Although Nicoli et al suggested that Crlr was induced by Hh in the somites and acted via VEGF, they could not exclude that direct effects of Crlr on ECs might occur.¹⁹ Indeed, our experiments indicate that such direct effects are probable because down-regulation of VEGF signaling is not sufficient to block arterial differentiation, arguing against a simple Hh-Crlr-VEGF pathway. If somitic VEGF was the sole effector acting on ECs to induce arterial fate, then inhibition of VEGF signaling in *ptc1;ptc2* mutants should

have resulted in the absence of arterial differentiation. Furthermore, it follows that *vegfa* overexpression would also be predicted to phenocopy the vascular defects of *ptc1;ptc2* mutants, a situation that did not occur (supplemental Figure 6B).¹⁷

The expression of *crlra* both in somites and ECs prompted us to analyze its potential role in bypassing a normal requirement for VEGF in double mutants. Indeed, *crlra* expression was also induced in double mutants. Importantly, knockdown of *crlra* did not affect VEGF up-regulation in the somites; thus, it is likely that Crlr acts in the ECs in VEGF-inhibited double mutants. This finding raises several questions: most importantly, how Crlr signaling is regulated. A recent report indicated that in certain cells CRLR might act to “transactivate” the VEGF receptor independently of the VEGF ligand.⁵² However, these authors also show that the VEGF inhibitor we used (SU5416) should block the action of both CRLR and VEGF receptor activation. Our data show that CRLR functions in the presence of SU5416, and thus this mechanism does not appear to be acting in zebrafish. A second simple assumption would be that signals via VEGF and Crlr could both independently induce arterial fate in ECs. This is not supported by experiments by us (supplemental Figure 9) and others.¹⁹ *crlr* overexpression is not sufficient to induce ectopic arterial fates in wild-types or rescue arterial fates in Hh signaling-deficient embryos. In contrast, VEGF has been shown to be an instructive factor with respect to arterial induction, and injection in wild-type or Hh signaling-deficient embryos can induce ectopic arteries (supplemental Figure 6B).¹⁷ One interesting assumption concerning the role of Crlr may be that it acts to enhance or modify Hh signaling in ECs to induce arteries rather than act directly in the induction steps. We have looked at mRNA expression of the classic Hh target *ptc1* in *crlr* MO-injected embryos but did not see changes (R.N.W., unpublished observations, February 2010). Another possibility is that *crlr* overexpression by itself is not sufficient to increase signaling via this pathway and other crucial components of this pathway also need to be up-regulated, such as RAMPs or adrenomedullin ligands.⁵³ Then we have to assume that Hh activation in *ptc1;ptc2* mutants is not just up-regulating *crlr* but also those other components required for its activation. We are currently evaluating this possibility.

The key outcome of Hh and VEGF signaling appears to be activation of Notch signaling. To further position the Crlr signaling pathway within this hierarchy, we inhibited Notch using a *Su(H)* morpholino capable of inhibiting the Notch pathway sufficiently to block artery formation.³⁴ We find that knockdown of *Su(H)* in *ptc1;ptc2* mutants can block artery formation, arguing that Crlr acts upstream of Notch. Thus, we suggest the model shown in Figure 7O for the signaling hierarchies involved in artery formation. Our results show that, although under normal circumstances VEGF is an essential signal for correct vessel patterning in the zebrafish embryo, in situations of elevated Hh signaling, Crlr can bypass this requirement. It may be interesting to evaluate whether similar mechanisms are at work in other settings, for example, in tumors where Hh signaling is activated. It has been reported that *crlr* down-regulation inhibits tumor angiogenesis in a zebrafish xenograft model.¹⁹ If this is a more widespread mechanism, inhibition of Crlr in addition to VEGF inhibition could be important to evaluate as a therapeutic strategy, not only for anticancer therapy, but also in the treatment of diseases, such as diabetic retinopathy or macular degeneration. Furthermore, the remarkable conservation of the genetic hierarchies involved in arterial specification between zebrafish and mammals indicates that signaling via Crlr represents a strong candidate pathway for recently identified, but as yet

uncharacterized, mechanisms of vessel patterning shown to be Hh-dependent and VEGF-independent.¹⁸

Acknowledgments

The authors thank C. Pouget for reagents, assistance, and critical reading of the manuscript, and W. Clements for sharing unpublished data.

This work was supported by The Wellcome Trust (program grant 082962/Z/07/Z; P.W.I. and F.J.M.v.E.) and the Medical Research Council (R.K.P.). The CDBG is supported by the MRC Center (grant G0700091; P.W.I.).

References

- Bertrand JY, Chi NC, Santos B, Teng S, Stainier DY, Traver D. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature*. 2010;464(7285):108-111.
- Kissa K, Herbomel P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature*. 2010;464(7285):112-115.
- Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E, Robin C. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature*. 2010;464(7285):116-120.
- Vogeli KM, Jin SW, Martin GR, Stainier DY. A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. *Nature*. 2006;443(7109):337-339.
- Fouquet B, Weinstein BM, Serluca FC, Fishman MC. Vessel patterning in the embryo of the zebrafish: guidance by notochord. *Dev Biol*. 1997;183(1):37-48.
- Wang HU, Chen ZF, Anderson DJ. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell*. 1998;93(5):741-753.
- Lawson ND, Weinstein BM. Arteries and veins: making a difference with zebrafish. *Nat Rev Genet*. 2002;3(9):674-682.
- Zhong TP, Childs S, Leu JP, Fishman MC. Gridlock signalling pathway fashions the first embryonic artery. *Nature*. 2001;414(6860):216-220.
- Lawson ND, Scheer N, Pham VN, et al. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development*. 2001;128(19):3675-3683.
- Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development*. 2005;132(23):5199-5209.
- Gering M, Patient R. Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. *Dev Cell*. 2005;8(3):389-400.
- Hong CC, Peterson QP, Hong JY, Peterson RT. Artery/vein specification is governed by opposing phosphatidylinositol-3 kinase and MAP kinase/ERK signaling. *Curr Biol*. 2006;16(13):1366-1372.
- Herbert SP, Huisken J, Kim TN, et al. Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. *Science*. 2009;326(5950):294-298.
- Lin FJ, Tsai MJ, Tsai SY. Artery and vein formation: a tug of war between different forces. *EMBO Rep*. 2007;8(10):920-924.
- Zhong TP, Rosenberg M, Mohideen MA, Weinstein B, Fishman MC. gridlock, an HLH gene required for assembly of the aorta in zebrafish. *Science*. 2000;287(5459):1820-1824.
- Rowlinson JM, Gering M. Hey2 acts upstream of Notch in hematopoietic stem cell specification in zebrafish embryos. *Blood*. 2010;116(12):2046-2056.
- Lawson ND, Vogel AM, Weinstein BM. sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev Cell*. 2002;3(1):127-136.
- Coultas L, Nieuwenhuis E, Anderson GA, et al. Hedgehog regulates distinct vascular patterning events through VEGF-dependent and -independent mechanisms. *Blood*. 2010;116(4):653-660.
- Nicoli S, Tobia C, Gualandi L, De Sena G, Presta M. Calcitonin receptor-like receptor guides arterial differentiation in zebrafish. *Blood*. 2008;111(10):4965-4972.
- Lawson ND, Mugford JW, Diamond BA, Weinstein BM. Phospholipase C gamma-1 is required downstream of vascular endothelial growth factor during arterial development. *Genes Dev*. 2003;17(11):1346-1351.
- Habeck H, Odenthal J, Walderich B, Maischein H, Schulte-Merker S. Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. *Curr Biol*. 2002;12(16):1405-1412.
- Covassin LD, Villefranc JA, Kacergis MC, Weinstein BM, Lawson ND. Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish. *Proc Natl Acad Sci U S A*. 2006;103(17):6554-6559.
- You LR, Lin FJ, Lee CT, DeMayo FJ, Tsai MJ, Tsai SY. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature*. 2005;435(7038):98-104.
- Vokes SA, Yatskevich TA, Heimark RL, et al. Hedgehog signaling is essential for endothelial tube formation during vasculogenesis. *Development*. 2004;131(17):4371-4380.
- Pola R, Ling LE, Silver M, et al. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med*. 2001;7(6):706-711.
- Williams C, Kim SH, Ni TT, et al. Hedgehog signaling induces arterial endothelial cell formation by repressing venous cell fate. *Dev Biol*. 2010;341(1):196-204.
- Koudijs MJ, den Broeder MJ, Groot E, van Eeden FJ. Genetic analysis of the two zebrafish patched homologues identifies novel roles for the hedgehog signaling pathway. *BMC Dev Biol*. 2008;8:15.
- Koudijs MJ, den Broeder MJ, Keijser A, et al. The zebrafish mutants dre, uki, and lep encode negative regulators of the hedgehog signaling pathway. *PLoS Genet*. 2005;1(2):e19.
- Westerfield M. *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*. Eugene, OR: University of Oregon Press; 1993.
- Wilkinson RN, Pouget C, Gering M, et al. Hedgehog and Bmp polarize hematopoietic stem cell emergence in the zebrafish dorsal aorta. *Dev Cell*. 2009;16(6):909-916.
- Kwan KM, Fujimoto E, Grabher C, et al. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn*. 2007;236(11):3088-3099.
- Hammerschmidt M, Bitgood MJ, McMahon AP. Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo. *Genes Dev*. 1996;10(6):647-658.
- Villefranc JA, Amigo J, Lawson ND. Gateway compatible vectors for analysis of gene function in the zebrafish. *Dev Dyn*. 2007;236(11):3077-3087.
- Echeverri K, Oates AC. Coordination of symmetric cyclic gene expression during somitogenesis by Suppressor of Hairless involves regulation of retinoic acid catabolism. *Dev Biol*. 2007;301(2):388-403.
- Bahary N, Goishi K, Stuckenholtz C, et al. Duplicate VegfA genes and orthologues of the KDR receptor tyrosine kinase family mediate vascular development in the zebrafish. *Blood*. 2007;110(10):3627-3636.
- Lawson ND, Weinstein BM. In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev Biol*. 2002;248(2):307-318.
- Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI. Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat Immunol*. 2003;4(12):1238-1246.
- Chen W, Burgess S, Hopkins N. Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity. *Development*. 2001;128(12):2385-2396.
- Isogai S, Horiguchi M, Weinstein BM. The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev Biol*. 2001;230(2):278-301.
- Bussmann J, Bos FL, Urasaki A, Kawakami K, Duckers HJ, Schulte-Merker S. Arteries provide essential guidance cues for lymphatic endothelial cells in the zebrafish trunk. *Development*. 2010;137(16):2653-2657.
- Tucker B, Heppeler C, Kortschak D, et al. Zebrafish angiotensin II receptor-like 1a (agtr1a) is expressed in migrating hypoblast, vasculature, and in multiple embryonic epithelia. *Gene Expr Patterns*. 2007;7(3):258-265.
- Burns CE, Traver D, Mayhall E, Shepard JL, Zon LI. Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes Dev*. 2005;19(19):2331-2342.

Authorship

Contribution: R.N.W. designed and performed research, analyzed data, and wrote the paper; M.J.K. designed and performed research and analyzed data; F.J.M.v.E. designed research, analyzed data, and wrote the paper; and P.W.I., R.K.P., and S.S.-M. discussed the data and edited the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Fredericus J. M. van Eeden, MRC Centre for Developmental and Biomedical Genetics/Department of Biomedical Sciences, Western Bank, Sheffield, S10 2TN, United Kingdom; e-mail: f.j.vaneeden@sheffield.ac.uk.

43. Kalev-Zylinska ML, Horsfield JA, Flores MV, et al. Runx1 is required for zebrafish blood and vessel development and expression of a human RUNX1-CBF2T1 transgene advances a model for studies of leukemogenesis. *Development*. 2002;129(8):2015-2030.
44. Clements WK, Kim AD, Ong KG, Moore JC, Lawson ND, Traver D. A somitic Wnt16/Notch pathway specifies haematopoietic stem cells. *Nature*. 2011;474(7350):220-224.
45. Sinha S, Chen JK. Purmorphamine activates the Hedgehog pathway by targeting Smoothened. *Nat Chem Biol*. 2006;2(1):29-30.
46. Covassin LD, Siekmann AF, Kacergis MC, et al. A genetic screen for vascular mutants in zebrafish reveals dynamic roles for Vegf/Plcg1 signaling during artery development. *Dev Biol*. 2009;329(2):212-226.
47. Nasevicius A, Larson J, Ekker SC. Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant. *Yeast*. 2000;17(4):294-301.
48. Vajkoczy P, Menger MD, Vollmar B, et al. Inhibition of tumor growth, angiogenesis, and microcirculation by the novel Flk-1 inhibitor SU5416 as assessed by intravital multi-fluorescence video-microscopy. *Neoplasia*. 1999;1(1):31-41.
49. Serbedzija GN, Flynn E, Willett CE. Zebrafish angiogenesis: a new model for drug screening. *Angiogenesis*. 1999;3(4):353-359.
50. Fortini ME, Artavanis-Tsakonas S. The suppressor of hairless protein participates in notch receptor signaling. *Cell*. 1994;79(2):273-282.
51. Lewis KE, Concordet JP, Ingham PW. Characterisation of a second patched gene in the zebrafish *Danio rerio* and the differential response of patched genes to Hedgehog signalling. *Dev Biol*. 1999;208(1):14-29.
52. Guidolin D, Albertin G, Spinazzi R, et al. Adrenomedullin stimulates angiogenic response in cultured human vascular endothelial cells: involvement of the vascular endothelial growth factor receptor 2. *Peptides*. 2008;29(11):2013-2023.
53. Gibbons C, Dackor R, Dunworth W, Fritz-Six K, Caron KM. Receptor activity-modifying proteins: RAMPing up adrenomedullin signaling. *Mol Endocrinol*. 2007;21(4):783-796.