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Report

Artery/Vein Specification Is Governed by Opposing Phosphatidylinositol-3 Kinase and MAP Kinase/ERK Signaling

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Summary

Angioblasts are multipotent progenitor cells that give rise to arteries or veins [1]. Genetic disruption of the *gridlock* gene perturbs the artery/vein balance, resulting in generation of insufficient numbers of arterial cells [2]. However, within angioblasts the precise biochemical signals that determine the artery/vein cell-fate decision are poorly understood. We have identified by chemical screening two classes of compounds that compensate for a mutation in the *gridlock* gene [3]. Both target the VEGF signaling pathway and reveal two downstream branches emanating from the VEGF receptor with opposing effects on arterial specification. We show that activation of ERK (p42/44 MAP kinase) is a specific marker of early arterial progenitors and is among the earliest known determinants of arterial specification. In embryos, cells fated to contribute to arteries express high levels of activated ERK, whereas cells fated to contribute to veins do not. Inhibiting the phosphatidylinositol-3 kinase (PI3K) branch with GS4898 or known PI3K inhibitors, or by expression of a dominant-negative form of AKT promotes arterial specification. Conversely, inhibition of the ERK branch blocks arterial specification, and expression of constitutively active AKT promotes venous specification. In summary, chemical genetic analysis has uncovered unanticipated opposing roles of PI3K and ERK in artery/vein specification.

Results and Discussion

How arterial and venous cells arise from common angioblast progenitors to form a functional vasculature poses a challenging biological question. Artery versus vein (A/V) specification is established prior to the onset of circulation [2, 4, 5], and once specified, arterial and venous progenitors migrate to the appropriate locales and coalesce into functional vessels. The transcriptional repressor hey2, encoded by the *gridlock* gene in zebrafish, is an important determinant of the arterial fate, as evidenced by the fact that disruption of *gridlock* results in formation of insufficient numbers of arterial cells that leads to reduction or loss of the aorta [2, 6]. Consequently, zebrafish with a mutation (grl^{m145}) in *hey2*/*gridlock* lack trunk and tail circulation because of an aortic dysmorphogenesis that resembles congenital aortic coarctation in humans (Figure 1A) [7]. In the mouse, defects in the *hey2* gene contribute to vascular deformities as well [8, 9]. Beyond *gridlock*, other genetic and biochemical factors are likely involved in A/V specification [10]. Identifying additional factors via traditional genetic approaches may be difficult, particularly if such factors are necessary for vital biological processes that occur prior to formation of the vasculature. For the identification of novel factors that govern the artery/vein cell-fate decision, it will be important to employ a strategy that enables control over the dose and timing of gene inactivation. Therefore, instead of traditional genetic approaches, we have employed small-molecule screening to identify conditional modifiers of the artery/vein cell-fate decision.

Previously, chemical screening was performed with whole zebrafish embryos, and a small molecule, GS4012, was identified that suppresses the vascular defect in *gridlock* mutant embryos [3]. GS4012 was postulated to function via activation of the VEGF signaling pathway. We have used a similar screening approach to identify a distinct compound class that is also capable of suppressing the *gridlock* phenotype (Figure 1A). These compounds, represented by the compound GS4898, possess a mechanism of action that is distinct from that of GS4012. We have used these two classes of *gridlock* suppressors to reveal the biochemical basis for artery/vein specification during embryogenesis.

GS4898, which is structurally distinct from GS4012 (Figure 1B), was identified in a screen of 7000 uncharacterized small molecules on the basis of its ability to restore trunk and tail circulation to zebrafish embryos that are homozygous *gridlock* (grl^{m145}) mutants. GS4898 is a flavone structurally similar to several small-molecule inhibitors of protein kinases [11, 12], suggesting that GS4898 might function by targeting a kinase. We tested structurally related flavone kinase inhibitors and found that LY294002, a specific phosphatidylinositol-3 kinase (PI3K) inhibitor, is also capable of suppressing the *gridlock* phenotype (Figure 1B). In addition, the structurally unrelated PI3K inhibitor wortmannin suppresses the *gridlock* phenotype. Moreover, GS4898 inhibits activation of AKT, a downstream mediator of PI3K signaling [13], in whole zebrafish embryos (Figure 1C). These data strongly suggest that PI3K inhibition underlies the suppression of the *gridlock* phenotype by GS4898.

GS4898 is capable of suppressing the *gridlock* phenotype in the 1–5 μM range (Figure 1D). At higher doses, GS4898, LY294002, and wortmannin each causes severe vascular defects. As discussed below, such high doses may cause severe perturbations in angioblast cell-fate determination as a result of a complete inhibition of PI3K. Thus, suppression of the *gridlock* phenotype by GS4898 appears to require a partial inhibition of PI3K, sufficient to overcome deficient arterial cell

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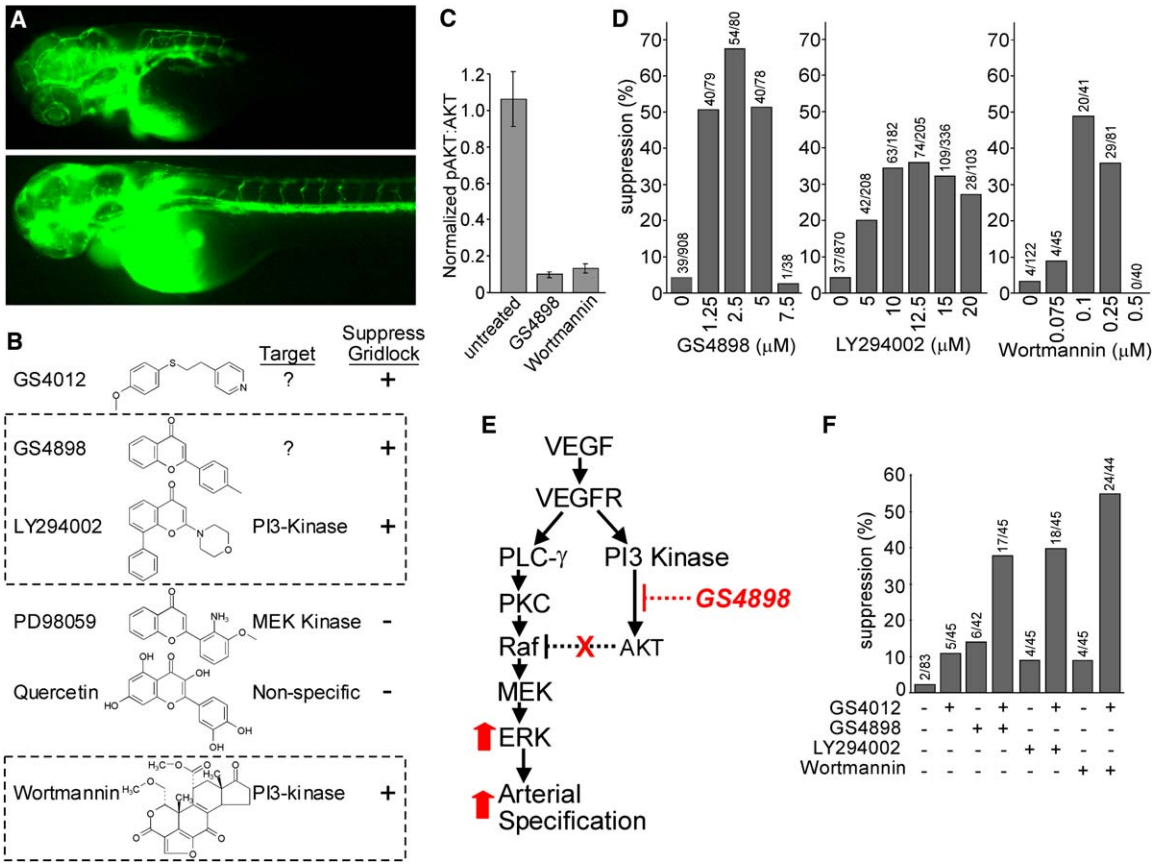


Figure 1. Suppression of the *gridlock* Phenotype by the Novel Flavone GS4898 Involves PI-3 Kinase Inhibition

(A) Suppression of the *gridlock* phenotype (atresia of the proximal aorta in *grl*^{m145} mutants) by GS4898 [2-(4-methylphenyl)-4H-chromen-4-one]. Above, a microangiogram of an untreated 60 hpf *grl*^{m145} mutant, in which arterial circulation to the tail is disrupted, is shown. Below, a microangiogram shows the restoration of tail circulation in a *grl*^{m145} mutant by GS4898 treatment.

(B) The *gridlock* phenotype is suppressed by GS4012, GS4898, LY294002, and wortmannin, but not PD98059 and quercetin. GS4898 and the PI3K inhibitors are enclosed in dashed boxes.

(C) Quantitative western analysis of activating AKT phosphorylation (at Ser472) in 20-somite-stage (ss) zebrafish embryos shows dramatic reduction in AKT phosphorylation by GS4898 (25 μM) or wortmannin (0.5 μM) treatment, starting at 10 hpf. Results were normalized from three independent experiments and achieved significance at $p < 0.004$ for each condition versus untreated. Error bars represent standard error.

(D) Dose response for GS4898, LY294002, and wortmannin. Compounds were added at 12 hpf and washed out at 27 hpf. Suppression (%) represents percentage of treated embryos that have tail circulation at 48 hpf. Number on top of each bar represents actual number of embryos with normal tail flow over the total number treated.

(E) Model for two VEGF-receptor-dependent signaling branches with opposing effects on arterial specification. The PLC-γ/ERK branch mediates arterial cell specification, whereas the PI3K branch exerts a negative effect on the PLC-γ/ERK branch, possibly via direct inhibition of Raf by Akt. By blocking PI3K, GS4898 lifts PI3K's inhibition of the PLC-γ/ERK pathway, leading to increased ERK activation and arterial specification.

(F) Synergy between GS4898 or PI3K inhibitors and GS4012. Single treatment involving a subeffective dose of GS4898 (0.75 μM), LY294002 (3 μM), wortmannin (0.075 μM), or GS4012 (0.7 μg/mL) does not suppress the *gridlock* phenotype, but cotreatment with GS4012 and GS4898, LY294002, or wortmannin at the same doses leads to significant suppression of the *gridlock* phenotype.

formation but not sufficient to grossly distort the artery/vein balance.

Suppression of the *gridlock* vascular defect by inhibition of PI3K, a well-known mediator of VEGF signaling, is intriguing because GS4012 was postulated to act via activation of the VEGF signaling pathway and VEGF cDNA injection can suppress the *gridlock* phenotype [3]. This apparent paradox led us to consider a hypothesis in which one of the two best-characterized branches of VEGF signaling [13], namely the PLC-γ/MAP kinase (ERK) pathway, triggers arterial fate specification, whereas the other, the PI3K/Akt branch, exerts an inhibitory effect on the PLC-γ/ERK branch (Figure 1E) [14]. Evidence for such crosstalk between the two signaling pathways has been observed at the level of direct phosphorylation of Raf by Akt in a human cancer cell line [15]. This model is attractive because PLC-γ is known to be required specifically for artery, but not vein, development in the zebrafish [16]. In this model, GS4898 compensates for the deficient artery formation in *gridlock* mutants by lifting PI3K's inhibition of the arteriogenic PLC-γ/ERK branch (Figure 1E). If this model is correct, GS4898 and the PI3K inhibitors LY294002 and wortmannin would be predicted to potentiate the effects of the putative VEGF pathway activator GS4012. Indeed, we find that GS4898, LY294002, and wortmannin synergize with GS4012 in suppressing the *gridlock* phenotype (Figure 1F). Moreover, GS4898 or LY294002 treatment significantly increases activation of ERK, a central downstream mediator of PLC-γ [13], in whole

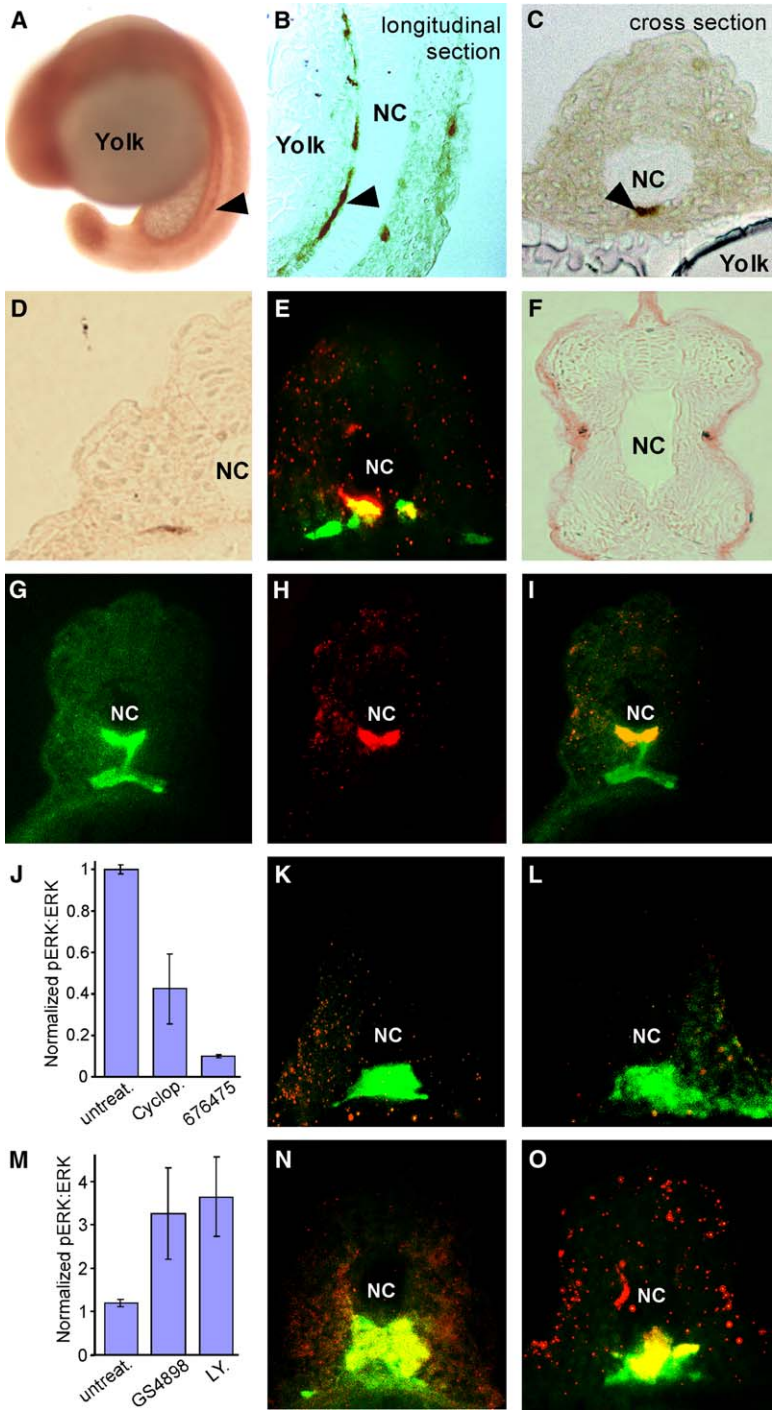


Figure 2. Diphosphorylated ERK Is a Specific Marker of Early Arterial Progenitors

(A–C) Immunostain for activated (diphosphorylated) ERK in 20-ss zebrafish embryos. Arrowhead shows activated ERK within the developing vasculature. In (B), dorsal side is to the right, and in (C) and in all other cross-sections, dorsal is to the top. NC denotes notochord, which lies dorsal to the angioblasts.

(D–F) Cross-sections of immunostain for activated ERK in 12-ss (15 hpf) embryos (D and E) and in a 24 hpf embryo (F). (E) shows a merged view of angioblasts, marked by GFP (green) expressed under the fli-1 vascular-specific promoter [26] and by activated ERK (yellow). At around 12 ss, activated ERK (yellow) is preferentially detectable in angioblasts that reach the midline earlier than the rest of the angioblasts (green). (F) By around 24 hpf, activated ERK is no longer detected in angioblasts.

(G–I) Cross-section of a 20-ss embryo. (G) shows angioblasts marked by vascular-specific GFP (green). (H) shows immunostain for activated ERK (red). (I) Merged view of activated ERK and GFP, with overlap in yellow. At this stage, detectable ERK activation is restricted to the dorsal-most angioblasts (yellow).

(J and M) Quantitative western analysis of ERK phosphorylation in 20-ss embryos. Error bars represent standard error. (J) Inhibition of ERK phosphorylation by cyclopamine (50 μM) or 676475 (25 μg/mL) treatment, starting at 10 hpf. Results were normalized from three independent experiments and achieved significance at $p = 0.03$ for cyclopamine and $p < 0.0001$ for 676475 versus untreated. (M) shows enhancement of ERK phosphorylation by GS4898 (10 μM) or LY294002 (15 μM) treatment, starting at 10 hpf. Results were normalized from seven independent experiments and achieved significance at $p = 0.04$ for GS4898 and $p = 0.009$ for LY294002 versus untreated.

(K and L) Merged view of activated ERK and GFP in embryos treated with cyclopamine (K) or 676475 (L). Note the loss of ERK activation with cyclopamine and 676475 treatments.

(N and O) Merged view of activated ERK and GFP in embryos treated with GS4898 (N) and LY294002 (O) showing the relative expansion in the overlap (yellow) between angioblasts and activated ERK.

embryos (Figure 2M). These data support a hypothesis in which GS4898 promotes arterial specification by lifting PI3K's inhibition of ERK signaling (Figure 1E).

The possible involvement of ERK signaling in arterial cell-fate specification led us to consider whether it might be activated specifically in the prearterial subpopulation of angioblasts. Using an antibody specific for ERK activated by dual phosphorylation at Thr-202 and Tyr-204 [15, 17], we found that activated ERK (dp-ERK) is preferentially localized to endothelial precursors fated to become arterial cells during a critical period in vasculogenesis (Figures 2A–2I). Starting around 15 hr postfertilization (hpf), activated ERK is detected in a subset of angioblasts that migrate from their origin in the lateral mesoderm toward the midline (Figures 2D and 2E). This pattern of activated ERK detected in the migrating angioblasts is reminiscent of the ERK activation in the migrating lateral tracheal branches in *Drosophila* [18]. Most remarkably, ERK activation persists in the dorsal-most angioblasts, adjacent to the notochord (Figures 2H and 2I), but not in ventral angioblasts. By 17 hpf, these dorsal-most angioblasts express the

arterial marker *ephrin-B2* as they differentiate into arterial endothelial cells of the aorta, which invariably forms dorsally in relation to the posterior cardinal vein [19]. By 24 hpf, activated ERK becomes undetectable in endothelial cells as the aorta matures (Figure 2F). The exquisite patterns of ERK activation in the arterial progenitors and the newly differentiated arterial endothelial cells raise the intriguing possibility that ERK activation is an early marker and/or determinant of the arterial cell fate [20].

If ERK activation is an arterial cell-fate marker, disruption of arterial specification should lead to perturbations in ERK activation. In the zebrafish embryo, two extracellular signals, sonic hedgehog (shh) and VEGF, expressed in the notochord and somites, respectively, are required for arterial specification [10, 21]. We found that blocking arterial formation with the hedgehog pathway inhibitor cyclopamine or the VEGF receptor (types 1 and 2) inhibitor 676475 greatly reduces ERK phosphorylation on quantitative western analysis of whole-embryo lysates and causes a loss of ERK activation within endothelial cells on whole-mount embryo immunostaining (Figures 2J–2L). Interestingly, treatment with GS4898 or LY294002 not only increases ERK activation on quantitative western analysis, but also causes an expansion of ERK activation within the endothelial cell population (Figures 2N and 2O). These data demonstrate that ERK activation is indeed an early marker of the arterial cell fate [20]. In addition, they suggest that PI3K may function to spatially limit ERK activation to the dorsal-most endothelial cells.

If ERK activation within a specific subset of angioblasts is a determinant of the arterial fate, changes in ERK activation should lead to perturbations in artery/vein specification. Indeed, we find that augmenting ERK activation increases artery and decreases vein formation. For example, treatment with GS4898 leads to occasional duplication of the aorta (Figure 3E and Figure 3G, middle) and to either loss or reduction of venous structures, such as the common cardinal vein (Figures 3C and 3E and Figure 3G, middle). In contrast, blocking ERK activation decreases artery and increases vein formation. For example, treatment with either SL327 or U0126, inhibitors of MEK, an upstream activator of ERK, leads to either loss or reduction of the aorta (Figures 3C and 3F and Figure 3G, right) and to an expansion of the common cardinal vein (Figure 3F and Figure 3G, right). In summary, stimulation of ERK activation shifts the artery/vein decision in favor of artery formation, whereas inhibition of ERK has the opposite effect. Therefore, activation of ERK in angioblasts is a key determinant of arterial specification.

Our findings from a chemical genetic approach indicate that PI3K and ERK have surprisingly opposing roles in artery/vein specification. Because PI3K and ERK are ubiquitously expressed signaling molecules utilized multiple times during embryonic development, traditional genetic disruptions of these pathways result in gross perturbation of the dorsoventral axial pattern [22] and in abnormal morphogenetic movements during gastrulation [23]. Nevertheless, mild defects such as deficient intersomitic vessel formation caused by a transient treatment with a VEGF inhibitor could be partially reversed by a low-dose injection of mRNA encoding constitutively active AKT [24]. In contrast, our attempts to overexpress constitutively active or dominant-negative forms of ERK, MEK, or AKT in embryos have resulted in early embryonic deaths that preclude an adequate assessment of their effects on artery/vein specification (unpublished data). These results highlight an inherent advantage of using small molecules for dissection of vascular development, because limiting compound exposure to critical times in vasculogenesis allows a relatively targeted perturbation of artery/vein specification while minimizing gross embryonic defects or lethality associated with complete disruption of pleiotropic factors (see the Supplemental Data available online).

To overcome the early-lethal effects of globally perturbing PI3K, we used mosaic expression of a dominant-negative form of AKT (DN-AKT) by injecting into 1- to 2-cell-stage embryos the expression plasmid pAd-TrackCMV.AA-AKT, which expresses both GFP and DN-AKT under separate CMV promoters [25]. Mosaic coexpression of constitutively active, myristoylated AKT (myr-AKT) and GFP was also achieved with a similar strategy. A low-dose (50 pg) injection, which permitted grossly normal development and establishment of circulation, resulted in a very small fraction (<1%) of cells that express GFP (Figure 4A). If inhibition of PI3K/AKT signaling in angioblasts promotes arterial specification, the rare angioblasts that express the dominant-negative AKT in such a mosaic setting would be predicted to become arterial endothelial cells. Conversely, if activation of PI3K/AKT signaling in angioblasts promotes venous specification, angioblasts that express myr-AKT would be predicted to become venous endothelial cells. Indeed, a blinded analysis of these GFP-positive cells localizing to either the dorsal aorta (Figure 4B) or the axial vein (Figure 4C) indicates that GFP-positive cells are preferentially found in the dorsal aorta when injected with the construct coexpressing DN-AKT (pAd-TrackCMV.AA-AKT), whereas GFP-positive cells are preferentially found in the vein when injected with the construct coexpressing myr-AKT (pAdTrackCMV.myr-AKT) (Figure 4D). These results provide further support for the model in which PI3K/AKT signaling in angioblasts functions to block ERK-mediated arterial specification (Figure 1E).

Conclusions

The two compound classes identified by unbiased screening in mutant zebrafish have been valuable tools for studying artery/vein specification. Not only have they confirmed the importance of VEGF signaling in this process, but they have also revealed that two downstream components of VEGF signaling surprisingly have opposite effects on artery/vein specification of endothelial progenitor cells. ERK signaling promotes the arterial cell fate, whereas PI3Kinase has an opposing effect by blocking ERK activation. GS4898 and known PI3K inhibitors shift the artery/vein balance in favor of arterial development. We note with interest that suppression of the *gridlock* vascular defect by GS4898 likely involves a partial inhibition of PI3K sufficient to overcome deficient arterial cell formation but not sufficient to grossly disrupt artery/vein balance. In light of this, it is doubtful that a typical loss-of-function genetic lesion in PI3K

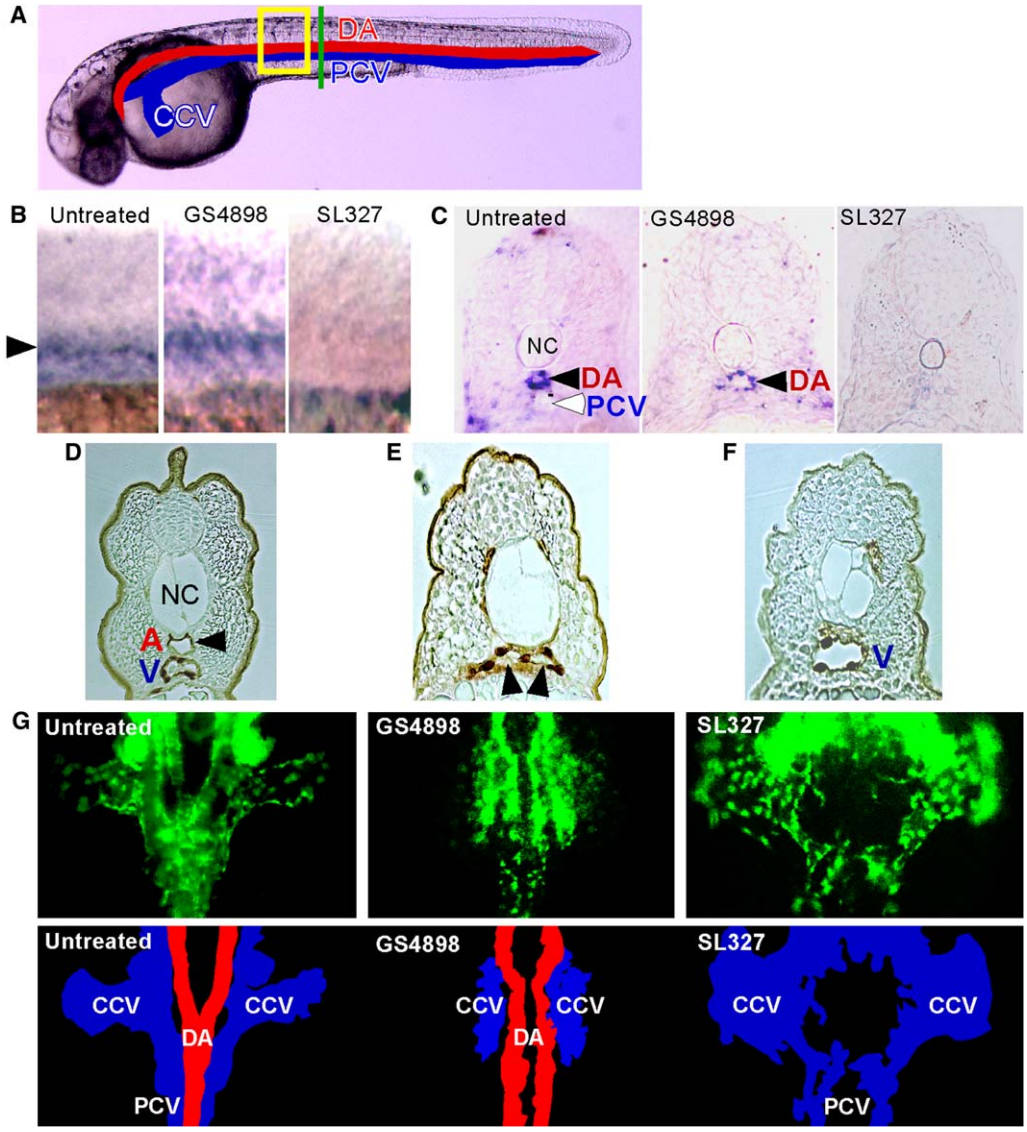


Figure 3. Diphosphorylated ERK is a critical determinant of the arterial fate

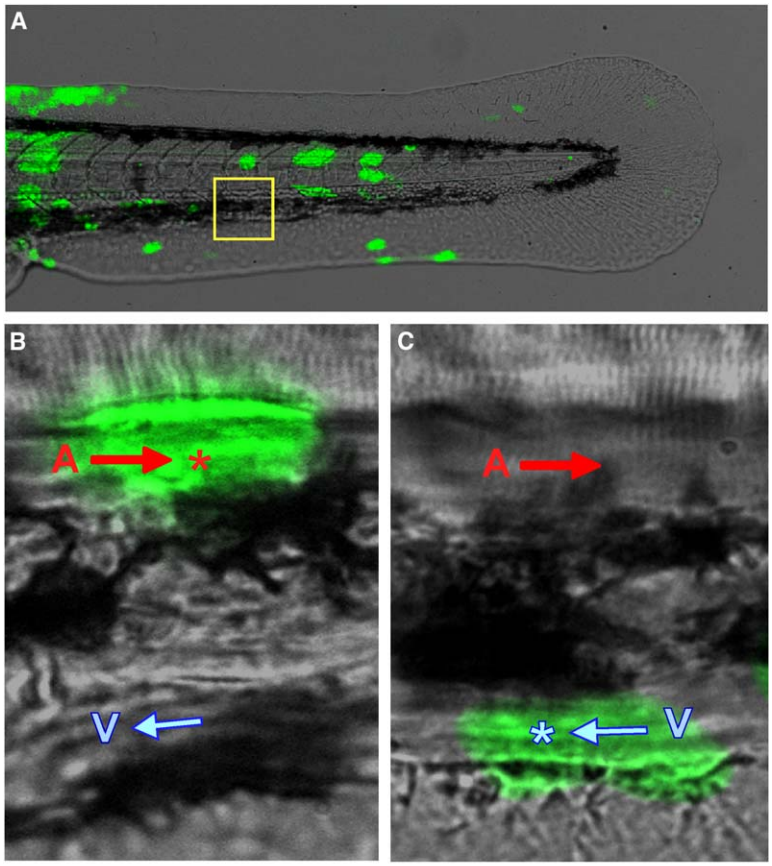
(A) A 30 hpf embryo. Red line indicates dorsal aorta (DA). Blue line indicates posterior cardinal vein (PCV), which terminates at the common cardinal vein (CCV). Yellow box indicates the region shown in (B). Green vertical line indicates cross-sections in (C)–(F).

(B) In situ hybridization of 30 hpf embryos with the arterial marker *ephrin-B2a*. At left, normal *ephrin-B2a* expression (arrowhead) in untreated embryos is shown. In the middle, intact *ephrin-B2a* expression in an embryo treated with high-dose (15 μM) GS4898 is shown. At right, *ephrin-B2a* expression is lost in SL327-treated (60 μM) embryos.

(C) Cross-sections of embryos shown in (B). At left, in untreated embryos, *ephrin-B2a* is expressed in the DA (black arrowhead), but not in the PCV (white arrowhead). In the middle, in GS4898-treated embryos, the *ephrin-B2a* expressing DA is prominent, but the PCV is not visible. At right, in SL327-treated embryos, neither *ephrin-B2a* expression nor the DA is observed.

(D–F) Cross-sections of 48 hpf embryos immunostained for GFP (brown) expressed in endothelial cells under the vascular-specific fli-1 promoter. Arrowheads indicate the aorta. (D) In untreated embryos, both the dorsal aorta (A) and the posterior cardinal vein (V) are prominent. (E) In embryos treated with 15 μM GS4898, duplication of the aorta is observed. (F) In embryos treated with 100 μM U0126, the aorta is greatly reduced, whereas the vein (V) is enlarged. (G) At top, fluorescent images of 48 hpf embryos expressing GFP in endothelial cells are shown. Dorsal view of the torso at the DA bifurcation and the PCV terminating at the CCV is shown. The head is above, and the tail is below. At bottom, cartoon representations of the fluorescent images are shown. At left, in untreated embryos, the CCV, via which blood from the tail drains to the heart, is situated lateral to the DA, which delivers blood to the tail. At the levels of the lower trunk and tail, the DA is situated dorsal to the PCV. In the middle, in GS4898-treated embryos, the DA is partially duplicated, and the CCV, situated lateral to the DA, is greatly reduced. At right, in SL327-treated embryos, the DA is missing and the CCV is significantly enlarged.

signaling could have suppressed the *gridlock* phenotype. Furthermore, the use of chemical suppressors allowed focused analysis of the role of PI3K and ERK in artery/vein specification, despite their pleiotropic requirement during early development [22, 23]. In summary, the chemical genetic approach employed here was instrumental in the discovery of previously unsuspected roles of two well-known VEGF signaling branches, demonstrating the utility and power of chemical genetics in the study of vertebrate development.



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	Artery	Vein	vs GFP only
dn-AKT, GFP	27	5	p=0.0283
myr-AKT, GFP	20	41	p=0.0039
GFP only	30	19	

Figure 4. Genetic Manipulation of AKT Activity Influences Artery/Vein Specification

(A–C) Overlay of GFP-fluorescence image onto bright-field image of a live 48 hp embryo injected with pAdTrackCMV.AA-AKT (dominant-negative AKT), pAdTrackCMV.myr-AKT (myristoylated AKT), or pAdTrackCMV (GFP alone). (A) Tail region of a plasmid-injected embryo expressing GFP in a small subset of cells is shown. Yellow box indicates the region represented in (B) and (C). (B) A high-magnification overlay image of a typical GFP-positive patch scored as arterial (red asterisk) is shown. It overlaps with the aorta (A), with a robust arterial flow (red arrow). (C) An overlay image of a typical GFP+ patch scored as venous (blue asterisk) is shown. It overlaps with the caudal vein, with a robust venous flow.

(D) Numbers of GFP-positive patches in the aorta and in the caudal vein in embryos injected with pAdTrackCMV.AA-AKT (DN-AKT), pAdTrackCMV.myr-AKT (myr-AKT), and pAdTrackCMV (GFP alone). A significant skewing of vascular GFP-positive cells in favor of aorta is noted in DN-AKT-injected embryos ($p = 0.028$, versus GFP alone), whereas a significant skewing of vascular GFP-positive cells in favor of vein is noted in myr-AKT-injected embryos ($p = 0.0039$, versus GFP alone).

Experimental Procedures

Screening for Suppressors of the *gridlock* Phenotype

The screen was performed as previous described with modifications [3]. Briefly, pairs of homozygous grl^{m145} zebrafish were mated, and fertilized eggs were arrayed in 96-well plates containing E3 embryo buffer. At the 6-somite stage, small molecules from the DiverSet E library (Chembridge) were added to each well, yielding a final concentration of 2.5 μM. Embryos were incubated at 28.5ºC for 48 hr. Circulation to the tail was assessed visually with a dissecting microscope.

Fluorescent Microangiography

Fluorescence microangiography was performed as previously described [7].

Quantitative Western Analysis

Control and treated 20 hr embryos were homogenized in SDS lysis buffer. Denatured lysates from equivalent numbers of embryos (typically 7) were loaded and separated on a 10% SDS polyacrylamide gel and transferred to a PVDF membrane. For Akt analyses, lysates were prepared from equivalent deyolked embryos. Membranes were blocked in 5% nonfat dry milk and incubated with rabbit polyclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-p44/42 MAP kinase, anti-phospho-Akt (Ser472), or anti-Akt (Cell Signaling Technology). The membranes were then washed and incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies. After several washes, membranes were developed with ECL+ chemiluminescence substrates and scanned with a Typhoon Imager (Amersham). Band intensities were quantified with image quantification software. After each immunoblotting, membranes were stripped according to manufacturer's instructions prior to incubation with other antibodies. For each membrane, the ratio of phospho-MAP kinase to total MAP kinase was calculated. The ratio between phospho-AKT and AKT was calculated in a similar fashion. For comparison between individual experiments, ratios were normalized to ratios obtained in control samples.

Immunostaining

Zebrafish embryos expressing EGFP under the fli-1 promoter [26] were fixed in 4% paraformaldehyde and stored in methanol at −20ºC until needed. Embryos were rehydrated, blocked in 2% sheep serum, and incubated with primary antibodies (rabbit polyclonal anti-phospho-p44/42 MAP kinase and mouse monoclonal anti-GFP). After several washes in blocking solution, embryos were incubated with secondary antibodies (goat anti-rabbit conjugated to either Alexa Fluor 594 or HRP, and goat anti-mouse conjugated to FITC, BD Biosciences). After extensive washes, embryos were embedded in plastic (JB-4) and sectioned. Immunofluorescent images of embryo sections were then analyzed with MetaMorph image analysis software. For specimens incubated with secondary antibody conjugated to HRP, color reaction was performed prior to plastic embedment.

Whole-Mount In Situ Labeling and Histology

Whole-mount RNA in situ hybridization for *ephrin-B2* expression was performed as described [2]. For sections, specimens were embedded in JB-4 plastic after color reaction.

cDNA Injections and Identification of GFP-Positive Cells within the Vasculature

Embryos were injected at the 1- to 2-cell stage with 1 nL of 50 μg/μL pAdTrackCMV.AA-AKT plasmid encoding GFP and DN-AKT, pAdTrackCMV.myr-AKT plasmid encoding GFP and myr-AKT, or pAdTrackCMV containing only GFP. More than 200 embryos were injected for each construct. Embryos were incubated at 28.5ºC for 48 hr, and surviving embryos with intact circulation were analyzed for the presence of GFP-positive cells within the vasculature with a fluorescent microscope by an observer blinded to the injected plasmid. A GFP-positive cell was scored as arterial only if it lay immediately adjacent to a strong arterial stream on the same focal plane. Likewise, a GFP-positive cell was scored as venous if it lay immediately adjacent to a strong venous stream on the same focal plane. Because of frequent intense fluorescence of the yolk, analysis was limited to the tail region caudal to the yolk extension.

Supplemental Data

Supplemental Data include one figure and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/13/1366/DC1/.

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