Essential Role of SH3-domain GRB2-like 3 for Vascular Lumen Maintenance in Zebrafish

Yan Zhao, Shuo Lin

Objective—Studying the underlying molecular mechanisms for maintaining stereotyped vascular lumen diameters should help toward a comprehensive understanding of vascular homeostasis and function. We aimed to determine the role of SH3-domain GRB2-like 3 (Sh3gl3) and its interacting pathways in dorsal aorta (DA) maintenance in zebrafish.

Approach and Results—Sh3gl3 and its binding partner, Cbl-interacting protein of 85K (Cin85), together regulate endocytosis and were expressed in the developing vasculature. Morpholino knockdown of either gene resulted in shrinkage of the DA lumen, although artery/vein specification and the initial formation of vascular lumens were unaffected. In addition, sh3gl3 and cin85 morpholinos exerted a synergistic effect in causing the vascular phenotypes. To identify the signaling pathways in which Sh3gl3/Cin85 may participate, we screened several candidate inhibitors for their ability to induce similar circulatory defects. Chemical inhibition of the epidermal growth factor receptor and the phosphatidylinositol 3-kinase/Akt cascade led to a loss of circulation and shrunken DA in zebrafish embryos. Furthermore, inhibition of the epidermal growth factor receptor/phosphatidylinositol 3-kinase pathway showed a functional cooperation with Sh3gl3 deficiency in impairing DA lumens.

Conclusions—These results identify 2 new factors, Sh3gl3 and Cin85, which are essential for DA lumen maintenance, and suggest that endocytosis, possibly involving epidermal growth factor receptor and phosphatidylinositol 3-kinase, is implicated in Sh3gl3/Cin85 function. (Arterioscler Thromb Vasc Biol. 2013;33:1280-1286.)

Key Words: Cbl-interacting protein of 85K ■ epidermal growth factor receptor ■ SH3-domain GRB2-like 3 ■ vascular lumen ■ zebrafish

The formation and maintenance of vascular lumens by endothelial cells (ECs) are essential for normal vascular function. Lumen size greatly impacts blood flow, blood pressure, and perfusion of tissues. However, the molecular mechanisms controlling the maintenance of blood vessel lumens are largely unknown, possibly because of the fact that early defects in vascularogenesis or angiogenesis usually impair lumen formation as well. Temporally controlled approaches, therefore, are required to elucidate mechanisms that specifically regulate vascular lumen size. The zebrafish seems to be a model system especially amenable to such investigation. Zebrafish embryos can survive and develop for ≈7 days without any blood circulation, providing a unique opportunity to examine lumen maintenance defects that usually cause lethality in mammalian embryos. Zebrafish embryos can be easily subjected to small-molecule chemical/drug treatment at designated time points. Furthermore, several EC-specific transgenic zebrafish lines have been generated using the kdrl or fli1a promoter, which facilitate real-time monitoring and recording of blood vessel maintenance in live embryos under a fluorescence microscope. 3,4

Recently, a zebrafish protein SH3-domain GRB2-like 3 (Sh3gl3) was identified as a potential regulator of blood vessel lumen maintenance. 5 Morpholino knockdown of Sh3gl3 caused a significant reduction in the diameter of the dorsal aorta (DA) in zebrafish embryos at 72 hours post fertilization (hpf). The mammalian SH3GL3, also referred to as endophilin A3 and extra eleven nineteen (EEN)-B2, belongs to a small family of SH3 domain containing proteins. SH3GL proteins are thought to regulate clathrin-coated vesicle formation by modifying membrane phospholipids and inducing negative curvature and invagination of the plasma membrane during the early steps of endocytosis. 6,7 Mammalian SH3GL3 proteins, which are preferentially expressed in the brain and testis, selectively interact with synaptojanin, dynamin, and exon 1 of the huntingtin protein. 7-9 Several in vitro studies have demonstrated that mammalian SH3GL3/endophilin A3 is constitutively associated with the adaptor protein Cbl-interacting protein of 85K (CIN85). The endophilin-CIN85 complex is recruited by the ubiquitin ligase Cbl to activated receptor tyrosine kinases, which subsequently promotes the internalization of these receptors. 10-12 To date, the in vivo function of SH3GL3 has yet to be addressed.

In the current study, we demonstrate that Sh3gl3 and Cin85 play a critical synergistic role in vascular lumen maintenance...
in zebrafish embryos. Furthermore, chemical inhibition of the epidermal growth factor receptor (EGFR) or the phosphatidylinositol 3-kinase (PI3K)/Akt signaling cascade showed a functional cooperation with Sh3gl3 deficiency in impairing blood vessel lumens, suggesting a possible involvement of Sh3gl3-Cin85 in the EGFR/PI3K signaling pathways.

Materials and Methods
Materials and Methods are available in the online-only Supplement. A full description of materials and methods used, including whole-mount in situ hybridization, morpholino knockdown, microangiography, RNA synthesis and injection, vibratome sections, measurement of DA diameter, chemical treatments, Western blots, and quantitative reverse transcriptase-polymerase chain reaction, is available in the online-only Data Supplement.

Results
Knockdown of Sh3gl3 Causes Collapse of Vascular Lumens
Zebrafish Sh3gl3 protein contains 386 amino acids and displays 59% identity (73% similarity) to the human SH3GL3 (endophilin A3). Protein alignment and phylogenetic analysis revealed the existence of another ortholog of mammalian SH3GL3, Zgc158742, in zebrafish embryos (Figure IA in the online-only Data Supplement). However, the expression of this homolog seemed to be ubiquitous, and no specific expression was observed in the vasculature. In contrast, Sh3gl3 RNA was specifically expressed in vascular ECs and their precursors. At the 12-somite stage, Sh3gl3 RNA was localized in 2 stripes of cells within the lateral mesoderm, likely EC precursors (Figure IC in the online-only Data Supplement). However, the expression of this homolog seemed to be ubiquitous, and no specific expression was observed in the vasculature. In contrast, Sh3gl3 RNA was specifically expressed in vascular ECs and their precursors. At the 12-somite stage, Sh3gl3 RNA was localized in 2 stripes of cells within the lateral mesoderm, likely EC precursors (Figure IC in the online-only Data Supplement). However, the expression of this homolog seemed to be ubiquitous, and no specific expression was observed in the vasculature. In contrast, Sh3gl3 RNA was specifically expressed in vascular ECs and their precursors. At the 12-somite stage, Sh3gl3 RNA was localized in 2 stripes of cells within the lateral mesoderm, likely EC precursors (Figure IC in the online-only Data Supplement).

Subsequently, 2 different Sh3gl3-specific morpholinos were used to knockdown the function of Sh3gl3. Injection of either morpholino resulted in similar circulatory defects, while having no effect on overall body morphology (Figure 1A). Most embryos injected with 5 ng Sh3gl3 morpholino-1 showed no circulation and developed pericardial edema between 48 and 72 hpf, and eventually became necrotic and died. Microangiography with fluorescein-labeled dextran showed the yellow remaining in the heart region and not entering the blood vessels in Sh3gl3 morphants (Figure 1B), although the heart morphology was unaffected. A closer examination of the axial vessels using Tg(kdr:GFP) transgenic fish embryos revealed a significant decrease in the DA diameter in Sh3gl3 morphants (arrow in Figure 1C compared with 1C, Figure 1G), implying lumen size reduction. Furthermore, vibratome cross-sections confirmed that Sh3gl3 morpholino indeed affected vascular lumens, as the DA lumen was almost absent (Figure 1D). As shown in Figure 1C in the online-only Data Supplement, 75 of 96 (78.1%) embryos injected with 4 ng Sh3gl3 morpholino-1 and 68 of 96 (70.8%) embryos injected with 10 ng Sh3gl3 morpholino-2 exhibited the reduced DA lumen phenotype, whereas only 2.1% of the standard control morpholino-injected embryos (2 of 96) had similar defects. Meanwhile, the vascular defects caused by Sh3gl3 morpholinos could be significantly rescued by coinjection of zebrafish sh3gl3 mRNA, demonstrating specificity of the observed phenotype (Figure IIC in the online-only Data Supplement).

Expression of both the arterial marker ephb2a and the venous marker flt-4 seemed normal at 33 hpf, indicating that the initial specification of the arterial/venous identity was unaffected by Sh3gl3 deficiency (Figure 1E and 1F). Previous studies have demonstrated that the definitive hematopoietic stem cells originate from the ventral wall of the DA in zebrafish embryos.11 Here, we also analyzed the expression of c-myc and rag-1 in Sh3gl3 morpholino-1–injected embryos. c-myc expression was significantly reduced in sh3gl3 morphants at 34 hpf. Similarly, rag-1 expression was greatly downregulated in response to sh3gl3 morpholinos (Figure III in the online-only Data Supplement). These results further suggested that the integrity of the DA was compromised by the knockdown of Sh3gl3 function.

To determine whether the effects of Sh3gl3 morpholino on DA lumen maintenance were secondary to lack of circulation, we carried out a closer examination of the cardiac function and circulation of sh3gl3 morphants. First, the contractile function of the heart indicated by heartbeat rate was not significantly affected by Sh3gl3 morpholino-1 at either 32 or 48 hpf (Figure IVA in the online-only Data Supplement). In addition, the initial blood flow in Sh3gl3 morphants seemed comparable with controls. Blood flow abnormality and DA defects became evident at 48 hpf. Next, we compared tnt2a/sih morphants,14,15 which lack heartbeats, with Sh3gl3 morphants. Injection of 1 ng of tnt2a/sih morpholino resulted in loss of circulation. Like Sh3gl3 morpholinos, tnt2a morpholino did not alter the initial arterial/venous specification and vascular lumen formation in zebrafish embryos. It seemed that vascular lumen expansion, which may occur between 26 and 40 hpf in wild-type zebrafish embryos, did not take place in tnt2a morphants. By 72 hpf, tnt2a morphants displayed collapse of the entire vasculature. DA, posterior cardinal vein, and intersomitic vessel lumens were all severely affected (Figure IVB in the online-only Data Supplement). In contrast, Sh3gl3 morpholino had more specific effects on DA diameter. Because Sh3gl3 morphants had normal heartbeat rate before 48 hpf, we also used a myosin-ATPase inhibitor 2,3-butanedione-2-monoxime treatment (15 mmol/L, from 48 to 72 hpf) to 72 hpf) did not lead to any significant DA defects (Figure 1D in the online-only Data Supplement). Moreover, we examined the expression level of Sh3gl3 gene in tnt2a morphants. Knockdown of tnt2a apparently did not alter Sh3gl3 expression either at 32 or at 48 hpf (Figure IVD in the online-only Data Supplement).

Cin85, the Binding Partner of Sh3gl3, Is Also Involved in Vascular Lumen Maintenance
To investigate the underlying mechanism of Sh3gl3 function in zebrafish vasculature, we identified the zebrafish homolog of Cin85 (XM_002666661) and examined its role during fish embryonic development. Whole mount in situ hybridization analysis showed that cin85 was expressed in 2 stripes of cells within the intermediate mesoderm at 14-somite stage (Figure
dent endocytosis,17,18 exhibited circulation defects and pericardial defects in zebrafish embryos. We found that embryos exposed to phenylarsine oxide, an inhibitor of clathrin-dependent endocytosis,17,18 exhibited circulation defects and pericardial defects in zebrafish embryos. We found that embryos tested whether inhibition of endocytosis could elicit any vascular defects. Wild-type embryos were injected with 4 ng of control morpholino (A) or sh3gl3 morpholino-1 (A') and analyzed at 48 hours post fertilization (hpf). Sh3gl3 morphants looked normal, except for blood cells stuck at the sinus venous. B, Microangiography analysis of the circulatory system in sh3gl3 morphants at 48 hpf. Note that sh3gl3 morpholino-1 led to a complete loss of circulation (B'). C, Knockdown of Sh3gl3 caused DA to reduce. Tg(kdr:GFP) embryos were injected with 4 ng of control morpholino (C) or sh3gl3 morpholino-1 (C'), and lateral-view confocal images were taken at 72 hpf. The trunk region above the yolk extension is shown. White arrows point to the DA. D, Sh3gl3 morpholino caused DA lumen to collapse. Tg(kdr:GFP) embryos were injected with 5 ng of control morpholino (D) or sh3gl3 morpholino-1 (D'), fixed at 72 hpf, cross-sectioned and imaged with a confocal microscope. White arrows point to DA lumen. E, Expression of the venous marker flt-4 appeared normal in sh3gl3 morphants (E') compared with control embryos (E) at 33 hpf. F, Expression of the arterial marker efnb2a was unaffected by sh3gl3 morpholino at 33 hpf. Wild-type embryos were injected with 4 ng of control morpholino (E and F) or sh3gl3 morpholino-1 (E' and F'). G, DA diameter was significantly reduced in sh3gl3 morphants. Tg(kdr:GFP) embryos were injected with 4 ng of control morpholino or sh3gl3 morpholino-1. DA diameter was measured at 72 hpf from confocal images, and the data from all the embryos in 1 group were averaged and shown as mean±SE. Sample sizes were n=10 for the control group and n=20 for sh3gl3 morphants. Double asterisks indicate significant difference (P<0.01, Student t test).

Figure 1. Morpholino knockdown of SH3-domain GRB2-like 3 (Sh3gl3) function causes collapse of dorsal aorta (DA) lumen in zebrafish embryos. A, Morphological analysis of live sh3gl3 morphants. Wild-type embryos were injected with 4 ng of control morpholino (A) or sh3gl3 morpholino-1 (A') and analyzed at 48 hours post fertilization (hpf). Sh3gl3 morphants looked normal, except for blood cells stuck at the sinus venous. B, Microangiography analysis of the circulatory system in sh3gl3 morphants at 48 hpf. Note that sh3gl3 morpholino-1 led to a complete loss of circulation (B'). C, Knockdown of Sh3gl3 caused DA to reduce. Tg(kdr:GFP) embryos were injected with 4 ng of control morpholino (C) or sh3gl3 morpholino-1 (C'), and lateral-view confocal images were taken at 72 hpf. The trunk region above the yolk extension is shown. White arrows point to the DA. D, Sh3gl3 morpholino caused DA lumen to collapse. Tg(kdr:GFP) embryos were injected with 5 ng of control morpholino (D) or sh3gl3 morpholino-1 (D'), fixed at 72 hpf, cross-sectioned and imaged with a confocal microscope. White arrows point to DA lumen. E, Expression of the venous marker flt-4 appeared normal in sh3gl3 morphants (E') compared with control embryos (E) at 33 hpf. F, Expression of the arterial marker efnb2a was unaffected by sh3gl3 morpholino at 33 hpf. Wild-type embryos were injected with 4 ng of control morpholino (E and F) or sh3gl3 morpholino-1 (E' and F'). G, DA diameter was significantly reduced in sh3gl3 morphants. Tg(kdr:GFP) embryos were injected with 4 ng of control morpholino or sh3gl3 morpholino-1. DA diameter was measured at 72 hpf from confocal images, and the data from all the embryos in 1 group were averaged and shown as mean±SE. Sample sizes were n=10 for the control group and n=20 for sh3gl3 morphants. Double asterisks indicate significant difference (P<0.01, Student t test).

EGFR/PI3K Pathway Is Possibly Involved in Sh3gl3-mediated DA Lumen Maintenance
Vascular endothelial growth factor (VEGF), the master regulator of angiogenesis, has also been shown to regulate lumen size of capillaries.1,19,20 It is likely that Sh3gl3 and Vegf may function in a common or converging pathway in zebrafish vascular development. To explore this possibility, we tested whether SU5416, a potent and selective inhibitor of VEGF signaling, could also reduce lumen size like sh3gl3 morpholinos. As shown in Figure V in the online-only Data Supplement, SU5416 treatment (2.5 μmol/L and 5 μmol/L) did not produce the thinner DA phenotype when added to fish embryos at 26, 30, 36, and 48 hpf, respectively. In addition, we found that sh3gl3 mRNA overexpression did not rescue arterial/venous differentiation defects caused by chemical inhibition of VEGF signaling (SU5416 5 μmol/L). Furthermore, ectopic induction of arterial marker expression (Efnb2a) by vegf overexpression did not require Sh3gl3 function (data not shown). These studies indicate that the function of Sh3gl3 in vascular lumen maintenance is independent of Vegf signaling.
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EGF, wingless/integrated (Wnt), and PI3K, for their ability to induce circulatory defects similar to those of sh3gl3 morphants. Inhibitor names and treatment conditions are listed in Table I in the online-only Data Supplement. The screen identified the PI3K inhibitor LY294002 and EGFR inhibitor AG1478 as positive hits.

EGFR is known to be an upstream activator of PI3K. Moreover, Cbl-CIN85–endophilin (SH3GL3) complex has been reported to be capable of promoting the internalization of activated EGFR in mammalian cell lines.10-12 Thus, it is likely that Sh3gl3 functions through regulating the endocytosis of Egfr protein in zebrafish vasculature. To test this hypothesis, we first checked the expression pattern of egfra in developing zebrafish embryos by whole mount in situ hybridization. From 18-somite to 36 hpf, when sh3gl3 was expressed in the vasculature, a ubiquitous distribution of a low level of egfra RNA was detected throughout the embryo (data not shown). When Tg(kdrl:GFP) transgenic embryos were exposed to 20 μmol/L LY294002 at 26 or 30 hpf, significant reduction of DA diameter was observed by 54 hpf (Figure 3A and 3E). Moreover, chemical inhibition of PI3K signaling by another potent inhibitor, wortmannin (0.2 μmol/L), produced similar DA defects, suggesting the specificity of these effects (Figure VIIA and VIIIB in the online-only Data Supplement). More importantly, obstruction of the DA by either LY294002 or wortmannin exhibited a robust synergistic effect with sh3gl3 morpholino-1 (Figure 3B–3D and Figure V, for their ability in the online-only Data Supplement). Combined use of subeffective doses of sh3gl3 morpholino-1 (2 ng) and egfra morpholino (2 ng) greatly reduced DA diameter as well (Figure IXA and IXB in the online-only Data Supplement). Combined use of subeffective doses of sh3gl3 morpholino-1 (2 ng) and egfra morpholino (2 ng) greatly reduced DA diameter as well (Figure IXA–IXE in the online-only Data Supplement). In addition, Akt phosphorylation level was downregulated in embryos treated with sh3gl3 morpholino-1 (Figure 3F), cin85 morpholino (Figure X in the online-only Data Supplement), or AG1478 (Figure 4F), respectively, further implying the involvement of EGFR/PI3K/Akt in Sh3gl3 function.

**Discussion**

Previous studies have indicated that the SH3GL/CIN85/Cbl complex regulates the internalization of activated receptor tyrosine kinases, including EGFR, in mammalian cell culture systems.10-12 In this report, we demonstrate that zebrafish sh3gl3 is specifically expressed in the developing vasculature, and that knockdown of Sh3gl3 function results in the collapse of the DA lumen without affecting the initial arterial/venous specification and vascular lumen formation in zebrafish embryos. It is quite unlikely that the vascular lumen defects in sh3gl3 morphants are because of the absence of blood flow and blood pressure. As a factor involved in endocytosis, Sh3gl3 is specifically expressed in the vasculature, not in the heart or blood cells. Knockdown of Sh3gl3 did not alter the
EGFR signaling, in vascular lumen maintenance. Inhibition of PI3K signaling by 2 structurally unrelated chemical inhibitors, after the establishment of arterial/venous specification, also caused defective circulation and severe DA lumen reduction. The inhibitory effect seemed to synergize with Sh3gl3 deficiency. In addition, knockdown of Sh3gl3 function greatly downregulated Akt phosphorylation. The function of PI3K in DA lumen maintenance is unexpected, because PI3K/Akt signaling has been shown to promote a venous fate in zebrafish.\textsuperscript{21}

Our results suggest that PI3K may play distinct roles at multiple stages during zebrafish vascular development. Finally, chemical inhibition of zebrafish Egfr signaling or knockdown of Egfr function by morpholino injection also led to a loss of circulation and significant DA lumen reduction, and had a synergistic effect with \textit{sh3gl3} morpholino during the process.

Although our results support the involvement of EGFR/PI3K pathway in \textit{sh3gl3}-mediated DA maintenance, we cannot rule out the possibility that EGFR/PI3K and Sh3gl3 might be functioning in independent pathways. Several in vitro studies in mammalian cell lines have shown that Sh3gl3/Endophilin A3 and Cin85 affect endocytic trafficking of EGFR.\textsuperscript{11,12}

To demonstrate the direct involvement of Sh3gl3/Cin85 in Egfr endocytosis in vivo, we would like to examine whether endogenous Sh3gl3/Cin85 and Egfr proteins colocalize with certain endosome markers, such as Rab5 and Rab7, in fish embryos. Alternatively, multiple zebrafish transgenic lines for these factors could be generated and manipulated to demonstrate the direct interaction in future studies. In addition, effective rescue of the DA defects in \textit{sh3gl3} morphants by...
PI3K activation would indeed prove the involvement of PI3K in Sh3gl3 function. We are currently in search of antibodies that could specifically recognize Sh3gl3, Cin85, or Egfr in vivo, and appropriate PI3K activators that could elevate PI3K/Akt signaling in a timely manner in fish embryos.

Although the involvement of EGFR signaling in tumor angiogenesis has been intensively documented, its function in normal vascular development remains largely unknown. Our data raise the possibility that EGFR signaling regulates the maintenance of blood vessel lumens through the PI3K/Akt cascade. Goishi et al. previously reported that inhibition of zebrafish Egfr activity resulted in dilated heart chambers and impeded blood flow into the DA. Here, we observed collapse of DA lumens in addition to enlarged hearts on Egfr inhibition. More importantly, combined use of subeffective doses of the EGFR inhibitor and sh3gl3 morpholino led to DA shrinkage without significant dilation of the heart, suggesting that the cardiac phenotype could be secondary to blood vessel lumen reduction.

Numerous studies have been done to address the mechanisms of EGFR endocytosis and its roles in EGFR signaling, producing several nonreconcilable models of these processes. It has been reported that binding of EGF to EGFR results in rapid internalization of activated receptors and targeting of internalized EGF–receptor complexes to lysosomes for degradation; additionally, several in vitro studies indicate that the SH3GL/CIN85/Cbl complex promotes the internalization and subsequent downregulation of activated EGFR. However, activated EGFR can continue to send signals from endosomes. Interestingly, in a recent report, Goh et al. generated a novel and specific internalization-defective EGFR mutant, and subsequently demonstrated that EGFR internalization is required for the sustained activation of Akt. It is possible that distinct internalization mechanisms of EGFR may contribute in different cell types and under various experimental conditions, and the outcomes of EGFR endocytosis may vary in different systems as well. Our current results suggest a possible involvement of Sh3gl3/Cin85-mediated Egfr internalization for PI3K/Akt activation in maintaining vascular integrity in zebrafish.

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**Disclosures**

None.
Blood vessels usually maintain stereotyped lumen diameters, and their stable structures are essential for normal vascular function. Lumen size greatly impacts blood flow, blood pressure, and perfusion of tissues. However, very little is known about the molecular mechanisms controlling the maintenance of blood vessel lumens, possibly because of the fact that early defects in vasculogenesis and angiogenesis usually impair lumen formation as well. In the present study, we used transgenic zebrafish technology coupled with temporal chemical genetics approach to investigate this late function of blood vessels. We identified 2 new factors, SH3-domain GRB2-like 3 and Cbl-interacting protein of 85K, which are essential for dorsal aorta lumen maintenance. In addition, we demonstrated that the epidermal growth factor receptor/phosphatidylinositol 3-kinase pathway is involved in the function of SH3-domain GRB2-like 3/Cbl-interacting protein of 85K. Our finding provides a novel entry point toward analyzing mechanistic pathways involved in maintaining blood vessel lumen after the establishment of functional circulation.

Significance

Blood vessels usually maintain stereotyped lumen diameters, and their stable structures are essential for normal vascular function. Lumen size greatly impacts blood flow, blood pressure, and perfusion of tissues. However, very little is known about the molecular mechanisms controlling the maintenance of blood vessel lumens, possibly because of the fact that early defects in vasculogenesis and angiogenesis usually impair lumen formation as well. In the present study, we used transgenic zebrafish technology coupled with temporal chemical genetics approach to investigate this late function of blood vessels. We identified 2 new factors, SH3-domain GRB2-like 3 and Cbl-interacting protein of 85K, which are essential for dorsal aorta lumen maintenance. In addition, we demonstrated that the epidermal growth factor receptor/phosphatidylinositol 3-kinase pathway is involved in the function of SH3-domain GRB2-like 3/Cbl-interacting protein of 85K. Our finding provides a novel entry point toward analyzing mechanistic pathways involved in maintaining blood vessel lumen after the establishment of functional circulation.
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**Supplemental Material**

**Figure I. Zebrafish sh3gl3 exhibited vascular-specific expression.** (A) Phylogenetic analysis of vertebrate Sh3gl3 proteins. There are two Sh3gl3 orthologues in zebrafish, Sh3gl3 and Zgc158742, possibly due to the zebrafish genome duplication. (B-D) Expression analysis of sh3gl3 by whole mount in situ hybridization (WISH). (B) Maternally deposited sh3gl3 transcripts were present at one-cell stage. (C) At 12-somite stage, sh3gl3 RNA was detected in two bilateral stripes presumptively within the lateral mesoderm in the posterior part of an embryo. Dorsal view; anterior is to the left. (D) At 36 hpf stage, sh3gl3 was expressed in the notochord and axial blood vessels (Black arrow). Lateral view of the tail; anterior is to the left.
Figure II. Forced expression of *sh3gl3* mRNA rescued the vascular phenotypes in *sh3gl3* morphants. (A) *sh3gl3* MO2 targeted the exon5/intron5 boundary and resulted in aberrant inclusion of intron 5 (labeled in yellow) into the splicing product. This misspliced transcript introduced a premature stop codon (asterisk), causing a truncation in Sh3gl3 protein. Primer 1 (5’- TGGATATCTTCAGCGGAACC -3’) and primer 2 (5’- AGACCTGAGGTTGCTGCAT -3’) were used to amplify the splicing products. (B) RT-PCR analysis of *sh3gl3* transcripts at 36 hpf in control and *sh3gl3* MO2-injected embryos. In embryos injected with 10 ng *sh3gl3* MO2, the majority of *sh3gl3* transcripts were misspliced (upper band). (C) Co-injection of 300 pg of *sh3gl3* mRNA with *sh3gl3* MO2 rescued the vascular defects. 75 out of 96 (78.1%) embryos injected with 4 ng *sh3gl3* MO1 and 68 out of 96 (70.8%) embryos injected with 10 ng *sh3gl3* MO2 exhibited the reduced DA lumen/defective circulation phenotype, while only 17.8% of the embryos (16 out of 90) injected with both *sh3gl3* MO2 and *sh3gl3* mRNA had similar defects.
Figure III. Expression of hematopoietic stem cell (HSC) markers was dramatically reduced in \textit{sh3gl3} morphants. (A) Normal \textit{c-myb} expression in control embryos at 34 hpf. (B) \textit{c-myb} expression was significantly decreased in \textit{sh3gl3} morphants (4 ng) at 34 hpf. (C) Normal \textit{rag-1} expression in the thymus in control embryos at 96 hpf. (D) \textit{Rag-1} expression was greatly reduced in response to \textit{sh3gl3} MO1 (4 ng) at 96 hpf.
Figure IV. *tnnt2a* MO injection resulted in vascular defects distinct from *sh3gl3* morphants. (A) Effect of *sh3gl3* MO1 on heart rate in zebrafish embryos. Heart rate was measured in beats per minute and the data from all the embryos in a group were averaged and shown as mean ± standard error. Unlike *tnnt2a/sih* MO, *sh3gl3* MO1 (4ng) did not induce significant change in heart rate at either 32 hpf (p=0.3, student’s t-test) or 48 hpf (p=0.17, student’s t-test). (B) Knockdown of *tnnt2a* caused collapse of the entire vasculature by 72 hpf. *Tg(kdrl:GFP)* embryos were injected with 1 ng of *tnnt2a* MO at 1-2 cell stage and analyzed at 72 hpf. (C) Temporary arrest of heart beats and circulation by BDM treatment did not cause significant DA defects. *Tg(kdrl:GFP)* embryos were exposed to BDM (15 mM) at 48 hpf and analyzed at 72 hpf. (D) *tnnt2a/sih* MO did not alter the expression of *sh3gl3* at either 32 hpf or 48 hpf. Wild type embryos were injected with 1ng of *tnnt2a* MO or 4ng of control MO at 1-2 cell stage and collected at 32 hpf and 48 hpf, respectively. Subsequently the expression of *sh3gl3* gene in *tnnt2a* morphants and control embryos was measured by quantitative RT-PCR, with β-actin used as the reference gene.
Figure V. Endocytosis inhibition with PAO significantly reduced DA diameter. Tg(kdrl:GFP) embryos were subjected to PAO treatment (4 µM) at 28 hpf and analyzed at 72 hpf. PAO-treated embryos (B) had a DA significantly thinner than that of controls (A). White arrows point to the DA. (C) Quantification of DA diameter. Sample sizes were n=10 for the control group and n=15 for PAO-treated embryos. Double asterisks indicate significant difference (p<0.01, student’s t-test).
Figure VI. Chemical inhibition of Vegf signaling after 24 hpf did not alter the size of DA lumens. Tg(kdrl:GFP) embryos were subjected to SU5416 treatment (5 µM) at 30 hpf and analyzed at 96 hpf.
Figure VII. Inhibition of BMP signaling did not alter the size of DA lumens. Heterozygous Tg(hsp70l:dnBmpr-GFP)\textsuperscript{w30} zebrafish were crossed to Tg(kdrl:GFP) fish. Embryos were heat shocked (HS) at 38.5°C for 45 minutes at 26 hpf, 30 hpf and 36 hpf, respectively, to induce the expression of a dominant-negative form of the Bmp receptor type 1a fused to GFP. Embryos were subsequently sorted by GFP expression 2 hours after the HS treatment and imaged at 48 hpf and 72 hpf, respectively. Inhibition of BMP signaling by the dominant-negative Bmp receptor did not result in significant circulation/DA defects at either 48 hpf (A and B) or 72 hpf (C and D).
Figure VIII. PI3K inhibition with Wortmannin significantly reduced the diameter of DA. (A) Wortmannin treatment resulted in a shrunken DA. Tg(kdrl:GFP) embryos were exposed to Wortmannin (0.2 µM) at 26 hpf and analyzed at 72 hpf. (B) Quantification of the DA diameter. Sample sizes were n=10 for the control group and n=15 for Wortmannin-treated embryos. Double asterisks indicate significant difference (p<0.01, student’s t-test). (C-E) Wortmannin treatment and sh3gl3 MO had a synergistic effect on reducing DA lumen size. 0.1 µM of Wortmannin (C) or 2ng of sh3gl3 MO1 (D) alone did not produce any DA defects. However, application of both treatments caused a great reduction of the DA diameter in 73.8% (48/65) of treated embryos (E).
Figure IX. Knockdown of egfра function significantly reduced DA diameter.

(A) egfра MO injection led to a shrunken DA. Tg(kdrl:GFP) embryos were injected with 5 ng of egfра MO at 1-2 cell stage and analyzed at 72 hpf. (B) Quantification of the DA diameter. Sample sizes were n=10 for the control group and n=15 for egfра MO-injected embryos. Double asterisks indicate significant difference (p<0.01, student’s t-test). (C-E) egfра MO and sh3gl3 MO had a synergistic effect on reducing DA lumen size. Tg(kdrl:GFP) embryos were injected at 1-2 cell stage and analyzed at 76 hpf. 2ng of egfра MO (C) or 2ng of sh3gl3 MO1 (D) alone did not produce any circulation/DA defects. However, application of both treatments caused a great reduction of the DA diameter in 70.7% (41/58) of injected embryos (E).
**Figure X. Akt phosphorylation was reduced in cin85 morphants.** Wild type embryos were injected with 6 ng of *cin85* MO, or 2 ng of *sh3gl3* MO1 and 3 ng of *cin85* MO, collected at 54 hpf, and subjected to western blot analysis of total Akt and phospho-Akt.
Figure XI. Knockdown of Sh3gl3 function did not significantly affect Erk phosphorylation. Wild type embryos were injected with 4 ng of sh3gl3 MO1, collected at 40 hpf and 54 hpf, respectively, and subjected to western blot analysis of total Erk1/2 and phospho-Erk1/2.
**Table I**

<table>
<thead>
<tr>
<th>Chemical inhibitor</th>
<th>Pathway</th>
<th>Concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsomorphin</td>
<td>BMP</td>
<td>5 µM, 10 µM, 20 µM</td>
</tr>
<tr>
<td>AG1478</td>
<td>EGFR</td>
<td>1 µM, 2µM, 5 µM</td>
</tr>
<tr>
<td>Cyclopamine</td>
<td>Sonic Hedgehog</td>
<td>10 µM, 25 µM, 50 µM, 100 µM</td>
</tr>
<tr>
<td>LY294002</td>
<td>PI3K</td>
<td>10 µM, 20 µM, 40 µM</td>
</tr>
<tr>
<td>SU5416</td>
<td>VEGF</td>
<td>2 µM, 5µM, 10µM</td>
</tr>
<tr>
<td>XVA939</td>
<td>Wnt/β-catenin</td>
<td>10 µM, 20µM, 40µM</td>
</tr>
</tbody>
</table>

* Each inhibitor was applied to *Tg(*kdrl:*GFP*) embryos at 26hpf/28 hpf, 30 hpf, 36hpf and 48hpf, respectively, and the treated embryos were examined at 72 hpf and 96 hpf, respectively.
Materials and Methods

**Zebrafish Husbandry.** Wild-type AB strain and Tg(kdrl:GFP) transgenic zebrafish (Danio rerio) were maintained at 28.5 °C. Embryos were raised and staged under standard laboratory conditions as described by Kimmel et al. (1)

**Whole-mount in situ hybridization (WISH).** WISH was performed as described (2). The following riboprobes were used: sh3gl3, c-myb, rag-1, flt-4, efnb2a and cin85.

**Morpholino (MO) knockdown.** Two sh3gl3-specific MOs targeting either the ATG (sh3gl3 MO1: 5’-TTAAACCCGACCTGACATCCTTC-3’) or the exon5/intron5 boundary (sh3gl3 MO2: 5’-AGCATTATATACAAGACTAACCCTCA-3’) were obtained to inhibit the function of Sh3gl3 protein (Gene Tools, LLC). One MO oligo (cin85 MO: 5’-ACTCCACAATGGCTTCACTCCATACTT-3’) was used to block the translation of the Cin85 protein. One egfra-specific MO targeting the 5’-UTR (egfra MO: 5’-AGCTCTCGGGCTTCACTCCATACTT-3’) was used to knockdown Egfra function. One tntt2a-specific MO (tntt2a MO: 5’-CATGTTTGCTCTGATCTGACACGCA-3’) was obtained to block Tntt2a translation. A standard MO control was obtained from Gene Tools as well. MOs were dissolved in sterile water to make 1 mM stock solution and stored at room temperature.

For phenotypic and marker analysis, 2–2.5 nl of 2 ng/nl sh3gl3 MO1 solution, 2.5 nl of 4 ng/nl sh3gl3 MO2 solution, 2 nl of 3 ng/nl cin85 MO solution, 2 nl of 0.5 ng/nl tntt2a MO solution, 2-3 nl of 2 ng/nl egfra MO solution, or 2-3 nl of 2 ng/nl standard control MO solution was injected into the blastomere of one-cell stage embryos, using a PicoInjector PLI-90 (Harvard Apparatus) as described (3). For synergistic effect analysis, 1.5-2 nl of 1 ng/nl sh3gl3 MO1 solution, 2 nl of 1.5 ng/nl cin85 MO solution or 2 nl of 1 ng/nl egfra MO solution was injected.

**Microangiography.** Tetramethylrhodamine dextran (MW=2000 KDa, Invitrogen) dissolved in double-distilled water was microinjected into the sinus venous of zebrafish embryos at 48 hpf. Subsequently the injected embryos were photographed using a color CCD camera (Axiocam, Zeiss) mounted on an Axioskop2 microscope.

**RNA synthesis and injection.** The full-length coding sequence of sh3gl3 was amplified by reverse transcription PCR using a forward primer, 5’-aaggatccggccgcatgtggtggtta-3’, containing a 5’ BamHI site and the Kozak sequence, and a reverse primer, 5’-taacctctacgtgctggcagaggtaa-3’ containing an XhoI restriction site. The PCR product was subsequently cloned into a pCS2 vector. To synthesize mRNA, sh3gl3-pCS2 was linearized with NotI and transcribed using SP6 mMessage mMachine Kit (Ambion) according to the manufacturer’s suggested protocol. Approximately 300 pg of sh3gl3 mRNA was injected into the blastomere of one-cell stage embryos. Sterile water was used for the control experiments.

**Vibratome sections.** Tg(kdrl:GFP) transgenic zebrafish embryos injected with sh3gl3 MO1, cin85 MO or control MO were fixed with 2% paraformaldehyde (PFA) (in PBS) overnight at 4°C, washed in PBS, and mounted on their lateral side in 4% low melting agarose (Fisher Scientific) in PBS. Thin 100 µm slices were cut using a vibratome (Vibratome 1000 Plus) and stored in PBS until imaging.

**Chemical treatments.** Embryos were incubated in the following: 15 mM 2, 3-butanedione-2-monoxime (BDM) (Sigma-Aldrich); 5 µM SU5416 (Sigma-Aldrich) from
a 0.5 mM stock in DMSO; 10 or 20 µM LY294002 (Promega) from a 10 mM stock in DMSO; 0.1 or 0.2 µM Wortmannin (Sigma-Aldrich) from a 1 mM stock in DMSO; 3 or 4 µM Phenylarsine oxide (PAO) from a 10 mM stock in DMSO; and 1 or 2 µM AG1478 (Sigma-Aldrich) from a 10 mM stock in DMSO. Controls were treated with equivalent volumes of vehicle. All dilutions were made with fresh fish water. Treatments were carried out in 4 ml volumes in 6-well plates with 40-50 embryos per well or 2 ml volumes in 12-well plates with 20-25 embryos per well. At least three independent chemical treatments were performed for each experiment.

**Western Blot.** For western blot, forty embryos per treatment group were deyolked, homogenized in SDS lysis buffer and boiled for 5 min. Denatured lysates from equivalent numbers of embryos (typically 2-3 embryos) were loaded and separated on a precast 4 to 20% gradient SDS polyacrylamide gel. Western blotting was performed using a standard protocol as described (4). Anti-Akt, anti-phospho-Akt (Ser473), anti-Erk1/2 and anti-phospho Erk1/2 (Thr202/Tyr204) antibodies were obtained from Cell Signaling Technology. Membranes were developed with enhanced chemiluminescence (ECL) Western Blotting substrates (Thermo Scientific Pierce).

**Imaging.** WISH stained embryos were washed three times with PBST, further processed by a serial dehydration in ethanol, followed by rehydration into PBS. Subsequently embryos were imaged in 2% methylcellulose in depression microscope slides. Images were captured with a color digital CCD camera (AxioCam, Zeiss) mounted on a dissecting microscope (Stemi 2000-C, Zeiss) with Openlab 4.0 software (Improvision). Adobe Photoshop was used to adjust brightness and contrast and assemble composite images. Confocal imaging of live Tg(kdrl:GFP) transgenic embryos and vibratome sections was done on a Zeiss LSM510 confocal microscope system.

**Measurement of dorsal aorta (DA) diameter.** Confocal images of 72 hpf Tg(kdrl:GFP) larvae were used to measure the diameter of DA. The actual diameter was found by drawing a straight line across the vessel using ImageJ software. Each DA was measured at five locations within one image and the mean of these measures was used as the vessel diameter. 10 embryos in each control group and 15-20 embryos in each morpholino/chemical treated group were imaged and measured.

**Quantitative RT-PCR.** Total RNA was extracted with Trizol reagent (Invitrogen) from 40 tnt2a morphants and 40 control embryos, respectively. Single strand cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen). Real-time PCR was performed using the iCycler iQ Real-Time PCR Detection System (BioRad) with iQ SYBR Green Supermix (BioRad). Sh3gl3 expression levels were measured by the ΔΔ Ct method, comparing tnt2a morphants to controls, with β-actin used as the reference gene.

**References**