Syx, a RhoA Guanine Exchange Factor, Is Essential for Angiogenesis In Vivo

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Abstract—Rho GTPases play an important and versatile role in several biological processes. In this study, we identified the zebrafish ortholog of the mammalian Rho A guanine exchange factor, synectin-binding guanine exchange factor (Syx), and determined its in vivo function in the zebrafish and the mouse. We found that Syx is expressed specifically in the vasculature of these organisms. Loss-of-function studies in the zebrafish and mouse point to a specific role for Syx in angiogenic sprouting in the developing vascular bed. Importantly, vasculogenesis and angioblast differentiation steps were unaffected in syx knockdown zebrafish embryos, and the vascular sprouting defects were partially rescued by the mouse ortholog. Syx knockdown in vitro impairs vascular endothelial growth factor-A–induced endothelial cell migration and angiogenesis. We have also uncovered a potential mechanism of endothelial sprout guidance in which angiomotin, a component of endothelial cell junctions, plays an additive role with Syx in directing endothelial sprouts. These results identify Syx as an essential contributor to angiogenesis in vivo. (Circ Res. 2008;103:710-716.)

Key Words: vascular  ■  zebrafish  ■  PDZ  ■  knockdown  ■  intersomitic vessels

Two distinct processes, vasculogenesis and angiogenesis, characterize vascular development in vertebrates.1,2 During vasculogenesis, vessels form de novo from endothelial cell (EC) precursors, or angioblasts, that coalesce at the midline to form luminalized tubes. Once primary vessels have formed, ECs sprout from preexisting vasculature to form secondary vessels via angiogenesis. ECs proliferate, migrate, and differentiate to form mature vasculature. Directed cell migration is necessary for angiogenesis and is governed in part by Rho-family GTPases, including RhoA, Cdc42, and Rac1.3 Activation of Rho GTPases at the cell periphery by Rho guanine exchange factors (GEFs)4 leads to remodeling of the actin cytoskeleton and consequently to cell migration.5 Recently, a novel Rho-GEF, synectin-binding RhoA exchange factor (Syx), also named GEF720, PLEKHG5, and Tech,6 was identified by yeast 2-hybrid analysis using Synectin as bait7 (M. Simons, personal communication). The mouse Syx ortholog is expressed as 2 splice variants that differ by only 2 C-terminal amino acid residues.8 Full-length Syx1 contains a PDZ-binding (postsynaptic density 95, disk large, zona occludens-1) motif9 required for the interaction of Syx1 with Synectin and localization to the plasma membrane. The shorter variant, Syx2, lacks the PDZ motif and is diffusely distributed in the cytoplasm. Syx1 augments EC migration and tube formation, whereas Syx2 does not.8 The first report on Syx localized it to band 1p36, in the distal region of human chromosome 1.6 This chromosomal region is rearranged in several types of cancer, suggesting that Syx may be involved in malignant transformation. The same report detected high expression levels of Syx in the brain and the heart, both highly vascularized organs.6 More recently, a missense mutation in Syx was linked with a degenerative motor neuron disease.10 Given the well-known similarities between signaling pathways in the nervous and vascular systems,11 the expression and functions of Syx in both system is not surprising. The most recent annotations of the human genome classified Syx as belonging to a 7-member family of GEFs, PLEKHG5 (pleckstrin homology domain containing, family G [with Rho-GEF domain] member 5). The functions of most members of this family are still unknown.

To investigate the function of Syx in vertebrate vascular development, we identified the Syx ortholog in zebrafish and performed whole mount in situ hybridization (ISH). Syx is expressed in the dorsal aorta (DA) and intersomitic vessels (ISVs). Syx vascular expression is recapitulated in mammals where the mouse Syx protein is observed in the DA and coronary vessels. Both gain- and loss-of-function analyses in zebrafish demonstrated specific defects in ISV sprouting. Injection of mouse syx mRNA partially rescued these defects, suggesting evolutionary conservation of Syx function. Fur-
thermore, we found that Syx and angiomotin, a regulator of EC migration, act additively during angiogenesis. These results implicate for the first time a Rho-GEF that functions specifically in vertebrate angiogenesis in vivo.

Materials and Methods

Zebrafish Stocks and Reagents

Zebrafish were maintained at 28.5°C under Medical College of Wisconsin guidelines (protocol no. 312-06-2). Mating was carried out at 28.5°C, and embryos were staged according to established protocols. Morpholino (MO1 and MO2 were designed by Gene Tools (Philomath, Ore) to target a intron–exon boundary and the ATG start codon, respectively. MO1, AGCTGTTTCTTGTTG-GCCTGCTGA; MO2, CATGCCCTCGCCCAATAGAACACGTG. Splice site nucleotides are italicized. The Amot MO sequence has been published previously.

Whole Mount ISH

Wild-type (WT) embryos were grown in 0.003% phenylthiourea until the desired stage, fixed overnight at 4°C in 4% paraformaldehyde, dechorionated, and stored in 100% methanol at −20°C until use. Whole mount ISH was performed as described using etsrp, flk, fltl, gfa, and syx probes. Digoxigenin-labeled sense and antisense syx probes were transcribed from a XmnI-linearized vector containing 541 bp of syx using T7 and SP6 RNA polymerases, respectively. Zebrafish syx cDNA used to make the RNA probes was obtained from Open Biosystems (Clone: 65260512, Huntsville, Ala).

For information regarding Tg (fltl: EGFP/Hucy)−/− zebrafish and Cre/loxP syx+/− mouse generation, refer to the online data supplement, available at http://circres.ahajournals.org.

Microcomputed Tomography

We used WT and syx knockout mice fixed at end diastole. The mice were anesthetized by ketamine/xylazine, heparinized, and euthanized by perfusion with citric KCl. After a PBS wash, mice were fixed by perfusion with 4% paraformaldehyde. A MV-132 (Flow-Tech) contrast medium was infused into the coronary system via the aorta and into the renal arteries. The hearts were imaged by a General Electric eXplore Locus SP apparatus at a resolution of 13 μm, and the vasculature was reconstructed by Micoview (GE) software.

Results

Two zebrafish National Center for Biotechnology Information sequences (accession numbers XM_686228.1 and XM_686228.2, encoding 1143-aa and 858-aa proteins, respectively) match mouse and human Syx, which are 1073 and 1091 aa, respectively. Amino acids 14 to 858 of the shorter zebrafish sequence (Figure IB in the online data supplement) are identical to amino acids 297 to 1141 of the longer sequence (supplemental Figure IA), suggesting that alternative start sites generate 2 proteins with different N termini. We have also detected multiple isoforms of syx by RT-PCR (data not shown). The 1143-aa Syx protein contains a DbI-homology (DH) (supplemental Figure IA, black arrows) and pleckstrin homology (PH) domains (supplemental Figure IA, gray arrows), and a PDZ-binding motif (ASEV) at its C terminus (Figure IA). Its amino acid sequence is 60% and 59% homologous to mouse and human syx, respectively (Figure IIA and IIB). Expression continues in blastomeres at 3 hours postfertilization (hpf) (supplemental Figure IIC) and is ubiquitous in 12-somite embryos (supplemental Figure IID). At 18 somites (supplemental Figure IIE), syx expression is

Figure 1. Syx expression in zebrafish and mouse embryos. A, The scheme shows the structure of the zebrafish Syx Rho-GEF with a Dbl-homology (DH) (AA 423 to 612), pleckstrin homology (PH) (amino acids 671 to 772), and a C-terminal PDZ-binding domain. B through E, Whole mount syx ISH zebrafish embryos at 24 hpf in DA (B, black arrow, and C) sites of sprouting ISVs at 26 hpf (D, E, asterisks). F through H, Micrographs of an E12.5 mouse sagittal section labeled with Syx and PECAM antibodies. The yellow-colored areas indicate merged image (G) indicates extensive colocalization of the 2 signals, which were originally red (Syx) and green (PECAM). Scale bars: 500 μm; 100 μm (in inset). Insets are high-power images of the aorta and circumflex coronary. ao indicates aorta; cco, circumflex coronary; la, left atrium; lv, left ventricle; ra, right atrium.
Figure 2. *Syx* loss-of-function causes sprouting defects in zebrafish arteries. A through F, Images of the trunk region of 24 hpf (A, C, and E) or 28 hpf (B, D, and F) Tg(*fli1*: EGFP) embryos. A and B, Uninjected (UI) embryos. C through F, Age-matched MO1-injected (C and D) and MO2-injected (E and F) embryos, respectively. Syx KD embryos show truncated (C, asterisk) and blunted ISVs (D through F, asterisks). G, Graphic representation of percentage of defective embryos (y axis) at 24 hpf: 63% (n = 41) of MO1-injected, 38% (n = 80) of MO2-injected, and 11% of uninjected (n = 47) Tg(*fli1*: EGFP) embryos show ISV defects; 42% of MO1-injected Tg(*flk*: GRCF) embryos show ISV defects compared to only 7% of uninjected embryos. The head is to the left.

fairly ubiquitous and is observed along the midline (supplemental Figure IIIF). Starting at 23 to 24 hpf (Figure 1B and 1C), *syx* transcripts are restricted to axial vessels (Figure 1B, black arrow). At 24 to 26 hpf (Figure 1D and 1E), *syx* expression is noted in the ISVs (Figure 1E, white asterisks). We also checked for Syx expression in mammalian tissue by probing cryosections of embryonic day (E)12.5 mouse embryos with anti-Syx (a custom-made antibody) (Figure 1F) and anti-platelet endothelial cell adhesion molecule (PECAM)-1/CD31 (Figure 1H), an EC marker. We found that Syx is expressed in the endoderm of the aorta, coronary vessels, and endocardium of the atria and left ventricle (Figure 1F and 1G). Syx was also detected in the ascending aorta (data not shown) but not in the vena cava, suggesting that Syx is an artery-specific protein. Both zebrafish and mouse share Syx aorta expression.

The endocardial expression of Syx in the mouse prompted us to investigate whether *syx* is differentially expressed in the cardiovascular genetic mutant cloche (*clo*) in zebrafish, which lacks the endocardial layer. Real time PCR analysis for *syx* showed that *syx* levels are reduced in *clo*−/− embryos compared to WT embryos (supplemental Figure IIIG). We injected *syx* mRNA into Tg(*fli1*: EGFP) embryos, compared to WT embryos (supplemental Figure IIIG). We injected *syx* mRNA into Tg(*fli1*: EGFP)*clo*−/− embryos, and found that *syx* expression is not affected in the *clo* mutants. *Syx* mRNA–injected embryos from Tg(*fli1*: EGFP)*clo*−/− mating pairs did not show significant differences in Mendelian ratios for edema or gain of the vascular marker *fli1*-EGFP compared to embryos from uninjected mating pairs (supplemental Figure IIIH), suggesting that *syx* expression may be indirectly affected in the *cloche* mutant.

**Knockdown of syx Perturbs ISV Development in the Zebrafish**

We designed 2 MOs to knockdown (KD) *syx* transcripts in zebrafish. MO efficacy is shown in supplemental Figure III and discussed in the text of the online data supplement. The effect of *syx* KD on zebrafish vascular development was analyzed in transgenic fish carrying an endothelial-specific promoter of vascular endothelial growth factor receptor-2 (vegfr2 or *flk*) or friend leukemia integration factor-1 (*fli1*), which drive expression of green fluorescent protein (GFP) specifically in the vasculature. Transgenic embryos were injected with 8 ng of MO1 or MO2 at 1-cell stage, and vessel development was monitored via fluorescence microscopy. By 24 hpf, uninjected embryos showed ISVs that migrated dorsally between somites (Figure 2A, asterisk). In MO1-injected embryos, the ISVs were truncated, and the leading end of the tip cell was blunt (Figure 2C, asterisk). Occasionally, 1 or 2 ISVs were completely absent (Figure 2C). At 28 hpf, the growth of ISVs in MO1-injected embryos remained stagnate (Figure 2D, asterisk), whereas those of uninjected embryos continued to grow to form the dorsal longitudinal anastomotic vessel (Figure 2B, asterisk). A second MO targeting the *ATG* translational start site, MO2 (8 ng), also caused growth arrest of ISVs (Figure 2E and 2F, asterisk). Quantification of MO-injected embryos at 24 hpf (Figure 2G) revealed that 62% of Tg(*fli1*: EGFP) and 42% of Tg(*flk*: G-RCPF) MO1-injected embryos displayed ISV defects compared to only 10% and 7% of their age-matched uninjected siblings, respectively. Thirty percent of MO2-injected Tg(*fli1*: EGFP) embryos had ISV growth defects (Figure 2G), suggesting some variability in MO efficacy, which is expected because MO1 targeted both forms of the *syx* transcript, whereas MO2 targeted only the long form.

To determine if gastrulation delays were caused by MO injection, we performed ISH with the gastrulation marker *myod* in MO1-injected (supplemental Figure IIIIE) and uninjected (supplemental Figure IIIID) embryos at 24 hpf. We observed no difference in *myod* expression patterns between samples, suggesting that the vascular phenotype caused by MO1 injection did not result from delayed gastrulation.

To investigate whether vascular defects originated at a developmental stage that preceded ISV sprouting, we performed ISH with *etsrp*, an early angioblast marker. At 14 hpf (Figure 3A through 3D) or 18 hpf (Figure 3E through 3H), all of the sample groups displayed normal *etsrp* expression pattern. To confirm the phenotypes observed in Tg(*fli1*: EGFP) embryos, we performed ISH with the *flk* probe at 24 hpf in uninjected (Figure 3I) and MO-injected (Figure 3J and 3K) embryos. We observed a darker staining of the DA in MO1-injected embryos, similar to the more intense GFP signal in the Tg(*fli1*: EGFP) zebrafish (Figure 2C), suggesting the presence of more number of ECs. In MO1-injected embryos at 24 hpf, secondary *flk*+ ISVs sprouting from the DA appeared defective and were truncated (Figure 3J, white asterisk) or misguided (Figure 3J, white arrowhead). Some ISV ends were marked by darker staining in both MO1- and MO2-injected embryos similar to the swollen ends of GFP-expressing ISVs (asterisks in Figure 3J and 3K). Quantification at 24 hpf (Figure 3M) showed that 11% of the MO1-injected embryos had no more than 5 ISVs, whereas 42% had truncated ISVs. In MO2-injected embryos, 2% had no ISVs, 5% had less than five ISVs, and 44% had truncated ISVs. Similar results were observed in MO-injected *flk*− embryos at
and performed ISH using etsrp, flk, and fli probes. At 14 hpf, etsrp-probed syx RNA-injected (Figure 3D) embryos appeared normal, showing angioblast patterning comparable to uninjected and the MO1- and MO2-injected embryos. Similarly, at 18 hpf, we observed no vascular defects in flk expression (data not shown). In syx mRNA–injected fli ISH embryos, vascular defects were observed at 24 hpf where ISVs had blunt ends (Figure 3L, asterisk), mimicking the phenotype of the MO-injected embryos at the same time point. These result suggest that gain- and loss-of-function phenotypes are not complementary but similar.

syx Does Not Affect Artery Versus Vein Specification

In 24 hpf syx KD (Figure 3J and 3K) or syx mRNA–injected (Figure 3L) embryo, we observed an intense blue staining in the DA upon fli ISH. In particular, the fli⁺-stained region in syx mRNA–injected embryos expanded into both the DA and posterior cardinal vein (Figure 3L). Therefore, we checked the arterial marker gridlock (grl) at 24 hpf and the venous marker flt4 at 30 hp to determine whether artery versus vein differentiation is affected in syx KD embryos. ISH with grl and flt4 antisense probes showed no difference in staining between MO or RNA-injected embryos and uninjected embryos (data not shown), suggesting that syx is not required for angioblast differentiation. Collectively, our ISH results confirm the transgenic embryo data indicating that syx plays an exclusive role in directing ISV sprout growth from the DA.

Vascular Defects Are Partially Rescued by syx Gene Complementation

To demonstrate MO specificity, we coinjected Tg(fli1: EGFP) embryos with syx sense mRNA and MO1 and compared them to embryos injected with MO1 alone. Initially, we performed rescue experiments with 100 pg of syx mRNA and observed no rescue in MO1-injected embryos (data not shown). Subsequently, we increased the dose of mRNA to 150 or 200 pg and observed a concomitant reduction in ISV defects in coinjected embryos when compared to MO1-injected embryos (Figure 3N). However, we were only able to partially rescue the phenotype with the 2 mRNA doses (12% and 15% rescue, respectively). When the amount of mRNA was further increased to 300 pg, the number of rescued embryos was reduced to 6%, suggesting a saturation effect. These results indicate that ISV sprouting requires a precise level of syx expression. Because the injection of mouse syx mRNA partially rescued the endogenous syx KD effects in zebrafish, we conclude that syx function is conserved across vertebrate species.

Disruption of syx Expression in the Mouse Causes Angiogenic Defects

To study Syx function in mammals, Cre loxP syx⁺/⁻ mice were generated and crossed with germ-line Cre mice, producing a global disruption of syx expression. Syx⁻/⁻ mice were viable up to at least 12 months and appeared grossly normal. Because syx is expressed in the cardiac and vascular tissue in the mouse (Figure 1F), and syx KD zebrafish shows defective ISVs, we imaged the coronary and kidney arterial systems by

Figure 3. Syx loss- and gain-of-function embryos show similar defects. A through L, Whole mount ISHs for etsrp at 14 hpf (A through D) and 18 hpf (E through H) and fli at 24 hpf (I through L). In 14- and 18-hpf etsrp ISH embryos, no distinct difference is noted across sample groups. At 24 hpf (I through L), truncated and/or blunt (asterisks) and misrouted ISVs (white arrowhead) appeared in MO- and RNA-injected embryos. M shows quantification of embryos (y axis) displaying particular morphological defects (x axis) observed in fli ISH embryos for each sample group. N shows percentage defects (y axis) in sample groups of (x axis): uninjected (UI) (n=55), MO1-injected (MO1) (n=42), or coinjected (MO1+200 pg syx mRNA) (n=26) Tg(fli1: EGFP) embryos.
Both the coronary (Figure 4A) and kidney (Figure 4B) arterial systems of the WT and syx–/– mice. The recently published ISV Sprouting and Angiomotin and syx function additively during ISV sprouting suggest that the defect is specific to the angiogenic stage of arterial development, without affecting arteriogenesis, similar to the vascular defect in zebrafish treated with MOs. The ISVs of embryos injected with 4 ng of syx MO (Figure 5B) or amot MO (Figure 5C) injected embryos were stunted. When amot and syx MOs were coinjected at 4 ng each, truncation of ISV sprouts (Figure 5D, white asterisk) was more severe than those in embryos injected with a single MO. In some cases, the ISVs in embryos coinjected by amot and syx MOs were completely absent (Figure 5E, white asterisk). Injection of syx or amot MO (4 ng) alone resulted in approximately half (23% and 28%, respectively) as many ISV-defective embryos (Figure 5I) as injection of double the dose (8 ng) of each MO (60% and 45%, respectively; Figure 5I). High-power images of ISVs in uninjected embryos at 28 hpf revealed elongated filopodia protruding out of their tip cells (Figure 5F), whereas the tip cells of ISVs in embryos injected by

Angiomotin and syx Function Additively During ISV Sprouting

The recently published angiomotin (amot) KD phenotype in zebrafish is strikingly similar to that of syx. To determine whether amot and syx regulate ISV sprouting additively, we coinjected half-doses of MOs for each gene (4 ng each) or each MO alone (4 ng or 8 ng) into Tg(flk: GRCFP) fish and compared the images of the vascular system in each sample. The ISVs of uninjected embryos followed the somite borders

Figure 4. Vascular defects in syx–/– mouse. A and B, µCT image reconstructions of WT and syx–/– mouse arterial coronary vasculature (A) and kidney arterial vasculature (B). C, Lectin-labeled sections of WT and Syx–/– myocardium. D, Capillary density in WT and syx–/– myocardia. Capillaries were counted in 4 sections, six 360° × 360 μm fields per section (P<10−6).

microcomputed tomography (µCT) of WT and syx–/– mice. Both the coronary (Figure 4A) and kidney (Figure 4B) arterial systems of the syx–/– mice were sparser than those in the WT mouse. On close examination, these systems appeared to be deficient in small diameter vessels, but the major coronary and kidney arteries were intact. This defect is analogous to the syx KD zebrafish, where the DA was intact but ISVs were truncated (Figure 2D and 2F). To quantify the coronary arterial defect, we used µCT images to measure the total volume of the contrast medium infused into the coronary arteries of the WT and syx–/– mice. We found that the ratio between the volumes of the syx–/– and WT coronary arterial systems was 0.75±0.11 (n=5, P=0.002).

Because the µCT technique cannot image capillaries due to the viscosity of the contrast medium, we probed sections of WT and syx–/– myocardia by histochemistry. The images clearly showed that the density of capillaries in the syx–/– was significantly lower than in the WT myocardium (Figure 4C). Together with the µCT images, these results demonstrate that the syx–/– mouse harbors a vascular defect affecting multiple organs. The major arteries were intact, but the growth of secondary arteries and capillaries was defective. We conclude that the defect is specific to the angiogenic stage of arterial development, without affecting arteriogenesis, similar to the vascular defect in zebrafish treated with syx MOs. The similarity of secondary arteries and capillaries in the zebrafish and the mouse suggests that the function of syx in angiogenesis is highly conserved throughout evolution.

Figure 5. Additive function of amot and syx in ISV sprouting. A through E, The trunk region of Tg(flk: GRCFP) embryos at 28 hpf after injection with MOs indicated in the bottom right of each image. The double-injected embryo in E was completely devoid of ISVs. Red asterisks indicate truncated ISVs, and white asterisks indicate missing ISVs. F through H, High-power trunk images of uninjected (UI), syx MO1-injected (syx MO), and syx MO- and Amot MO-injected (syx+amot MO) Tg(flk: GRCFP) embryos. I is a graphic representation of the percentage of defective embryos (y axis) within each sample group (x axis) (uninjected [ui], n=46; syx 8 ng: syx MO 8 ng, n=42; amot 8 ng: amot MO 8 ng, n=56; uninjected [ui], n=17; syx 4 ng: syx MO 4 ng, n=22; amot 4 ng: amot MO 4 ng, n=18; amot+syx [4 ng each]: amot MO 4 ng+syx MO 4 ng, n=42). The compass indicates the orientation of the embryos.
Syx KD Impairs EC Migration and Angiogenesis in Response to Vascular Endothelial Growth Factor-A

Vascular endothelial growth factor (VEGF)-A is a major agonist of angiogenesis. Because the vascular defects in the syx mouse and in syx KD zebrafish were angiogenic in nature, we tested the effect of silencing syx expression on EC vessel formation, namely VEGF-A165-induced migration and in vitro tube formation. Transfection by syx small interfering (si)RNA significantly depleted Syx and reduced EC migration under basal conditions and in response to VEGF-A165 (Figure 6A). The tubular network formed by ECs transfected with syx siRNA, either with or without VEGF-A165, was sparser than the network formed by cells transfected with control siRNA, indicating that Syx depletion impaired angiogenesis (Figure 6B). However, ECs transfected by syx siRNA did not show a difference in invasive behavior compared with control siRNA-transfected ECs to either VEGF or serum stimulus indicating no function for Syx in invasion (supplemental Figure IVB). These results indicate that Syx has an essential role in specific steps of angiogenesis such as EC migration, in agreement with our previous results.

Discussion

This study identifies the function of a novel Rho-GEF, Syx, in vascular development in vivo. We report three important findings. First, syx is specifically expressed in the vasculature of teleosts and mammals. Second, gain- and loss-of-function analyses show that syx plays a specific role in orchestrating directional migration of endothelial sprouts during vertebrate angiogenesis. Third, a novel signaling axis between syx and amot necessary for proper patterning of angiogenic vessels has been uncovered.

Vessel patterning in vertebrates is a complex process that involves the formation of primary axial vessels by vasculogenesis and the sprouting of secondary vessels by angiogenesis. This study focuses on the angiogenic mechanism of ISV sprouting. Syx has been identified as a novel RhoA-specific GEF, which contributes to EC migration in vitro. This function of Syx is consistent with previously published reports suggesting an important role for Rho GTPases in axon and endothelial tip cell guidance.

We have extended the EC expression profile of syx to mouse and zebrafish vessels. In both species, syx is expressed in the vasculature and in zebrafish the expression is almost exclusive to the DA and emerging sprouts. Because we did not detect Syx expression in the zebrafish posterior cardinal vein and in the mouse vena cava, it is likely that the Syx is specifically involved in arterial angiogenesis.

In the mouse, global disruption of the syx gene was accompanied by a significant reduction in the density of secondary arteries and of capillaries in the heart. We observed a similar reduction in the density of secondary arteries of the kidneys. Although we have not characterized additional vascular beds, we suspect that the angiogenic defect in the syx+/− mouse is likely to extend to all tissues and organs. Given the pivotal function of Syx in EC migration, it is surprising that vascular defects in the syx+/− mouse and the syx KD zebrafish are not severe. In vascular development, isoform-specific function of signaling molecules is frequently confined to specific developmental stages. For example, in zebrafish, the KDα isoform of the VEGF-A receptor is specifically involved in arterial angiogenesis.

Two lines of evidence suggest that maintaining optimal levels of syx is critical for directional migration of ISV sprouts. First, both KD and overexpression of syx resulted in
similar phenotypes. In both experiments, ISVs had sprouting defects, suggesting that ISV growth requires a strict balance of guidance cues. When there is either a deficiency or overabundance of guidance molecules, the ISVs stall until a proper migratory path is established. These results mimic those observed for robo4, although we did not find evidence for a genetic interaction with syx. Second, in our complementation experiments, we injected mouse syx mRNA together with the syx MO1 to demonstrate MO specificity and evolutionary conservation of syx function. Because 100 pg of syx mRNA did not rescue MO-induced vascular defects, we increased the dose of mRNA to 150 and 200 pg and saw a proportional increase in rescued embryos. However, when the dose of mRNA was increased to 300 pg, the percentage of rescued embryos declined. These results also suggest that syx levels have to be maintained within a particular range to support proper vascular patterning.

A recent study demonstrated that amot KD caused ISV defects phenotypically similar to that of syx. In the present study, we found that syx and amot function additively to regulate ISV sprouting. The genetic interaction between amot and syx is accounted for, at least in part, by the coupling of the corresponding proteins via the adaptor protein MUPP1 (elsewhere and supplemental Figure IV). Both Amot and Syx are involved in mediating EC migration in response to VEGF (14 and Figure 6A), further substantiating the functional cooperation between these proteins. The inhibitory effect of Syx depletion on in vitro EC migration and tube formation in response to VEGF-A provides a potential molecular mechanism to explain the angiogenic defects in the syx-/- mouse and the syx KD zebrafish. The genetic interaction between Amot and Syx implies that the role of Syx in VEGF-A–induced angiogenesis is part of a more complex scenario involving additional signaling pathways. The binding of Syx to MUPP1, a component of tight junctions and its involvement in cell migration.

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**Disclosures**

None.

**References**

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LEGENDS:

Online Figure I
(A) Mouse Syx (AY605057) and the 1143 AA zebrafish (Danio) Syx (XP_693210.1) protein sequences were aligned using the CLC Free Workbench 3.2.1 program. The zebrafish Syx short (Danio Syx-S) and Syx long (Danio Syx-L) forms were aligned and is shown in the lower panel in A. Identical residues are indicated in red and gaps are indicated by dashes. Black arrowheads indicate the Rho GEF domain (423-612 AA) and grey arrowheads indicate the PH domain (671-772 AA). For most current annotation of syx please visit: http://www.genecards.org/cgibin/carddisp.pl?id=O94827&id_type=uniprot

Online Figure II
(A-F) show whole mount syx in-situ hybridization images across zebrafish embryonic development with stages indicated on the bottom right of each panel. y: yolk. (G) Quantification of the expression level of syx mRNA in WT and clo<sup>−/−</sup> zebrafish. (H) Comparison of the number of uninjected (UI) (n=72) and syx MO-injected clo<sup>−/−</sup> zebrafish with edema and vascular defects (GFP-defect) (n=98).

Online Figure III
(A) shows the efficacy of splice MO1 targeting by RT-PCR. Syx transcripts produced from corresponding primer sets encompassing the MO1 target sites are shown. The syx transcript is downregulated in MO-injected embryos in the expected targeted regions (F1-R1 and F1-R2). No change in actin transcript was noted between uninjected (UI) and MO1-injected samples. (B) A truncated western blot with mouse Syx antibody on uninjected (UI) or MO2-injected zebrafish lysates shows absence of Syx protein (127 kDa) at 24 hpf. The actin (42 kD) blot demonstrates equal loading. (C) A full western blot of zebrafish lysates of uninjected (UI) and Syx MO2 injected (MO) embryos probed with mouse syx antibody is shown. The arrowheads show multiple protein bands ranging in size in between 100-150 kDa. The red arrowhead shows a truncated protein band at or less than 100 kDa in lane UI, which are all absent or fainter in lane
MO. A high molecular weight band (black arrowhead) is also observed in between 150 and 250 kDa marker bands that shows strong cross reactivity to mouse Syx antibody in lane UI, which is absent in Syx MO2, lane MO. (D) and (E) are myod in situ on uninjected or syx MO1-injected embryos at 24 hpf, respectively. (F) shows percent defect (y-axis) of flk in situ embryos at 22 hpf for different sample groups (x-axis).

Online Figure IV

(A) An immunoblot with anti-MUPP1 (BD Biosciences Pharmingen; used according to manufacturer’s instructions) of rat fat pad endothelial cells expressing YFP-fused Syx1 or Syx2, following immunoprecipitation by anti-Syx (protocol and antibody described in 1). (B) Graphical representation of control and syx siRNA transfected rat fat endothelial cells in response to VEGF (50 ng/mL) or serum (10% FBS) in a modified Boyden chamber matrigel invasion assay. The error bars represent SD and n=3.

RESULTS:

Efficacy of MO-mediated knockdown of syx

We designed two MOs to knockdown (KD) syx transcripts. MO1 was designed to target splicing junction of exon 5 of the longer syx transcript, which contains the functional DH domain. MO2 was designed to inhibit the ATG translational start site in exon 1. MO1 should target both the long and short forms of syx, while MO2 should selectively target the long form. To demonstrate MO1 efficacy, we performed RT-PCR using a primer set (Online Fig. IIIA, arrows) flanking MO1’s target site regions of syx. The syx transcript levels were reduced in MO1-injected embryos (Online Fig. IIIA, lane MO) compared to uninjected embryos (Online Fig 3A, lane UI) and no difference is noted in actin (Online Fig. IIIA, actin) transcript levels. Immunoblotting with a mouse Syx antibody shows endogenous Syx protein of approximately 127 kDa size, which was significantly lower in MO2-injected zebrafish compared to uninjected embryos (Online Fig. IIIB). In addition, we observed multiple bands in uninjected embryos that
likely represent at least four Syx isoforms (Online Fig. IIIC, arrow heads, lane UI), and these were reduced in MO2 embryos (Online Fig. IIIC, lane MO).

METHODS:

Creation of Tg (fli: EGFP)$^{clo39+/+}$: To establish a line of viable zebrafish expressing both the desired $clo^{+/+}$ heterozygosity and $fli^{+/+}$ homozygosity, a series of breedings and screenings were performed. An identified $clo^{+/+}$ fish was crossed with a known Tg (fli: EGFP) fish whose genotype is irrelevant since GFP is expressed in both heterozygous and homozygous carriers. The offspring of this original cross were screened for GFP expression, and GFP$^+$ embryos were raised to adulthood. Outcrossing these adults in 1:1 ratios to known $clo^{+/+}$ fish resulted in identification of the $clo$ trait and the $clo^{+/+}$ $fli^{+/+}$ carrier. Incross of these adult carriers resulted in a F2 generation with mosaic expression of the two traits, which were screened for GFP. F2 fish were outcrossed 1:1 to a known $clo^{+/+}$ and the resulting F3 generation was screened. If GFP expression was 100% and $clo^{-/-}$ was ~25%, the adult was positively identified as a $clo^{+/-}$ $fli^{+/-}$. Subsequent crossing of these fish consistently produces offspring in Mendelian fashion of ~50% $clo^{+/-}$, ~25% $clo^{-/-}$ (lethal), ~25% $clo^{+/-}$ and 100% $fli^{+/-}$.

Generation of a Cre/loxP $syx^{-/-}$ mouse: The mouse was raised in the laboratory of Dr. Jay Baraban, Johns Hopkins Medical School and details of mouse characterization will appear in a separate manuscript. The loxP sites flanking exons 9-13 (containing the catalytic domain of Syx) were inserted by homologous recombination. Heterozygous mice were crossed with germline Cre mice to excise the targeted region in all mouse tissues. Mice homozygous for the mutant $syx$ allele were viable up to at least 12 months, and appeared grossly normal. Western blot with antibody recognizing the N-terminus of Syx showed a complete loss of the band that corresponds to full-length Syx in Cre/loxP $syx^{-/-}$ mice. However, we observed a new band (with lower intensity than the full-length in the wt) that corresponds to a truncated Syx protein made from a transcript that is missing exons 9-13 (data not shown).
**RT-PCR:** To determine MO efficacy, RT-PCR was performed on 1.0 µg of total RNA isolated from embryos at 24 hours post fertilization (hpf) using oligo dT primers. PCR was performed using the following cycling parameters: 94°C for 2 min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min (34 cycles), 72°C for 5 min. Primers: F1, ATGGGAACCTCCTGCAGACCGAAT; F2, CGACGAGGTATTCCAAACGGA; R1, CCATGCAGTAACGGATGTAAGGCTTG; R2, CCACAGCCTCATAACGCTCGATA; R3, AGTCAGTGTTGAATTGAGCA.

**Real time PCR:** Total RNA was prepared from a single 24 hpf wild type or clo embryo using RNAqueous®-4PCR Kit (Ambion, Austin, TX). Random hexamer-primed first-strand cDNA synthesis was performed with SuperScript™ RNaseH Reverse Transcriptase (Invitrogen, Carlsbad, CA). The genotype of each embryo was confirmed by the relative expression of Flk. A cDNA pool of 7 wild type embryos and 7 mutant embryos were used in the quantitative PCR analysis. The cDNA was then amplified in an ABI 7300 Real-Time PCR System using the DyNAmo HS Syber Green qPCR Kit (Finnzymes Oy, Espoo, Finland). Specificity of RT-PCR products was verified by checking the product melting curves each time. To further ascertain the specificity and size of PCR products, the products were run on a 2% agarose gel with molecular weight markers. Relative expression values were calculated by SDS software 1.3.1 using the comparative Ct (2^ΔΔCt) method. Primer sequences used for Syx real time PCR: F, CTGCTGCTCAAAAGTGTGCT; R, TCCTCCCTGTGCCTCATCT.

**Microinjections:** Microinjections were performed as described previously 2. MOs were reconstituted in nuclease-free water to a stock concentration of 2 mM. MOs were diluted in 5x injection dye (100 mM Hepes, 1M KCl, 1% phenol red) to a final concentration of 4-7 ng/nL, and 2 nL were injected into 1-cell embryos. In RNA overexpression and rescue experiments, 100-300 pg of capped syx mRNA were injected into 1-cell embryos. Full-length mouse syx cDNA was linearized with XhoI and transcribed with T7 polymerase to make sense RNA. For double MO injections, MOs were combined prior to injection.
Western blot analysis: Thirty MO-injected or uninjected embryos were anesthetized with tricaine and were de-yolked prior to making protein lysates in RIPA buffer (Sigma). Equal amounts of protein were resolved in a 7.5% PAGE gel and wet-transferred to a PVDF membrane to perform western analysis. A custom-designed polyclonal antibody raised against the mouse sequence QHRKLTLAQLYRIRTT was used as the primary antibody. Actin antibody from Sigma was used for the equal loading control.

Histochemistry of mouse myocardium sections: Similarly oriented 8 µm sections were cut from the left ventricular walls of 4% paraformaldehyde perfused mice sacrificed according to NIH guidelines. The sections were labeled with GSL I-B4 isolectin subunit, 50 µg/ml, applied according to the manufacturer’s instructions (Vector Labs). Capillary density was calculated by manually counting the number of capillaries in six 360×360 µm fields per section.

Gap closure and in-vitro tube formation assays: Rat fat pad endothelial cells\(^3\) (RFPEC), the protocols, YFP-Syx1 and YFP-Syx2 constructs, and the siRNA sequences were described previously\(^1\). The gap closure experiments were performed on fibronectin-coated (25 ng/ml, BD Biosciences) 6-well plates. Tube formation was quantified by counting the total number of nodes per field.

Modified Boyden chamber matrigel invasion assay: RFPEC were cultured in DMEM medium supplemented with 10% FBS and plated in a 6 well plate. Upon 70 % confluence the cells were transiently transfected with control and syx siRNA (80 pmol each) for a period of 36 h. Prior to performing the assay, matrigel (BD biosciences) was diluted 1:3 in serum free DMEM medium and applied to the upper surface of the filter insert (8 µm) and kept in laminar air flow hood overnight. RFPEC cells in each group were loaded with Calcein AM 5 µg/mL in DMEM media with 0.5% FBS for 2 h. Calcein-stained cells were harvested with 0.5 mM EDTA and re-suspended in DMEM with 0.5% FBS.
Chemoattractants VEGF (50ng/mL) and FBS (10%) were added to the bottom chamber and cells plated in the upper chamber at the concentration of 100,000 cells/well in DMEM with 0.5% FBS and incubated for 4 h. The assay was performed in a 24 well format and read using a bottom plate microplate fluorimeter (CytoFluor 2350, Millipore) reader (Exi = 485 nm and Emi = 528 nm).

Statistical Analyses: Fisher’s exact test was used to determine statistical significance of experimental groups of zebrafish data. T-tests were used otherwise. P values less than or equal to 0.05 were considered significant. In Fig. 2, panel G P-values for fli: UI to MO1 = 5.2 X 10^{-15}, UI to MO2 = 6.6 X 10^{-6}, flk: UI to MO1 = 3.3 X 10^{-9}. In Fig. 3, panel M P-values for UI to MO1 = 2.5 X 10^{-21}, UI to MO2 = 7.4 X 10^{-20}, UI to RNA = 1.1 X 10^{-59}. In Fig. 3, panel N P-values for UI to MO1 = 7.1 X 10^{-11}, UI to MO1+RNA = 6.3 X 10^{-5}, MO1 to MO1+RNA = 2.2 X 10^{-3}. For Fig. 5, panel I P-values for UI to syx MO (8 ng) = 5.1 X 10^{-15}, UI to amot MO (8 ng) = 4.5 X 10^{-9}, UI to syx MO (4 ng) = 5.1 X 10^{-4}, UI to amot MO (4 ng) = 2.4 X 10^{-5}, UI to syx plus amot MO (4 ng) = 6.41 X 10^{-13}. 
References


