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Syk and Zap-70 function redundantly to promote angioblast migration

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ABSTRACT

Spleen tyrosine kinase (Syk) plays critical roles in B-cell and T-cell development, the maintenance of vascular integrity, and proper partitioning of the blood vascular and lymphatic vascular system. Here, we utilize the zebrafish as an *in vivo* system to demonstrate novel roles for Syk and the related kinase Zeta associated protein (Zap-70) in promoting angioblast migration. Partial knockdown of either gene results in early angiogenic delay of the intersegmental vessels, dorsal intersegmental vessel patterning defects, and partial loss of the thoracic duct. Higher dose knockdown of both genes results in little to no angiogenic sprouting of the intersegmental vessels, a phenotype which resembles knockdown of *vegfa*. Di-phosphorylated ERK, an effector of the *vegfa* pathway, is also downregulated in the aorta of *syk:zap* double morphants. Over-expression of *syk* under the control of a blood-specific or vascular-specific promoter rescues sprouting defects after loss of *vegfa*. Together these results suggest that *syk* and *zap-70* function redundantly in an early progenitor to promote the migration of intersegmental vessel angioblasts and lymphangioblasts that contribute to the thoracic duct, either downstream of, or in parallel to *vegfa*.

Introduction

Regulation of vascular sprouting and growth involves multiple overlapping molecular pathways, and is still incompletely understood. Spleen tyrosine kinase (*Syk*) is a cytosolic kinase important for vascular, lymphatic, B-cell, and T-cell development (Abtahian et al., 2003; Cheng et al., 1995; Cornall et al., 2000; Sebzda et al., 2006; Turner et al., 1995; Yanagi et al., 2001). *Syk* is also critical for vascular development as most null mice die shortly after birth with systemic hemorrhage and edema (Cheng et al., 1995; Turner et al., 1995) due to improper partitioning of the developing lymphatic system from the blood vascular system, resulting in blood-filled lymphatics (Abtahian et al., 2003; Sebzda et al., 2006). *Slp-76* serves as a substrate for *Syk*, and null mice display a similar phenotype but also develop a network of dilated and tortuous blood vessels in the small intestine suggesting a role in vascular patterning and migration (Abtahian et al., 2003). In cell culture, *Syk* is involved in endothelial-cell proliferation and migration (Inatome et al., 2001), while lymphatic defects in *Syk* null embryos result from loss of signaling in circulating endothelial precursor cells (EPCs), which contribute to the developing lymphatic vasculature (Sebzda et al., 2006).

Zeta associated protein 70 (Zap-70) is a cytosolic tyrosine kinase, structurally related to *Syk*, that functions in hematopoietic activation and differentiation by signaling through T-cell antigen receptors (Arpaia et al., 1994). ZAP-70 deficiency in humans results in severe compromised immune deficiency syndrome due to the loss of signaling in T-cells (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1995; Elder et al., 1994; Gelfand et al., 1995). Although related in structure and function (as *syk* and *zap-70* are functionally interchangeable (Fallah-Arani et al., 2008; Gong et al., 1997)) lymphatic or blood endothelial defects have not been detected after loss of *zap-70*.

In B- and T-lymphocytes, Syk and Zap-70 bind to an 'immunoreceptor tyrosine-based activation' motif (ITAM) consisting of a consensus sequence with two tyrosines about 10–12 amino acids apart on immune receptors (Turner et al., 2000). Upon receptor activation these tyrosines become phosphorylated allowing cytoplasmic tyrosine kinases, including Syk and Zap-70, to bind the phosphorylated ITAM motifs through their SH2 domains and initiate downstream signaling. *In vitro*, Syk and Zap-70 have been shown to activate PLCγ1 (Law et al., 1996; Williams et al., 1999) and ERK (Griffith et al., 1998; Parsa et al., 2008; Shan et al., 2001; Slack et al., 2007). PLCγ1 and ERK are also important downstream components of the *vegfa* pathway in endothelial cells, and are involved in angiogenesis (Hong et al., 2006; Lawson et al., 2003). However, it is not known whether Syk or Zap-70 functions in endothelial cells *in vivo*, and if they function through similar pathways in endothelial cells as they do in B-cells and T-cells.

The external fertilization, optical clarity and availability of fluorescent transgenic animals make zebrafish an excellent model system for many developmental studies. The recent discovery of a functional lymphatic system in zebrafish has opened the door to take advantage of the unique benefits of zebrafish genetics and development to decipher the molecules and processes involved in blood–lymphatic vascular separation *in vivo* (Hogan et al., 2009; Kuchler et al., 2006; Yaniv et al., 2006). Although many of the genes involved in

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angiogenesis and lymphangiogenesis are conserved between mammals and zebrafish, the lymphatic vasculature of zebrafish develops by an alternative mechanism (Kuchler et al., 2006; Yaniv et al., 2006). In mammals, lymphatic endothelial cells bud off from the main cardinal vein and migrate to form the primary lymphatics (Wigle et al., 2002; Wigle and Oliver, 1999). While zebrafish lymphatics are still venous in origin, individual angioblasts do not directly pinch off from the major vein. Instead, they initially migrate as individual cells to the horizontal myoseptum before migrating dorsally to contribute to the dorsal longitudinal lymphatic vessels, or ventrally to contribute to the main thoracic duct (Hogan et al., 2009; Yaniv et al., 2006). This novel mechanism of zebrafish lymphatic development prompted us to analyze the function of zebrafish *syk* and *zap-70* in the developing vasculature to see if they play similar roles in fish angiogenesis and lymphangiogenesis as they do in mammals. Here we show that zebrafish *syk* and *zap-70* play unexpected and redundant roles to promote the migration of angioblasts to form both blood vascular and lymphatic vascular systems.

Materials and methods

Zebrafish husbandry

Embryos were raised at 28.5 °C in E3 embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) supplemented with 0.25 mg/l methylene blue, and staged by hours post fertilization (hpf) or days post fertilization (dpf) according to Kimmel et al. (1995). Chorions were removed by incubation in 20 mg/ml pronase (Sigma, St. Louis, MO) and pigmentation was blocked at 24 hpf by the addition of 0.003% 1-phenyl-2-thiourea (Sigma). *Tg(fli1a:egfp)^{y1}* (Lawson and Weinstein, 2002), *Tg(fli1a:nEGFP)^{y7}* (Roman et al., 2002) or *Tg(kdr-1:GFP)^{la116}* (Choi et al., 2007) embryos on a wild-type Tupfel long fin (TL) background were used for all experiments.

Identification, phylogenetic, and structural analysis of the zebrafish syk and zap-70 orthologs

The full length zebrafish (*Danio rerio*) *syk* ortholog has previously been identified: AF253046 or ZFIN:ZDB-GENE-040702-3. The full length *D. rerio zap-70* ortholog is *zgc:110383* (ZFIN:ZDB-GENE-050522-257). Nucleotide sequences of *D. rerio syk* (NP_998008.1), *Homo sapiens* SYK (NP_003168.2), *Mus musculus* SYK (NP_035648.2), *Tetraodon nigroviridis* Syk (CAF96564), *Gallus gallus* Syk (NP_001026601), *Xenopus tropicalis* Syk (NP_001086665), *Takifugu rubripes* Syk (SINFRUG00000143646), *D. rerio zap-70* (NP_001018425.1), *H. sapiens* ZAP-70 (NP_001070), *M. musculus* ZAP-70 (NP_033565.2), *T. nigroviridis* Zap-70 (CAG00734.1), *G. gallus* Zap-70 (NP_001026601.1), and *T. rubripes* Zap-70 (SINFRUG00000148610) were analyzed with the CLUSTAL W (1.81) multiple sequence alignment program.

Morpholino injection and angiography

The following splice site targeted morpholinos were synthesized by GeneTools LLC (Philomath, OR): *syk^{e2i2}* 5′-AGTGAAGAAGACTTACAGAA-ATTTG-3′; *syk^{e7i7}* 5′-TGAAGCACACCAACCTGAATCCAAG-3′; *zap-70^{i1e2}* 5′-TGGCATCCCCCTAAAAAGGTAACAA-3′; *zap-70^{e3i3}* 5′-TTGATGGG-CAAAACGTACCTGCCAC-3′; *vegfa3* 5′-TAAGAAAGCGAAGCTGCTG-GGTATG-3′ (Nasevicius et al., 2000). Morpholino efficiency was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) with gene specific primers over the morpholino target site (Supplementary Fig. 1). All primer sequences are listed in Supplementary Table 1. Equal loading of products was confirmed by RT-PCR with primers specific to elongation factor 1α (*eF1αF*/*eF1αR*, Supplementary Fig. 1). Off-target effects of morpholinos were assayed by incubation of live embryos in 5 μg/ml acridine orange (Sigma) for 30 min at 28 °C.

Blood flow in wild-type and morphant embryos was assessed by angiography through the injection of 2 MDa rhodamine dextran (Molecular Probes, Carlsbad, CA) into the sinus venosus of 5 dpf embryos. For fluorescent images embryos were mounted in 3% methyl cellulose (Sigma, St. Louis, MO), and photographed with a Stemi SV 11 microscope and an AxioCam HRc camera with AxioVision software (Carl Zeiss Canada Ltd., Toronto, ON). Confocal images were obtained by embedding embryos in 1.2% agarose, and photographed with an Olympus FV5-PSU. Some images taken at high resolution were pieced together with Adobe Photoshop.

Probe synthesis and whole-mount in situ hybridization

syk and *zap-70* probe templates were amplified from adult zebrafish fin cDNA with gene specific primers *sykF*/*T7sykR* and *zap-70F*/*T7zap-70R* (Supplementary Table 1). Products were purified with the QIAGEN PCR purification kit (QIAGEN, Mississauga, ON) prior to transcribing with T7 RNA polymerase (Invitrogen, Burlington, ON). Whole-mount *in situ* hybridization was performed on embryos as previously described (Oxtoby and Jowett, 1993). All post hybridization washes were performed with the aid of the BioLane™ HTI Robot (Holle and Huttner, Tübingen, Germany). The embryos were developed in BCIP/NBT at room temperature for 5–7 days (Roche Applied Science, Lavalle, QC). Whole-mount images were captured as above. A second set of full length antisense probes were also created against the *syk* and *zap-70* genes and resulted in the same expression patterns (data not shown).

Genetic rescue with gata1:syk and fli:syk

Multisite Gateway® cloning (Invitrogen), as developed by Kwan et al. (2007), was used to create full length, C-terminal myc-tagged transcripts of zebrafish *syk* under the control of a blood-expressed *gata1* promoter (Long et al., 1997) and endothelial-cell expressed *fli1* promoter (Buchner et al., 2007). Full length *syk* was amplified from wild-type cDNA with primers incorporating a portion of the attB1 and attB2 recombination sites required for Gateway cloning (*sykFB1*/*sykRB2*, Supplementary Table 1). A second round of PCR was performed to incorporate the entire attB1 and attB2 recombination sites, prior to Gateway cloning into the entry vector pDONOR221 with BP Clonase (Invitrogen). These middle entry constructs, plus a 5′ entry clone with either 5.4 kb of the *gata1* promoter (Long et al., 1997) or 2 kb of the *fli1* promoter (Buchner et al., 2007), and a 3′ entry clone with 6× C-terminal myc tag fusion and SV40 late polyA (p3E-MTpA), were combined with the multisite destination vector pDestTol2pA2 (Invitrogen) to obtain *gata1:syk* and *fli1:syk*. For rescue experiments, 50 pg of transposase and 10 pg of DNA were co-injected into single cell embryos.

Whole-mount immunohistochemistry

Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2 h at room temperature, permeabilized in methanol for 30 min at 4 °C, and then blocked in 10% normal sheep serum (NSS) in PBS with the addition of 0.1% Tween-20 (PBT). Immunostaining was performed overnight at 4 °C in 10% NSS/PBT with a 1:100 dilution of αphospho-p44/42 Map Kinase (Thr202/Tyr204) (αERK-P) polyclonal antibody (Cell Signaling, Danvers, MA), or a 1:20 dilution of αcMyc-9E10 (Developmental Studies Hybridoma Bank, Iowa City, IA). Embryos were washed thoroughly with PBT prior to incubation with a 1:250 dilution of goat α–rabbit Alexa555 secondary antibody (Invitrogen) in PBT. Embryos were imaged as for whole-mount *in situ* hybridization.

Results

Zebrafish syk and zap-70 are orthologs of mammalian SYK and ZAP-70

To identify the roles of *syk* and *zap-70* in zebrafish, we first identified their gene sequences from the genomic database. We find that there is a single zebrafish *syk* ortholog, located on chromosome 10. Likewise, there is a single *zap-70* ortholog on chromosome 8. By PCR, we identified a single transcript for *syk* and a single transcript for *zap-70*. Mammalian SYK and ZAP-70 contain two N-terminal SH2 domains and a C-terminal tyrosine kinase domain (reviewed in Turner et al., 2000). Similar to mammals, an NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer A, 2004) predicts that zebrafish Syk and Zap-70 contain two C-terminal SH2 domains and an N-terminal tyrosine kinase domain (PTK, Fig. 1A). Mammalian SYK is alternatively spliced and the less abundant, shorter isoform of mammalian SYK is more similar in structure to mammalian ZAP-70 (Turner et al., 2000). Nucleotide sequence alignment of *syk* and *zap-70* with orthologs in other species show clear clustering of zebrafish *syk* with the other Syk orthologs, and *zap-70* with the other Zap-70 orthologs (Fig. 1B).

syk and zap-70 function redundantly to promote trunk angiogenesis

To assess the biological function of *syk* and *zap-70* in zebrafish vascular development, two independent morpholino antisense oligonucleotides (MOs) were targeted to block splicing of each gene. Independently targeted morpholinos were used to confirm that the observed phenotype was due to specific downregulation of these genes. The morpholinos were targeted to exon 2–intron 2 (e2i2) of *syk* (*syk*^{e2i2}), e7i7 of *syk* (*syk*^{e7i7}), e3i3 of *zap-70* (*zap-70*^{e3i3}) and intron 1, exon 2 of *zap-70* (*zap-70*^{i1e2}). Single MOs against each gene were injected into *Tg(fli1a:EGFP)*^{y1} embryos expressing GFP in all endothelial cells, and development of the vasculature was monitored by fluorescence. Morpholino efficiency was confirmed by RT-PCR with primers designed over the targeted exon–intron boundary to test for omission of the targeted exon from the transcript (Supplementary Fig. 1), and showed that all morpholinos efficiently inhibited splicing of *syk* and *zap-70* at both 30 and 48 hpf with the exception of *zap-70*^{i1e2} which was more efficient at 30 hpf than at 48 hpf. The phenotypes were indistinguishable after injection of either of the two *syk* morpholinos or two *zap-70* morpholinos, suggesting specificity. At 30 hpf, ISV sprouts in control embryos have reached the dorsal aspect of the embryo, and are joining with adjacent ISVs to form the dorsal longitudinal anastomotic vessel (DLAV) (Fig. 1C). Control embryos also possess a fully formed dorsal aorta (DA) and posterior cardinal vein (PCV) at this time. Low dose injection of either morpholino (~1.5 ng of *syk*^{e2i2} morpholino (*syk*^{LO}), or ~3.4 ng of *zap-70*^{e3i3} morpholino (*zap*^{LO})) into *Tg(fli1a:EGFP)*^{y1} embryos resulted in delayed sprouting (i.e. migration) of angioblasts to form the trunk intersegmental vessels (ISVs) (Figs. 1D–E). In some instances, the sprouting of individual ISVs was completely suppressed (asterisks in Fig. 1). 89% of wild-type ISVs have reached the DLAV at 30 hpf ($n=287$), while only 3% of *syk*^{e2i2} ($n=79$), 10% of *syk*^{e7i7}($n=122$), 9% of *zap-70*^{e3i3} ($n=74$), and 14% of *zap-70*^{i1e2} ($n=189$) morphant ISVs have reached the DLAV (Supplementary Fig. 2). The morphology of these morphants is shown in Supplementary Fig. 3. Co-injection of *syk*^{LO} and *zap-70*^{LO} suggests *syk* and *zap-70* function redundantly since *syk*^{LO}*:zap-70*^{LO} double morphants show a similar but more pronounced phenotype, with increased suppression of ISV sprouts (Fig. 1F). Higher doses of *zap-70*^{e3i3} (>8 ng) resulted in a more penetrant phenotype, while injection of doses higher than 4 ng of *syk*^{e2i2} morpholino resulted in severely malformed embryos and death (results not shown).

Delay in migration of ISV angioblasts in *syk*^{LO}*:zap-70*^{LO} double morphants may result from defects in migration or proliferation. To assess whether loss of *syk* and *zap-70* leads to proliferation defects, we counted the number of angioblasts per ISV sprout in *Tg(fli1a:nEGFP)*^{y7} embryos. We have previously demonstrated that a single ISV is comprised of three to four endothelial cells with distinct fates; the ventral stalk cell, a connector cell that runs the length of the somite boundary, and a dorsal T-shaped cell that contributes to the DLAV (Childs et al., 2002). These cells can be visualized in *Tg(fli1a:nEGFP)*^{y7} embryos expressing GFP in the nuclei of their endothelial cells

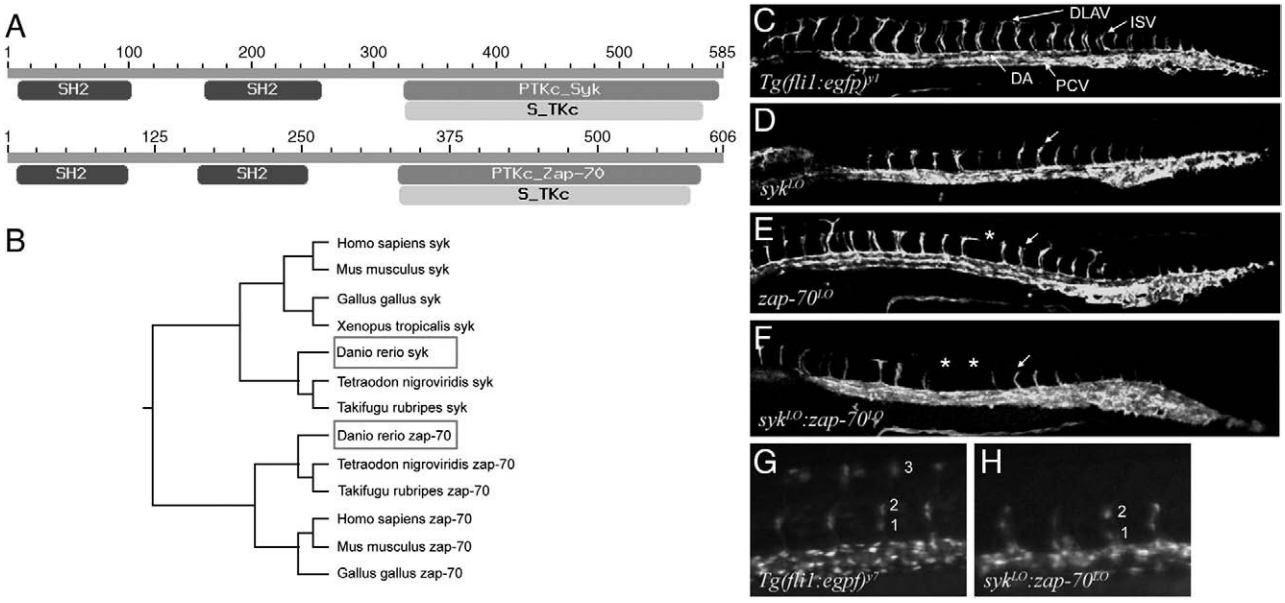

Fig. 1. The zebrafish orthologs of *syk* and *zap-70* mediate angioblast migration to form the ISVs. (A) Zebrafish *syk* and *zap-70* have similar domain structures with N-terminal SH2 domains, and a C-terminal tyrosine kinase domain (PTK). (B) Alignment of the nucleotide sequences of *syk* and *zap-70* with orthologs in other species shows a clear grouping of zebrafish *syk* with the other *syk* orthologs, and *zap-70* with the other *zap-70* orthologs. (C) At 30 hpf, ISVs in *Tg(fli1a:EGFP)*^{y1} embryos have reached the dorsal aspect of the somite and are contributing to the DLAV in wild-type embryos. (D–E) Injection of a low dose of *syk* morpholino (*syk*^{LO}, D) or *zap-70* morpholino (*zap-70*^{LO}, E) results in delayed (arrows) or completely suppressed (asterisk) angioblast migration to form the ISVs. (F) Co-injection of low doses of both morpholinos results in a more severe stalling and suppression of angioblast migration (*syk*^{LO}*:zap-70*^{LO}). (G–H) *Tg(fli1a:EGFP)*^{y7} embryos with endothelial-expressed nuclear GFP show fewer cells in the ISVs of *syk*^{LO}*:zap-70*^{LO} embryos. Anterior is to the left in all embryos. DA: dorsal aorta; PCV: posterior cardinal vein; ISV: intersegmental vessel; DLAV: dorsal longitudinal anastomotic vessel; hpf: hours post fertilization.

(Siekmann and Lawson, 2007). We found that control *Tg(fli1a:nEGFP)^{y7}* embryos show a median of four nuclei per ISV ($n=100$ ISVs; Fig. 1G) while *syk^{LO}:zap-70^{LO}* double morphants only show a median of two nuclei per ISV at the same developmental stage ($n=100$ ISVs; Fig. 1H, Supplementary Fig. 4) suggesting the cell division and migration process had not yet occurred. The angiogenic delay is not likely due to altered trunk architecture as *syk^{LO}:zap-70^{LO}* morphants with delayed vessels displayed normal somite structure as visualized by F59 fast-twitch muscle fiber staining (Devoto et al., 1996) (data not shown). We also observed that the number of cells in the trunk axial vessels (dorsal aorta and posterior cardinal vein) was reduced. An average of 95 endothelial cells was observed in the axial vessels between a region under 10 somites in wild-type embryos ($n=50$ ISVs) while an average of 71 cells was observed in the equivalent region in *syk^{LO}:zap-70^{LO}* morphants ($n=70$ ISVs; Supplementary Fig. 5). Acridine orange staining was used as a marker of cell death and to observe possible off-target effects. A small increase in cell death was observed although it did not appear to be localized to vessels (Supplementary Fig. 6). Surprisingly by 48 hpf, morphants showed some recovery to form full ISVs and the DLAV, although the morphology of the vessels was abnormal (data not shown). Since it is only angiogenesis that was delayed and not the overall growth of the embryo, and since the morpholinos still effectively inhibited splicing of *syk* and *zap-70* at this timepoint, this suggests that while *syk* and *zap-70* function in the initial migration of angioblasts from the artery, a second pathway promoting angioblast migration drives the angioblasts to their correct final position.

syk^{LO} and zap-70^{LO} morphants display late stage dorsal vascular patterning defects

At 5 dpf, wild-type *Tg(fli1a:EGFP)^{y1}* embryos show regularly patterned ISVs between somites (Fig. 2A). The dorsal-most endothelial cell in the ISV forms a clear T shape, contributing to the DLAV. There is a clear distinction between the dorsal aorta and posterior cardinal vein. The main lymphatic vessel, the thoracic duct (TD), is visible between the dorsal aorta and posterior cardinal vein. In contrast, at 5 dpf, *syk^{LO}*, *zap-70^{LO}*, and *syk^{LO}:zap-70^{LO}* double morphants have pericardial and general body edema (data not shown). Few embryos survive to 5 dpf and maintain circulation, but in those embryos with circulation we found that the ISVs appear abnormal and have mild dorsal patterning defects (asterisks, arrows, Figs. 2B–D). Although the dorsal T-cell still contributed to the DLAV, branching of the cell into the T shape was abnormal, and sometimes occurred midsomite. These defects resulted in a misshapen DLAV in morphants. Not surprisingly, the dorsal patterning defects were more pronounced in the *syk^{LO}:zap-70^{LO}* double morphants (Fig. 2D). The vertebral artery and parachordal lymphangioblasts appeared normal in the morphants.

Since *SYK* has been implicated in the development of the mammalian lymphatic system, 5 dpf morphants with a strong heart beat and circulation were analyzed for the presence of a thoracic duct by injection of 2 MDa rhodamine dextran into the vascular, but not lymphatic, circulation via the sinus venosus. Angiography depends on blood flow and can only be performed on embryos with robust

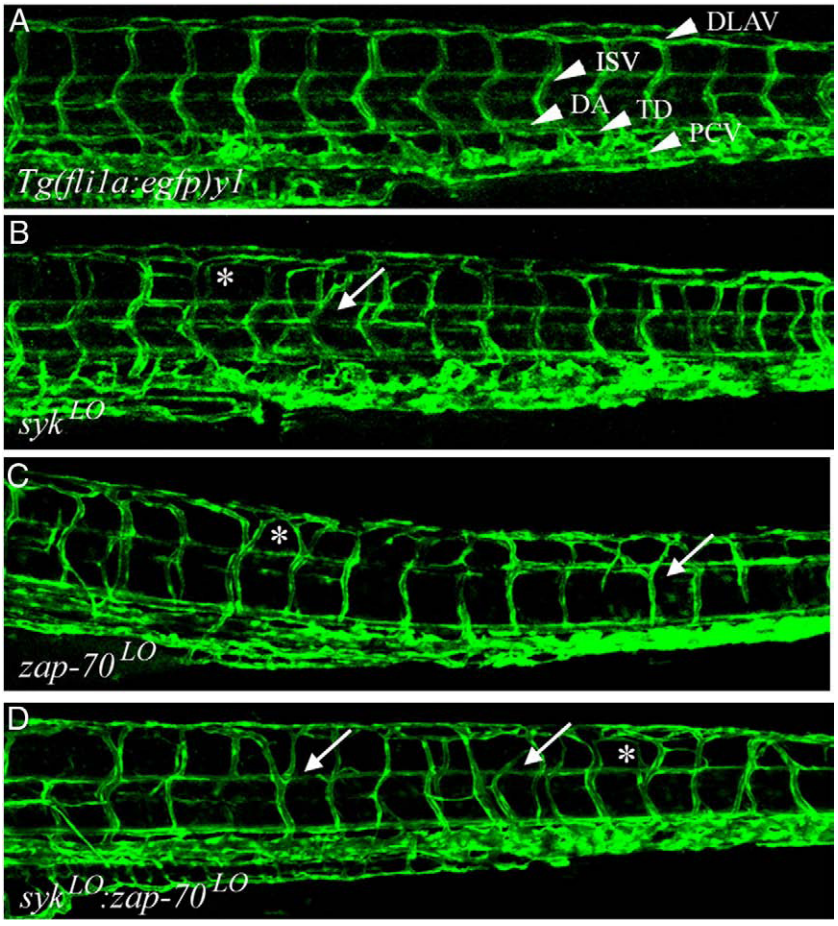

Fig. 2. *syk^{LO}* and *zap-70^{LO}* morphants display late stage ISV patterning defects. (A) The trunk of 5 dpf wild-type *Tg(fli1a:EGFP)^{y1}* embryos display regularly patterned ISVs between somites, with the dorsal-most ISV endothelial cell forming a clear T shape and contributing to the DLAV. In contrast, 5 dpf *syk^{LO}* (B), *zap-70^{LO}* (C), and *syk^{LO}:zap-70^{LO}* (D) morphants display abnormal ISV vasculature with mild patterning defects. The ventral stalk ISV endothelial cell is relatively normal, and the dorsal T-cell still contributes to the DLAV, but the branching of the cell into the T shape is abnormal (asterisks) and can occur as ventrally as midsomite (arrows). These dorsal patterning defects are more pronounced in the *syk^{LO}:zap-70^{LO}* morphants.

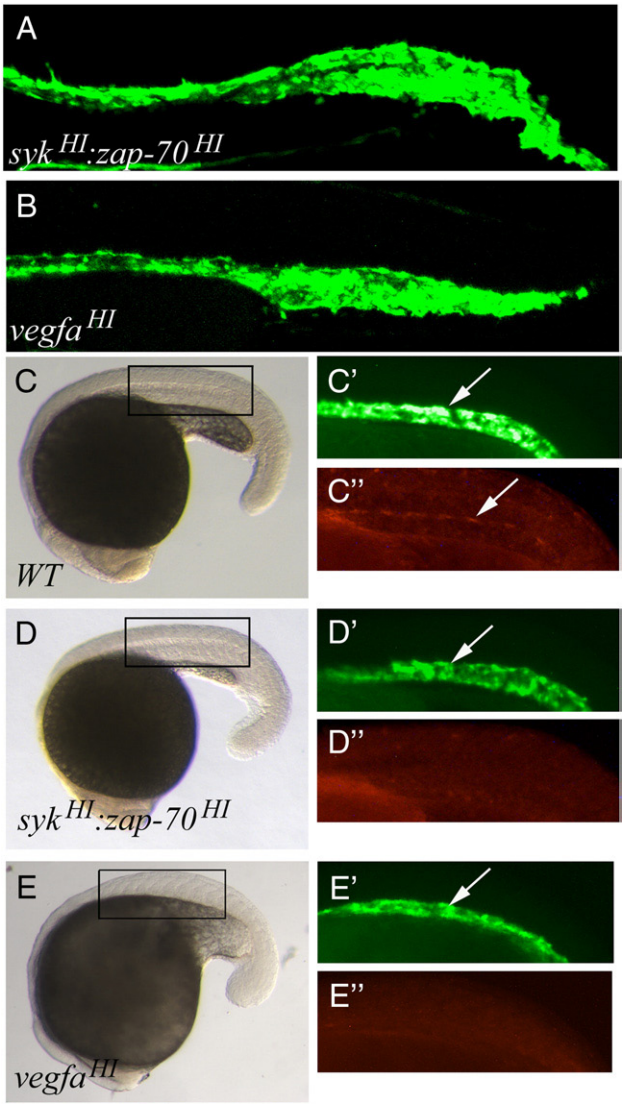

Fig. 3. *syk*^{HI}*:zap-70*^{HI} morphants resemble *vegfa* morphants and lack activated ERK. (A) *syk*^{HI}*:zap-70*^{HI} *Tg(fli1a:EGFP)*^{y1}morphants have a complete suppression of ISV sprouting which is phenotypically similar to the ISV stalling observed in high dose *vegfa* morphants (B). While the artery of 20s wildtype (WT) embryos (arrow, C') shows activated ERK (arrow, C") comparable staged *syk*^{HI}*:zap-70*^{HI} morphants (D) show a strong downregulation of activated ERK (arrow D' = artery, D"). The level of downregulation of activated ERK in the artery (arrow, E') is similar to *vegfa*^{HI} morphants (E, E"). C', D', E' are enlargements of GFP signal in the boxed region of C, D, E, respectively. C", D", E" are enlargements of the αERK-P immunohistochemistry in the boxed regions of C, D, and E respectively.

circulation. Angiography in wild-type *Tg(fli1a:EGFP)*^{y1} embryos showed a clear separation between the dorsal aorta and posterior cardinal vein, and the presence of the thoracic duct lacking dye between the two vessels, while angiography in *syk*^{LO}, *zap-70*^{LO}, and *syk*^{LO}*:zap-70*^{LO} double morphants demonstrated the clear lack of a thoracic duct between the artery and vein (Supplementary Fig. 7).

syk functions downstream of vegfa to mediate vascular growth

To observe the effect of redundancy between *syk* and *zap-70*, we co-injected higher doses of *syk*^{e2i2} and *zap-70*^{e3i3} morpholino (~2.5 ng and ~4.5 ng respectively). *syk*^{HI}*:zap-70*^{HI} double morphants had a nearly complete knockdown of both genes resulting in a severe vascular phenotype with a complete lack of ISV angioblast migration from the aorta (Fig. 3A). *syk*^{HI}*:zap-70*^{HI} double morphants did not survive past 2 dpf. This severe effect is reminiscent of the lack of sprouting observed in *vegfa* morphants (Nasevicius et al., 2000) after injection of 4 ng of morpholino targeted against *vegfa* (*vegfa*^{HI}, Fig. 3B). *vegfa*, signaling through PLCγ1 and ERK, has been previously shown to be critical for arterial ISV growth and specification (Hong et al., 2006). As both *syk* and *zap-70* can phosphorylate PLCγ1 (Law et al., 1996; Williams et al., 1999), and alterations in *syk* and *zap-70 in vitro* can affect levels of phosphorylated ERK (Griffith et al., 1998; Parsa et al., 2008; Shan et al., 2001; Slack et al., 2007) we investigated whether *syk* influences ISV growth through a pathway involving *vegfa*. Compared to wildtype embryos (Fig. 3C, C', C") di-phosphorylated ERK was strongly downregulated in the dorsal aorta of *syk*^{HI}*:zap-70*^{HI} double morphants (Fig. 3D, D', D"), similar to what is seen in *vegfa*^{HI} morphants (Fig. 3E, E', E").

The phenotypic similarity of the *syk*^{HI}*:zap-70*^{HI} double morphants to *vegfa*^{HI} morphants leads us to investigate if the lack of ISV sprouting observed in *vegfa* morphants could be rescued by over-expression of *syk*. In wild-type embryos *syk* and *zap-70* are co-expressed and are ubiquitous (Figs. 4A–D). At 16 somites (s) *syk* and *zap-70* are highly expressed in the head, with low levels of expression throughout the rest of the embryo (Figs. 4A, C). This pattern of expression continues through to 21s when the signal increases in the ventral trunk (Figs. 4B, D). *syk* and *zap-70* are thus expressed at the proper time to interact with the VEGF pathway in sprouting angiogenesis.

To determine whether *syk* is required in the blood and/or endothelial cells for vascular growth, we used the *gata1* promoter (blood) or the *fli1* promoter (endothelium) to drive expression of a C-terminal

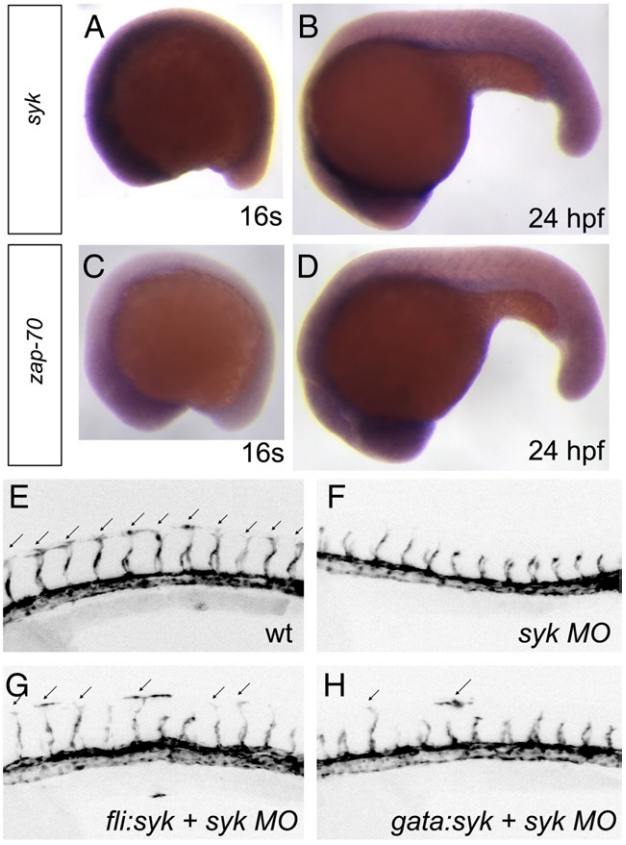

Fig. 4. *syk* rescues angioblast migration defects after *syk* knockdown. (A–D) *syk* and *zap-70* are expressed in a ubiquitous manner and have overlapping expression profiles. At 16 somites (16s) *syk* (A) and *zap-70* (C) are highly expressed in the head, with low levels of expression throughout the rest of the embryo. (B, D) By 21s increased expression is seen in the ventral trunk. (E–F) By 30 hpf, the ISVs of wildtype *Tg(fli1:egfp)*^{y1} embryos have reached the dorsal portion of the embryo to form the DLAV (E) while the ISVs of *syk* morphants are delayed at the mid-trunk (F). (G–H) Expression of a C-terminal myc-tagged *syk* in the vasculature (G, *fli1:syk:myc*) or blood (H, *gata1:syk:myc*) partially rescues the number of ISVs that have reached the DLAV (black arrow).

myc-tagged *syk* (*gata1:syk*,or *fli1:syk*). As *syk* and *zap-70* were interchangeable in previous studies (Fallah-Arani et al., 2008; Gong et al., 1997), we used *syk* in this rescue. After injection of *fli1:syk* or *gata1:syk* into *syk* morphants we observed a statistically significant increase in the number of ISVs that reached the DLAV in comparison to *syk* morphants (Figs. 4. E–H, Supplementary Table 2 and 3 respectively), suggesting that the specific lack of *syk* in an endothelial or blood population causes defects in *syk* morphants. As DNA injections are mosaic and cell autonomous, the rescued cells are seen as single cells in the DLAV without necessarily being connected to the full ISV.

We next set out to rescue *vegfa* morphants using the same constructs with the hypothesis that expression of *syk* in either tissue could rescue the *vegfa* morphant phenotype. We found that injection of *fli1:syk* or *gata1:syk* into *vegfa* morphants resulted in a significant increase in the number of endothelial cells that reached the DLAV ($p = 0.004$ or $p < 0.001$ respectively, Fig. 5; Supplementary Tables 4 and 5). Myc staining of the injected protein demonstrated that *syk* expressed under the *fli1a* promoter was clearly visible in rescued ISV cells (Fig. 5C), while myc was not detectable in ISV cells in *gata1:syk* injected embryos (Fig. 5D). This could reflect the different timings of expression of each promoter where the transgene is no longer expressed at this timepoint in *gata1:syk* embryos, or an indirect effect in promoting sprouting by *gata1:syk* (i.e. a blood cell or EPC paracrine effect on angioblasts) since expression is still observed in the dorsal aorta. The significant rescue of angioblast migration after loss of *vegfa* suggests *syk* functions in blood to effect vascular migration downstream of, or in a parallel pathway, to *vegfa*. Increased expression of *syk* in blood and endothelial cells has no effect on the growth of the ISVs in wild-type embryos (data not shown).

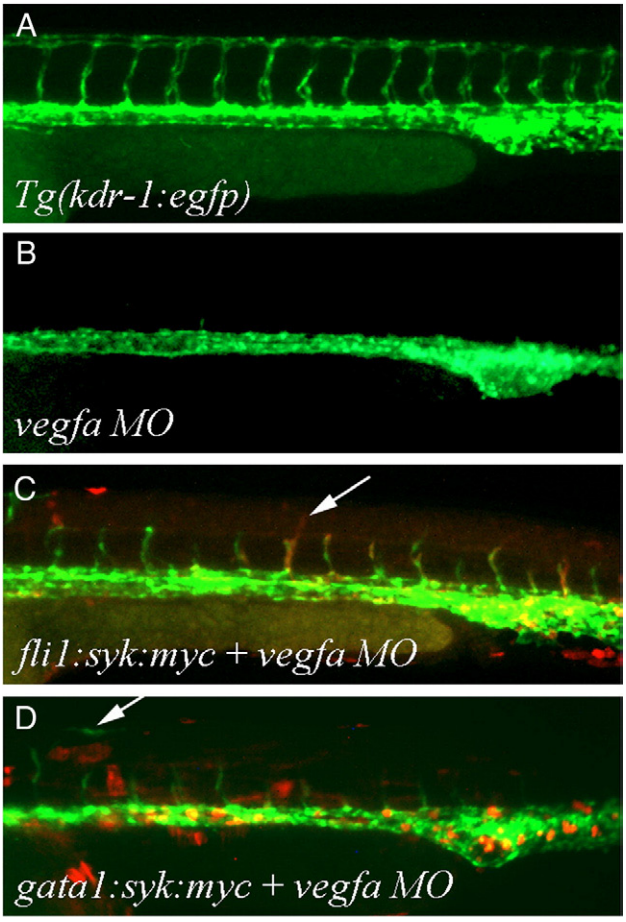

Fig. 5. Endothelial and vascular expression of *syk* rescues angioblasts migration in *vegfa* morphants. (A–B) Compared to 30 hpf wildtype *Tg(fli1:egfp)^{y1}* embryos with complete ISVs (A), *vegfa* morphants lack any ISV sprouting (B). (C–D) Expression of a C-terminal tagged *syk* under the control of a vascular *fli1* promoter (*fli1:syk:myc*, C) or a blood *gata1* promoter (*gata1:syk:myc*, D) in single celled embryos injected with 4 ng of *vegfa* morpholino results in increased number of ISV sprouting (white arrow). Expression of the *fli1:syk:myc* construct is detected by immunohistochemistry against the myc tag (red, C, D). The large myc-expressing cells visible in the dorsal and mid-trunk of *gata:syk* injected embryos are superficial and do not represent expression of the transgene in blood vessels, while ventral myc expression cells are within the axial vessels.

Discussion

The zebrafish is a powerful model system to identify genes affecting vessel growth since the vessels of the trunk contain few angioblasts and develop in a characteristic manner. Several genes have been identified to play roles in ISV migration, including *vegfa* and its receptors (Covassin et al., 2006), and downstream components of the pathway such as PLCγ (Lawson et al., 2003). We show a genetic interaction between *syk* and the *vegfa* pathway through rescue of *vegfa* morphants with over-expression of *syk* in blood and endothelial cells. *In vitro*, *Syk* has been shown to play an important role in endothelial-cell proliferation and migration in HUVEC cells in response to bFGF and VEGF (Inatome et al., 2001). A kinase dead *Syk* mutant results in decreased cell proliferation, survival, and migration, while over-expression of *Syk* results in increased levels of activated ERK (Inatome et al., 2001). Ours is the first *in vivo* evidence of *syk* affecting the migration of angioblasts, and the first evidence that *zap-70* plays a similar role.

Although downregulation of *syk* and *zap-70* results in a strong phenotype at 30 hpf, this phenotype resolves itself by 48 hpf, similar to resolution in low dose *vegfa* morphants, and *plcγ1^{y10}* mutants (Yaniv et al., 2006) which also recover from initially stalled ISV growth. This could result from genetic redundancy resulting from expression of unknown genes which compensate for the loss of *syk* and/or *zap-70*.

Here we show that *syk* and *zap-70* function redundantly in zebrafish to regulate angioblast migration to form the ISVs. After the ISVs finally finish growing in the low dose morphants, dorsal patterning defects still result. This is a common phenotype among embryos with delayed ISV sprouting and may reflect that delayed sprouting prevents angioblasts from receiving the necessary signals for late stage angiogenic patterning.

Similarly, taking into account the manner in which the zebrafish thoracic duct develops, angiogenic delay may also explain the differences in lymphatic vessel phenotypes between zebrafish *syk* morphants and *Syk* null mice. *Syk* null mice present with superficial vascular malformations resulting from the improper partitioning of the developing lymphatic system (Abtahian et al., 2003). In mammals, the main lymphatic thoracic duct develops directly off of the cardinal vein (Wigle et al., 2002). *Syk* and its substrate *Slp-76* are involved in the process that segregates these two vascular systems (Abtahian et al., 2003; Sebzda et al., 2006). If the blood and lymphatic system do not segregate properly, the blood will be free to enter the lymphatic system, resulting in blood-filled lymphatics. In zebrafish the main lymphatic thoracic duct does not directly bud off of the venous system, but instead develops by the *de novo* migration of individual cells which originate from the main venous axial vessel (Hogan et al., 2009; Yaniv et al., 2006) These cells migrate to their final location between the dorsal aorta and posterior cardinal vein to form the functional thoracic duct. In zebrafish *syk* and/or *zap* morphants, the thoracic duct is partially or completely missing. Although this might indicate that *syk* and *zap-70* play roles in both blood and lymphatic vascular patterning in the zebrafish, we suggest that the delay in angiogenesis of the blood vascular system results in a lack of formation of lymphatic vessels. For instance, if precursors are not in the correct position at the right time they may fail to receive the proper signals for their subsequent differentiation and migration.

Since knockdown of both *vegfa* and *syk*/*zap-70* affect ISV arterial angiogenesis, we hypothesized that *syk* and *zap-70* might function

redundantly through a *vegfa*/ERK pathway. We found there was a downregulation of activated ERK in syk^{HI}:zap^{HI} double morphants, and we were able to rescue the *vegfa* morphant phenotype by overexpression of *syk* under the control of a blood or endothelial-expressed promoter. These results are consistent with previous studies in mice that show Syk functions in endothelial precursor cells during the separation of the blood and lymphatic vasculature (Sebzda et al., 2006). The *vegfa* pathway is a key regulator of ISV migration and arterial specification that signals through the receptors Vegfr1/2 to activate PI3K, or PLCγ1 and ERK (Hong et al., 2006; Lawson et al., 2003). In addition, recent data shows that PLCγ2 is also necessary for lymphatic separation from the blood vasculature as mouse mutants display blood-filled lymphatics (Ichise et al., 2009). Both Syk and Zap-70 can phosphorylate PLCγ1 (Law et al., 1996; Williams et al., 1999) and activate ERK (Griffith et al., 1998; Parsa et al., 2008; Shan et al., 2001; Slack et al., 2007) *in vitro*. Although we find changes in ERK phosphorylation, we did not find any defects in markers of arterial–venous identity in *syk*/*zap-70* double morphants (data not shown). ERK is important in arterial identity of zebrafish ISVs (Hong et al., 2006), but has also been shown to regulate cell migration through direct activation of myosin light chain kinase (Klemke et al., 1997). Our results suggest *syk* and *zap-70* activate the ERK pathway that regulates cell migration, but not cell identity.

Mammalian *Syk* was first characterized as an abundant protein tyrosine kinase in thymus and spleen (reviewed in Turner et al., 2000). Though *Syk* has mostly been studied in hematopoietic tissues such as fetal liver cells and human platelets, *Syk* is also found at low levels in nonhematopoietic cells including endothelial cells, hepatocytes, neuronal cells, breast tissue and fibroblasts. We find that both *syk* and *zap-70* are ubiquitously expressed throughout the zebrafish. Interestingly, although ubiquitously expressed, downregulation of *syk* and/or *zap-70* results in specific vascular defects. Syk has been detected in endothelial cells *in vivo*, HUVECs, BAECs and endothelial precursor cells (Furuhata et al., 2007; Inatome et al., 2001; Sebzda et al., 2006; Yanagi et al., 2001). Recent studies show *syk* likely functions in circulating endothelial precursor cells which contribute to lymphatic development. EPCs have not been characterized in zebrafish, but our rescue data suggests that autonomous expression of *syk* in blood (a source of EPCs) is sufficient to promote angioblast migration. Yanagi et al. (2001), have noted that Syk-deficient mice have decreased numbers of endothelial cells and abnormal morphology (similar to the phenotype we identify in *syk* or *syk:zap-70* knockdown zebrafish embryos), but did not suggest a mechanism. Autocrine signaling of VEGF in endothelial cells has also been shown to be critical for promoting the survival of endothelial cells (Lee et al., 2007). Whether *syk* and/or *zap-70* function directly in a VEGF pathway, or indirectly by influencing ERK or other downstream signaling molecules such as PLCγ will be interesting to determine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.01.011.

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