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The Spinster homologue, Two of Hearts, is required for sphingosine 1-phosphate signaling in zebrafish

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Summary

The bioactive lipid sphingosine 1-phosphate (S1P) and its G protein-coupled receptors play critical roles in cardiovascular, immune and neural development and function [1–6]. Despite its importance, many questions remain about S1P signaling, including how S1P, which is synthesized intracellularly, is released from cells. Mutations in the zebrafish gene encoding the S1P receptor Miles Apart (Mil)/$S1P_2$ disrupt the formation of the primitive heart tube [5]. We find that mutations of another zebrafish locus, *two of hearts (toh)*, cause phenotypes that are morphologically indistinguishable from those seen in *mil/s1p2* mutants. Positional cloning of *toh* reveals that it encodes a member of the Spinster-like family of putative transmembrane transporters. The biological functions of these proteins are poorly understood, although phenotypes of the *Drosophila spinster* and zebrafish *not really started* mutants suggest that these proteins may play a role in lipid trafficking [7,8]. Through gain- and loss-of-function analyses, we show that *toh* is required for signaling by $S1P_2$. Further evidence indicates that Toh is involved in the trafficking or cellular release of S1P.

Keywords

Sphingosine 1-phosphate; G protein-coupled receptor; Spinster; cardiac development

Results and Discussion

The lysophospholipid sphingosine 1-phosphate (S1P) has emerged as a key cellular signaling molecule. Many of the relevant signaling properties of S1P are mediated via its interaction with a family of G protein-coupled receptors (GPCRs). The interaction of these receptors with

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S1P is known to affect numerous processes including the development and function of the vertebrate cardiovascular system [1,3–5]. In addition, S1P has been identified as a mediator of immune function [6]. The S1P analog FTY720 has recently been shown to function as a potent immune modulator, promising to improve the treatment of organ transplant recipients [9] as well as those suffering from pathological immune responses following infection [10]. Despite the importance of S1P signaling, little is known about the processes that make this lipid available to bind its receptors.

Previously, we have shown that the *miles apart (mil)* gene, which is required for the formation of the primitive heart tube in zebrafish, encodes the orthologue of the mammalian receptor $S1P_2$, initially called Edg5 and also known as S1PR2 [5,11]. Screening mutagenized lines of zebrafish, we have identified another recessive mutation, *two of hearts (toh)*, which causes phenotypes indistinguishable from those caused by *mil/s1p2* mutations.

At 36 hours post fertilization (hpf), wild-type embryos have a functioning heart (Figure 1A) while *toh* (Figure 1D) and *mil/s1p2* (Figure 1G) mutants exhibit pericardial edema (arrows) indicating circulatory defects. Circulatory failure and consequential pericardial edema in *toh* and *mil/s1p2* mutants is in fact the result of a defect in early heart tube formation. In zebrafish, as in all vertebrates, the primitive heart tube is formed from bilateral groups of anterior mesodermal cells. These two cell populations migrate to the embryonic midline and fuse to form a single heart tube [12]. In wild-type zebrafish embryos at 19 hpf, the ring shaped primitive heart tube has formed (Figure 1B). At 19 hpf, the myocardial cells of *toh* (Figure 1E) or *mil/s1p2* (Figure 1H) mutants have not migrated to the midline, resulting in the formation of bilateral heart-like structures, a phenotype called cardia bifida. Despite the cardia bifida, differentiation of the myocardial cells in *toh* mutants appears unaffected, as the bifid heart structures have wild-type-like chamber-specific gene expression and are infiltrated by endocardial cells (data not shown). However, the bifid heart structures in *toh* and *mil/s1p2* mutants are not appropriately connected to the vasculature and thus cannot support circulation.

In addition to cardiac defects, *toh* and *mil/s1p2* mutants also display blistering in the tip of the tail, first evident around 26 hpf (Figure 1D and G, respectively; arrowheads). This tail blister phenotype is not shared with other cardia bifida mutants in zebrafish [12], suggesting that *toh* and *mil/s1p2* may function in the same pathway. As *mil/s1p2* mutant embryos have also been shown to have defects in the morphogenesis of the anterior endoderm [5], we analysed the endoderm in *toh* mutants. The anterior endoderm of wild-type embryos, visualized at 18 hpf by the expression of the *−0.7her5:EGFP* transgene [13], forms a contiguous sheet across the embryonic midline (Figure 1C). Embryos lacking *toh* function display holes in their anterior endodermal sheet (Figure 1F), similar to *mil/s1p2* mutants (Figure 1I). These endodermal morphogenesis defects in *toh* and *mil/s1p2* mutants were also observed by examining the expression of the endodermal markers *foxa1* and *foxa2* (data not shown). Since the endoderm is required for precardiac mesoderm migration [14–17], the cardia bifida phenotype seen in *toh* and *mil/s1p2* mutants is likely due to these endodermal defects.

Due to the similarities between the *toh* and *mil/s1p2* mutant phenotypes, we hypothesized that Toh was involved in signaling by Mil/$S1P_2$. In order to test this hypothesis, we isolated the *toh* gene (Figure 2A). Positional cloning of *toh* (Figure 2A) is described in detail in the Experimental Procedures. *toh* encodes a 12 pass transmembrane domain protein of the major facilitator superfamily (MFS) of non-ATP dependent transporters (Figure 2B). The protein is a predicted 504 amino acid member of theSpinster-like family of proteins (Figure S1). This family is named after the *Drosophila spinster* gene, also called *benchwarmer* [18, 19]. The lesions found in the *spinster-like* gene in the *toh* mutant alleles are described in Figure S2.

The identification of the *spinster-like* gene as the *toh* locus was further verified by loss- and gain-of-function analyses. First, injection of a morpholino antisense oligonucleotide (MO) blocking *toh* mRNA splicing at the boundary between exon 4 and intron 4 resulted in the phenotypes seen in *toh* mutants (Figure 2C). Second, mRNA encoding the putative Toh protein rescued migration of the precardiac mesoderm when injected into maternal-zygotic toh^{s8} mutants (*MZ*toh^{s8}; generation of these embryos is described in the Experimental Procedures) and zygotic toh^{s420} mutants (data not shown), leading to functional hearts. Overexpression of the *toh* mRNA had no effect on wild-type development and did not rescue *mil/s1p2* mutants. These loss- and gain-of-function experiments together with the tight genetic linkage and presence of molecular lesions show that we have isolated the *toh* gene. Interestingly, precardiac mesoderm migration in *toh* mutants cannot be rescued by overexpressing *Drosophila spinster (spin)* or either of the two additional *spinster-like* genes found in the zebrafish genome (Figure S1), *not really started (nrs)* [20] and a gene we here name *spinster-like 3 (spinl3)*. These data indicate that the Spinster-like proteins have acquired divergent functions.

The *toh* gene is expressed dynamically during early development (Figures 3 and S3). *toh* mRNA is maternally provided (Figure S3A) and appears to be distributed ubiquitously during cleavage stages (Figure S3B). At the onset of gastrulation, the expression of *toh* changes. Cells that have undergone involution appear to express heightened levels of *toh* (Figure S3C, arrowhead). In addition, transcripts can be seen in the yolk syncytial layer, an extraembryonic tissue (Figure 3A and B, arrowhead). At the conclusion of gastrulation, *toh* is expressed strongly in tissues adjacent to the yolk cell and remains evident in the YSL (Figure 3C and D, arrowhead). This expression continues through early somitogenesis. However, as somitogenesis proceeds, expression domains of *toh* become evident in the somitic mesoderm and in the endoderm adjacent to the yolk extension. By 24 hpf, *toh* is strongly expressed in a distinct compartment of the somites (Figure S3D and E; arrowhead), in the endoderm (Figure S3D) and in the heart (Figure S3F and G).

Because *mil/s1p2* and *toh* mutants have clear defects in the morphogenesis of the anterior endoderm and both genes are expressed in the developing endoderm, we hypothesized that they might function cell-autonomously in the anterior endoderm to regulate precardiac mesoderm migration. To test this hypothesis we performed endoderm transplantation [17] in *mil/s1p2* and *toh* MO injected embryos (morphants). This technique allows one to populate the endoderm of morphants with wild-type cells while leaving other tissues untouched. In four cases, we were able to rescue the *mil/s1p2* mutant heart phenotype (data not shown). In these cases, the majority of the anterior endoderm had been replaced with wild-type cells. In cases of partial replacement of the anterior endoderm, or replacement of only posterior endoderm, no rescue was observed. However, in the case of *toh* morphants, rescue was never observed regardless of the level of endoderm replacement. Altogether, these data indicate that while *mil/s1p2* functions cell-autonomously in the endoderm to regulate precardiac mesoderm migration, *toh* functions in another cell type.

toh is clearly expressed in the YSL (Figure 3A–D), a tissue previously implicated in precardiac mesoderm migration in zebrafish [21]. In order to test whether the YSL expression of *toh* regulates precardiac mesoderm migration, we injected *toh* MO into the YSL. Injection of *toh* MO (Figure 3F), but not mock carrier solution (Figure 3E), into the YSL resulted in cardia bifida at high frequency (84%, n = 56). We also injected 200pg of *toh* mRNA into the YSL of *toh* morphants. YSL injection of *toh* mRNA was able to rescue precardiac mesoderm migration in some *toh* morphants (38%, n = 32). However, injection of equivalent amounts of *nrs* mRNA into the YSL never rescued precardiac mesoderm migration. Altogether, these data indicate that Toh function in the YSL is necessary and sufficient for precardiac mesoderm migration.

Little is known about the mechanism by which Spinster-like proteins carry out their function. However, the common phenotypes of *toh* and *mil/s1p2* mutants suggest that *toh* and *mil/s1p2* function in the same pathway. To test this hypothesis, we injected suboptimal amounts of *mil/s1p2* and *toh* MOs alone and in combination. When injected singly, 0.4 ng of *mil/s1p2* MO and 0.2 ng of *toh* MO caused cardia bifida in 2.5% (n=40) and 2.4% (n=85) of the embryos, respectively. However, when embryos were co-injected with 0.4 ng of *mil/s1p2* and 0.2 ng of *toh* MO, 85.7% (n=70) of the embryos exhibited cardia bifida. Therefore, partial loss of function of both Mil/$S1P_2$ and Toh caused cardia bifida much more frequently than the partial loss of either protein alone. We also observed that *mil/s1p2; toh* double mutants have no additional phenotypes compared to *mil* or *toh* single mutants. Together, these data suggest that Mil/$S1P_2$ and Toh may function in a common genetic pathway.

In order to test directly whether *toh* is required for Mil/$S1P_2$ signaling, we overexpressed mRNA encoding Mil/$S1P_2$ in wild-type and *toh* mutant embryos. Injection of 100 pg of *mil/s1p2* mRNA at the 1-cell stage caused severe morphological defects in wild-type embryos (89%, n = 208; Figure 4A), including cyclopia (asterisk) and disorganized body axis formation. Defects caused by *mil/s1p2* overexpression can be traced to problems with cell movements during gastrulation, as embryos overexpressing *mil/s1p2* exhibited defects in convergence, extension and epiboly movements (Figure 4D). It is likely that the gastrulation defects seen in *mil/s1p2* overexpressing embryos are due to the antagonism between *mil/s1p2* and *silberblick/wnt11* signaling [22], as mutations in *slb/wnt11* cause defects in convergence and extension movements similar in nature to those seen in *mil/s1p2* overexpressing embryos [23, 24]. If Toh function is required for signaling by Mil/$S1P_2$, one would expect that loss of Toh function would suppress the phenotypes caused by *mil/s1p2* overexpression. Indeed, *toh* mutants or morphants overexpressing *mil/s1p2* only rarely showed morphological defects similar to those seen in control *mil/s1p2* overexpressing embryos, as assessed both at 30 hpf (7%, n = 195; Figure 4B) and 8 hpf (Figure 4E). Therefore, Toh function is required for Mil/$S1P_2$ signaling in this overexpression assay. Note that these *mil/s1p2* overexpressing embryos still, however, display the phenotypes seen in *toh* mutants or morphants, including cardia bifida and blister formation in the tail, indicating that *mil/s1p2* mRNA is incapable of rescuing loss of *toh* function.

One manner by which Toh might affect the function of the Mil/$S1P_2$ receptor is by regulating the availability of the receptor's ligand, S1P. To determine whether the phenotypes of Mil/$S1P_2$ overexpression are dependent on receptor-ligand interaction, we generated a mutant form of Mil/$S1P_2$. The E129A mutation in Mil is analogous to mutations that have been shown for other S1P receptors to block the receptor's ability to interact with S1P without affecting the receptor's stability or ability to interact with downstream signaling components [25]. Embryos injected with 100 or 200 pg of *milE129A* mRNA gastrulated normally and displayed none of the phenotypes observed in embryos injected with 100 pg of wild-type *mil/s1p2* mRNA. Thus, it appears that the effects seen in Mil/$S1P_2$ overexpressing embryos require an interaction between the exogenous receptor and S1P.

Because the yolk cell contains nutrients as well as some developmental signals necessary for embryonic development [26], we hypothesized that Toh in the YSL was required to make S1P available from the yolk cell to the embryo. One prediction of this hypothesis is that the Mil/$S1P_2$ receptor should be capable of responding to exogenously applied S1P even in the absence of Toh function. To test this prediction, embryos were injected with *mil/s1p2* mRNA alone or in combination with *toh* MO. At the onset of gastrulation (~5 hpf) the embryos were also injected at the animal pole with a mock carrier solution or carrier solution containing 10 μM S1P. Embryos injected with S1P alone showed no effect (Figure 4F). However, embryos injected with *mil/s1p2* mRNA showed immediate morphogenetic effects from the application of exogenous S1P regardless of whether or not they had intact Toh expression (Figure 4H, J).

These effects were S1P dependent, as *mil/s1p2* overexpressing embryos injected with the carrier solution alone did not show this response (Figure 4G and 4I). The presence or absence of Toh function had no effect on the severity of a *mil/s1p2* overexpressing embryo's response to exogenously supplied S1P. In both groups of overexpressing embryos, gastrulation movements paused and the cells of the embryo retreated back to the animal pole, causing a thickening of the embryo (Figure 4H and 4J, asterisks). These data suggest that the reason *toh* mutants or morphants do not exhibit any defects upon *mil/s1p2* overexpression is because of a lack of interaction between overexpressed Mil/$S1P_2$ and endogenous S1P.

The above results and the fact that Toh is a member of a superfamily of transporters raise the possibility that Toh functions as a transporter of S1P. However, *Drosophila* embryos with mutations in the *toh* homologue *spin* display a dramatic expansion of the acidified compartment of the cell, as visualized by Lysotracker staining [7], accompanied by inappropriate accumulations of lipids and sugars in those cellular compartments [19,27]. Therefore, *toh* mutations might be affecting S1P release by affecting the storage and trafficking of many lipids, not just S1P or its precursors. We examined the levels of Lysotracker staining in *toh* morphants and *MZtoh*^{s8} mutants, and they appeared equivalent to those of wild-type embryos (data not shown), indicating normal lysosomal structure in *toh* mutants. We also examined the early and recycling endocytic compartments of wild-type and *toh* MO injected embryos to determine whether the earlier stages of the endocytic pathway might be disrupted in *toh* mutants. The architecture of the early and recycling endosomal compartments appeared indistinguishable between wild-type and *toh* MO injected embryos when visualized with a yellow fluorescent protein (YFP) tagged Rab5c protein [28] (data not shown). Therefore, in contrast to *Drosophila spin* mutants and zebrafish *nrs* heterozygotes [7,8], *toh* mutants do not appear to exhibit gross defects in lipid or carbohydrate trafficking. These findings, together with the failure of *Drosophila spin* and zebrafish *nrs* genes to rescue *toh* mutants, indicate that vertebrate *spinster-like* genes have diverged in function and that Toh may have a specific role in the trafficking or release of S1P.

In this study, we identify Toh as a novel component of signaling via the zebrafish $S1P_2$ orthologue, Mil. The combination of shared phenotypes between *toh* and *mil/s1p2* mutants, the fact that *toh* loss-of-function suppresses the deleterious effects of *mil/s1p2*overexpression, and the synergistic effects of suboptimal *mil/s1p2* and *toh* MO injections suggest that *toh* is a critical component of the Mil/$S1P_2$ signaling pathway. Furthermore, we have shown that the Mil/$S1P_2$ receptor is capable of signaling in the absence of Toh function when exogenous S1P is applied. Therefore, Toh appears to be a novel contributor to the biosynthesis, trafficking or release of S1P.

Previous analysis has shown that the YSL is critical for precardiac mesoderm migration [21]. This study suggested that the transcription factor gene *mtx1*, which is expressed exclusively in the YSL, regulates the deposition at the embryonic midline of Fibronectin (FN), an extracellular matrix component critical for precardiac mesoderm migration in mouse [29] and zebrafish [30]. Interestingly, the deposition of FN in *mil/s1p2* morphants is also deficient (Figure S4). Furthermore, injection of FN into the midline of *mil/s1p2* deficient embryos appears to rescue precardiac mesoderm migration [31]. Thus, *mtx1* might regulate *toh* expression or function in the YSL. The absence of Mtx1 would lead to an absence of Toh function in the YSL and therefore a lack of S1P release from the yolk. Lack of S1P release would, in turn, prevent Mil/$S1P_2$ signalingand the downstream deposition of FN required for endoderm and precardiac mesoderm morphogenesis.

The exact biochemical function of Toh and other Spinster-like proteins remains unclear. The homology of these proteins to small solute transporters suggests the interesting possibility that Toh is involved in the trafficking or actual cellular release of S1P, and that *toh* mutations affect

Mil/$S1P_2$ signaling by limiting available S1P. Our data showing that overexpressed Mil/$S1P_2$ is capable of responding to exogenously supplied S1P strongly supports the idea that the defect observed in animals lacking functional Toh/Spinl2 transporter are due to a reduction in endogenous ligand production or release. Thus, Toh is a strong candidate for a transmembrane transporter that, as previously claimed for ABCC1 [32], may be capable of moving S1P across cellular membranes to make it available for receptor-ligand interactions.

Conclusions

As the relevance of S1P signaling to both basic and clinical sciences becomes more evident, it is critical that the signaling partners of S1P receptors be identified. $S1P_2$ signaling in mammalian systems has been shown to play a vital role in activation of mast cells [33], a cell type thought to contribute to the pathogenesis of asthma [34]. In addition, $S1P_2$ receptor function is known to affect vascular tone [35] and may contribute to the protective effects S1P demonstrates during ischemic challenge to the heart during myocardial infarction [36]. The identification of Toh as a component of $S1P_2$ signaling in zebrafish suggests that the mammalian orthologues of *toh* may have similar functions in S1P mediated signaling. Therefore, Toh and its orthologues may represent new targets for the manipulation of specific S1P signaling pathways in both normal and pathological states.

Experimental procedures

Zebrafish Strains and Care

Adult and embryonic zebrafish were raised and cared for using standard laboratory procedures [37]. We used the following zebrafish mutant and transgenic strains: toh^{s8}, toh^{s220}, toh^{sk12}, toh^{s420}, mil^{m93}, $Tg(-0.7her5:egfp)^{ne2067}$ [13] and $Tg(cmlc2:\text{EGFP})^{f1}$ [38]. Maternal-zygotic *s8 (MZ*toh^{s8}*)* mutant embryos were generated by mating heterozygous toh^{s8} fish. Escaping homozygous toh^{s8} embryos were then raised to adulthood.

Immunohistochemistry, Fluorescence microscopy and confocal analysis Embryos were fixed at room temperature for 1 hour in 4% Paraformaldehyde or overnight at 4°C in 2% Paraformaldehyde in PBS. Lysotracker DND-99 (Molecular Probes) was diluted 1:200 in 1/10 Hanks Basic Salt Solution and staining was carried out on live embryos for 1 hour at 28°C, after which embryos were washed 3 times for 5 minutes with 1/10 Hanks Basic Salt Solution, then fixed and processed as above. Images were acquired using a Zeiss LSM5 Pascal confocal microscope. Wholemount fluorescence microscopy was performed with a Zeiss SteREO Lumar.V12 microscope.

Endoderm Transplantation

Endodermal cell transplantation was carried out as described [17], using 100 pg *cas/sox32* mRNA to force donor cells into the endodermal lineage. Host embryos were injected with 2 ng *mil/s1p2* MO or 1 ng *toh* MO along with 1 ng *cas/sox32* MO to deplete their endoderm.

In situ hybridization

Wholemount in situ hybridization was carried out as described [39] using the following probes: *cmlc2* [40], *foxa2* [41] and *toh.* The *toh* probe was generated using the primers 5′-TTG GAG CCA TCA CAT GTG TGA – 3′ and 5′ – TTA CTT GTT TGG CGG CTT TGT – 3′ to PCR a 516 base pair fragment of *toh*. The primers were engineered with T3 and T7 promoters, respectively to allow the generation of sense and antisense probes directly from the PCR product.

RNA overexpression and morpholino oligonucleotide generation

All capped mRNA for injection was generated using mMessage mMachine kits (Ambion). *mil* mRNA was generated as described [5]. pCS2+ *mil E129A* was generated using the QuikChange II Mutagenesis kit (Stratagene). mRNA encoding *toh* was generated by linearizing pCS2+ *toh* with NotI and transcribing with SP6 polymerase. Zebrafish *spinl3* mRNA was generated by subcloning the coding region of *spinl3* into pCS2+, linearizing this construct with NotI and transcribing with SP6 polymerase. *Drosophila spinster* mRNA was generated by subcloning *spinster-RFP* from pUAS *spinster-RFP* (gift of Sean Sweeny and Graeme Davis) into pCS2+, linearizing with NotI and transcribing with SP6 polymerase. Zebrafish *nrs* was generated from pCS2+ *nrs* [20]. *rab5c-YFP* mRNA was generated from pCS2+ Rab5c-YFP (gift of C–P. Heisenberg) as described [42] and embryos were injected with 100 pg of mRNA. The *toh* MO (5′-GCA GCT CTT ACC CTC AGT GCC CAG T –3′) was designed by Gene-Tools, Inc. YSL injections of 4ng of *toh* MO were carried out as described [43].

Exogenous S1P application

S1P (Sigma-Aldrich cat #S9666) was dissolved in 100% methanol at 1 mg/mL. This stock solution was further diluted to 10 μM concentration into a carrier solution of 250 mM potassium chloride containing 0.5% w/v Fatty Acid Free Bovine Serum Albumin (Calbiochem/EMD cat # 126575). Embryos were injected with either 100 pg *mil/s1p2* mRNA or *mil/s1p2* mRNA plus 1 ng *toh* MO at the one cell stage. Controls were not injected at this stage. At 5 hpf, the embryos were then injected into the animal pole, amongst the cells of the embryos, with 2.3 nL of 10 μM S1P solution or the carrier solution. They were aged for 30 minutes at 28°C and then imaged.

Cloning of the *toh* locus

Using bulk segregant analysis, the *toh* locus was localized to zebrafish chromosome 5. 1197 diploid *s220 toh* mutant embryos were used to narrow the affected locus to a 295 kilobase region between the CA repeat marker z9419 and a single nucleotide polymorphism (SNP) in a homologue of the mammalian *ankhzn* gene. This SNP was designated ankdCAP4 and was amplified using primers designed by the dCAPS 2.0 web-based program and detected with HinfI cutting [44]. Zebrafish CA repeat microsatellite primers were obtained from the Massachusetts General Hospital MGH/CVRC Zebrafish Server website (http://zebrafish.mgh.harvard.edu/). The 295 kilobase region is covered entirely by three bacterial artificial chromosomes (BACs): CHORI-211 134D21, CHORI-211 138A6 and DanioKey 7B17. These BACs have been sequenced and assembled by the Sanger Centre *Danio rerio* Sequencing Project. Full length sequences are available at ftp://ftp.sanger.ac.uk/pub/sequences/zebrafish. Six putative open reading frames (ORFs) were found between z9419 and ankdCAP4 using a combination of the GENSCAN exon prediction software [45] and analysis of conservation of synteny between zebrafish, mouse and human genomic sequence. These ORFs were sequenced from cDNA and genomic DNA in wild-type and mutant samples.

Supplementary Material

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Acknowledgments

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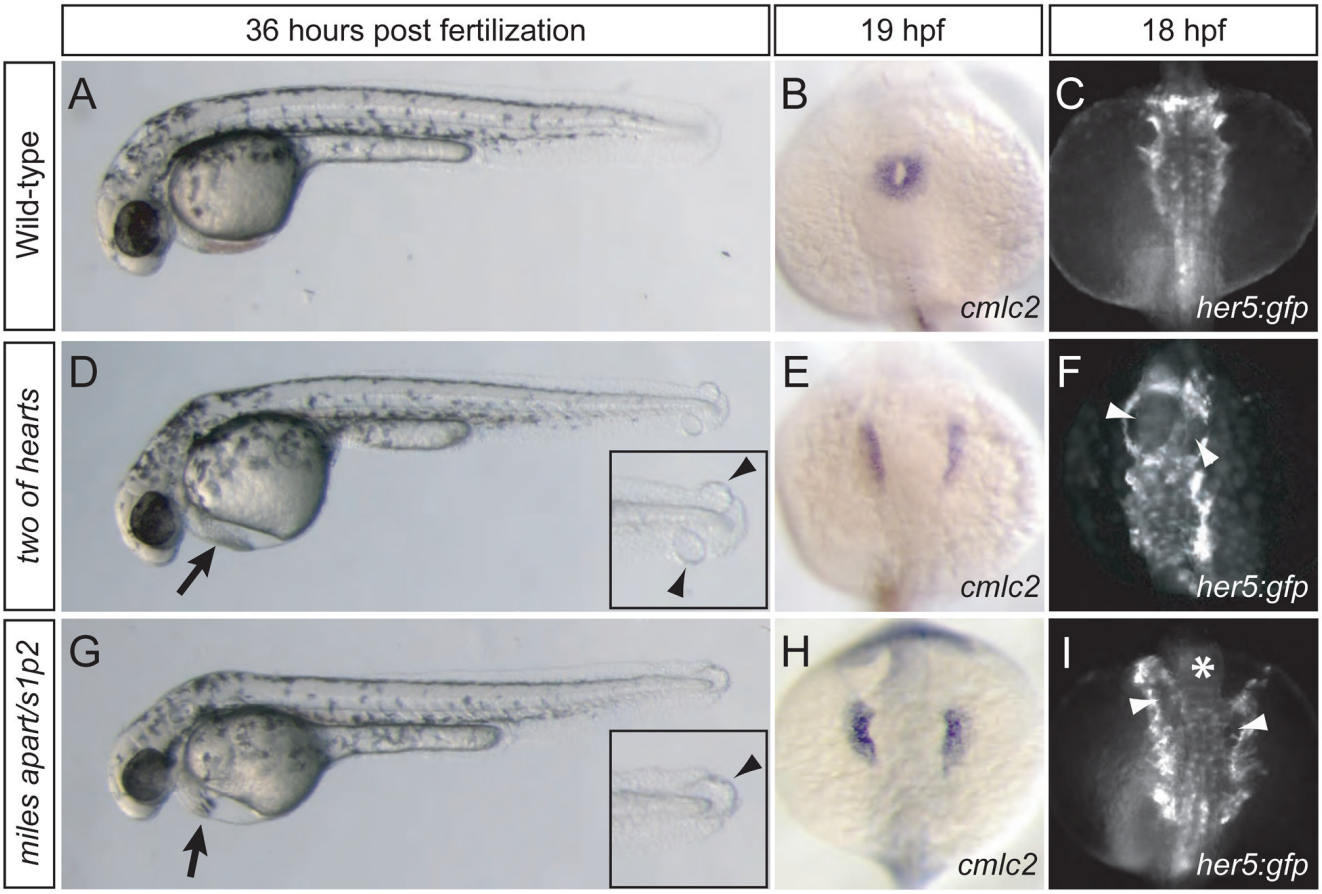

Figure 1. two of hearts and *miles apart* mutant phenotypes

Comparison of wild-type (A–C), *toh* mutant (D–F), and *mil/s1p2* mutant (G–I) embryos. (A, D and G) Lateral brightfield images, anterior to the left, at 36 hpf show pericardial edema (arrow) and epidermal blisters (insets, arrowheads) in the tails of toh^{sk12} (D) and $mil/s1p2^{m93}$ (G) mutants. (B, E and H) Examination of *cmlc2* expression at 19 hpf shows heart-ring formation in wild-type embryos (B) and a failure in precardiac mesoderm migration in toh^{s420} (E) and $mil/s1p2^{m93}$ (H) mutant embryos. Dorsal views, anterior up. (C, F and I) Visualization of anterior endoderm by $Tg(-0.7her5:EGFP)^{ne2067}$ expression at 18 hpf. In embryos injected with *toh* (F) and *mil/s1p2* (I) MOs, numerous gaps (arrowheads) appear in the endodermal sheet which is also irregularly shaped. The most anterior region of *mil/s1p2* morphants lack GFP positive endodermal cells at the midline (asterisk). Dorsal views, anterior up.

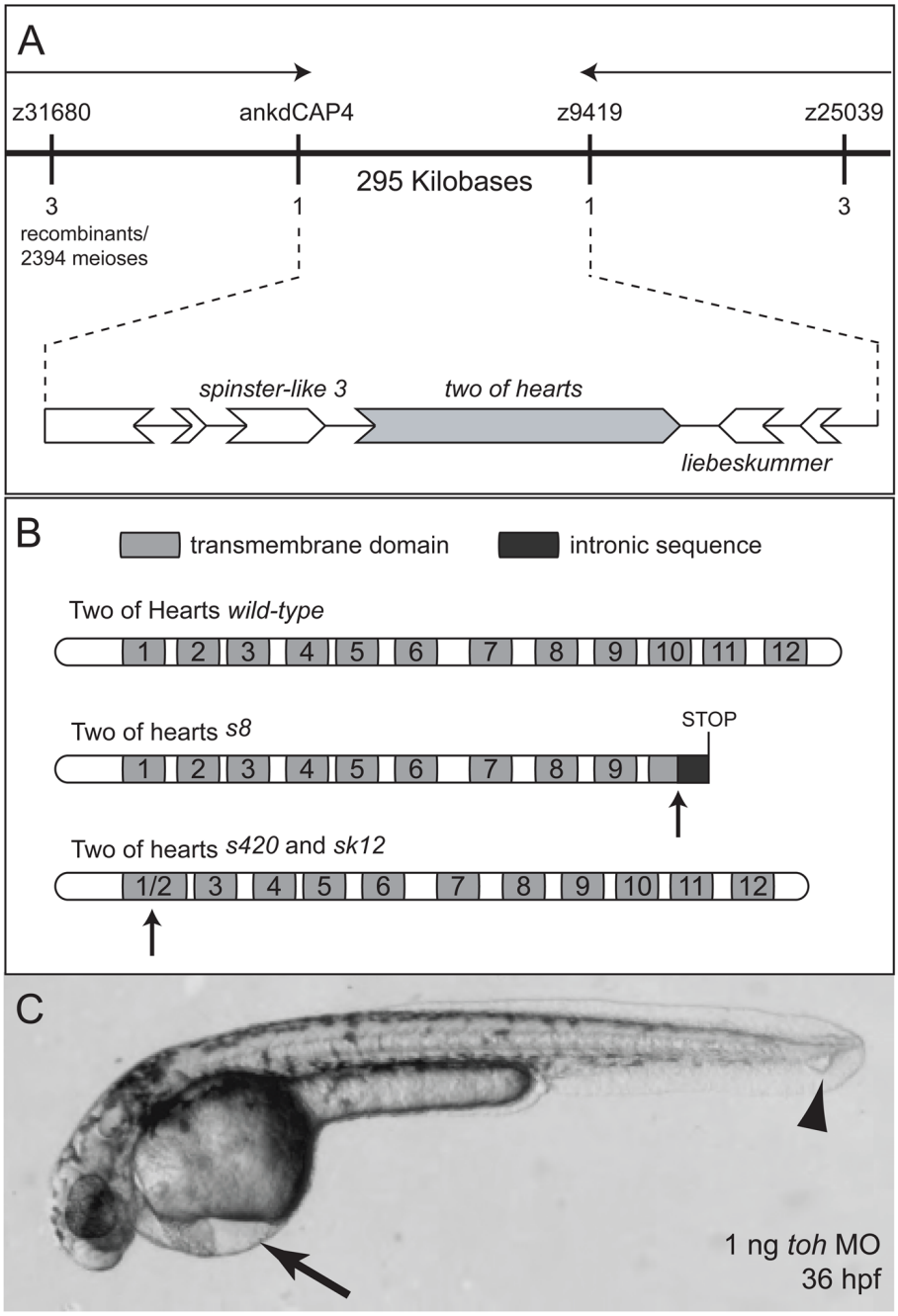

Figure 2. Isolation of the *two of hearts* gene

(A) Positional cloning of *toh*. Direction of the chromosomal walk is indicated with black arrows above the marker names. Markers used for mapping are indicated above the line representing the genomic region. The numbers of recombination events out of 2394 meioses found at each marker are indicated below the genomic region. A magnification of the critical region depicts portions of 6 open reading frames (open arrows) in the identified genetic interval, including the previously cloned locus *liebeskummer/reptin*. The critical region contains two *spinster-like* genes, one of which was identified as *toh* (gray filled arrow) and the other we name here *spinl3*. (B) Schematic diagram of Toh proteins produced from each allele. Arrows point to the site affected in the mutant alleles. Transmembrane domains are indicated in blue and the

predicted translated intronic sequence in the *s8* allele is indicated in red. Numbers also identify the individual transmembrane domains. (C) Injection of a splice blocking MO against *toh* into wild-type embryos phenocopies the *toh* mutations, leading to pericardial edema (arrow) and blistering in the tail (arrowhead).

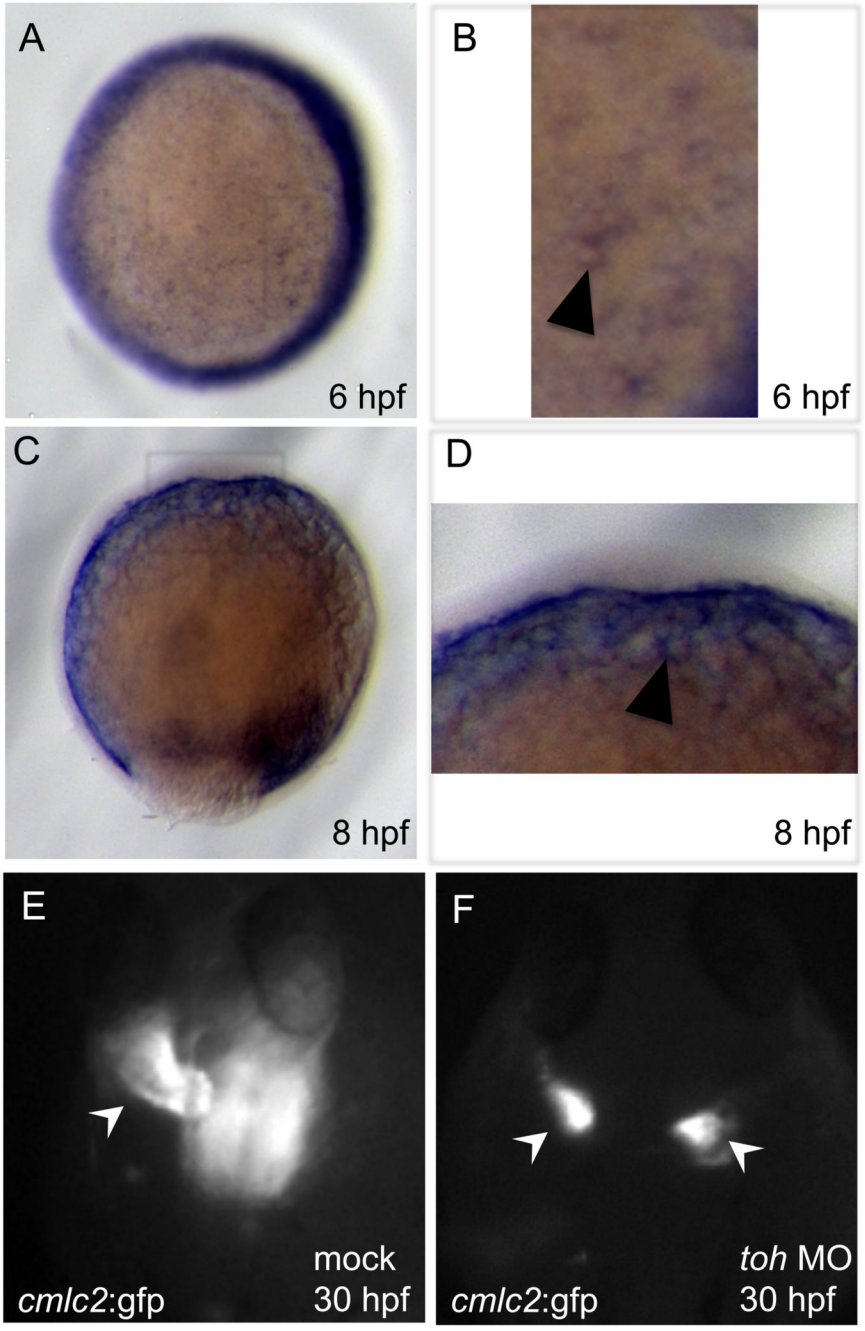

Figure 3. Toh function in the YSL is required for precardiac mesoderm migration

toh expression at 6 (A and B) and 8 (C and D) hpf, dorsal to the right. (A) Animal pole view, 6 hpf, showing *toh* expression around the margin and diffusely throughout the YSL. (B) Magnified view of the box in (A) showing *toh* expression around the YSL nuclei (arrowhead). (C) Lateral view, 8 hpf, showing continued *toh* expression in cells that have involuted, as well as in the YSL. (D) Magnified view of the box in (C) showing pronounced *toh* expression in the YSL (arrowhead). (E and F) Dorsal images of *Tg(cmlc2:GFP)*^{f1} embryos injected into the YSL with mock solution (E) or 4 ng *toh* MO (F) and visualized at 30 hpf. Mock injected embryos have a single heart tube (E; arrow), while embryos with loss of Toh function in the YSL very frequently (84%, n = 56) display cardia bifida (F; arrows).

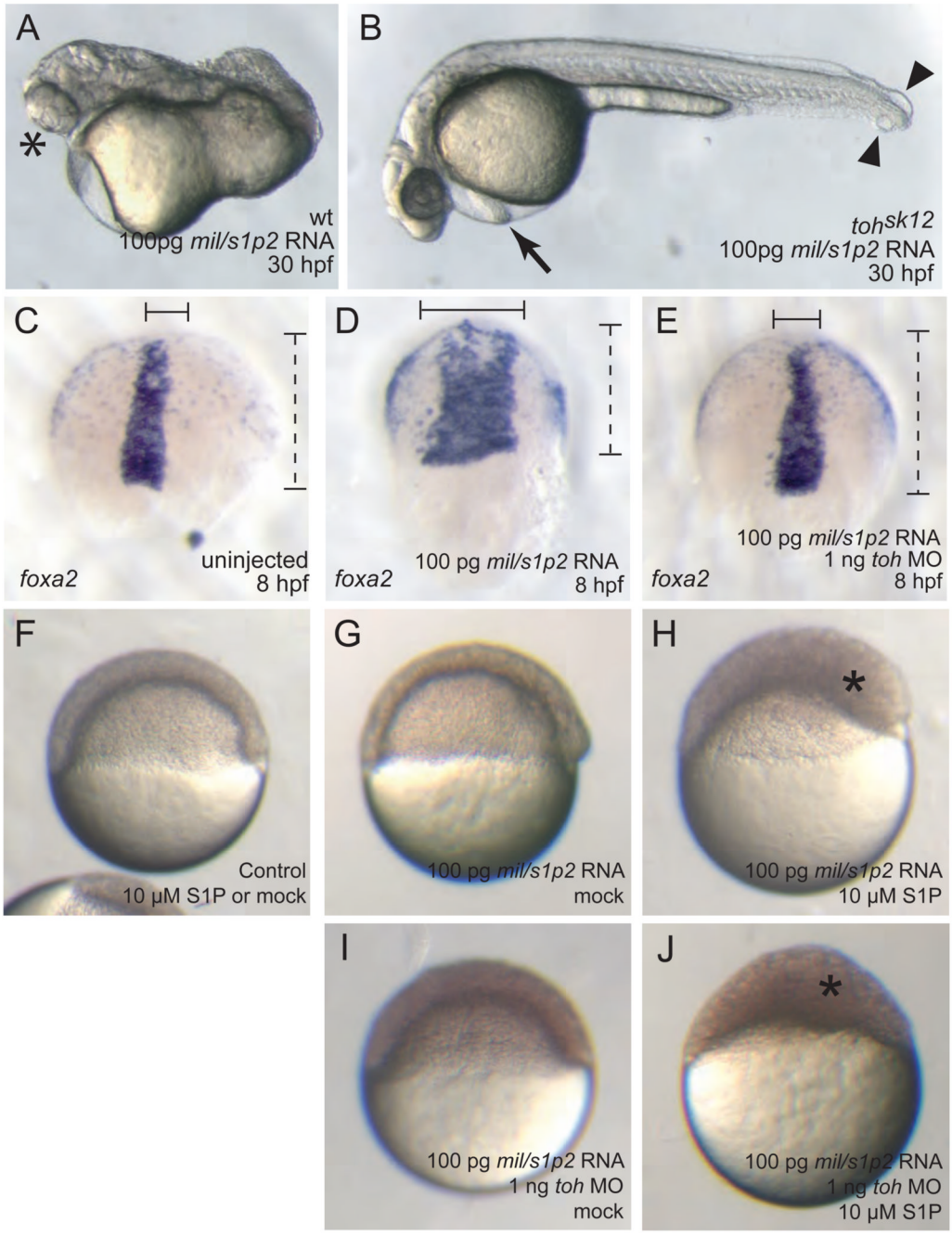

Figure 4. Exogenous S1P substitutes for Toh function in Mil/S1P$_2$ overexpressing embryos

(A and B) Lateral views, anterior to the left, of embryos overexpressing *mil/s1p2* at 30 hpf. Wild-type embryos injected with 100 pg of *mil/s1p2* RNA (A) display a shortened body axis and cyclopia (asterisk). *MZtoh*^{s8} mutants injected with 100 pg of *mil/s1p2* RNA have no body axis defects (B) but develop pericardial edema (arrow) and tail blisters (arrowheads). (C–E) Visualization of axial mesoderm and endoderm by expression of *foxa2* at late gastrula stages, dorsal views, anterior up. Wild-type embryos overexpressing *mil/s1p2* (D) have broadened axial mesoderm (solid line) compared to uninjected embryos (C). Progression of epiboly (dashed line) is also impaired in embryos overexpressing *mil/s1p2* as compared to uninjected siblings. *toh* MO injected embryos overexpressing *mil/s1p2* (E) are indistinguishable from

uninjected siblings (C). (F–J) Lateral views of 5.5 hpf embryos injected with mock carrier solution or 10 μM S1P. Control embryos show no response to exogenous S1P (F). Embryos overexpressing Mil/$S1P_2$ do not show more severe phenotypes when injected with mock carrier solution at 5 hpf (G). Mil/$S1P_2$ overexpressing embryos injected with a 10 μM S1P solution at 5 hpf exhibit severe phenotypes (H), including an exaggerated thickened layer of cells at the animal pole (asterisk). Mil/$S1P_2$ overexpressing embryos coinjected with *toh* MO show no phenotypes when injected with mock solution (I) and resemble controls (F). When injected with a 10 μM S1P solution, Mil/$S1P_2$ overexpressing embryos lacking Toh function show severe defects (J), including a thickened layer of cells at the animal pole (asterisk).