Supporting Online Material for

The Sphingolipid Transporter Spns2 Functions in Migration of Zebrafish Myocardial Precursors

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Published 11 December 2008 on Science Express
DOI: 10.1126/science.1167449

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Materials and Methods

Isolation of ko157 and genotyping: The ko157 was an embryonic lethal mutant presenting cardia bifida. For meiotic mapping, the ko157 was crossed to TL strain. The ko157 locus was defined by genotyping of ko157 mutant embryos (400 diploid) using SSLP markers on the linkage group 5. We identified single amino acid substitution (R153S) in Spns2 protein. Genomic DNA from ko157 mutant and wild-type was sequenced to confirm the mutation. For genotyping of the ko157 mutant, the ko157 locus was amplified by PCR using the following primers: ko157-S; 5’-AACGTTCTGATCATGCAAAC-3’, ko157-AS; 5’-ATTTTTGGGACTCACCTTCC-3’. Genotypes were determined by direct sequencing of the amplified fragments.

Morpholinos: spns2 MO (targeting for the exon3-intron3 splicing site for spns2), 5-mis MO (5 base mis-matched control morpholino for spns2 MO), mil MO and spns1 MO morpholinos (Gene Tools, LLC) were used; spns2 MO, 5’-GGAGGGAATATGTGATGCTTACTTC-3’; 5-mis MO, 5’-GGAGCGAATTTGTCATGGTTAGTTC-3’; mil MO, 5’-CCGCAAACAGACGGCAAGTAGTCAT-3’; spns1 MO, 5’-ATCTGCTTGTGACATCAGCTGCTGGA-3’. Morpholinos (spns2 MO or 5-mis MO) were injected into the yolk of 3 hpf stage embryos or into the YSL of shield stage embryos. mil MO was injected into the yolk of 1-2 cell stage embryos or into the YSL of shield stage embryos. Both spns2 and spns2(R153S) mRNAs were synthesized using
mMESSAGEmMACHINE kit (Ambion). Synthetic mRNA of either spns2 or spns2(R153S), was injected into the blastomere of 1-2 cell stage embryos or into the YSL of shield stage embryos. Morpholinos and synthetic mRNAs were dissolved in injection buffer (40 mM HEPES [pH 7.4], 240 mM KCl and 0.5% phenol red). Injection of mRNAs or morpholinos in the YSL was confirmed by the distribution of co-injected FITC-dextran and/or phenol red.

**Transgenic zebrafish:** We constructed a reporter plasmid DNA pTol-cmlc2P-mRFP with the promoter region of cmlc2 fused to mRFP. We established two transgenic lines cmlc2:RFP^ko07 and cmlc2:RFP^ko08 that stably expressed mRFP in developing myocardial cells. We used both Tg(cmlc2:mRFP) transgenic lines throughout the experiments in the present study.

**RNA probes:** Antisense RNA probes labeled with digoxigenin (DIG) for spns2, cmlc2, nkx2.5, fli1, amhc, vmhc, sox17, foxa2, hand2, gata1 and pax2 were prepared by using the RNA labeling kit (Roche). Whole-mount in situ hybridization was performed as previously described (1).

**Measurement of S1P export from the cells:** To examine the S1P export, the CHO cells constitutively expressing mouse sphingosine kinase 1 (mSphk1) were generated (CHO-SphK cells) by using the flp-in system (Invitrogen). Cells were cultured under 5% CO2 at 37°C. Expression plasmids (Spns1-EGFP or Spns2-EGFP) were transfected into the CHO-SphK cells (1x10^5 cells) using the Lipofectamine 2000 reagent (Invitrogen). After
6-16 h incubation, medium was changed to F12 medium (Sigma) with 10% fetal bovine serum (FBS) and 10 mM fumonisine B. The cells were incubated with $[^{3}H]$sphingosine for 30 min in F12 medium with 10% FBS. Subsequently, medium (supernatant: S) and cells (pellet: P) were separately subjected to lipid extraction. Extracted sphingolipids were analyzed by thin layer chromatography (TLC) and radioactive bands corresponding each spingolipid were obtained by FLA-3000 Bioimaging Analyzer (Fuji Film). We used F12 medium with 10% FBS for $[^{3}H]$S1P release assay, because the amount of the $[^{3}H]$S1P released in F12 medium with 10% FBS was greater than that with 1% BSA.

**Measurement of endogenous S1P by HPLC:** The endogenous S1P released from the cells was measured by the o-phthalaldehyde (OPA) modification followed by HPLC analysis as a modified protocol from Min et al. (2). Briefly, CHO-SphK cells (4x10⁵ cells) were transfected with either EGFP, zSpns2-EGFP or zSpns2-R153S-EGFP expression plasmid. After 24 h, the medium (F12 medium with 10% FBS) was changed to releasing medium (F12 medium with 1% BSA, 10 mM sodium glycerophosphate, 5 mM sodium fluoride and 1 mM semicarbazide). After 12 h incubation, 100 μl aliquots of the medium were transferred to new tubes and subjected to lipid extraction in alkaline chloroform condition. C₁₇-S1P (30 pmol) was added to each sample as an internal standard. Extracted S1P was dephosphorylated with calf intestinal alkaline phosphatase (30 units) for 90 min at 37°C. The resulting sphingosine was extracted with chloroform, dried and resuspended in ethanol. OPA modification was performed at room temperature for 1 h. After centrifugation of the samples, 15 μl out of total sample (135 μl) was analyzed by HPLC (Hitachi) with Cosmosil
Determination of amount of the cellular sphingolipids: Cellular S1P and sphingosine contents were measured with OPA modification and HPLC analysis by the aforementioned method. For quantification of sphingosine, C17-sphingosine was added to each sample as an internal standard.

The amount of cellular ceramide and sphingomyeline was determined by the diacylglycerol kinase (DGK) assay and sphingomyelinase assay, respectively. Cellular lipids were extracted with Bligh and Dyer extraction. For DGK assay, lipids were suspended in assay buffer (40 mM HEPES-KOH [pH7.3], 80 mM KCl, 1 mM MgCl2) with mixed micelles, 0.1 unit of \textit{E. coli}. DGK and 1 μCi of [\textsuperscript{32}P]-ATP (370 GBq/mmol), and incubated for 1 h at 37°C. Lipids were extracted again and separated by Silica Gel 60 TLC plate (Merck) in Chloroform/acetone/methanol/acetic acid /water (10/4/3/2/1). Corresponding bands of ceramide 1-phosphate derived from ceramide were quantified by using FLA3000 Bioimager (Fuji Film). Sphingomyelinase assay was performed with Amplex Red sphingomyelinase kit (Invitrogen) or sphingomyelin assay kit (Cayman) according to the manufacture’s protocol.

For quantification of cellular ceramide 1-phosphate content, cells were metabolically labeled with \([\textsuperscript{3}H]\)serine (1.07 TBq/mmol) for 1 to 2 days. Cells were washed with PBS and detached from the plate with trypsin treatment. Cell suspensions (500 μl) were subjected to lipids extraction with 1.875 ml of chloroform/methanol/HCl (100:200:1), 625 μl of chloroform and 625 μl of 1% KCl. Extracted lipids were separated by TLC in
chloroform/methanol/acetic acid (65:15:5) and radioactive bands were quantified with FLA3000 Bioimager (Fuji Film).

**Cell death assay:** CHO cells (4x10⁵ cells) were transfected with each expression plasmid (4 μg). After 24 h, cells were stained with 0.1 mg/ml propidium iodide (PI) solution for 15 min at 37°C. Subsequently, fluorescence images were collected with BioRevo fluorescent microscopy (Keyence, Japan). The number of PI-positive cells (> 500 cells) was counted in each well to examine the effect of either Spns1-EGFP or Spns2-EGFP on cell death.

Apoptotic cells in zebrafish embryos were detected by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay (3) and acridine orange staining. For TUNEL assay, the fixed embryos were incubated overnight at room temperature with TdT buffer containing TdT (terminal deoxynucleotidyl transferase) (Invitrogen) and DIG-dUTP (Roche). Embryos were incubated with 1 mM EDTA in PBS at 65°C for 30 min and washed with PBS four times. Embryos were incubated overnight at 4°C with anti-DIG alkaline phosphatase (Roche) in blocking solution (0.1 M maleic acid [pH 7.5], 150 mM NaCl, 5% sheep serum and 2% blocking reagent). After washing with PBS, apoptotic cells were detected by incubation with NBT (4-nitroblue terazolium chloride) and BCIP (5-bromo-4-chloroindolyl-phosphate) in reaction buffer (100 mM Tris [pH9.5], 50 mM MgCl₂, 100 mM NaCl and 0.1% Tween 20). For acridine orange staining, live embryos were stained for apoptotic cells with the vital dye acridine orange (5 μg/ml in E3 medium) for 20 min in the dark. Embryos were washed with E3 medium and analyzed under a fluorescence microscopy.
RT-PCR: Total RNA was prepared from the embryos injected with spns2 MO (10 ng) or 5-mis MO (10 ng) and reverse-transcribed. spns2 was amplified by PCR using the following primers: 5’-GACATACAGAAACAATTC-3’, 5’-TTTAGCAATTGATCCCAG-3’. To amplify EF-1α, the following primers were used; 5’-TCACCCTGGGAGTGAAACAGC-3’ and 5’-ACTTGCAGCGATGTGAGCAG-3’.

Transplantation: Donor embryos were labeled by the injection of 5% fluorescein isothiocyanate (FITC)-dextran (ICN Biomedicals, Inc) at 1-2 cell stage embryos. Labeled cells were removed from the lateral margin of shield stage embryos and were transplanted into the lateral margin of recipient embryos.

Transverse section: Embryos stained with antisense spns2 probe were dehydrated with acetone. Embryos were embedded in Technovit 8100 (Heraeus Kulzer) and 10 μm thick transverse sections were sliced by a microtome (Carl Zeiss, 4HM325). Sections were visualized by optical microscopy (Carl Zeiss, Axioplan2).
**Figure S1** Schematic representation of Spns2 function. (A) Spns2 can mediate the secretion of S1P as a S1P transporter. Binding of S1P to S1P2 initiates cellular responses. (B) Spns2 in the YSL is required for the migration of myocardial precursors. The activation of S1P2 by S1P in the mesoderm just lateral to the midline would contribute to the migration of myocardial precursors.

**Figure S2** Expression of cardiac markers and vascular network formation in *ko157* mutant. (A to F) Whole-mount *in situ* hybridization with the probes indicated at the bottom right. Wt embryos express cardiac markers, *atrial myosin heavy chain (amhc)*, *ventricular myosin heavy chain (vmhc)* and *nkx2.5* as one cylinder shape signal in the heart (A to C; arrowheads) at 24 hpf. In contrast, these cardiac markers were expressed bilaterally in *ko157* mutants (D to F; arrowheads). (G to J) Vascular development visualized by EGFP expression under the control of *fli1* (an endothelial cell specific gene) promoter at 34 hpf was comparable between Wt and *ko157*.

**Figure S3** Expression of mesoderm and endoderm markers in *ko157* mutants. (A to D) Whole-mount *in situ* hybridization with double probes. (A and B) *hand2* (lateral plate mesoderm marker; black arrows) and *pax2* (pronephric marker; yellow arrows) were expressed bilaterally in Wt and *ko157* embryos at 24 hpf. (C) *gata1* were expressed at the midline (erythroid marker; blue arrows) in Wt and *ko157* embryos at 24 hpf. (D) *foxa2* expression in pharyngeal endoderm (orange arrows) at 24 hpf was comparable between Wt and *ko157*. *ko157* mutant embryos were determined by the bilateral expression of cardiac
marker cmle2 (A to D; red arrowheads). (E and F) Whole-mount in situ hybridization with antisense sox17 (E) and foxa2 (F) probes. Endoderm markers sox17 (E) and foxa2 (F) were expressed in endoderm in all embryos from the intercross of ko157 heterozygous carriers (sox17; n=30, foxa2; n=26) at bud stage. foxa2 is also expressed in the notochord (green arrowheads). Twenty-five percent of the embryos from the intercross of ko157 heterozygous carriers were expected to be ko157 mutants.

**Figure S4** Morphological phenotypes of mil MO-injected embryos. (A and B) Two swollen pericardial sacs (B; arrowheads) at 54 hpf were observed in mil MO (15 ng)-injected embryo. (C) Cardia bifida (arrowheads) in mil MO (15 ng)-injected embryo at 28 hpf. (D) Tail blister (arrow) in mil MO (15 ng)-injected embryo at 54 hpf.

**Figure S5** Predicted protein sequence of the Spns2s. Sequence alignment of human (hSpns2), mouse (mSpns2) and zebrafish (zSpns2) Spns2 proteins. Identical amino acids are boxed. Asterisk shows the conserved arginine that is substituted to serine in the ko157 mutant. Dashes indicate gaps to optimize sequence alignment.

**Figure S6** Depletion of Spns1 does not affect cardiac morphogenesis. (A to D) Uninjected embryos. (E to H) spns1 MO (8 ng)-injected embryos. Cardiac morphology was comparable between uninjected (A) and spns1 MO-injected (E) embryos at 28 hpf. Cardia bifida was observed in both ko157 (C) and spns1 MO-injected ko157 (G) embryos. The efficacy of spns1 MO was confirmed at 50 hpf by an accumulation of an opaque substance in the yolk of the embryos injected with spns1 MO (F and H: arrows). After examining the morphology, genotypes of the embryos were analyzed.
Figure S7 Effect of spns2 MO on the production of the mature form of spns2 mRNA. spns2 MO (10 ng) or 5-mis MO (10 ng) was injected in the yolk of 1-2 cell stage embryos. RT-PCR analysis showed that spns2 MO injection, but not 5-mis MO injection, prevented the production of the mature form of spns2 mRNA. The amplification of EF-1α was comparable in uninjected, 5-mis MO-injected and spns2 MO-injected embryos.

Figure S8 Overexpression of high doses of spns2 mRNA causes severe defects in the trunk and tail. (A and E) Uninjected embryos. (B, D and F) spns2 mRNA (500 pg)-injected embryos. (C, G and H) spns2 mRNA (1 ng)-injected embryos. Injection of high doses of spns2 mRNA (500 pg or 1 ng) resulted in severe defects with abnormal vascular patterning in the trunk and the tail at 28 hpf. In contrast, morphology of the head and heart in the spns2 mRNA-injected embryos seems to be normal.

Figure S9 Rescue of the tail blisters in ko157 mutant by spns2 mRNA injection. (A and B) Synthetic spns2 mRNA (250 pg) or spns2(R153S) mRNA (250 pg) was injected into the embryos derived from ko157 heterozygous carriers. Tail blisters in the ko157 mutant at 54 hpf were substantially recovered by mRNA injection of spns2, but not spns2(R153S). After examining the morphology, genotypes of the embryos were analyzed.

Figure S10 Rescue of the cardiac defect in ko157 mutant by human Spns2 mRNA injection. (A to C) Synthetic mRNA (250 pg) for hSpns2(R199S), hSpns2 or hSpns1-EGFP was
injected into the embryos from the intercross of *ko157* heterozygous carriers. The morphological heart defect in the *ko157* mutant at 54 hpf was rescued by mRNA injection of *hSpns2*, but neither *hSpns2(R199S)* nor *hSpns1-EGFP*. After examining the morphology, genotypes of the embryos were analyzed.

**Figure S11** Depletion of Mil/S1P2 does not affect the *spns2*<sup>ko157</sup> phenotypes. (A to D) Overall morphology and cardiﬁda are comparable between *mil* MO (15 ng)-injected Wt embryo and *mil* MO (15 ng)-injected *spns2*<sup>ko157</sup> embryo at 28 hpf. After examining the morphology, genotypes of the embryos were analyzed.

**Figure S12** Effect of *spns2* mRNA injection on the *mil/S1P2* morphant. Injection of *mil* MO (15 ng) in the yolk at 1-2 cell stage induced cardiﬁda at 26 hpf (A). Cardiﬁda in *mil* MO (15 ng)-injected embryo was not restored by the subsequent injection of *spns2* mRNA (250 pg) in blastomere at 2-4 cell stage.

**Figure S13** A time-dependent spontaneous S1P release from the CHO-SphK cells expressing Spns2-EGFP. The CHO-SphK cells transfected with indicated expression vector were incubated with [³H]sphingosine. [³H]S1P was converted from [³H]sphingosine by cellular SphK and the secreted [³H]S1P in the medium from the cells was measured at the indicated time point. The increased release of [³H]S1P was observed in a time dependent manner in zSpns2-EGFP-transfected cells (open circle), but neither EGFP-transfected cells (open diamond) nor zSpns2(R153S)-EGFP-transfected cells (filled circle). Each point represents the mean of three individual experiments with SD.
Figure S14 Endogenous S1P release from cells expressing Spns2-EGFP. The endogenous S1P released from the CHO-SphK cells expressing EGFP, zSpns2-EGFP or zSpns2(R153S) was separated and calculated with C_{17}-S1P (internal standard) by HPLC. The release of endogenous S1P was observed in zSpns2-EGFP-transfected cells, but neither in EGFP- nor in zSpns2(R153S)-EGFP-expressing cells. The S1P concentration in the medium of zSpns2-EGFP-transfected CHO-SphK cells was approximately 0.36 μM.

Figure S15 Overexpression of Spns2-EGFP does not alter overall sphingolipid metabolism. We quantified cellular sphingolipids as described in "Materials and Methods". The amount of cellular sphingolipids, sphingomyelin, ceramide, ceramide 1-phosphate (C1P), sphingosine and sphingosine 1-phosphate (S1P) in the CHO-SphK cells expressing either EGFP, zSpns2-EGFP- or zSpns2(R153S) is shown. The amount of each sphingolipid was divided by the amount of proteins of the cells from which each lipid were extracted. Comparable cellular sphingolipid metabolism was observed among the three groups. The column represents the mean of two or three independent experiments with SD.

Figure S16 Introduction of Spns1-EGFP induces cell death to an extent comparable to that of Spns2-EGFP. Cell death induced by Spns2-EGFP or Spns1-EGFP was examined by propidium iodide (PI) staining and the percentage of PI-positive dead cells was expressed.

Figure S17 Overexpression of Spns2-EGFP does not alter the Sphingosine kinase activity
in culture medium. Sphingosine kinase activity outside of the cells expressing either EGFP, zSpns2-EGFP or zSpns2(R153S)-EGFP was measured in the culture medium of those cells. The activity of Sphingosine kinase was analyzed by the conversion of $[^3]H$sphingosine to $[^3]H$S1P. Lipid extracts were separated on a TLC plate and bands corresponding to $[^3]H$S1P were quantified. The relative amount of $[^3]H$S1P in the medium of the cells expressing zSpns2-EGFP or zSpns2(R153S)-EGFP to those expressing EGFP was indicated at the bottom of the TLC plate image.

**Figure S18** Expression pattern of *spns2* during early embryogenesis. (A to J) Whole-mount *in situ* hybridization with antisense *spns2* (A to H) and *sox 17* (I and J) probes. (C and D) Transverse sections of *spns2*-stained embryos. *spns2* expression initiated in the blastoderm margin at dome stage (A; black arrowheads) and in the YSL at shield stage (B). *spns2* expression was detected in the YSL (C; red arrowheads), somites (C; yellow arrowheads) and at the tip of the tail (D; blue arrowheads) at the 15s stage. *spns2* was expressed in the myocardial precursors (E; orange arrowhead) and in the intermediate cell mass (K; green arrowheads) at 24 hpf. *spns2* expression was not suppressed in *sox32* MO (10 ng)-injected embryo (H), while *sox17* expression was completely suppressed in *sox32* morphant (J).

**Figure S19** Apoptotic cells in *ko157* mutant. (A to C and F to H) Acridine orange staining. (D, E, I and J) TUNEL assay. Apoptotic cells detected by acridine orange staining or TUNEL assay were increased in the tail of *ko157* mutant (H and J: brackets) compared to Wt, while the number of apoptotic cells in the heart(s), head and trunk was comparable between *ko157* mutant and Wt. Red arrows indicate the position of hearts. White and black
arrowheads indicate apoptotic cells.
Supporting Online Material

Figure S1

A

sphingosine → S1P

SphK1

Spns2

S1P2

G proteins

cellular responses

B

Figure S2

A    Wt

B    Wt

C    Wt

D    ko157

E    ko157

F    ko157

G    Wt

H    Wt

I    ko157

J    ko157

amhc

vmhc

nkh2.5

amhc

vmhc

nkh2.5
Figure S3

A. Wt vs ko157 for hand2 + cmic2 at 24hpf
B. Wt vs ko157 for pax2 + cmic2 at 24hpf
C. Wt vs ko157 for gata1 + cmic2 at 24hpf
D. Wt vs ko157 for foxa2 + cmic2 at 24hpf

Figure S4

A. mil MO (15ng) at 54hpf
B. mil MO at 54hpf
C. mil MO at 54hpf (Tg(cmlc2:mRFP))
D. mil MO at 54hpf
Figure S6
Figure S7

<table>
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<th>5-mis MO</th>
<th>spns2 MO</th>
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spns2

EF1α

Figure S8

![Image](image4.png)

A uninj B spns2 (500pg) C spns2 (1ng) D spns2 (500pg) Tg(cmlc2:mRFP)

E uninj F spns2 (500pg) G spns2 (1ng) H spns2 (1ng) Tg(fli1:EGFP) Tg(fli1:EGFP) Tg(fli1:EGFP) Tg(cmlc2:mRFP)
Figure S12

A  mil MO  B  mil MO+spns2

Tg(cmlc2:mRFP)  Tg(cmlc2:mRFP)
Figure S13

- ○ zSpns2-EGFP
- ● zSpns2(R153S)-EGFP
- □ EGFP

% of secreted S1P vs. Time (min)
Figure S14

EGFP

$C_{17}$-S1P

zSpns2
-EGFP

S1P

zSpns2(R153S)
-EGFP
### Table S1
Phenocopy of ko157 by spns2 MO
Wild-type X Tg(cmlc2:mRFP)

<table>
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<th>morpholino (amount)</th>
<th>Number</th>
<th>Number of cardia bifida cases</th>
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<tbody>
<tr>
<td>uninjected</td>
<td>97</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>spns2 MO (10 ng)</td>
<td>69</td>
<td>59 (86%)</td>
</tr>
<tr>
<td>5-mis MO (10 ng)</td>
<td>68</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>mil MO (15 ng)</td>
<td>49</td>
<td>44 (90%)</td>
</tr>
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Morpholino was injected at the indicated concentrations into the yolk of 1-2 cell (mil MO) or 3 hpf stage embryos (spns2 MO or 5-mis MO).

### Table S2
Rescue of ko157 by spns2 mRNA or S1P injection
ko157 X ko157

<table>
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<th>mRNA or S1P (amount)</th>
<th>Number</th>
<th>Number of cardia bifida cases</th>
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<tbody>
<tr>
<td>uninjected</td>
<td>44</td>
<td>11 (25%)</td>
</tr>
<tr>
<td>spns2 (250 pg)</td>
<td>95</td>
<td>8 (8%)</td>
</tr>
<tr>
<td>spns2(R153S) (250 pg)</td>
<td>41</td>
<td>11 (27%)</td>
</tr>
<tr>
<td>MeOH (1 nl)</td>
<td>44</td>
<td>11 (25%)</td>
</tr>
<tr>
<td>S1P (1 nl; 1 ng)</td>
<td>54</td>
<td>6 (11%)</td>
</tr>
</tbody>
</table>

Synthetic mRNA (250 pg) was injected at into the blastomere of 1-2 cell stage embryos. S1P (1 ng) was injected into deep area of blastomeres of blastula stage embryos. Logically, one-fourth of embryos are ko157/ko157.

### Table S3
Rescue of ko157 by hSpns2 mRNA injection
ko157 X ko157

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<th>RNA (amount)</th>
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<tr>
<td>uninjected</td>
<td>64</td>
<td>14 (22%)</td>
</tr>
<tr>
<td>hSpns2 (250 pg)</td>
<td>70</td>
<td>8 (11%)</td>
</tr>
<tr>
<td>hSpns2(R199S) (250 pg)</td>
<td>59</td>
<td>14 (24%)</td>
</tr>
<tr>
<td>hSpns1-EGFP (250 pg)</td>
<td>55</td>
<td>15 (27%)</td>
</tr>
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</table>

Synthetic mRNA (250 pg) was injected into the blastomere of 1-2 cell stage embryos. Logically, one-fourth of embryos are ko157/ko157.
Table S4
Positive genetic interaction between spns2 and mil/S1P2

Wild-type X Tg(cmlc2:mRFP)

<table>
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<th>Morpholino (amount)</th>
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<tbody>
<tr>
<td>uninjected</td>
<td>50</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>mil MO (2 ng)</td>
<td>55</td>
<td>4 (7%)</td>
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Low dose of mil MO (2 ng) was injected into the blastomere of 1-2 cell stage embryos (Wt/Wt).

ko157 X Tg(cmlc2:mRFP)

<table>
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<tr>
<th>Morpholino (amount)</th>
<th>Number</th>
<th>Number of cardia bifida cases</th>
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</thead>
<tbody>
<tr>
<td>uninjected</td>
<td>49</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>mil MO (2 ng)</td>
<td>18</td>
<td>8 (44%)</td>
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Genotyping results of mil MO-injected embryos

<table>
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<th>Normal one heart</th>
<th>Number</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>Wt/Wt</td>
<td>8</td>
<td>Wt/Wt=8, Wt/ko157=2</td>
</tr>
<tr>
<td>Wt/ko157</td>
<td>6</td>
<td>Wt/ko157=6</td>
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</table>

Low dose of mil MO (2 ng) was injected into the blastomere of 1-2 cell stage embryos.

Table S5
spns2 MO injection in the YSL

Wild-type X Tg(cmlc2:mRFP): YSL injection at shield stage

<table>
<thead>
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<th>morpholino (amount)</th>
<th>Number</th>
<th>Number of cardia bifida cases</th>
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<tbody>
<tr>
<td>uninjected</td>
<td>22</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>5-mis MO (10 ng)</td>
<td>48</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>spns2 MO (10 ng)</td>
<td>43</td>
<td>13 (30%)</td>
</tr>
<tr>
<td>mil MO (15 ng)</td>
<td>57</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Morpholino (10 ng) was injected into the YSL of shield stage embryos.

Table S6
spns2 mRNA injection in the YSL

ko157 X ko157: YSL injection at shield stage

<table>
<thead>
<tr>
<th>RNA (amount)</th>
<th>Number</th>
<th>Number of cardia bifida cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>uninjected</td>
<td>54</td>
<td>14 (26%)</td>
</tr>
<tr>
<td>spns2 (250 pg)</td>
<td>37</td>
<td>5 (14%)</td>
</tr>
<tr>
<td>spns2(R153S) (250 pg)</td>
<td>39</td>
<td>9 (23%)</td>
</tr>
</tbody>
</table>

Synthetic mRNA (250 pg) was injected into the YSL of shield stage embryos. Logically, one-fourth of embryos are ko157/ko157.
Movie S1 Transplantation of Wt-derived donor cells into Wt recipient. Wt-derived donor cells labeled with FITC-dextran (green cells) are incorporated into the beating heart of Wt recipient. Similar results were obtained in nine independent embryos.

Movie S2 Transplantation of ko157-derived donor cells into Wt recipient. ko157-derived donor cells labeled with FITC-dextran (green cells) are incorporated in the beating heart of Wt recipient. Since myocardial precursors lacking Spns2 (ko157 donor cells) can participated in the formation of the heart in Wt embryo, Spns2 acts at least partially in a cell non-autonomous manner. After examining the incorporation of donor cells into the recipient, the genotype of the donor embryo was found to be ko157/ko157. Similar results were obtained in three independent embryos.

Movie S3 Transplantation of Wt-derived donor cells into ko157 recipient. Wt-derived donor cells labeled with FITC-dextran (green cells) were incorporated into one of two beating hearts of ko157 recipient. After examining the incorporation of donor cells into the recipient, the genotype of the recipient embryo was found to be ko157/ko157. Similar results were obtained in three independent embryos.

References