S1pr2/Gα13 signaling controls myocardial migration by regulating endoderm convergence

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SUMMARY
A key process during vertebrate heart development is the migration of bilateral populations of myocardial precursors towards the midline to form the primitive heart tube. In zebrafish, signaling mediated by sphingosine-1-phosphate (S1P) and its cognate G protein-coupled receptor (S1pr2/Mil) is essential for myocardial migration, but the underlying mechanisms remain undefined. We show that suppression of Gα13 signaling disrupts myocardial migration, leading to the formation of two bilaterally located hearts (cardia bifida). Genetic studies indicate that Gα13 acts downstream of S1pr2 to regulate myocardial migration through a RhoGEF-dependent pathway. Furthermore, disrupting any component of the S1pr2/Gα13/RhoGEF pathway impairs endoderm convergence during segmentation, and the endodermal defects correlate with the extent of cardia bifida. Moreover, endoderm transplantation reveals that the presence of wild-type anterior endodermal cells in Gα13-deficient embryos is sufficient to rescue the endoderm convergence defect and cardia bifida, and, conversely, that the presence of anterior endodermal cells defective for S1pr2 or Gα13 in wild-type embryos causes such defects. Thus, S1pr2/Gα13 signaling probably acts in the endoderm to regulate myocardial migration. In support of this notion, cardiac-specific expression of Gα13 fails to rescue cardia bifida in the context of global Gα13 inhibition. Our data demonstrate for the first time that the Gα13/RhoGEF-dependent pathway functions downstream of S1pr2 to regulate convergent movement of the endoderm, an event that is crucial for coordinating myocardial migration.

KEY WORDS: S1pr2, Mil, Gα13, Myocardial migration, Endoderm convergence, Zebrafish

INTRODUCTION
During vertebrate development, two groups of myocardial precursors are specified at lateral regions of the gastrula and migrate towards the embryonic midline where they merge and form a single primitive heart tube during segmentation (Stainier, 2001; Evans et al., 2010; Bakkers, 2011). This process is essential for formation of the heart tube, the foundation for all subsequent cardiac morphogenesis. Its disruption might lead to cardiac defects that include the formation of two bilaterally located hearts – a condition known as cardia bifida (Stainier et al., 1996; Yelon, 2001), which has catastrophic consequences for cardiac function. Studies in numerous model organisms, including Xenopus, zebrafish, chick and mouse, have shown that proper myocardial migration requires the intrinsic properties of myocardial precursors (including the proper differentiation, correct number and epithelial organization of cardiomyocytes) (Horne-Badovinac et al., 2001; Trinh and Stainier, 2004; Rohr, 2006; Garavito-Aguilar et al., 2010), as well as an environment conducive to migration. One such extracellular requirement is the presence of adjacent endoderm. Endoderm-deficient mouse and zebrafish mutants display cardia bifida (Kuo et al., 1997; Molkentin et al., 1997; Alexander et al., 1999; Reiter et al., 1999; David and Rosa, 2001). Similarly, in chick and Xenopus embryos, surgical removal of anterior endoderm impairs the migration of myocardial precursors, as well as heart formation (Rosenquist, 1970; Nascone and Mercola, 1995; Withington et al., 2001). It has been proposed that the endoderm serves as a substrate that allows cardiac mesoderm to migrate, and that it releases either cues that guide the migration of cardiac progenitor cells or signaling molecules that promote cardiac differentiation (Schultheiss et al., 1997; Andree et al., 1998; Lough and Sugi, 2000; David and Rosa, 2001; Alsan and Schultheiss, 2002; Nijmeijer et al., 2009). Additionally, the endoderm might provide a mechanical force that drives the migration of myocardial cells (Varner and Taber, 2012). Thus, despite the extensive evidence that the endoderm is important for cardiomyocyte movement during heart tube formation, its exact roles remain to be determined.

The zebrafish mutant miles apart (mil), which lacks a functional G protein-coupled receptor S1pr2 [which responds to sphingosine-1-phosphate (S1P), a bioactive lysophospholipid], displays defects in myocardial migration that lead to cardia bifida (Kupperman et al., 2000; Osborne et al., 2008; Kawahara et al., 2009). Although the s1pr2 transcript was detected in the lateral plate mesoderm, which is where the myocardial cells are located, transplantation experiments indicate that S1pr2/Mil may function outside cardiomyocytes to make the environment permissive for myocardial precursor migration (Kupperman et al., 2000). This notion is supported by the findings that S1pr2 regulates fibronectin (Fn) expression in the local environment, to facilitate the cell-Fn interaction needed for myocardial migration (Matsui et al., 2007; Osborne et al., 2008). Furthermore, embryos defective for S1pr2 signaling have defects in endoderm morphogenesis, and this may affect myocardial migration (Kupperman et al., 2000; Osborne et al., 2008). However, which downstream effectors of S1pr2 regulate endoderm development and how endoderm defects impair myocardial migration, remain unknown.

Here, we demonstrate that specifically the G protein isoform Gα13 acts downstream of S1pr2, and that it regulates myocardial migration through a RhoGEF-dependent pathway. Moreover, our analyses using a transgene that expresses Gα13 specifically in cardiomyocytes and endoderm transplantation provide evidence that...
the S1pr2/Gα13/RhoGEF signaling pathway functions within the endoderm to regulate convergent movement of this tissue, and that defects in this movement impair proper myocardial migration. Together, our studies uncover, for the first time, the signaling pathway by which S1P regulates myocardial migration, and establish S1pr2/Gα13-regulated endoderm convergence as having a crucial role in myocardial migration.

**MATERIALS AND METHODS**

**Zebrafish strains and maintenance**

AB**/Tuebingen, transgenic Tg(my7:EGFP) (Huang et al., 2003), and Tg(sox17:EGFP) (Mizoguchi et al., 2008) and miR1710 zebrafish strains (Kupperman et al., 2000) were used. Embryos were obtained by natural mating and staged according to morphology, or hours post fertilization (hpf) at 28.5°C or 32°C unless otherwise specified, as described previously (Kimmel et al., 1995).

**RNA expression and morpholinos**

mRNA and MOs were injected at the one-cell stage at the doses indicated. Capped mRNA was synthesized using the mMessage mM Machine Kit (Ambion). The RNAs encoding the following genes were used: gna13a (100 pg) and GNA13 (100 pg) (Lin et al., 2005); the RGS domain of PDZ-RhoGEF (arghef1RGS) (500 pg) or a dominant-negative mutant Arhgef1 lacking the DHPH domain (arghef1ADHPH) (500 pg) (Panizzi et al., 2007; Lin et al., 2009); s1pr2/mil (200 pg) (Osborne et al., 2008); and sox32 (400 pg) (Stafford et al., 2006). The previously validated morpholino antisense oligonucleotides (MOs) targeting the following genes were used: gna12 (4 ng), gna13a and gna13b (2 ng each) (Lin et al., 2005), and s1pr2/mil (15 ng) (Kawahara et al., 2009).

**Whole-mount in situ hybridization and immunofluorescence assay**

Digoxigenin-labeled antisense RNA probes were synthesized by *in vitro* transcription. *In situ* hybridization was performed as described (Lin et al., 2005; Thiese and Thissee, 2008). After *in situ* hybridization, the embryos were re-fixed in 4% PFA and sectioned at 10 μm, as described previously (Barthel and Raymond, 1990). An immunofluorescence assay was performed as previously described (Trinh and Stainier, 2004) using an anti-GFP antibody (1:200, Santa Cruz).

**Endoderm transplantation**

Endoderm transplantation was performed as described previously (Stafford et al., 2006; Chung and Stainier, 2008). Wild-type embryos injected with sox32 RNA (confers an endodermal identity to all cells) and 1% rhodamine-dextran (70,000 MW, lysine fixable, Invitrogen), with or without gna13ab MOs or s1pr2 MO served as the donors. Tg(sosx17:EGFP) or Tg(sosx17:EGFP)/my7:EGFP embryos, a subset of which were injected with gna13ab MOs, served as hosts. At mid-blastula stage, two independent groups of donor cells were transplanted into the host embryos along the blastoderm margin, at an angle of ~120–120° from one another (to increase the chances that they would contribute to the anterior region of the endoderm).

**Microscopy, time-lapse imaging and image processing**

For still images, live or fixed embryos were photographed using a Leica DMI 6000 microscope. For time-lapse experiments, embryos were embedded in a dorsal-mound imaging mold as previously described (Megason, 2009) and images were taken in the anterior region of endoderm of Tg(sosx17:EGFP) at 25°C, at 5-minute intervals with a 5 x/NA 0.15 objective on a Leica DMI 6000 microscope. The images were processed using the Metamorph software and cell tracking was analyzed by ImageJ. Data was exported to Excel where cell migration speed, paths and direction were determined as previously reported (Lin et al., 2005).

**Statistical analysis**

Data were compiled from two or three independent experiments, and are presented as the mean ± s.e.m. The number of embryos analyzed is indicated. Statistical analyses were performed using unpaired Student’s *t*-tests with two tails and unequal variance.

**RESULTS**

**Gα13 signaling is required for myocardial migration**

S1pr2 has been shown to couple with diverse G proteins, in particular Gα12 and Gα13, to mediate a variety of cellular responses (Skoura and Hla, 2009; Suzuki et al., 2009). We previously identified one Gα12 and two Gα13 (Gα13a and Gα13b) isoforms in zebrafish, and demonstrated that they are essential during gastrulation (Lin et al., 2005; Lin et al., 2009). Here, we show that Gα13 is also required for myocardial migration. Injection of morpholinos (MOs) that inhibit the translation of gna13a and gna13b genes (Lin et al., 2005), but neither alone, caused cardia bifida, a lack of circulation and pericardial edema (Fig. 1C-D). Notably, the two hearts in gna13ab MO-injected embryos (morphants) beat and expressed the myocardial markers nkb2.5 (Serbedzija et al., 1998) and myl7 (formerly known as cardiac myosin light chain 2, cmlc2), as well as the chamber-specific markers atrial myosin heavy chain (amhc; myh6 – Zebrafish Information Network) and ventricular myosin heavy chain (vmhc) (Yelon et al., 1999) (Fig. 1G-J; not shown).

Together, these data indicate that Gα13 is required for the migration, but not the differentiation, of cardiomyocytes. In addition, the morphants exhibited blistering in the tailfin (Fig. 1C′,C′′, arrowheads), a feature unique to embryos defective for S1P signaling and not observed in other cardia bifida mutants (Kupperman et al., 2000; Osborne et al., 2008; Kawahara et al., 2009).

Both the severity and penetrance of cardia bifida and tail blistering increased with the MO dose (not shown). Expressing either human Gα13 or a zebrafish Gα13a form that is insensitive to the MOs significantly suppressed cardia bifida and tail blistering in gna13ab morphants (Fig. 1K; not shown). These findings demonstrate that the observed phenotypes are specific consequences of interference with Gα13 function, and indicate that the functions of the zebrafish and human Gα13 proteins are conserved (Lin et al., 2005).

Interestingly, although Gα12 belongs to the Gα12/13 subfamily of G proteins and exhibits functions similar to those of Gα13 in mammalian cell culture (Buhl et al., 1995; Lin et al., 2005), we found that suppression of Gα12 alone caused neither cardia bifida nor tail blistering (supplementary material Fig. S1; not shown). However, inhibiting Gα12 significantly enhanced the defects resulting from Gα13 inhibition, as judged by increases in the frequency of cardia bifida and the distance between the two hearts (supplementary material Fig. S1). These data indicate that Gα12 and Gα13 play partially redundant roles in myocardial migration.

**Gα13 acts downstream of S1pr2 to regulate myocardial migration**

The striking phenotypic similarities between embryos depleted of Gα13 and those deficient for S1P signaling (Fig. 1C,D versus 1E,F) led us to hypothesize that Gα13 may act downstream of S1pr2 to regulate myocardial migration. To test this, we first examined whether Gα13 overexpression can suppress the defects resulting from the S1pr2 deficiency. We found that when embryos were co-injected with s1pr2 MO and an RNA encoding either zebrafish Gα13a (Fig. 2E) or human Gα13a (not shown), the frequencies of cardia bifida and tail blistering were significantly reduced. Examination of myl7 expression revealed that, in the co-injected embryos, the bilateral populations of cardiomyocytes migrated to a point much closer to the midline (Fig. 2C versus 2B). Thus, Gα13...
overexpression can partially compensate for S1pr2 deficiency, suggesting that Gα13 acts downstream of S1pr2 to regulate myocardial migration.

To gain further support for the hypothesis that S1pr2 and Gα13 function in the same pathway, we carried out co-injection experiments and tested for synergistic effects. Specifically, we injected MOs targeting s1pr2 or gna13ab at suboptimal doses, alone and together, and evaluated the location of the heart and the morphology of the tailfin. At the doses used, defects caused by injecting the individual MOs were very mild (supplementary material Table S1); those caused by co-injection of the two MOs were more severe than the additive effects of each manipulation alone (higher frequencies of both cardia bifida and tail blistering, as well as increased distance between the two populations of cardiomyocytes) (supplementary material Table S1; not shown). These data are consistent with the notion that S1pr2 and Gα13 function in a common genetic pathway.

We reasoned that if Gα13 acts downstream of S1pr2, its function should be required for S1P signaling. To directly test this hypothesis, we examined whether Gα13 inhibition blocks the gastrulation defects resulting from S1pr2 overexpression (Osborne et al., 2008). S1pr2 overexpression induced defects in convergence and extension (C&E) movements (revealed by broadening of the epiboly defects resulting from Gα13 overexpression (Lin et al., 2009). In addition, it has been reported that disrupting RhoGEF or RhoA function causes cardia bifida (Matsui et al., 2005; Panizzi et al., 2007). Thus, we reasoned that Gα13 may signal through Arhgef11/RhoA to effect S1pr2-triggered myocardial migration.

To test this possibility, we first re-evaluated the functions of RhoGEF in myocardial migration and tailfin development by overexpressing dominant-negative forms of Arhgef11 (Arhgef11RGS – the RGS domain of Arhgef11 that competes for Gα13 binding but contains no functional RhoA-activating DH and PH domains; and Arhgef11ΔDHPH – which lacks the DH and PH domains) significantly suppressed the epiboly defects resulting from Gα13 overexpression (Lin et al., 2009). In addition, it has been reported that disrupting RhoGEF or RhoA function causes cardia bifida (Matsui et al., 2005; Panizzi et al., 2007). Thus, we reasoned that Gα13 may signal through Arhgef11/RhoA to effect S1pr2-triggered myocardial migration.

**S1pr2/Gα13 signaling regulates myocardial migration via a RhoGEF/Rho-dependent pathway**

Next, we investigated the molecular effectors of S1pr2 and Gα13. Gα13 regulates the remodeling of the myocardium and the development of the tailfin. To gain further support for the hypothesis that S1pr2 and Gα13 function primarily by activating RhoGEF and then RhoA (Suzuki et al., 2009). We have previously shown that Gα13 inhibits a PDZ-RhoGEF known as Arhgef11, via its regulator of G protein-coupled signaling (RGS) domain, such as a PDZ-RhoGEF known as Arhgef11, via its regulator of G protein-coupled signaling (RGS) domain, and that Gα13a binds to Arhgef11, via its regulator of G protein-coupled signaling (RGS) domain, and that Gα13 regulates zebrafish epiboly through a RhoGEF-dependent pathway. Specifically, overexpression of dominant-negative forms of Arhgef11 (Arhgef11RGS – the RGS domain of Arhgef11 that competes for Gα13 binding but contains no functional RhoA-activating DH and PH domains; and Arhgef11ΔDHPH – which lacks the DH and PH domains) significantly suppressed the epiboly defects resulting from Gα13 overexpression (Lin et al., 2009). In addition, it has been reported that disrupting RhoGEF or RhoA function causes cardia bifida (Matsui et al., 2005; Panizzi et al., 2007). Thus, we reasoned that Gα13 may signal through Arhgef11/RhoA to effect S1pr2-triggered myocardial migration.

To test this possibility, we first re-evaluated the functions of RhoGEF in myocardial migration and tailfin development by overexpressing dominant-negative forms of Arhgef11. We found that 60% of the embryos (n=73) expressing Arhgef11RGS displayed cardia bifida and pericardial edema (Fig. 3G-I). In addition, these embryos exhibited tail blistering (Fig. 3G, arrowheads), a phenotype that had not been appreciated previously (Panizzi et al., 2007). Moreover, the bilateral hearts observed in these embryos were properly differentiated, as evident from the expression of nkx2.5 and myl7 (Fig. 3I; not shown). Similar results were obtained in embryos expressing Arhgef11ΔDHPH (not shown). Based on the similar cardia bifida and tail blistering phenotypes that result from disrupted RhoGEF function and depleted S1P signaling, we postulated that RhoGEF and S1pr2 probably function in the same genetic pathway. Accordingly, a synergistic effect was observed when RhoGEF and S1pr2 were
simultaneously inhibited. Partial inhibition of both proteins (by injecting suboptimal doses of the s1pr2 MO and the Arhgef11RGS RNA) resulted in cardiabifida and tail blistering that were much more severe than that produced by partial inhibition of either protein individually (supplementary material Table S1). We could not overexpress Arhgef11 to assess whether it can rescue the defects in s1pr2 morphants, as such expression caused severe morphological defects during early development (not shown) (Panizzi et al., 2007). However, we found that Arhgef11 is required for s1pr2 signaling. Similar to inhibition of Gα13 expression, interference with Arhgef11 function (by expressing Arhgef11RGS) suppressed the gastrulation defects otherwise caused by S1pr2 overexpression, as judged by ntl expression at 8 hpf and shortening of the body axis at 35 hpf (Fig. 3D; not shown). Specifically, embryos co-expressing S1pr2 and Arhgef11RGS had a body axis of nearly normal length and two eyes (Fig. 3E), in contrast to S1pr2-overexpressing embryos, which featured a shortened body axis and cyclopia (Fig. 3D). However, the co-injected embryos remained labeled cardiabifida, pericardial edema and tail blistering (Fig. 3E; not shown), features that were not observed in S1pr2-overexpressing embryos; these defects presumably resulted from the expression of Arhgef11RGS. Taken together, these results support the model that S1pr2 signals through Gα13 to activate RhoGEF/Rho, and to thereby regulate myocardial migration.

**S1pr2/Gα13 signaling regulates convergent movement of the anterior endoderm**

Next, we examined through which tissue S1pr2/Gα13 signaling controls myocardial migration. Previous studies have suggested that S1P signaling regulates morphogenesis of the anterior endoderm, thereby indirectly affecting the migration of myocardial precursors (Osborne et al., 2008). However, how this signaling pathway regulates endoderm development and how the endodermal defects relate to those in myocardial migration is unclear. As the first step in investigating the role of S1pr2/Gα13 signaling in the endoderm, we tested for the expression of s1pr2 and gna13 in this tissue at the 14-somite (14s, 16 hpf) and 18s (18 hpf) stages, during which myocardial precursors migrate toward the midline (Matsui et al., 2005; Holtzman et al., 2007). We visualized the endoderm using transgenic Tg(sox17:EGFP) embryos, in which specifically the endodermal cells are labeled with EGFP (Mizoguchi et al., 2008). Performing a combination of in situ hybridization (to detect s1pr2 and gna13 transcripts) and immunofluorescence (to identify GFP-labeled endoderm) analyses, we found that gna13a, gna13b and s1pr2 transcripts are present in the GFP-expressing endodermal cells at both the 14s (data not shown) and 18s stages (Fig. 4A,B; the expression of gna13a is not shown, but is identical to that of gna13b). Interestingly, the s1pr2 transcript is highly enriched in the endoderm (Fig. 4A, arrowheads), whereas the gna13 transcripts are present uniformly in all tissues including the endoderm (Fig. 4B). These results suggest that S1pr2 and Gα13 may function within the endoderm.

We further evaluated how S1pr2/Gα13 signaling affects endoderm development using Tg(sox17:EGFP) embryos. We found that the anterior endodermal sheet was significantly widened in S1pr2/Gα13-deficient embryos during segmentation, which was not reported previously (Osborne et al., 2008). By the end of gastrulation, endodermal morphology in s1pr2 or gna13 mutants was indistinguishable from that in control embryos, as assessed by the sox17 expression pattern (supplementary material Fig. S2) and sox17:EGFP distribution (not shown). As development proceeded, however, differences became obvious. By mid-segmentation (8s stage), the anterior endodermal sheet in mil-Δ/Δ embryos (in sox17:EGFP background) or gna13ab morphants was significantly wider than that in their controls (Fig. 5A-C,J). The endodermal sheet remained widened throughout late segmentation, the period during which the myocardial precursors migrate towards the midline to fuse at the 20-somite stage (Fig. 5D-I). These data indicate that S1pr2/Gα13 signaling is required for endoderm convergence.

To further evaluate the role of S1pr2 signaling in endoderm migration, we performed endoderm transplantation (Stafford et al., 2006; Chung and Stainier, 2008) as outlined schematically in Fig. 5K, assessing how S1pr2-deficient endodermal cells migrate in the wild-type endodermal environment. We selected embryos in which donor endodermal cells had been transplanted into one side of the host endoderm, and performed time-lapse experiments on the anterior endoderm region from the 3-somite to the 10-somite stage. We then compared the extent of convergent movement in the transplanted donor versus host endodermal cells. Our analyses revealed that transplanted wild-type cell populations migrated to the midline at a speed similar to that of the wild-type host cells and that, at the end of the time-lapse experiment, the width of the transplanted endodermal populations was similar to that of the host endodermal populations (Fig. 5L,M,P; supplementary material Movie 1). By contrast, the transplanted s1pr2-deficient cells moved towards the midline at a slower speed, and the width of the sheet they formed at the end of the time-lapse experiment was wider than on the wild-type side of the embryo (Fig. 5N-P, supplementary material Movie 2). Together, these results suggest that S1pr2/Gα13 signaling is required autonomously for convergent movement of the anterior endoderm.

In addition to the impaired endoderm convergence, gna13ab morphants or mil mutants featured ‘holes’, i.e. regions devoid of endodermal cells, within the most anterior endoderm (Fig. 5A-I), a phenotype similar to that reported previously for embryos defective for S1P signaling (Osborne et al., 2008). Notably, the endodermal...
paths (consistent with participation in the process of convergence) (Fig. 6; supplementary material Movie 3). Although in mil mutants the cells in the anterior region likewise migrated predominantly in the anterior direction, they did so at reduced speed (82% of wild-type total speed). Strikingly, analysis of the paths and directions of cell migration revealed that mil cells tended to migrate away from the midline laterally. Thus, their net anterior migration (extension) was especially compromised, reaching only 50% of the wild-type net anterior speed, whereas the convergence velocity towards the lateral was increased (Fig. 6B-G; supplementary material Movie 4). Similarly, mil cells in the posterior region migrated less directly towards the midline and tended to move in the posterior direction, as reflected by a significantly reduced net speed of convergence and an increase in extension towards the posterior (Fig. 6; supplementary material Movie 4). Interestingly, the total migration velocity of the posterior mil cells was similar to that of control cells. Together, these data showed that S1pr2/Mil function is required for the directed cell migration that underlies C&E movements during segmentation.

Interestingly, despite the defects in endodermal C&E in S1pr2/Gna13-deficient embryos, the C&E movements of the mesoderm and ectoderm appeared normal, as judged by the length of the body axes (Fig. 1C,E) and the expression patterns of various tissue markers (supplementary material Fig. S3). These findings suggest that S1pr2/Gna13 signaling is required to control the migration of endodermal, but not ectodermal and mesodermal, cells.

Endoderm differentiation appeared to be unaffected in embryos defective for S1pr2/Gna13 signaling, as the endodermal marker sox17 was expressed normally during gastrulation (supplementary material Fig. S2). Moreover, analyses by TUNEL assay indicated that S1pr2/Gna13 deficiency did not lead to excessive apoptosis in the anterior endoderm (supplementary material Fig. S4). Taken together, these results demonstrate that during segmentation, S1pr2/Gna13 signaling regulates the migration, but not differentiation or apoptosis, of endodermal cells.

**The endoderm defects resulting from S1pr2/Gna13 deficiency impair myocardial migration**

Given that the endoderm converges as the myocardial precursors migrate towards the embryonic midline, and both processes were defective in S1pr2/Gna13-deficient embryos, we reason that these phenotypes are causally linked. To test this, we simultaneously analyzed endoderm morphology and cardiomyocyte location in Tg(sox17:EGFP/myl7:EGFP) embryos. We found that disrupting any component of the S1pr2/Gna13/RhoGEF pathway resulted in a similarly deformed and widened endodermal sheet, as well as cardia bifida (Fig. 7B,E,F versus 7A). Quantitation of endodermal defects and cardia bifida revealed that in embryos defective for S1pr2/Gna13 signaling both the frequency of endodermal holes and widening of the anterior endodermal sheet corresponded to the severity of cardia bifida (Fig. 7H,I). Notably, some of these embryos displayed cardia bifida even in the absence of an endodermal hole (not shown), and the frequency of holes in the endoderm was lower than that of cardia bifida (Fig. 7H). These findings suggest that the endoderm deformity may not be the sole defect underlying cardia bifida, as speculated previously (Osborne et al., 2008). Rather the impairment in endoderm convergence could contribute to cardiac bifida. Whereas overexpression of human Gna13 in wild-type embryos did not induce visible defects in the endoderm (Fig. 7D,J), its expression in the gna13ab morphants significantly reduced the frequency of cardia bifida and the incidence of endodermal holes, and decreased endoderm width and the distance between the two populations of
myocardial precursors (Fig. 7C,H,I). Thus, there is a strong correlation between the endodermal and myocardial defects.

Importantly, although we detected low levels of s1pr2 and gna13ab expression in cardiomyocytes at the 20-somite stage (Fig. 4C,D), expression of Gα13 specifically in cardiomyocytes (using the myl7 promoter) failed to rescue cardia bifida caused by global inhibition of Gα13 (injection of gna13ab MOs; supplementary material Fig. S5). These data suggest that S1pr2/Gα13 signaling in the endoderm, but not cardiomyocytes, is important for myocardial migration. Considering that the defects in endoderm convergence in S1pr2/Gα13-deficient embryos occur at mid-segmentation (8 somites, 13 hpf, Fig. 5), which is well before myocardial migration takes place (16-19 hpf) (Stainier, 2001; Holtzman et al., 2007), we hypothesize that the endodermal defects contribute to the myocardial defects. To assess directly the effects of endodermal defects on myocardial migration, we performed transplantation experiments as described in Fig. 5K, except that Tg(sox17:EGFP/myl7:EGFP) embryos were used as hosts, and endoderm morphology and cardiomyocyte locations were analyzed at 26-28 hpf (Fig. 8A). High-resolution 3D reconstruction of confocal images confirmed that donor endodermal cells were not incorporated into the myocardium (supplementary material Fig. S6);
thus, the phenotypes resulting from endoderm transplantation were not due to the presence of donor cells in the myocardium.

When wild-type endodermal cells were transplanted into gna13ab morphants, all the embryos (n=46) in which the endoderm did not include donor wild-type cells displayed cardia bifida (not shown), whereas the 21.4% of embryos (n=15/70) in which the endoderm included wild-type donor cells had a single heart (Fig. 8B). In the rescued embryos (15/70), the majority of the anterior endoderm was replaced with wild-type donor cells (red cells), and the endodermal sheet lacked holes and was significantly narrower than in non-rescued embryos. In the remaining embryos with cardia bifida (55/70), the anterior endoderm was not, or was only partially, populated by wild-type donor cells (Fig. 8C versus 8B). Strikingly, whereas all wild-type embryos (n=42) in which endoderm transplanted with wild-type donor cells exhibited single hearts (supplementary material Fig. S6; not shown), 60.5% of those transplanted with cells defective for gna13ab (n=23/38; Fig. 8D) and 61% of those transplanted with cells defective for s1pr2 (n=11/18; Fig. 8F; supplementary material Fig. S6) displayed cardia bifida. Notably, in the wild-type hosts with cardia bifida, a large proportion of the anterior endoderm contained morphant cells, and the overall width of the anterior endoderm was significantly greater than that in embryos with one heart (Fig. 8D,F versus 8E,G). In addition, in some of these wild-type hosts, holes were present in regions highly populated by transplanted morphant cells (Fig. 8F, white asterisks), suggesting that the endodermal phenotypes resulted from defects within the endoderm rather than from defects in the surrounding tissues. Overall, these experiments indicate that S1pr2 and Gα₁₃ function autonomously in the endoderm, and that endodermal defects are sufficient to impair myocardial migration.

**DISCUSSION**

**Gα₁₃ acts downstream of S1pr2 to regulate myocardial migration through a RhoGEF-dependent pathway**

In this study, we have elucidated the signaling mechanism by which S1pr2 regulates myocardial migration: S1pr2 activates a Gα₁₃/RhoGEF-dependent pathway. We show that depletion of Gα₁₃ results in cardia bifida and tail blistering (Fig. 1), phenotypes that are reminiscent of those in embryos deficient for either S1pr2 or the S1P transporter Spns2 (Kupperman et al., 2000; Osborne et al., 2008; Kawahara et al., 2009), suggesting that Gα₁₃ is involved in the S1P signaling pathway. Indeed, overexpression of Gα₁₃ can significantly rescue both cardia bifida and tail blistering that results from MO-mediated inhibition of S1pr2 (Fig. 2). Furthermore, our epistasis experiments demonstrate that inhibiting Gα₁₃ can largely suppress the gastrulation defects induced by S1pr2 overexpression (Fig. 3), and that Gα₁₃ and S1pr2 have synergistic effects on myocardial migration and tail fin development (supplementary material Table S1). Together, these results indicate that Gα₁₃ and S1pr2 function in the same genetic pathway, and that Gα₁₃ acts downstream of S1pr2 to regulate myocardial migration.

S1pr2 belongs to the S1P family of G protein-coupled receptors (S1P-S1P₅), which are activated by S1P (Skoura and Hla, 2009). In vitro studies in mammalian cultured cells show that S1pr2 can couple to various G protein isoforms, including Gα₂₅, Gα₁₃ and Gα₁₂/13, but that its coupling to Gα₁₂/13 is the most efficient (Ancellin and Hla, 1999; Windh et al., 1999). However, we found that only interference with Gα₁₃ results in cardia bifida (not shown), indicating that Gα₁₃ is the predominant G-protein isoform that mediates S1pr2-regulated myocardial migration. Interestingly, the finding that Gα₁₃ is only partially redundant with Gα₁₂ with respect to regulating myocardial migration suggests that the functions of these two G protein isoforms are tissue specific, as our previous findings on gastrulation movements of zebrafish indicated that functions of Gα₁₂ and Gα₁₃ are fully redundant in that context. These findings are consistent with those from mouse studies: Gα₁₃-deficient mice die at E9.5 owing to defective angiogenesis, whereas mice lacking Gα₁₂ have no overt phenotypes (Offermanns et al., 1997), but mice doubly deficient for Gα₁₂ and Gα₁₃ die at an earlier stage (E8.5), and mice that are deficient for Gα₁₂ and carry only...
one allele of Gα13 also die in utero (Offermanns, 2001; Gu et al., 2002). Thus, Gα12 and Gα13 appear to have differential functions in developmental processes.

In cultured mammalian cells, Gα12/13 function primarily through a RhoGEF-regulated RhoA signaling pathway (Kozasa et al., 1998; Hart et al., 2000; Worzfeld et al., 2008). We showed previously that Gα12/13 also signal through a RhoGEF/Rho-dependent pathway to regulate epiboly in zebrafish (Lin et al., 2009). Our current data indicate that Gα13 additionally employs this conserved signaling pathway to control myocardial migration. We show that interference with Arhgef11 function leads to cardiac bifida and tail blistering, as do defects in S1pr2 and Gα13 signaling (Fig. 3). Moreover, simultaneous partial inhibition of the functions of S1pr2 and Arhgef11 had synergistic effects on myocardial migration (supplementary material Table S1). Finally, interference with Arhgef11 function suppresses S1pr2-induced gastrulation defects (Fig. 3E), suggesting that S1pr2 signals through Arhgef11 to regulate the development of multiple tissues in zebrafish.

**S1pr2/Gαα13/PDZRhoGEF signaling is required for convergent movement of the anterior endoderm during segmentation**

Previous work suggests that S1pr2 may function within the endoderm to regulate myocardial migration (Osborne et al., 2008). Consistent with this notion, we found the s1pr2 transcript is highly enriched in the endoderm (Fig. 4), suggesting that S1pr2 has specific roles in the endoderm. Indeed, in addition to the appearance of endodermal holes in S1P-deficient embryos, as previously reported (Osborne et al., 2008), our analyses of the endodermal morphology of S1pr2/Gαα13-deficient embryos and transplanted embryos indicate that S1pr2/Gαα13 signaling is autonomously required for convergent movement of the endoderm, a defect that was not identified and appreciated previously (Figs 5-8).

Consistent with previous findings in mil (Kupperman et al., 2000) and toh (Kawahara et al., 2009) mutants, we found that S1P/Gαα13 signaling is not required for the C&E movements of mesodermal cells, suggesting that this signaling pathway functions specifically in the endoderm. During zebrafish embryonic development, the endodermal cells display unique migratory characteristics distinct from those of mesodermal cells, and require different signaling pathways (Warga and Nüsslein-Volhard, 1999; Pezeron et al., 2008). During gastrulation, endoderm migration is controlled by chemokine signaling (mediated by Cxcl12b and its receptor Cxcr4a), and this is not required for the migration of mesodermal cells (Mizoguchi et al., 2008; Nair and Schilling, 2008). Although Wnt/PCP and Vegf signaling have been shown to regulate morphology of the anterior endoderm during segmentation (Ober et al., 2004; Matsui et al., 2005), how these pathways influence endoderm development has not been defined. Thus, the mechanisms whereby endodermal cells migrate during segmentation remain uncharacterized. Additionally, defective Wnt/PCP signaling severely impairs the C&E of mesodermal cells (Matsui et al., 2005), and depletion of Vegf function affects endoderm morphogenesis and specification (Ober et al., 2004). Thus, our findings that S1pr2/Gαα13 signaling is required for endoderm migration at segmentation represent a novel regulatory pathway.

Our time-lapse experiments indicate that S1pr2 signaling is required for the efficient directed migration that underlies the C&E movements of the endoderm (Fig. 6). Further studies are needed to dissect the mechanisms by which S1pr2/Gαα13 signaling controls the migratory behaviors of endodermal cells. The presence of holes lacking endodermal cells in S1pr2/Gαα13-deficient embryos is intriguing. Given that neither dying cells nor an excessive number of apoptotic cells were found in the endoderm of S1pr2/Gαα13-deficient embryos (supplementary material Fig. S4; not shown), this phenomenon is unlikely to be a consequence of cell death/apoptosis.
Time-lapse experiments suggest that abnormal migration of the cells (away from the midline) could contribute to the formation of holes (Fig. 6; supplementary material Movies 3, 4). Furthermore, given that the endoderm developed as a cohesive sheet from mid-segmentation (not shown), defects in cell-cell adhesion may also contribute. Further studies will be required to address this possibility.

The role of endoderm convergence in myocardial migration

Endoderm convergence and the migration of myocardial precursors to the midline both occur during segmentation, leaving open the possibility that these two events are linked. Indeed, we found that the extent of endodermal convergence defects correlates with the severity of cardia bifida, and that the cardiac and endodermal defects in gna13ab morphants are simultaneously rescued by the overexpression of G\(\alpha_{13}\)a (Fig. 7). Moreover, our endoderm transplantation assays reveal that while the replacement of anterior endoderm in gna13ab morphants with wild-type endodermal cells simultaneously rescues the defects of both endoderm and myocardial migration, the transplantation of endoderm defective for either S1pr2 or G\(\alpha_{13}\) into wild-type embryos leads to endoderm defects as well as to cardia bifida (Fig. 8). These data support the notion that S1pr2 and G\(\alpha_{13}\) function within the endoderm to influence myocardial migration during heart-tube formation, and that impaired endoderm convergence is likely to be the root cause of the myocardial defects observed in the context of S1pr2/G\(\alpha_{13}\) deficiency. In support of a role for S1pr2/G\(\alpha_{13}\) in the microenvironment, we found that stably overexpressing G\(\alpha_{13}\) specifically in cardiomyocytes fails to rescue either cardia bifida or the endodermal defects in gna13ab morphants (supplementary material Fig. S5; not shown).

Interestingly, a recent study indicates that S1pr2 signaling in the endoderm is crucial for jaw development in zebrafish (Balczerski et al., 2012), further supporting the roles of this signaling pathway in endoderm development.

The fact that the endoderm is crucial for regulating myocardial-precursor migration in a variety of organisms, including Xenopus, zebrafish, chick and mouse, is well recognized, yet the underlying mechanisms remain elusive (Lough and Sugi, 2000; Stainier, 2001). The endoderm has been shown to produce growth factors such as BMP2 and FGF8, and thereby to control the specification and differentiation of cardiac mesoderm, and indirectly influence myocardial migration (Nascone and Mercola, 1995; Schultheiss et al., 1997; Andree et al., 1998; Lough and Sugi, 2000; Alsan and Schultheiss, 2002). However, it is unlikely that this mechanism accounts for the cardia bifida in embryos defective for S1pr2/G\(\alpha_{13}\) signaling because cardiomyocytes differentiate normally in these embryos (Fig. 1G-J) (Kupperman et al., 2000). Based on the fact that the endoderm is adjacent to the myocardial mesoderm and that myocardial precursors fail to migrate in the absence of the endoderm, the endoderm has been proposed to serve as a physical substrate that enables the myocardial cells to migrate (David and Rosa, 2001). Alternatively, it may produce migratory cues to guide the migration of cardiomyocytes towards midline (David and Rosa, 2001), although such factors have not been identified. Recent studies in chick suggest that endoderm migration may provide an active mechanical force that pulls the myocardial cells towards the midline, and that cardiomyocytes exhibit minimal autonomous migration during this process (Cui et al., 2009; Varner and Taber, 2012). Our finding of a correlation between impaired endoderm convergence and cardia bifida in zebrafish embryos defective for S1pr2/G\(\alpha_{13}\) signaling is consistent with such a mechanical role for the anterior endoderm in myocardial...
migration. Future work is required to determine whether the endoderm indeed exerts such an effect on cardiomyocytes, and how it does so.

In summary, the present study delineates the signaling pathway whereby S1pr2 regulates myocardial migration (Fig. 7G). Specifically, we show that S1pr2 functions through a G\alpha\_13/RhoGEF-dependent pathway to regulate myocardial migration. Moreover, we demonstrate that S1pr2/G\alpha\_13 signaling is required autonomously for the convergence and integrity of the anterior endoderm, and that convergent movement of the endoderm is crucial for the medial migration of myocardial precursors. This work represents an important step towards understanding the crucial roles of the endoderm in promoting myocardial migration during vertebrate heart development. Studies are underway to further elucidate how S1pr2/G\alpha\_13 signaling regulates endoderm morphogenesis, and how the endoderm in turn influences the migration of myocardial precursor cells.

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Competing interests statement
The authors declare no competing financial interests.


