A Slit/miR-218/Robo regulatory loop is required during heart tube formation in zebrafish

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SUMMARY
Members of the Slit family of secreted ligands interact with Roundabout (Robo) receptors to provide guidance cues for many cell types. For example, Slit/Robo signaling elicits repulsion of axons during neural development, whereas in endothelial cells this pathway inhibits or promotes angiogenesis depending on the cellular context. Here, we show that miR-218 is intronically encoded in slit2 and slit3 and that it suppresses Robo1 and Robo2 expression. Our data indicate that miR-218 and multiple Slit/Robo signaling components are required for heart tube formation in zebrafish and that this network modulates the previously unappreciated function of Vegf signaling in this process. These findings suggest a new paradigm for microRNA-based control of ligand-receptor interactions and provide evidence for a novel signaling pathway regulating vertebrate heart tube assembly.

KEY WORDS: Slit/Robo, Vegf, MicroRNA, Heart tube formation, Zebrafish

INTRODUCTION
During vertebrate cardiac morphogenesis, myocardial and endocardial precursors are specified in two bilateral domains within the anterior lateral plate mesoderm and subsequently migrate toward the embryonic midline, where they fuse into a linear heart tube consisting of an outer ring ofcardiomyocytes surrounding an inner core of endocardial cells (Bussmann et al., 2007; Holtzman et al., 2007). Analysis of several zebrafish mutants has shed light on the requirements for myocardial migration, including the specification and development of the endoderm (Alexander et al., 1999), the deposition of extracellular matrix proteins such as fibronectin (Trinh and Stainier, 2004; Sakaguchi et al., 2006) and signaling through both heterotrimeric G proteins (Kupperman et al., 2000; Osborne et al., 2008) and Rho family GTPases (D’Amico et al., 2007). By contrast, much less is known about the mechanisms that control endocardial migration. For example, in embryos mutant for the transcription factor Scl/Tal1, endocardial cells migrate to the midline but then fail to migrate posteriorly, resulting in endocardial cell aggregation at the arterial pole (Bussmann et al., 2007). Importantly, midline-derived cues that guide endocardial (or myocardial) cells toward the midline have not yet been identified.

Several guidance molecules are expressed at the embryonic midline, including the Slit family of secreted extracellular matrix proteins. Although Slits and their Roundabout (Robo) receptors were initially characterized as repulsive guidance cues for neuronal axons (Kidd et al., 1998a; Kidd et al., 1999), they are also involved in the development of several other organs systems (Liu et al., 2003; Grieshammer et al., 2004; Strickland et al., 2006). In Drosophila, Slit functions via Robo1/2 as a repulsive cue to position cardioblasts relative to the midline (Qian et al., 2005). Subsequently, Slit/Robo signaling is required to downregulate E-cadherin cell-surface localization during lumenization of the heart tube (Medioni et al., 2008; Santiago-Martinez et al., 2008). It is unclear whether these observations can be extended to vertebrates, considering that Drosophila lacks the endocardium, which is required for vertebrate heart tube assembly (Holtzman et al., 2007).

In vertebrates, the Robo family comprises four known members (Robo1–4), whereas the Slit family has three members (Slit1-3). Here, we show that a highly conserved microRNA, miR-218, is encoded intronically in slit2 and slit3 and negatively regulates Robo1 and Robo2. Knockdown experiments indicate that Slit2, Robo1 and miR-218 are required for the formation of the linear heart tube in zebrafish. Further analyses indicate that Vegf is also required for migration of the heart fields to the midline, and that Slit/Robo signaling regulates the response of endocardial cells to Vegf. Thus, we provide evidence that a novel Slit/miR-218/Robo/Vegf signaling axis controls heart tube formation in zebrafish.

MATERIALS AND METHODS
Plasmids
HA-tagged rat ROBO1 (Li et al., 1999) and Myc-tagged rat ROBO1 (Stein and Tessier-Lavigne, 2001) have been described previously. mCherry was from Timothy Gomez (University of Wisconsin, Madison).

Morpholinos (MOs)
MOs (Gene Tools or Open Biosystems) were titrated to avoid toxic effects of excess MO.

Control MO, CCTCTAACCTCAGTTACAATTATA; miR-218 MO1, TGCATGGTTAGATCAAGCACAAGGG; miR-218 MO2, CATATGGTTAGATCAAGCACAAGGG; robo1 ATG MO, ATCCAAATTATTTCTCCCGTCATCGT; robo4 ATG MO, GCAGACACCTGCATCCTACCCCTTAAA; slit2 ATG MO, GCCACCTATGTTTCAACACAACAT; slit3 ATG MO, CCCCCATACTTTTACCCCGCATC;
vegfa ATG MO, CTCGCTTAATTTCCGTGACTTTT (Ober et al., 2004).

Zebrafish embryology, MO injections and treatment
Tg(kdrl:ras-mCherry)<sup>966</sup> (Chi et al., 2008), Tg(myl7:GFPr<sup>2a26</sup> (Huang et al., 2003), Tg(kdrl:GFP)<sup>psal</sup> (Jin et al., 2007), Tg(gata1:dsRED)<sup>al2</sup> (Traver et al., 2003) and Tg(axial:GFP)<sup>psata</sup> (Chung and Stainier, 2008) zebrafish lines were used in these studies. Embryos were injected at the 1-cell stage with 8-12 ng of miR-218 MO<sup>1</sup>, 5-10 ng of miR-218 MO<sup>2</sup>, 4 ng robo1 MO, 1 ng robo4 MO, 2 ng slit2 MO, 3 ng slit3 MO or 2.5 ng vegfa MO. For gene interaction studies, sub-phenotypic doses of robo1 (2 ng) and vegfa (1 ng) MOs were used in combination, and for rescue experiments sub-phenotypic doses of robo1 MO (1 ng) were used in combination with a phenotypic dose of miR-218 MO<sup>1</sup> (12 ng). To inhibit Vegf receptor signaling, embryos were treated with the indicated concentration of Vatalanib (LC Laboratories) in embryo water. After transient pulses of the drug, embryos were washed extensively in embryo medium and allowed to develop until analyzed. To better visualize the embryos, pigment development was inhibited with 0.003% phenylthiourea.

Slit2 heat-shock experiments
Tg(hsp70:slit2-GFP)<sup>psata</sup> zebrafish (Yeo et al., 2004) were crossed to the Tg(kdrl:ras-mCherry)<sup>966</sup> line. At the 5-somite stage, embryos were heat shocked for 1 hour at 38°C then returned to 28.5°C and raised until the 20-somite stage. Embryos were fixed and endocardial morphology was assessed by confocal microscopy as described below.

Robo1 overexpression experiments
Rat Robo1 mRNA was synthesized from pSecTag2-Robo1-Myc with T7 polymerase (mMessage mMachine Kit, Ambion) and 75 pg of mRNA was injected. As a control, a similar amount of mCherry mRNA was injected in parallel.

Confocal and fluorescence microscopy and time-lapse analysis
For live time-lapse imaging, embryos were injected with the indicated MO and allowed to develop at 25°C until 15-16 somites. They were then embedded in 1% low-melting-point agarose and imaged at 25°C on a Nikon C1si spectral confocal microscope with a 40×/0.8 NA Apo water-dipping lens. Every 5 minutes, z-stacks of 20-30 sections at 4-μm intervals were acquired. Images were denoised in collaboration with John Sedat (UCSF) with software developed by Jerome Boulanger (Kervrann and Boulanger, 2006). Default parameters were used, except for the patch size, which was 5×5 pixels for the mCherry channel and 8×8 pixels for the GFP channel. Maximum projections were made using ImageJ (NIH).

Cell migration parameters were quantified using the Manual Tracking plug-in in ImageJ. For fixed embryos, 4% paraformaldehyde was used (4°C overnight), and embryos were processed for indirect immunofluorescence. Embryos were imaged on a Zeiss LSM 5 confocal microscope with a 40×/0.75 NA Achrom plan water-dipping lens. For analysis of axial vasculature and heart structure, fixed 48 hours post-fertilization (hpf) embryos were embedded in 1% low-melting-point agarose and sectioned with a Vibratome (250-μm sections). Confocal imaging was performed as above. For analysis of cardiovascular function, live 48 hpf embryos were embedded in 1% low-melting-point agarose and movement of Tg(gata1:dsRED)<sup>al2</sup>-positive cells was observed. Images of live zebrafish were acquired using a Leica MZ16F microscope with a DFC500 camera.

In situ hybridization
Fluorescent in situ hybridization was performed as described (Schoenebeck et al., 2007) using riboprobes for slit2 (Hutson and Chien, 2002), robo1 (Lee et al., 2001) and robo4 (Bedell et al., 2005). miR-218 in situ hybridization was performed as described (Sweetman et al., 2006) using a double DIG-labeled mir-218a LNA probe (Exiqon).

Fluorescence-activated cell sorting (FACS)
FACS was performed essentially as described (Fish et al., 2008). Embryos were manually dechorionated at the 18- to 20-somite stage and digested to a single-cell suspension with TrypLE (Invitrogen). Total RNA was isolated from cell pellets (RNAeasy Micro Kit, Qiagen).

Sprouting assays
Tg(kdrl:GFP) embryos were fixed at the 26-somite stage. Embryos were immunostained with F59 (Developmental Studies Hybridoma Bank) to visualize the somites and with anti-GFP (Invitrogen) to label the blood vessels. The number of intersomitic vessel sprouts present from somites 4-16 (starting from the head and moving posteriorly) and the number of sprouts crossing the myoseptum were quantified.

Transfection and electroporation of plasmids, siRNAs and microRNA mimics
HeLa cells (ATCC) were transfected using Lipofectamine 2000 (Invitrogen) and HUVEC (ScienCell) were electroporated using the Amaxa Nucleofector according to the manufacturer’s recommendations. For transfection of endothelial cells with mir-218 mimics (20 nM), RNAiMax (Invitrogen) was used. Cells were analyzed at 48 hours post-transfection. For small interfering RNA (siRNA)-mediated knockdown of ROBO1 and ROBO4, SilencerSelect siRNAs (50-100 nM, Ambion) were used.

Cell treatments
Recombinant human SLIT2-N and VEGF were purchased from PeproTech and BD Biosciences, respectively. HUVEC were serum-starved overnight in basal medium with 0.1% BSA and pretreated with 10 nM SLIT2-N for 1 hour or were left untreated, followed by stimulation with 10 ng/ml VEGF for 10 minutes.

Migration and scratch wound assays
The migration of endothelial cells was assessed as described (Fish et al., 2008).

Cell polarity experiments
After 16 hours of migration in a scratch wound assay, HUVEC were immunostained for Gigantin (Abcam) and were counterstained with FITC-phalloidin (Invitrogen) and DAPI. The position of the Golgi apparatus (Gigantin staining) with respect to the nucleus was determined. Cells in which the Golgi was less than 45° from the wound face were classified as being oriented towards the wound.

ROBO1 rescue of mir-218 mimic-transfected cells
HUVEC (0.5×10<sup>6</sup>) were electroporated with 2.5 μg of control or Robo1 expression plasmids with the Amaxa Nucleofector apparatus. After 24 hours, cells were transfected with control or mir-218 mimics (20 nM, Dharmaco) using RNAiMax (Invitrogen). Cells were analyzed 48 hours later.

Luciferase assays
 Confirmation of the targeting of robo1/ROBO1, robo2/ROBO2 and srgap2/SRGAP2 by miR-218 was performed as described (Fish et al., 2008). Mutagenesis of the predicted microRNA seed sequence in robo1/ROBO1 and robo2/ROBO2 was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The miR-218 seed sequence UGUCGUU was mutated to UGACGUU.

Western blotting
Western blots were performed as described (Fish et al., 2008) using the following antibodies: anti-phospho-ERK1/2 (MAPK3/1) (Thr202/Tyr204, Cell Signaling), anti-ERK2 (Santa Cruz), anti-GAPDH (Santa Cruz), anti-ROBO1 (Abcam, ab7279), anti-phospho-VEGFR2 (KDR) (Tyr1175, Cell Signaling) and anti-VEGFR2 (Cell Signaling).

Quantification of gene expression by real-time quantitative reverse transcriptase PCR (qRT-PCR)
MicroRNA and mRNA expression analyses were performed as described (Fish et al., 2008). Primer sequences are available upon request. For absolute quantification experiments, a mir-218 mimic, or PCR amplicons encompassing the gene of interest, were utilized to generate a standard curve. For expression analysis of zebrafish mir-218a-1/mir-218a-2 and mir-218b, RNA was reverse transcribed using the QuantiMir Kit from Systems Biosciences and primers specific for mir-218a or mir-218b were utilized.
miR-218 regulates Slit/Robo signaling

Statistical analysis

Unless otherwise stated, all experiments were performed a minimum of three times and data represent the mean ± s.e.m. Statistical analyses were performed using a Student’s t-test, ANOVA and the Newman-Keuls post-hoc test, or a Fisher’s exact test, as appropriate. P<0.05 was considered statistically significant.

RESULTS

miR-218 is intronically encoded in SLIT2 and SLIT3 and targets the SLIT receptors ROBO1 and ROBO2

We found that a family of highly conserved microRNAs – miR-218a-1 and miR-218a-2 – is encoded intronically in SLIT2 and SLIT3, respectively (Fig. 1A). Unlike in mammals, zebrafish have three genomic copies of miR-218. Whereas miR-218a-1 and miR-218a-2 are located in slit2 and slit3, respectively, miR-218b is intergenic. Quantitative real-time PCR with primers specific to miR-218a or miR-218b revealed that miR-218b is expressed at ~60-fold lower levels than miR-218a in 20-somite stage zebrafish embryos (data not shown), suggesting that expression of miR-218 in zebrafish is likely to be primarily directed by the slit2 and slit3 loci.

A bioinformatic search for potential targets of miR-218 identified conserved complementary sequences in the 3’ UTRs of ROBO1 and ROBO2 (Fig. 1B). Human and zebrafish ROBO1/robo1 or ROBO2/robo2 3’ UTRs were sensitive to miR-218-dependent repression in luciferase assays and mutation of their miR-218 target sites alleviated this repression (Fig. 1C). Overexpression of miR-218 in human umbilical vein endothelial cells (HUVEC) decreased endogenous ROBO1 and ROBO2 mRNA (Fig. 1D). ROBO1 protein levels were also decreased (Fig. 1E), whereas ROBO2 protein was undetectable in HUVEC (data not shown). These findings suggest that SLIT2/3-encoded miR-218 negatively regulates ROBO1/2 expression.

SLIT-ROBO Rho GTPase activating protein 2 (SRGAP2), a downstream component of Slit/Robo signaling, was also identified as a potential target of miR-218. In contrast to ROBO1 and ROBO2, its putative target site in the 3’ UTR was not as highly conserved across phyla (see Fig. S1A in the supplementary material). However, human and zebrafish SRGAP2/srgap2 3’ UTRs were repressed by miR-218 in luciferase assays (see Fig. S1B in the supplementary material). Similar to other microRNAs that target multiple components of a common pathway (Fish et al., 2008; Cordes et al., 2009), miR-218 might regulate Slit/Robo signaling at multiple nodes.

Slit/Robo signaling regulates cardiovascular function in zebrafish

Examination of miR-218 expression during zebrafish development revealed that it was upregulated at ~24 hpf and remained highly expressed at the subsequent developmental stages analyzed (Fig. 2A). In situ hybridization revealed strong expression of miR-218 in the heart and neural tissue at 72 hpf (Fig. 2B). miR-218 expression in neural tissue, in light of the enriched expression of the host genes slit2 and slit3 in these same tissues (Holmes and Niswander, 2001), further implies that miR-218a is likely to be processed from slit2/3. To investigate the in vivo function of miR-218, we designed two morpholinos (MOs) to knock down miR-218 expression. The first MO (miR-218 MO1) targeted the Drosha (Rnasen) cleavage site of miR-218a-1, miR-218a-2 and miR-218b (see Fig. S2A in the supplementary material). The second Drosha-blocking MO (miR-218 MO2) was more specific for miR-218a-1 and miR-218a-2 than for miR-218b (see Fig. S2A in the supplementary material), as our previous experiments revealed a lower level of expression of miR-218b (data not shown). Injection of either of these MOs at the 1- to 2-cell stage resulted in a dose-dependent knockdown of mature miR-218, without affecting slit2 and slit3 mRNA levels (Fig. 2C and see Fig. S2B in the supplementary material). miR-218 MO1-injected embryos were morphologically indistinguishable from control embryos, with the notable exception of severe pericardial edema at 48 hpf (Fig. 2D; for quantification, see Fig. S3 in the supplementary material). This same phenotype occurred at a similar penetrance in embryos injected with miR-218 MO2 (see Fig. S2C and, for quantification, Fig. S3 in the supplementary material).

Direct visualization of blood flow revealed reduced circulation in miR-218 MO1 morphants at 48 hpf, although gross vascular patterning appeared normal (Fig. 2D; for quantification see Fig. S3 in the supplementary material). Injection of miR-218 MO2 resulted in similar circulation defects (see Fig. S2C in the supplementary material), and a small percentage of embryos also had minor vascular defects (for quantification see Fig. S3 in the supplementary material). Because of the apparent lower toxicity, we used miR-218 MO1 for all further phenotypic analyses.

The sprouting of the intersomitic vessels (ISVs) at 22 hpf was unperturbed in miR-218 MO1-injected embryos (see Fig. S4A,B in the supplementary material), and axial vessels were lumenized (see Fig. S4C in the supplementary material). However, we also found no evidence of hemorrhage in miR-218 morphants (Fig. 2D; see Fig. S2C and Fig. S3 in the supplementary material). The hearts of miR-218 morphants were morphologically abnormal at 48 hpf (Fig. 2E). The combination of a dysmorphic heart and pericardial edema and the absence of severe vascular defects suggested that miR-218 might be required for cardiogenesis.

As miR-218 is embedded within the slit2/3 genes and directly represses Robo1 and Robo2 in vitro, we assessed the role of the ligands Slit2 and Slit3 and the receptors Robo1, Robo2 and Robo4 in zebrafish cardiovascular development. Importantly, MO-mediated knockdown of slit2 did not affect miR-218 expression (data not shown). At 48 hpf, slit2 morphants had pericardial edema and circulation defects but apparently normal vascular patterning (see Fig. S5A and, for quantification, Fig. S3 in the supplementary material). By contrast, slit3 morphants did not develop pericardial edema; however, slit3 morphants displayed highly penetrant vascular defects, characterized primarily by missing or detached ISVs and/or dorsal longitudinal anastomotic vessels (DLAVs) (see Fig. S3 and Fig. S5A in the supplementary material). Similar to slit2 morphants, robo1 and robo4 morphants also developed pericardial edema and circulation defects (see Fig. S3 and Fig. S5A in the supplementary material). By contrast, examination of robo2 homozygous loss-of-function mutants (astray; ast272z) (Fricke et al., 2001) revealed no obvious defects in the development or function of the cardiovascular system [at 72 hpf, 6% of astray mutants (n=18) and 7% of control embryos (n=31) had mild pericardial edema; see Fig. S5B in the supplementary material]. Sprouting angiogenesis at 22 hpf (see Fig. S4A,B in the supplementary material) and vascular patterning at 48 hpf (see Fig. S5A in the supplementary material) appeared grossly unaffected in slit2, robo1 or robo4 morphants, suggesting that these genes do not play a major role in vascular development. These data suggest that Slit2, Robo1, Robo4 and miR-218 appear to regulate cardiac development and function, whereas Slit3 participates in vascular development.

Slit/Robo signaling regulates migration of the heart fields in zebrafish

In Drosophila, Slit/Robo signaling is required for proper migration and alignment of cardioblasts at the midline (Qian et al., 2005; Medioni et al., 2008; Santiago-Martinez et al., 2008).
Since cardiac function was impaired in Slit/Robo morphants at late developmental time points (i.e. 48 hpf), we examined whether these late defects arise from an earlier requirement for Slit/Robo signaling during heart field fusion. First, we examined the expression of several Slit/Robo signaling components by mRNA in situ hybridization at stages when the heart fields are fusing at the midline (18-20 somites; see Fig. S6A in the supplementary material). 

\textit{slit2} appeared to be expressed in midline structures, including the floor plate of the neural tube. Additionally, \textit{slit2} was expressed in the endoderm and in endocardial cells. \textit{robo1} appeared to be expressed in the endoderm, myocardium and endocardium, whereas \textit{robo4} expression was detected in the dorsal neural tube and at low levels in the endocardium. Quantification of \textit{miR-218}, \textit{slit2}, \textit{slit3}, \textit{robo1}, \textit{robo2} and \textit{robo4} expression was performed by real-time qRT-PCR analysis of FACS-isolated endothelial [\textit{Tg(kdrl:GFP)}], myocardial [\textit{Tg(myl7:GFP)}] or endodermal [\textit{Tg(sox17:GFP)}] cells from 18- to 20-somite stage embryos (see Fig. S6B in the supplementary material). Each transcript, with the exception of \textit{robo2}, was expressed at appreciable levels in each of these tissue types, confirming that they are present in cell types that are required for heart formation, namely the endothelium/endocardium, myocardium and endoderm.

We examined the functional role of the Slit/Robo signaling pathway in the migration of the bilateral heart fields to the midline. In vivo time-lapse confocal microscopy revealed that knockdown of \textit{slit2} resulted in abnormal migration of endocardial cells (Fig. 3A; see Fig. S7A in the supplementary material). Individual endocardial cells migrated significantly faster in \textit{slit2} morphants than in controls (Fig. 3B) and also exhibited a loss of directionality (Fig. 3C; see Fig. S7A and Movies 1 and 2 in the supplementary material). \textit{slit2} knockdown also disrupted collective cell migration, as individual migrating endocardial cells did not contact neighboring cells and they extended numerous filopodia in multiple directions (Fig. 3A, arrows; see Movies 1 and 2 in the supplementary material). However, the speed (Fig. 3B) and directionality (see Fig. S7B in the supplementary material) of myocardial migration were not affected in \textit{slit2} morphants compared with controls, suggesting that Slit2 primarily regulates endocardial migratory behavior. As a consequence of the disrupted migration of the heart fields, multiple lumens appeared to form in 43% of morphants ($n=57$; see Fig. S7C in the supplementary material).

In contrast to \textit{slit2} morphants, \textit{miR-218} knockdown reduced the migration rate of endocardial cells (Fig. 3A,B), perhaps owing to a loss of \textit{miR-218}-mediated repression of the Slit receptors Robo1 and/or Robo2. Myocardial cell migration was also reduced after
miR-218 knockdown (Fig. 3A,B) and these morphants showed an increase in incomplete heart field fusion at the 20-somite stage (40% of miR-218 morphants (n=53) versus 8% of control embryos (n=125)). Although we were unable to detect a significant change in robo1 or robo2 expression by qRT-PCR analysis of whole miR-218 morphant embryos (data not shown), in situ hybridization suggested increased robo1 expression in the endocardium and myocardium of miR-218 morphants at the 20-somite stage (Fig. 3D). These data indicate that miR-218, potentially through its regulation of Robo1 and/or Robo2, controls heart field migration.

As robo2 is expressed at low levels in the endocardium/myocardium and robo2 mutants displayed no cardiovascular abnormalities, we focused our attention on the other miR-218 target, robo1, and its role in heart field migration. robo1 knockdown revealed an unanticipated inhibition of endocardial and myocardial migration (Fig. 3A,B), and many morphant embryos (47%; n=108) had unfused heart fields at 20 somites. Unlike slit2 morphants, which had hyper-migratory endocardial cells with multiple filopodial projections, the endocardial cells in robo1 morphants exhibited a rounded, non-migratory morphology (Fig. 3A, arrow).

Whereas Drosophila have three Robo family members, vertebrates have a fourth, Robo4, which is enriched in the endothelium and expressed at low levels in the endocardium in zebrafish (see Fig. S6A,B in the supplementary material). Migration of the heart fields to the midline was not delayed in robo4 morphants (Fig. 3A). Only 15% of robo4 morphants (n=68) displayed delayed heart field fusion and endocardial cell migration speed was unaffected (Fig. 3B). However, endocardial cell morphology (Fig. 3A) and directionality (Fig. 3C) were affected, suggesting that Robo4 might play a role in heart tube formation. This is consistent with the pericardial edema and circulation defects observed in robo4 morphants at 48 hpf (see Fig. S3 and Fig. S5A in the supplementary material). The phenotypic differences between robo1 and robo4 morphants suggest that these Robo family members have distinct roles in regulating the migration of the heart fields to the midline.

To test the sensitivity of zebrafish heart field migration to Robo1 dosage, we performed Robo1 gain-of-function experiments by injecting rat Robo1 mRNA (or mCherry mRNA as a control) into 1-cell stage embryos and observed heart field migration at 20 somites. Injection of Robo1 mRNA resulted in incomplete heart field fusion compared with mCherry injection (47% of Robo1 mRNA-injected (n=19) versus 15% of mCherry mRNA-injected (n=13) embryos had delayed fusion; Fig. 4A). We reasoned that if the miR-218 morphant phenotype was due, at least in part, to increased Robo1 expression, robo1 knockdown should rescue the miR-218 morphant phenotype. Since robo1 knockdown also

Fig. 2. miR-218 regulates heart formation and function. (A) Expression of miR-218 (relative to 0.5 hpf timepoint) was monitored by real-time qRT-PCR during zebrafish development. (B) In situ analysis of miR-218 expression at 72 hpf reveals expression in the heart and neuronal tissue. (C) Expression of miR-218, slit2 and slit3 were quantified by real-time qRT-PCR in miR-218 morphants (2.5, 5 and 10 ng of miR-218 MO1) at 48 hpf. Data are mean + s.e.m. (D) Control and miR-218 morphants were assessed at 48 hpf by live imaging of embryos. Phase-contrast images (top) demonstrate pericardial edema in the miR-218 morphant (arrow). Fluorescent images of the same embryos show labeling of endothelial/endocardial cells in Tg(kdrl:GFP) (middle) and labeling of blood cells in Tg(gata1:dsRed) (bottom). Vascular patterning appears normal in miR-218 morphants, but circulation is reduced. (E) Cardiac morphology at 48 hpf was assessed by confocal microscopy (ventral view). Tg(myt7:GFP) expression labels the myocytes and Tg(kdrl:ras-mCherry) labels the endocardium. miR-218 morphant hearts contain myocytes and endocardium but exhibit severe morphological defects. ht, heart; nt, neural tube; a, atrium; v, ventricle; ba, branchial arches; h, head; isv, intersomitic vessel; da, dorsal aorta; pcv, posterior cardinal vein. Scale bars: 100 μm in B,D; 25 μm in E.
induced heart field migration defects, we utilized a sub-phenotypic dose of robo1 MO (1 ng). Whereas miR-218 knockdown (n=64) delayed heart field migration compared with a control MO (n=71), a combination of a sub-phenotypic dose of robo1 MO with a phenotypic dose of miR-218 MO (n=38) significantly rescued the miR-218 phenotype (P<0.0001, Fig. 4B). These results suggest that elevated Robo1 levels are at least partly responsible for the observed heart field migration defects in miR-218 morphants, perhaps through an increase in Robo1/Slit2 repulsion, and that heart field migration is sensitive to Robo1 dosage.

In a similar fashion, we examined Slit2 gain-of-function in vivo. In control embryos, endocardial cells were arranged in a disc with a core of densely packed cells centered at the embryonic midline (Fig. 4C). However, in embryos overexpressing Slit2-GFP (beginning at the 5-somite stage) endocardial cells did not form a disc at the 20-somite stage, but were medially located diffuse and less densely packed at the midline [70% of Slit2-GFP embryos displayed this phenotype (n=16) compared with 16% of controls (n=12); Fig. 4C]. Endocardial cells also appeared larger and less rounded than in control embryos. These results, combined with the slit2 knockdown data and robo1 loss- and gain-of-function data, suggest that the dosage of Slit-Robo signaling components is important in endocardial cell migration.

**Vegf signaling controls heart tube fusion and is regulated by Slit/Robo**

The migration defects observed in slit2 morphants led us to hypothesize that the Slit/Robo signaling pathway acts to control the responsiveness of endocardial cells to a midline chemotactrant. Slit2 inhibits Vegf signaling in endothelial cells (Jones et al., 2008; Jones et al., 2009; Marlow et al., 2010) and vegfa knockdown induces profound pericardial edema by 48 hpf (Nasevicius et al., 2000). However, it is unclear whether this edema is exclusively attributable to defective angiogenesis from 22 hpf onward, or if Vegf also plays a primary role in cardiac morphogenesis. We therefore examined whether Vegf is involved in recruiting endocardial cells to the midline in zebrafish.

vegfa knockdown delayed heart field fusion in 63% of morphants (n=16). Both endocardial and myocardial cells migrated more slowly in vegfa morphants than in controls (Fig. 5A). Transient inhibition of Vegf signaling with a Vegfr inhibitor during heart field migration (14-20 somites; 16-19 hpf), but before the onset of angiogenesis, delayed heart tube fusion at the 20-somite stage [Fig. 5A; 41% of inhibitor-treated embryos had delayed migration (n=17)] and resulted in severe cardiac dysfunction at 48-72 hpf (Fig. 5B). This phenotype occurred in the absence of vascular patterning defects, as the drug was removed.
before ISV sprouting. By contrast, continued inhibition of Vegf signaling from 16 to 48 hpf resulted in profound pericardial edema and severe defects in vascular patterning, including a failure to form distinct arterial and venous axial vessels (Fig. 5B). Consistent with an early role for Vegf in cardiac morphogenesis, inhibiting Vegfr after heart tube fusion (24-48 hpf) did not result in edema or cardiac defects, despite obvious angiogenic defects (Fig. 5B). These experiments reveal a temporal window during which Vegf signaling appears to regulate heart formation, prior to its role in blood vessel patterning.

Whereas directional migration was compromised in slit2 morphant endocardial cells, perhaps owing to enhanced Vegf motogenic activity, robo1 morphants had reduced endocardial motility. We hypothesized that, in contrast to Robo4, which inhibits Vegf signaling, Robo1 might potentiate the responsiveness of the heart fields to Vegf. We injected sub-phenotypic doses of vegfa and robo1 MOs, either alone or in combination, at the 1- to 2-cell stage and assessed heart field fusion at 20-22 somites (Fig. 5C). At the low doses used, robo1 or vegfa MO alone had only subtle effects on heart field fusion. However, co-injection of sub-phenotypic doses of robo1 and vegfa MOs dramatically increased the percentage of embryos with delayed heart field fusion. In addition, 22% of the co-injected embryos (n=51) had a delayed migration phenotype resembling cardia bifida, compared with only 2.5% of vegfa-only (n=40) and no robo1-only (n=37) or control MO-injected (n=23) embryos. These results reveal an in vivo interaction between robo1 and vegfa in the regulation of heart field migration.

To determine the mechanisms by which the Slit/miR-218/Robo axis might regulate Vegf signaling, miR-218 was overexpressed in cultured endothelial cells (HUVEC). In a wound closure assay, miR-218-overexpressing cells failed to respond to VEGF-induced migration (Fig. 6A). miR-218 overexpression also disrupted the polarization of the cells towards the wound edge (Fig. 6B). We next examined MAP kinase pathway activation in response to VEGF stimulation and found that endothelial cells overexpressing miR-218, and thus expressing less ROBO1, failed to robustly activate the MAP kinase pathway after VEGF treatment (Fig. 6C). Pretreatment of endothelial cells with recombinant SLIT2-N [the bioactive, N-terminal cleavage fragment of SLIT2 (Nguyen Ba-Charvet et al., 2001)] blunted the Vegf-induced activation of the MAP kinase pathway, and this SLIT-dependent effect was enhanced in miR-218 mimic-transfected cells (Fig. 6D). ROBO1 knockdown (Fig. 6E) recapitulated the MAP kinase signaling defect seen in miR-218-overexpressing cells, especially in SLIT2-N pretreated cells (Fig. 6F).

Although SLIT2 directly interacts with ROBO1 (Suchting et al., 2005), it is not clear whether SLIT2 directly interacts with ROBO4 (Park et al., 2003; Suchting et al., 2005; Zhang et al., 2009). However, SLIT2 inhibits VEGF signaling through a ROBO4-dependent pathway (Jones et al., 2008; Jones et al., 2009; Marlow et al., 2010), perhaps by binding to a ROBO4 co-receptor, such as ROBO1 or Syndecan (Sheldon et al., 2009). To determine whether ROBO1 promotes VEGF signaling directly or by antagonizing SLIT2/ROBO4 inhibition of VEGF signaling, we knocked down ROBO4 in HUVEC (Fig. 6E). ROBO4 knockdown increased VEGF signaling, as reported (Jones et al., 2008; Jones et al., 2009). Combinatorial knockdown of ROBO1 and ROBO4 partially rescued the ROBO1 knockdown signaling defect (Fig. 6F). This result suggests that, although ROBO1 and ROBO4 may compete for SLIT2-dependent pathway activation, ROBO1 also exerts a positive effect on VEGF signaling that is independent of ROBO4.

To determine if ROBO1 downregulation was responsible for the MAP kinase signaling defects in miR-218-overexpressing cells, we transfected Robo1 (lacking the 3' UTR) into endothelial cells. Exogenous ROBO1 largely rescued the defect in MAP kinase activation in miR-218-overexpressing cells and restored VEGF signaling in SLIT2-N-pretreated cells (Fig. 6G). To elucidate how SLIT2/ROBO1 signaling impinges on the VEGF signaling pathway, we examined signaling events proximal to VEGF-induced activation of the VEGF receptor VEGFR2. VEGF-dependent autophosphorylation of VEGFR2 (Y1175) was diminished in ROBO1 knockdown cells, and this effect was even more dramatic in SLIT2-N-pretreated cells (Fig. 6H). ROBO4 knockdown did not appear to affect VEGFR2 phosphorylation, in agreement with a previous report (Jones et al., 2008).

**Fig. 4. Heart field migration is sensitive to Robo1 dosage.** (A) Endocardial migration was defective at 20 somites in zebrafish embryos injected with 75 pg Robo1 mRNA (mCherry mRNA was utilized as a control). (B) Rescue of migration defects in miR-218 morphants was accomplished by co-injecting sub-phenotypic doses of robo1 MO. Quantification of migration defects is shown beneath. *, P<0.05 (Fisher’s exact test for a 2 x 3 contingency table). (C) Analysis of endocardial migration at the 20-somite stage in Tg(hsp70l:slit2-GFP) embryos that were heat shocked at 5 somites. Embryos lacking the transgene were used as a control. Disorganization of the endocardium was evident in Slit2-overexpressing embryos. Scale bars: 50 μm.
DISCUSSION

The data presented here indicate that Slit/Robo signaling controls migration of the heart fields to the midline in zebrafish, in part by modulating Vegf signaling in the endocardium (Fig. 7). The titration of Vegf activity may be further refined by miR-218, which is intronically encoded in slit2 and slit3 and directly targets robo1 for repression. This Slit/miR-218/Robo/Vegf feedback regulatory loop provides an elegant mechanism for precise regulation of the migration of the heart fields to form the linear heart tube.

Vegf signaling is tightly regulated in endothelial cells. This regulation includes crosstalk with other signaling pathways, such as Notch and TGFβ (for a review, see Holderfield and Hughes, 2008), as well as Vegf retention in the extracellular matrix (Park et al., 1993; Chen et al., 2010; Purushothaman et al., 2010). Vegf signaling can also be regulated by microRNA-mediated control of signaling molecules that act downstream of the Vegf receptor (Fish et al., 2008; Wang et al., 2008; Nicoli et al., 2010). Our data lend further evidence to a functional interplay between the Slit/Robo pathway and Vegf signaling (Jones et al., 2008). Using temporal inhibition of Vegf activity, we found that Vegf is a mediator of midline heart field migration in zebrafish. We additionally showed that robo1 and vegfa interact in vivo to control heart field migration, and that Robo1 facilitates the phosphorylation of Vegfr2 in response to Vegf. Our data suggest that the robo1 gene dosage is critical for the proper migration of the heart fields to the midline. Although Robo1 appears to be a positive regulator of Vegf signaling and robo1 morphants displayed a delayed heart field migration phenotype, the phenotype of miR-218 morphants resulted in profound vascular defects observable at the 20-somite stage. Quantification of phenotypes is shown beneath. Mild delay was defined as the contralateral heart fields contacting at the posterior end but remaining unfused anteriorly. Severe delay was defined as a complete absence of heart field fusion at the midline. *, P<0.05 (Fisher’s exact test for a 2×3 contingency table). h, head; ht, heart; isv, intersomitic vessel; da, dorsal aorta; pcv, posterior cardinal vein. Scale bars: 50 μm in A,C; 100 μm in B.

Fig. 5. Vegf regulates the migration of the endocardium/myocardium towards the midline. (A) Defects in heart field migration in zebrafish vegfa morphants and embryos treated with Vegfr inhibitor (10 μM Vatalanib at 14-20 somites, 16-19 hpf). Quantification of migration velocity of the myocardium [Tg(my7:GFP) expression] and endocardium [Tg(kdrl:ras-mCherry) expression] in vegfa morphants is indicated beneath. *, P<0.05 compared with control. Data are mean ± s.e.m. (B) The patterning of the intersomitic vessels (ISVs) and the head vasculature is severely disrupted by inhibiting Vegfr after heart field migration has occurred (1 μM Vatalanib at 22-48 hpf, left panels), but pericardial edema does not occur and blood flow through the dorsal aorta and posterior cardinal vein is robust. By contrast, edema (arrow) and circulation defects occur when Vegfr is inhibited during heart field migration (10 μM Vatalanib at 16-19 hpf, middle panels), despite the normal patterning of the vessels after removal of the drug at 19 hpf. Continued inhibition of Vegfr (10 μM Vatalanib from 16-48 hpf, right panels) results in profound edema (arrow), which appears to result from severe vascular defects, including a fused dorsal aorta/posterior cardinal vein. Quantification of circulation phenotypes is shown beneath. A circulation defect was defined as reduced blood flow in the axial vessels. (C) Genetic interaction between vegfa and robo1 was assessed by use of sub-phenotypic levels of vegfa and robo1 MOs. The combination of these MOs resulted in profound migration defects observable at the 20-somite stage. Quantification of phenotypes is shown beneath. Mild delay was defined as reduced blood flow in the axial vessels.
responses to motogenic stimuli such as Vegf. Since Vegf receptors are expressed on endocardial cells (Bussmann et al., 2007), Vegf is likely to signal directly to the endocardium to regulate cell migration to the midline. The defects in myocardial migration in vegfa morphants might therefore be due to endocardial-myocardial crosstalk (Holtzman et al., 2007). Since miR-218 and Slit/Robo components are expressed in the endothelium/endocardium, our data also suggest that Slit/Robo signaling might play a cell-autonomous role within the endocardium to control Vegf responsiveness (Fig. 7). The distinct contributions of autocrine versus paracrine Slit/Robo signaling in the control of heart field migration remain to be determined.

The conserved genomic organization of Slit2/miR-218-1 and Slit3/miR-218-2 in vertebrates suggests an important and conserved function for the embedding of this microRNA in the Slit2/3 genes. Whether a similar Slit/miR-218/Robo signaling network also controls mammalian heart formation remains to be determined; however, a similar regulatory loop appears to control postnatal vascular growth in mice (Small et al., 2010). Interestingly, dynamic regulation of Robo1 is crucial during Drosophila development (Kidd et al., 1998b). The Drosophila genome does not encode miR-218, but Robo1 expression is controlled by a Drosophila-specific protein, Commiesureless, that regulates the cell-surface localization of Robo1. Our findings provide insight into how Robo1 titration can be achieved during development in the absence of a vertebrate Commiesureless homolog.

In summary, we have elucidated a novel regulatory pathway that controls the migration of the heart fields to the midline in zebrafish. Our findings provide a new paradigm of receptor/ligand regulation, in which a ligand-encoded microRNA regulates the expression of its own receptor. Given the importance of the Slit/Robo pathway in the development and pathology of other organ systems (Kidd et al., 1999; Wu et al., 2001; Wang et al., 2003; Marlow et al., 2010; Tie...
et al., 2010), our findings are likely to have broad implications for understanding the contribution of the Slit/Robo pathway to the regulation of developmental and pathological processes.

Acknowledgements
We thank Christopher Jones for valuable discussions; the laboratory of Dean Y. Li (University of Utah) for providing protocols and reagents; Ana Ayala and Milly Alva for fish care; Jan Moulton (Gene Tools) for morpholino design; Wendy Staub (Hervig Baur laboratory, UCSF) for technical assistance with microRNA in situ; the Nikon Imaging Center at UCSF for imaging equipment and software; Shiel Dandekar and Orion Weiner (UCSF) for help with image denoising; and Gary Howard and Bethany Taylor for editorial assistance. J.E.F. was supported by postdoctoral fellowships from the Canadian Institute of Health Research and the Heart and Stroke Foundation of Canada; J.D.W. and S.W. were supported by postdoctoral fellowships from the National Institutes of Health (NIH T32HL007731); B.G.B. received funding from NHLBI (P01-HL089707-01A1) and the Lawrence J. and Florence A. DeGeorge Charitable Foundation; and D.S. received funding from the NHLBI/NIH, the HL089707-01A1) and the Lawrence J. and Florence A. DeGeorge Charitable Foundation. B.G.B. received funding from NHLBI (P01-HL089707-01A1) and the Lawrence J. and Florence A. DeGeorge Charitable Foundation; and D.S. received funding from the NHLBI/NIH, the HL089707-01A1) and the Lawrence J. and Florence A. DeGeorge Charitable Foundation.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl?URL=10.1242/dev.060046

References
miR-218 regulates Slit/Robo signaling

RESEARCH ARTICLE 1419

cardiac defects associated with Slt3-deficiency in mice. Mech. Dev. 120, 1059-1070.