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Slit coordinates cardiac morphogenesis in *Drosophila*

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Abstract

Slit is a secreted guidance cue that conveys repellent or attractive signals from target and guidepost cells. In *Drosophila*, responsive cells express one or more of three Robo receptors. The cardial cells of the developing heart express both Slit and Robo2. This is the first report of coincident expression of a Robo and its ligand. In *slit* mutants, cardial cell alignment, polarization and uniform migration are disrupted. The heart phenotype of *robo2* mutants is similar, with fewer migration defects. In the guidance of neuronal growth cones in *Drosophila*, there is a phenotypic interaction between *slit* and *robo* heterozygotes, and also with genes required for Robo signaling. In contrast, in the heart, *slit* has little or no phenotypic interaction with Robo-related genes, including Robo2, Nck2, and Disabled. However, there is a strong phenotypic interaction with Integrin genes and their ligands, including Laminin and Collagen, and intracellular messengers, including Talin and ILK. This indicates that Slit participates in adhesion or adhesion signaling during heart development.

Keywords: Vasculogenesis; Guidance signaling; Adhesion Integrin; Laminin; Robo; Migration

Introduction

Guidance cues are tethered or diffusible factors generated by intermediate and/or final target cells to direct cell migration during morphogenesis. Four classes of guidance cues (Ephrins, Netrins, Semaphorins and Slits) have been revealed in studies of axon pathfinding in the nervous system (Klagsbrun and Eichmann, 2005; Tessier-Lavigne and Goodman, 1996). These ligands and their receptors have diverse patterns of expression during development of many other tissues. Each class of ligand may act to attract or repel cell migration, depending upon the receptor or second messenger status of the guided cell (Englund et al., 2002; Hinck, 2004; Holmberg et al., 2000; Hopker et al., 1999; Song et al., 1998).

Blood vessels form a remarkable precise network that navigates through and around tissues much like peripheral nerves. In some tissues, arteries follow peripheral nerves as if guided by them. Indeed, recent reports have established that all four classes of guidance cues play a role in vasculogenesis or angiogenesis (Carmeliet and Tessier-Lavigne, 2005; Hinck, 2004; Klagsbrun and Eichmann, 2005). Of these, Slit is the least characterized guidance factor of the cardiovascular system. A novel Slit receptor, Robo4 is expressed by endothelia (Huminiecki et al., 2002). Robo4 mutant mice have no phenotype, but in vitro, Robo4 repels endothelial cells from a source of Slit2 (Park et al., 2003). In contrast, tumor cells also express Slit2, triggering an attractive response in Robo1 expressing endothelial cells, and triggering vascularization of growing tumors (Wang et al., 2003).

Many guidance factors were first identified in *Drosophila* and *C. elegans*, where a genetic approach has revealed much of what we know about these molecules (Jacobs, 2000; Tessier-Lavigne and Goodman, 1996). The tracheal system of *Drosophila* is an exquisitely patterned network analogous to the vascular system, and employs guidance cues to navigate tissue. In particular, Slit acts as an attractant to direct tracheal growth into the nervous system, and then acts as a repellent to keep trachea away from the midline (Englund et al., 2002; Gallio et al., 2004).

Drosophila is the simplest genetic model organism which has a heart. Early development of the *Drosophila* heart mirrors vertebrate heart formation, before looping morphogenesis (Bier and Bodmer, 2004; Bodmer and Venkatesh, 1998). In both

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organs, the bilateral strip of mesoderm most distant from the point of gastrulation migrates dorsally (*Drosophila*) or ventrally (vertebrates) and then medially to fuse into a single structure at the midline. Subsequently, these cells undergo vasculogenesis to form a lumen. The guidance factors regulating vertebrate heart formation have not been identified. Of the *Drosophila* guidance factors, Slit is the only one known to be expressed in the heart (Rothberg et al., 1990). While this work was in review, a study of the heart phenotype of mutations in *slit* and *robo* in the heart was published (Qian et al., 2005).

Genetic studies of Slit have exploited the dose sensitive nature of *slit*. *Drosophila* heterozygous for a mutation in *slit* are normal. However, reduced expression of a gene that acts in the same or converging function as *slit* will reveal a phenotype in *slit* heterozygotes (Kim et al., 2002; Stevens, 2000). In this study we characterize the requirement for Slit, and its receptor Robo2 in heart assembly. *slit* has a penetrant phenotypic interaction with genes for Integrin based function, and not for genes associated with Robo signaling, suggesting that adhesion or adhesion signaling is a major component of Slit function in heart formation.

Materials and methods

Drosophila stocks

Loss of function alleles of sli^{2}, $scb^{2,10288}$, mys^{1}, mew^{M6}, lea^{2}, $wb^{SF11,9437}$, $dock^{04723}$, $vkg^{177\text{-}2}$, dab^{M54}, $rhea^{1}$, and $ILK^{ZCL3111,\,2}$ were obtained from the Indiana Stock Centre. Stocks were maintained over marked balancers. The *laminin* allele $lanA^{9\text{-}32}$ and sli^{2990} were provided by C. Goodman (Berkeley), ras^{5703} by D. Montell (Johns Hopkins University), lea^{5418} by C. Klämbt (Münster), vkg^{p10388} by N. McGinnis (University of Massachusetts) and tig^{X} was provided by T. Bunch (University of Arizona). Multiple alleles of each gene were tested when possible.

Transposon exchange

The cardial cell GAL4 driver was created by converting the B2-3-30 *lac*Z enhancer trap to a GAL4 via P-element exchange (Sepp and Auld, 1999).

Rescue of slit²

Full-length and LRR (Leucine Rich Repeat) deletion transgenes in *p[UASt]* (Battye et al., 2001) were each established in a sli^{2}*/Cyo[eng-lacZ]* mutant background and then mated with flies containing the cardial cell GAL4 driver. Homozygous *sli* embryos were determined by lack of *lac*Z staining for the marked balancer and analyzed for a heart phenotype.

β-galactosidase antibody generation

Polyclonal α–βgal Ab was generated in chickens using β-galactosidase protein (Sigma). Purification of the yolk IgY was performed according to Bird and Thorpe (Bird and Thorpe, 2002). A CNBr sepharose (Pharmacia) β-galactosidase column was used to purify the β-gal IgY antibody, which was subsequently tested for specificity using both Western blot and immunohistochemistry.

In situ hybridization

Whole-mount *Drosophila* RNA in situ hybridization was performed according to Tautz and Pfeifle (1989). Dig-labeled RNA sense and α-sense probes were generated using a Dig RNA labeling kit (Roche), with probes of 200–300 base pairs in length. After incubation with α-Digoxigenin antibody (Roche), color reaction was performed using NBT and BCIP (Roche). Embryos were mounted in glycerol and visualized using a Zeiss Axiophot microscope.

Immunohistochemistry

Drosophila immunohistochemistry techniques were adapted from Patel (Patel, 1994). *Drosophila* embryos were collected, fixed and incubated with the appropriate primary antibody. α–βgalactosidase, α-Dmef2 (H. Nguyen, Albert Einstein College of Medicine), α-phosphotyrosine (Upstate) and Slit monoclonal antibody (6D.4 DSHB) were diluted in phosphate-buffered saline containing 0.5% Triton X-100. Embryos were then incubated in either a biotinylated secondary antibody (Vector Laboratories) followed by incubation in Vector Laboratories Elite ABC and reaction using 3,3-Diaminobenzidine Tetra hydrochloride (DAB, Gibco-BRL), or in a fluorescent secondary antibody (Alexa 488, Alexa 594, Molecular Probes). Whole embryos were mounted and visualized using either a Zeiss Axiophot microscope or analyzed via confocal microscopy using Zeiss LSM 510.

Electron microscopy

Dechorionated embryos were fixed in heptane equilibrated with 25% glutaraldehyde (Fluka) in 0.1M Sodium Cacodylate. Embryos were manually devitellinated in 4% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer, post-fixed in 1% osmium tetroxide, and stained in uranyl acetate before embedding in Epon./Araldite (Jacobs and Goodman, 1989). Lead stained 0.1 μm sections were examined on a JEOL 1200EXII microscope. We examined over 800 sections from specimens from two stages of each genotype.

Transgenic constructs

Full-length and ΔLRR *slit* transgenic constructs were created as described previously (Battye et al., 2001). Truncated LRR *slit* was generated from a full-length construct, engineered with a carboxy-terminal HA epitope tag. The transgenic constructs were subcloned into *P[UASt]*, injected into *Drosophila*

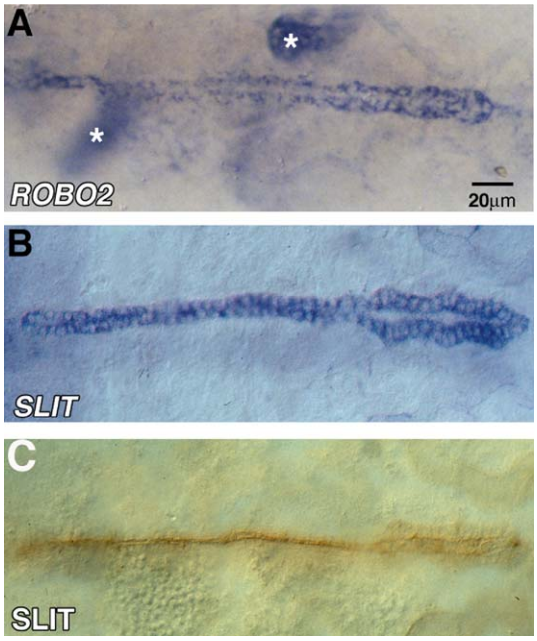


Fig. 1. *ROBO2* and *SLIT* expression in the larval heart. Stage 17 *Drosophila* hearts express transcript for *ROBO2* (A) and *SLIT* (B). Both transcripts are expressed in the cardial cells only, and are uniformly distributed through the cytoplasm. Midgut structures expressing *ROBO2* are also seen in panel A (asterisk). Slit protein is expressed almost exclusively in the lumen of the mature heart (C). Anterior is at left of all photomicrographs. Calibration: 20 μm.

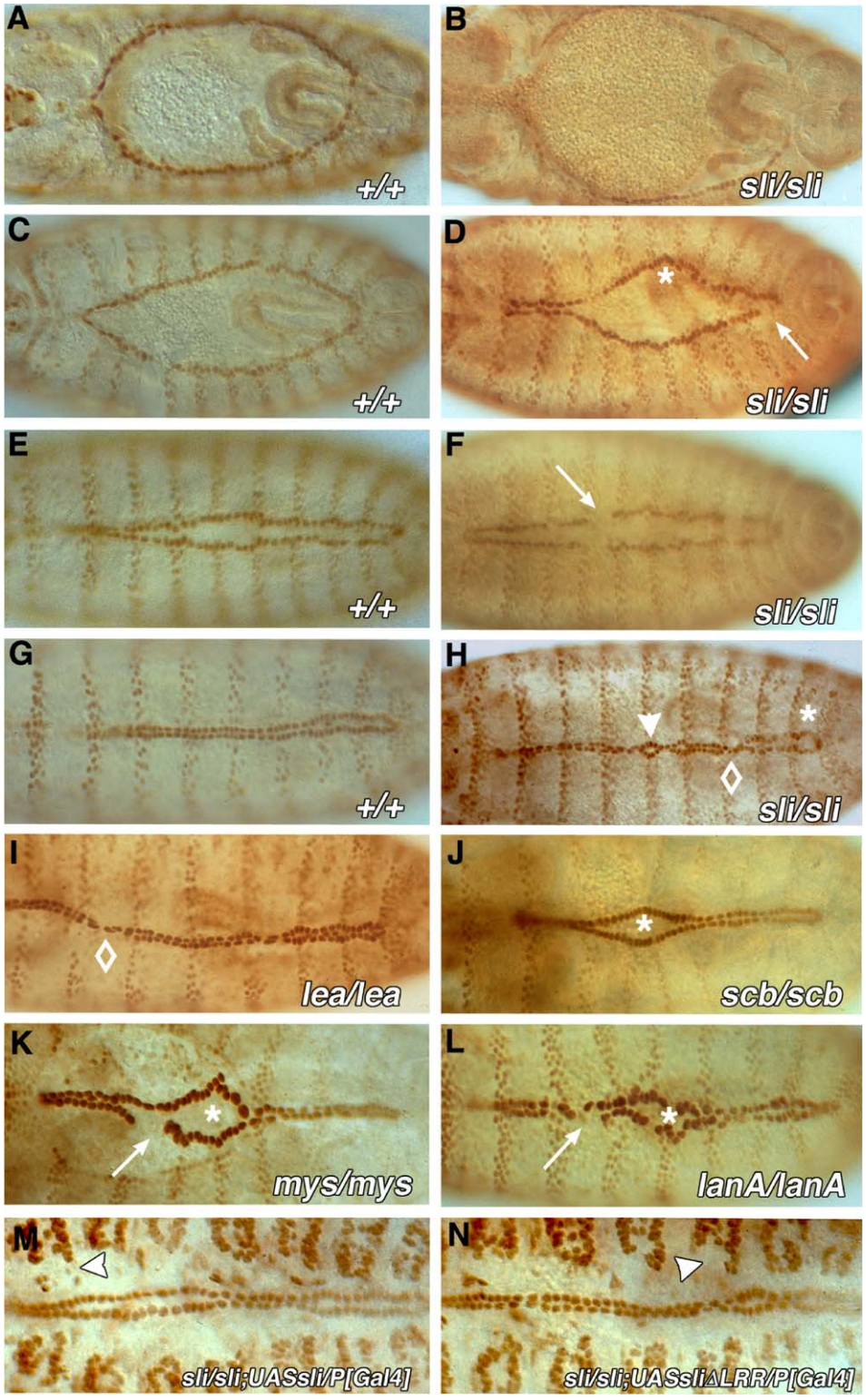


Fig. 2. Slit is required for the assembly of the heart. The dorsal and medial migration of the cardial cells in wild type (A, C, E, G) and *slit* mutant (B, D, F, H) embryos is shown for stages 13 (A, B) 14 (C, D) 16 (E, F) and 17 (G, H). Common mutant phenotypes include delayed migration (asterisk), gaps (arrows), blisters in the heart (arrowheads) and twists, or midline crossing of cardial cell nuclei (diamonds). The hearts of *robo2* mutants have similar, but less severe defects (I). Embryos mutant for αPS3 (*scab*; J), βPS1 (*mys*; L) and LamininA (*lanA*; K) share dorsal closure defects that result in a failure to close the heart tube (Asterisk) as well as cardial cell clumping and gaps (arrows). If full-length Slit (M) or Slit lacking the LRR domain is expressed in the cardial cells of embryos mutant for *slit* (*slit*^{2}*, p{UAS-slit}/sli*^{2}*,p{UAS-slit]; B2heartGAL4/+*), then significant recovery of morphogenesis in the heart is seen. *slit* is required for patterning of lateral muscle (arrowheads). Cardial cells are labeled with the B2-3-20 enhancer trap (A–L) and antibody to MEF-2 (M, N). The B2-3-20 enhancer additionally labels hindgut cells and a segmental stripe, 3 cells in width.

embryos and the resultant transformants were mapped according to standard procedures.

Results

slit and robo2 are co-expressed in the heart

Previous studies demonstrated that Slit acts as a morphogen by signaling through Robo proteins. Although *slit* expression in the heart has been described (Rothberg et al., 1990), it has not been linked to morphogenetic signaling. We were interested in knowing whether *robo* genes were expressed in or near the heart, as a means to reveal *slit* function in this organ. We detected transcripts for both *slit* and *robo2* in the cardial cells of the developing heart by in situ hybridization (Figs. 1A, B). Expression of Slit mRNA was first detected at the end of stage 12 in the lateral mesoderm, which becomes elevated in the cardial cells at stage 13 (data not shown). Robo3 transcripts were not localized in or near this tissue. Antibody labeling of Slit was restricted to the cardial cells, over the entire cardial cell surface, but concentrated at the apical surface. In the mature heart, Slit is concentrated in the lumen (Fig. 1C). All three Robo receptors are thought to respond to a gradient of Slit protein, and typically Robo expressing cells are tens or hundreds of microns away from a source of Slit (Gallio et al., 2004; Kramer et al., 2001). The coincident expression of both ligand and receptor was unexpected, and is suggestive of a different mode of Slit and Robo function in this context.

slit and robo2 are required for heart assembly

Assembly of the *Drosophila* heart proceeds by the migration of an aligned row of cardial cells underneath the ectoderm. The ectoderm moves dorsally and medially to enclose the aminoserosa. The cardial cells follow, two cell diameters behind, to fuse at the dorsal midline, and form a single vessel. The migration of cardial cells can be monitored with the B2-3-20 enhancer trap (Figs. 2A, C, E, G).

Embryos mutant for *slit* or *robo2* share features indicative of disrupted heart development. Cell migration, and final assembly of the heart vessel is slower than wild type. Embryos mutant for *slit* have breaks in the continuity of the adherent cardial cells during migration, which can lead to lesions in the final heart vessel (Figs. 2B, D, F, H). Cell counts indicate that cardial cells are not lost by apoptosis, but are displaced. In some instances, a few cardial cells are not incorporated into the heart. Nuclei may cross the midline, and irregular bulges in cardial cell alignment (blisters) are also seen. Embryos mutant for *robo2* have few delays in cell migration and the larval heart is more uniform in morphology (Fig. 2I; Table 1).

The cell migration and displacement phenotype of *slit* suggested to us that Slit may have a Robo independent function in the heart. Robo recognizes Slit by binding to the amino terminal Leucine Rich Repeat (LRR) domain (Battye et al., 2001; Nguyen Ba-Charvet et al., 2001). Slit transgenes that lack the LRR domain do not bind Robo, and cannot restore *slit* function in the nervous system (Battye et al., 2001). We similarly tested the ability of full length Slit, and Slit lacking the LRR to restore heart assembly in *slit* mutants. We directed expression of *UAS-slit* transgenes in the heart of *slit* mutants with a cardial cell GAL4, generated by transposon exchange with the B2-3-20 enhancer trap (Bier et al., 1989; Sepp and Auld, 1999). Full length Slit and SlitΔLRR expression restored heart morphology to a similar extent, indicating that Slit likely has Robo2 independent function in heart assembly (Figs. 2M, N).

Reduced Integrin function enhances the slit phenotype

The cardial cells of the heart express the αPS3/βPS1 Integrin dimer (Stark et al., 1997). Heart assembly defects similar to *slit* have been previously described in *Drosophila* mutant for Integrins, for example, βPS1 Integrin (*mys*), αPS3 (*scab*), or Integrin ligands, such as Laminin A (*lanA*), (Figs. 2J–L) (Martin et al., 1999; Stark et al., 1997). Therefore we sought to determine whether the function of Slit during heart morphogenesis was dependent upon Integrin activity.

In *Drosophila* heterozygous for *slit* mutations, *slit* function is reduced but still sufficient to support CNS development. However, *slit* output is close to threshold, such that partial reduction in the function of other genes that participate in the same biochemical pathway result in a deficit in axon guidance.

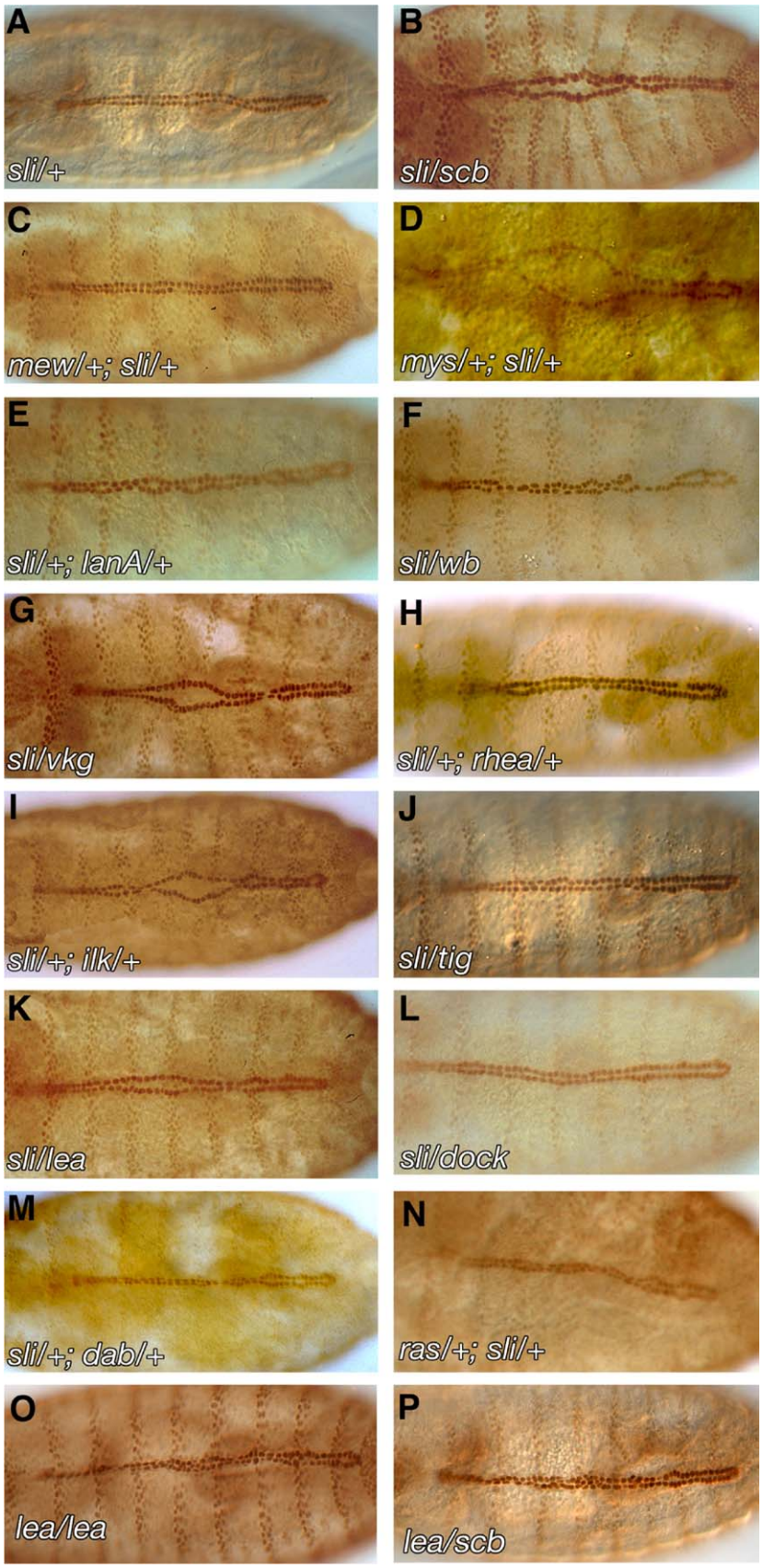
Table 1
Frequency of heart defects in interactions with sli^2

Genotype	+/+	sli^2	sli^2/+	sli^2/scb^2	sli^2/lea^2	sli^2/+;$lanA^{9\text{-}32}$/+	$sli^2/dock^{04723}$	$sli^2/vkg^{177\text{-}27}$	sli^2/wb^{SF11}	lea^2	lea^2/scb^2
Severity[a]	0.5	2.2	0.4	2.6	0.9	1.8	0.5	1.5	2.0	2.6	1.7
Penetrance	0.43	1.00	0.24	1.00	0.61	1.00	0.27	1.00	0.83	1.00	1.00
Twists	13	35	7	26	9	22	10	17	25	39	33
Gaps	3	26	2	9	12	8	6	9	13	29	4
Delays	0	30	2	39	9	26	4	15	27	15	5
Other[b]	29	44	32	47	27	30	31	36	39	48	51
Number of embryos	30	30	30	30	30	30	30	30	30	30	30

Genetic interactions with sli^2/+ in heart assembly.

[a] Severity: embryos were ranked on an ordinal scale from 0 to 4 for an increasing number of defects per embryo. Averaged value is shown. Penetrance: fraction of embryos with a severity greater than 0. Note that even "wildtype" embryos may have 1 or more cells out of position. Twists are contralaterally placed cardial cell nuclei. Gaps are cardial cells missing from a row of migrating cardial cells Delays are cardial cells more laterally placed than wildtype at that stage.

[b] Other phenotypes observed in the heart, including clumps of three or more cardial cells, cells displaced more than one cell diameter and ectopic alignment of cardial cells. Embryos stages 15 to 17 were scored.


A
sli/+
B
sli/scb
C
mew/+; sli/+
D
mys/+; sli/+
E
sli/+; lanA/+
F
sli/wb
G
sli/vkg
H
sli/+; rhea/+
I
sli/+; ilk/+
J
sli/tig
K
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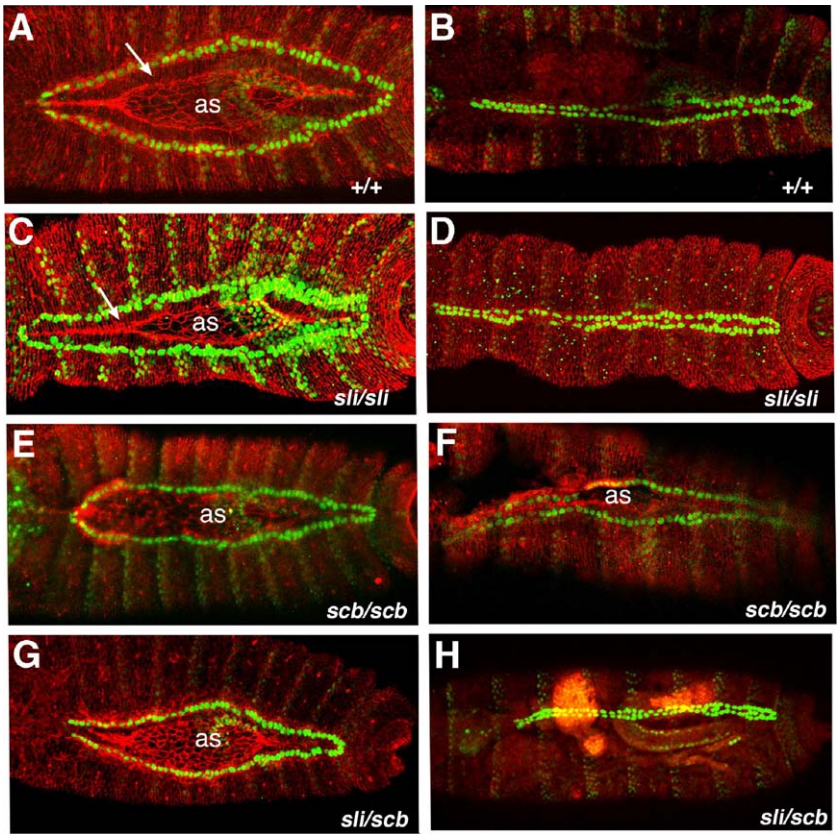

Fig. 4. Slit is not required for dorsal closure. In wild type (A) and *slit* (C) embryos, cardial cells (green) are 1 to 2 cell diameters away from the aminoserosa (as) at stage 15 (arrows). (Labeled cells under the aminoserosa in panel C are in the gut.) Dorsal closure is complete in wild type (A) and *slit* embryos (D). In contrast, the cardial cells of αPS3 mutants are adjacent to the aminoserosa at stage 15 (E) and 17 (F), and dorsal closure is incomplete. Embryos doubly heterozygous for *slit* and αPS3 have an intermediate phenotype at stage 15 (G). Dorsal closure is delayed, but complete at hatching (H). Embryos are labeled with α–phosphoTyrosine to outline cell membranes (red) and the heart enhancer trap B2-3-20 (green).

Thus slit heterozygotes ($slit^2$/+) have been effectively used as a sensitized genetic background to search for novel genes that may play a role in the same process (Fritz and VanBerkum, 2002; Stevens and Jacobs, 2002). Our hypothesis is that the mechanism of *slit* function in the heart is different from that in the CNS. In order to address this hypothesis and to identify the genetic network in which *slit* participates during heart morphogenesis, we undertook a genetic screen similar to that used previously for the CNS.

Drosophila heterozygous for a null allele of *slit* have a normal heart (Fig. 3A). Therefore, we examined mutations in genes that may have a heart assembly phenotype when homozygous, and assessed heart morphogenesis in *Drosophila* heterozygous for both *slit* and each candidate gene. We employed two cardial cell markers, the B2-3-20 reporter, and antibody to *Drosophila* MEF2. We confirmed interactions with other alleles of slit (sli^{GA20}, sli^{2990}) and other alleles of candidate genes when possible. Our results are based upon double-blind assessment of phenotype by counting the frequency of specific defects in embryo collections (Table 1).

Embryos doubly heterozygous for *slit* and Integrins or Integrin ligands had heart assembly phenotypes very similar to homozygous *slit* mutants. The most profound interactions were seen with *scab* and its dimerization partner *mys*, (Figs. 3B–D; Table 1). Of the Integrin ligands we examined, LamininA (*lanA*), Laminin α1,2 (*wb*), and collagen IV (*vkg*) interact genetically with *slit* heterozygotes, while Tiggrin (*tig*) did not (Figs. 3E–G, J). The αPS3/βPS1 Integrin binds Laminin, but not Tiggrin (Bunch et al., 1998; Stark et al., 1997). Collagen IV may be an Integrin ligand, and is also required for the stability and integrity of the ECM (Poschl et al., 2004; Yasothornsrikul et al., 1997). Therefore, these data are consistent with a

Fig. 3. *slit* interacts genetically with genes for adhesion. Stage 17 embryos heterozygous for a phenotypically null allele of *slit* have normal heart development (A). Embryos doubly heterozygous for *slit* and αPS3 (B) or βPS1 (D) have delayed migration of cardial cells, and clumping of heart cells. In contrast, αPS1 Integrin does not interact with *slit* (C). Integrin ligands Laminin A (E), Laminin α1,2 (F) and Collagen IV (G) interact genetically with *slit*, but ECM protein Tiggrin, that does not bind αPS3βPS1, does not (J). Integrin intracellular linkers ILK (I) and Talin (H) interact with *slit*. Robo2 (K) and downstream second messengers Disabled (M) and Ras (N) interact weakly with *slit*, and second messenger Nck2 (*dock*, L) has no interaction with *slit*. Embryos homozygous for *robo2* (*lea*) have a late assembly phenotype (O) and also interacts genetically with integrin αPS3 (P). All embryos labeled with the B2-3-20 enhancer trap.

requirement for Integrin based adhesion for the proper function of *slit*. Table 1 summarizes the type and degree of interactions between *slit*- and Integrin-related genes.

Integrin function is dependent upon coupling to intracellular proteins that mediate both linkage to the cytoskeleton, as well as signal transduction that regulates the organization of the cytoskeleton (Bokel and Brown, 2002). Integrins have a variety of intracellular adaptors, which function in both adhesive linkage and signaling. We examined three of these adaptors to determine if *slit* function was sensitive to any particular Integrin output. Embryos heterozygous for Talin (*rhea*), Integrin Linked Kinase (*ilk*) and embryos with a dominant negative form of Focal Adhesion Kinase (*FAK56D*) expressed in the heart (*dMEF2-GALK4; UAS-dFAK56D*) have normal heart assembly (Supplementary data, Fig. 1). We observed heart defects in embryos doubly heterozygous for *rhea* or *ilk*, and *slit* (Figs. 3H, I). Delays in midline fusion of *sli/+;ilk/+* cardial cells suggests a delay of dorsal closure is part of this interaction.

Genes required for Robo signaling do not interact with slit

The functional relationship between *slit* and *robo* was first revealed by a strong genetic interaction between *slit* and *robo1* (Battye et al., 1999; Kidd et al., 1999). When we performed a comparable test with embryos doubly heterozygous for *lea* and *slit*, defects in heart assembly were not severe (Fig. 3K; Table 1). Significantly, there is a genetic interaction between *lea* and *scb* (Fig. 3P; Table 1). Genes required for second messenger signaling downstream of Robo, such as Disabled (*dab*), Abelson (*abl*), Ras (*ras*), and Nck1 (*dock*) also interact genetically with *slit* in the nervous system (Forsthoefel et al., 2005; Stevens and Jacobs, 2002). A similar test in the heart revealed little or no genetic interaction (Figs. 3L–N, Table 1). These data indicate that *slit* function in the heart is more sensitive to changes in ECM adhesion than to *robo2* function or signaling, and that Robo2 function and adhesion interact.

Dorsal closure does not require slit

Dorsal closure is carried out by the dorsal migration of the ectoderm to enclose the gut and a transient dorsal structure, the aminoserosa. Migrating cardial cells maintain contact with the leading edge of this migrating epithelium, so that heart vessel formation cannot proceed unless dorsal closure is complete (Rugendorff et al., 1994). Both αPS3 and βPS1, which interact with *slit*, also affect dorsal closure in *Drosophila* embryos (Schöck and Perrimon, 2003; Stark et al., 1997). We have also tested *abl* and *ena*, which encode intracellular kinases required for dorsal closure, and they did not interact with *slit* in the heart. Nevertheless, to clarify the nature of the interaction of *slit* and Integrins in the heart, it is necessary to determine whether *slit* contributes to dorsal closure.

The actin cytoskeleton of the cells of the Leading Edge and Aminoserosa can be visualised simultaneously with cardial cell position in B2-3-20 embryos (Fig. 4). During early dorsal closure, the cardial cells are 1–2 cell diameters behind the Leading Edge cells, but the cardial cells abut the Aminoserosa at the final stages of dorsal closure (Figs. 4A, B). Although neither Slit nor Robo are expressed in the leading edge, or aminoserosa, it was important to determine whether the genetic interactions with *slit* in the heart were secondary to defects in dorsal closure. First, we established that dorsal closure was not affected in *slit* mutants. Heart assembly defects, without overlying changes in

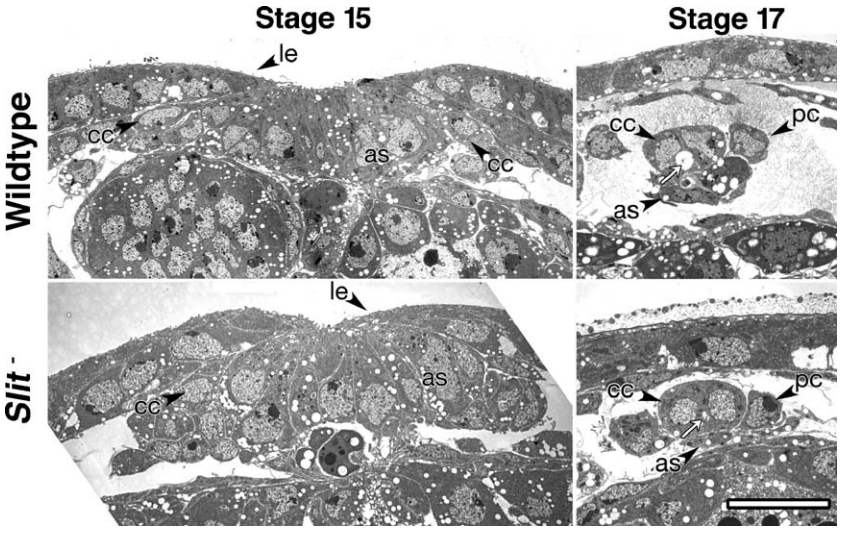

Fig. 5. The heart of *slit* mutants lacks a lumen. Transverse sections of a wildtype (A) and *slit* embryo at stage 15 reveals similar morphology. Aminoserosa cells (as) are invaginating ventrally, leaving a filopodia rich contact with the exterior. Leading edge cells of the ectoderm (le) migrate medially. Cardial cell nuclei trail 1–2 cell diameters behind (cc) but have a leading process in contact with both the aminoserosa and the leading edge. Cardial cell position is more irregular in *slit* mutants. On the left it is more ventral than wildtype, and could not be located on the right side. At stage 17, cardial cells develop an apical lumen (arrow), and are accompanied by pericardial cells (pc) and apoptotic aminoserosa cells. *slit* mutants have extensive basolateral adhesion, but a very small, or no lumen (arrow). Calibration, 10 μm.

dorsal closure, were seen in *slit* mutant embryos (Figs. 4C, D). In contrast to wild type, the gap between the Leading Edge and the cardial cells did not shrink as dorsal closure was completed. No changes in the ultrastructure of Leading Edge or Aminoserosa cells were seen in *slit* mutant embryos (Fig. 5C) relative to wildtype (Fig. 5A). However, the leading process of the migrating cardial cell was variable in size and position in *slit* mutants.

Dorsal closure is incomplete or delayed in *scab* mutant embryos, which arrests cardial cell migration just prior to closure of the heart vessel. In contrast to wildtype, the cardial cells abut the aminoserosa during early and late development, even if dorsal closure is incomplete (Figs. 4E, F). In embryos heterozygous for both *slit* and *scab*, the cardial cell nuclei are underneath the leading edge, as in *scab* homozygotes. Dorsal closure is complete at hatching (Figs. 4G, H). We conclude that the genetic interaction between *slit* and *scab* is not due to defect in dorsal closure.

slit is required for heart lumen formation

A second phase of *slit* function in heart morphogenesis occurs subsequent to cardial cell migration. During stage 16, cardial cells adhere to their contralateral partner, and develop a lumen by limiting adhesion to the basolateral surface, and expanding the non-adherent apical surface of the cell (Fig. 5B). Slit protein is localized to the lumen (Fig. 1C). *slit* mutant embryos have no lumen, or a very small one, and an expanded basolateral zone (Fig. 5D).

Discussion

In this study, we have shown that *slit* is required for the ordered migration of cardial cells to meet their contralateral partners at the dorsal midline. This function was independent of dorsal closure. The cells that secrete Slit also express the Slit receptor, Robo2. *robo2* has a mild heart phenotype; *Drosophila* doubly mutant for *robo1* and *robo2* have a more severe phenotype (Qian et al., 2005). When tested genetically, we found that the functional interaction between Robo2 and Slit was weak. In contrast, reduced levels of function of the αPS3/βPS1 Integrin, its ligands, or cytoplasmic linkers all acted to enhance the phenotype of *slit*.

Slit and Robo2 function in the heart

Defects in cardial cell morphogenesis were seen at all stages of heart development in *slit* mutants, suggesting a continuous requirement for Slit. Errors included gaps in the ribbon of migrating cells, delayed cell migration, gaps, clumping or blisters in the midline alignment of cardial cells, and the lack of a heart lumen. All of these defects reflect changes in cell interaction with the ECM, or cell polarization, and do not clearly define a role for Slit in attractive or repellent guidance signaling.

In ectodermal and mesodermal guidance, Robo2 participates in both attractive and repellent signaling (Englund et al., 2002; Schimmelpfeng et al., 2001; Simpson et al., 2000). The developing heart is the only tissue known where Slit and a Robo receptor are expressed in the same cell, and this complicates a model of cardial cell migration by attractive guidance. Similarly, ephrins and EphA receptors are co-expressed in retinal ganglion cells and in spinal motoneurons (Hornberger et al., 1999; Marquardt et al., 2005). It has not been resolved whether co-expression of ephrin and EphA silences receptors by desensitization (Piper et al., 2005), or if subcellular segregation of ligand and receptor prevents autocrine signaling.

Slit is concentrated at the apical surface, which reflects the apical location of its receptor (Qian et al., 2005). There is no source of Slit available to the basal surface of heart, so it is unlikely that Robo signals from the basal domain. Slit function as an attractive or repellent guidance ligand in the nervous system is reflected by a strong genetic interaction between *slit* and *robo*, and between *slit* and genes for molecules required for Robo signaling, such as Nck (*dock*), Disabled (*dab*), and Abelson (*abl*) (Stevens and Jacobs, 2002). We did not observe a similar genetic interaction in heart assembly, suggesting the functional relationship between Slit and Robo2 involves other signals, such as adhesion signals.

Robo2, which has 5 Ig and 3 fibronectin-like domains, is a member of the Ig Cell Adhesion Molecule (CAM) superfamily (Kidd et al., 1998). Heterophilic and homophilic adhesion between Robos has been demonstrated (Hivert et al., 2002; Liu et al., 2004). It is possible that Robo2 homophilic interactions, and Robo2-Slit binding act to adhere, align, and polarize migrating cardial cells. Heart morphogenesis was partially restored in *slit* mutants that express a *slit* transgene unable to bind Robo. Direct Slit–Robo interaction may not be essential to *slit* function in the heart.

Slit function and cardial cell adhesion

At the genetic level, we have established that Integrins and Integrin ligands interact with Slit in axon guidance in the nervous system (Stevens and Jacobs, 2002). In this context, decreased levels of Integrin function made axons more sensitive to changes in guidance signaling. We proposed that Integrin signaling or adhesion act to raise the threshold of growth cones to respond to guidance signals.

In the *Drosophila* heart, Slit function may include adhesion-related functions. This would account for the similarity of heart assembly phenotypes between *slit*, αPS3/βPS1 Integrin and Integrin ligands (Laminin and Collagen IV). Simultaneous reduction of Slit and Integrin function compromised heart assembly. This may involve adhesive signals from Integrins, given the genetic interaction between *slit* and two downstream Integrin linkers, talin (*rhea*) and ILK. Two models emerge from these data. The first is that Slit and Integrins function in parallel pathways, both of which converge upon adhesion dependent regulation of cell migration and morphogenesis. The second is that Slit and

Integrin function in a common pathway—perhaps as ligand and receptor.

Development of the lumen of the heart requires the ECM protein Pericardin, and cell surface receptors Toll, Faint sausage and DE-cadherin (Chartier et al., 2002; Haag et al., 1999; Wang et al., 2005). Possible outside-in signaling by these molecules remains to be explored.

Do Slit and Integrin signals converge?

There is strong evidence that other guidance molecules act in a parallel path to affect Integrin function. Sema3A expressed by vascular endothelia during angiogenesis acts to reduce Integrin function at focal adhesions, perhaps to facilitate branching angiogenesis (Serini et al., 2003). In contrast, activation of ephrin A2 or A5 results in increased adhesive function of Integrins (Huai and Drescher, 2001). In both cases, second messenger signaling is implicated to regulate Integrin function. The pattern of gene interactions with *slit* in the nervous system are consistent with a role for second messengers downstream of guidance cue receptors, including Nck2 (*dock*), Abelson, and Myosin Light Chain Kinase (Kim et al., 2002; Stevens and Jacobs, 2002). In contrast, our survey of modifiers of *slit* in the heart has only revealed Integrin linked proteins, implicating integrin dependent Slit function.

Does Slit have an adhesive function?

The structures of Slit and Netrin share similarities with Laminins, raising the possibility that these guidance molecules may link cells to the ECM. Like Laminin, Slit contains multiple EGF-like repeats, and a globular "G" (also known as ALPS) domain (Huber et al., 2003). Neither of these domains are linked to Robo signaling (Battye et al., 2001; Nguyen Ba-Charvet et al., 2001). The EGF domains of Laminin are functionally associated with linking to other ECM proteins like Nidogen (Gersdorff et al., 2005). The G domain is involved in association with Integrins and α-Dystroglycan (Ido et al., 2004; Timpl et al., 2000).

Slit contains domains that suggest association with the ECM, and this association may play a role in the formation of a Slit gradient. However, Slit localization determined by light or electron microscopy finds the protein on cell or axon surfaces (Rothberg et al., 1990), unlike the distribution of basement membrane proteins like Laminin or Perlecan (Friedrich et al., 2000; Montell and Goodman, 1989). Cell surface labeling for Slit has only been reported on Slit or Robo expressing cells (Rothberg et al., 1990). We suggest that Slit is localized on cardial cells by association with Robo, and possibly also αPS3/βPS1 Integrin. This positions Slit to participate in linking the cell to the ECM as well as to trigger intracellular signals through both Integrins and Robos. Slit likely associates with the ECM. Biochemical studies have implicated Laminin and Glypican as vertebrate Slit ligands (Brose et al., 1999; Liang et al., 1999). We suggest that Slit facilitates cardial cell adhesion, migration, polarization, and lumen formation by physical interaction with Robo2, integrin receptors and ECM ligands.

In contrast to other guidance receptor systems, motifs associated with structural proteins of the ECM prevail in the structure of Slit and Netrin and their receptors Robo and DCC/Neogenin (Hinck, 2004; Huber et al., 2003). The receptor–ligand binding domains have been identified, and the functions of other conserved domains remain undefined. Experiments aimed at uncovering the functions of the EGF and G domains of Slit and Netrin will clarify the multifunctional nature of these proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.01.027.

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