Roundabout Receptors Are Critical for Foregut Separation from the Body Wall

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SUMMARY

In mammals, precise placement of organs is essential for survival. We show here that inactivation of Roundabout (Robo) receptors 1 and 2 in mice leads to mispositioning of the stomach in the thoracic instead of the abdominal cavity, which likely contributes to poor lung inflation and lethality at birth, reminiscent of congenital diaphragmatic hernia (CDH) cases in humans. Unexpectedly, in Robo mutant mice, the primary defect preceding organ misplacement and diaphragm malformation is a delayed separation of foregut from the dorsal body wall. Foregut separation is a rarely considered morphogenetic event, and our data indicate that it occurs via repulsion of Robo-expressing foregut cells away from the Slit ligand source. In humans, genomic lesions containing Robo genes have been documented in CDH. Our findings suggest that separation of the foregut from the body wall is genetically controlled and that defects in this event may contribute to CDH.

INTRODUCTION

The mammalian gut tube gives rise to both the respiratory and digestive systems (Domyan and Sun, 2011). In mature mammals, the respiratory system (trachea and lungs) is positioned within the thoracic cavity, while the majority of the digestive system, with the exception of the esophagus, is positioned within the abdominal cavity. Proper allocation of organs into their appropriate compartments is critical for survival. This requirement is highlighted in congenital diaphragmatic hernia (CDH), a life-threatening birth defect. In CDH, a portion of the abdominal organs protrude into the thoracic cavity, thereby preventing lung expansion at birth (Ackerman et al., 2005; Ackerman and Greer, 2007; Liu et al., 2003; Pober et al., 1993; Yuan et al., 2003). Despite the importance of proper segregation of organs into their respective cavities, relatively little is known about how this is achieved during development.

Evidence suggests that final organ position is not solely a reflection of where organs are initially specified. For example, during early stages of foregut development (Embryonic day [E] 9.75 in mouse embryos), the lung and stomach emerge in close proximity to one another. Through a poorly understood process of differential growth and morphogenesis, the stomach gut tube rotates to the left and elongates posterior to the lungs. The subsequent formation of the diaphragm at E13.5 sequesters these organs in two separate cavities. Thus, precisely controlled morphogenesis of the organs is a prerequisite for accurate final positioning.

Roundabout (Robo) genes encode cell-surface receptors that respond to their secreted ligands, Slit proteins, in a wide variety of cellular processes (reviewed in Long et al., 2004; Ypsilanti et al., 2010). Four Robo genes and three Slit genes have been identified in the mouse genome (Brose et al., 1999; Kidd et al., 1998). First implicated in regulation of axon pathfinding (Kidd et al., 1998, 1999; Rothberg et al., 1988), Slit-Robo signaling has since been demonstrated to play a role in processes such as neural crest cell migration and sensory ganglia morphogenesis (De Bellard et al., 2003; Shiau and Bronner-Fraser, 2009), leukocyte chemotaxis (Ye et al., 2010), epithelial adhesion (Macias et al., 2011), and diaphragm and kidney formation (Griesshammer et al., 2004; Liu et al., 2003; Yuan et al., 2003). Functionally, Slit-Robo signaling has been shown to transmit migratory cues by modulating cell adhesion and actin polymerization (Lundström et al., 2004; Rhee et al., 2002, 2007; Shiau and Bronner-Fraser, 2009). These cues are largely repulsive, although they can be attractive or promote growth and branching in some cellular contexts (Englund et al., 2002; Ma and Tessier-Lavigne, 2007; Wang et al., 1999; Wang et al., 2003; Ye et al., 2010). Homophilic interactions between Robo receptors have also been shown to regulate cell adhesion and migration (Hlvert et al., 2002).

In this study, we report an unexpected requirement for Robo genes in foregut morphogenesis. In mice that are deficient in Robo1 and Robo2 (Robo1;2), the lungs fail to inflate and the animals die at birth. Accompanying these defects, the stomach protrudes through the diaphragm into the thoracic cavity. These phenotypes are reminiscent of the diaphragmatic hernia defect previously reported in Slit3 mutant mice (Liu et al., 2003; Yuan et al., 2003). The hernia phenotype in the Slit3 mutant was attributed to a primary defect in diaphragm formation. In this study, we demonstrate that the diaphragm defect in Robo, as well as in Slit, mutant embryos is preceded by a delayed separation of foregut tube from the body wall. Our findings implicate Slit-Robo
signaling as a key regulator of this poorly understood foregut morphogenesis process.

RESULTS

Perinatal Lethality of Robo1;2 Mutants

Multiple Robo and Slit genes are expressed in the embryonic foregut (Anselmo et al., 2003; Greenberg et al., 2004). To determine their potential roles in foregut development, we generated mice deficient for the principal receptor genes Robo1 and Robo2 (Robo1;2) using existing mutant alleles (Grieshammer et al., 2004; Long et al., 2004). Robo1 and Robo2 genes both reside on chromosome 16 about 1.1 centimorgans apart. We generated linked mutant alleles through recombination. All Robo1;2 homozygous pups examined became cyanotic quickly after birth, gasped for air, and died within minutes (Figures 1A and 1B). Upon further analysis, we found that Robo1;2 mutant lungs, but not heterozygous control lungs, failed to inflate at birth (Figures 1C–1F) and sank when placed in aqueous solution (data not shown). Immunostaining using an anti-Surfactant-C antibody showed that the major surfactant-producing cell population, type II cells, are present in normal numbers in the mutant

(data not shown). While it remains possible that Robo genes are required within the lung for gas exchange, we observed an extrinsic defect that may contribute to the failure of lung inflation: Robo1;2 mutants exhibit a striking, fully penetrant mispositioning of the abdominal organs, primarily the stomach. Specifically, rather than being located on the left side of the abdominal cavity, the stomach of Robo1;2 mutants was located at the midline and protruded through the esophageal hiatus into the thoracic cavity (Figures 1G and 1H). The diaphragm of Robo1;2 mutants was also malformed (Figures 1I and 1J). Because relatively little is known about how abdominal organ positioning is controlled, we sought to further characterize the stomach-protrusion phenotype.

We found that mispositioning of the stomach was already apparent at E11.5 prior to diaphragm formation. In control embryos, the stomach was rotated to the left of the midline and shifted posterior to the lung lobes (Figures 1K and 1M). In Robo1;2 embryos, however, the stomach remained at an anterior location at the midline between the lung lobes (Figures 1L and 1N). Other left-right asymmetric properties, such as heart, gut looping, and lung lobe number, were normal, suggesting that the midline placement of the stomach in Robo1;2 embryos is not due to a general left-right determination defect. Accompanying the stomach-position phenotype, the dorsal aortae did not fuse in the mutant and remained separated by a thick band of mesenchymal cells (Figure 1N). At E13.5, stomach mispositioning was more prominent, as it interrupted the newly formed diaphragm (Figures 1O and 1P). We found that the esophagus of

Figure 1. Gross Defects in Robo1;2 Mutants

(A and B) Newborn pups. The mutant is cyanotic. (C–F) Whole-mount (C and D) and hematoxylin-and-eosin (H&E)-stained sections (E and F) of postnatal day 0 (P0) lungs showing that the mutant lung failed to inflate. (G–J) Anterior views of whole-mount (G and H) or dissected diaphragms (I and J) from P0 pups cut at the approximate level indicated in (A) and (B). Black arrows in (H) and (J) indicate the protrusion of the stomach through the diaphragm in the mutant. (K–P) H&E-stained sagittal (K and L) and transverse (M–P) sections taken at the approximate planes indicated by dashed lines in the inset diagrams. The stomach remains between lung lobes in the mutant. (Q) Length of the trachea and esophagus of control and Robo1;2 embryos at E13.5 (trachea: 1.7 ± 0.1 mm for Robo1;2 versus 1.9 ± 0.04 mm for control, n = 4 each, p = 0.03; esophagus: 2.48 ± 0.65 mm for Robo1;2 versus 4.19 ± 0.07 mm for control, n = 4 each, p = 0.01). Data are presented as mean ± SD. *p < 0.05. bw, body wall; da, dorsal aorta; di, diaphragm; dm, dorsal mesentery; es, esophagus; he, heart; li, liver; lu, lung; nc, notochord; nt, neural tube; ph, pharyngeal arch; st, stomach.
Robo1;2 embryos was significantly shorter than in control embryos, while the trachea of Robo1;2 embryos was only slightly shorter than control (Figures 1Q [see legends for quantification], 4R, and 4S). These data suggest that in Robo1;2 mutants, organ-positioning defects precede the diaphragm defect.

**Robo and Slit Genes Are Expressed in Complementary Patterns during Early Foregut Morphogenesis**

To determine the primary role of Slit-Robo signaling during early foregut morphogenesis, we sought first to examine the expression of Robo1-2, Slit1-3 using RNA in situ hybridization (Figures 2 and 3). The overall expression patterns we detected throughout the embryo were congruent with previously published results, confirming specificity of our probes (Brose et al., 1999; Yuan et al., 1999). At E9.5 (24 somites), Robo expression was detected in the mesothelium, mesenchyme and epithelium of the foregut and in the dorsal mesentery connecting the foregut to the body wall (Figures 2A, 2B, 2D, and 2E). At E10.0, Robo1 was expressed in a similar pattern, while expression of Robo2 was primarily restricted to the mesothelium and the dorsal mesentery (Figures 2G, 2H, 2J, and 2K).

To confirm the RNA in situ results, we took advantage of the lacZ reporter insertions present in both Robo1 and Robo2 mutant alleles (Grieshammer et al., 2004; Long et al., 2004). Sites of β-galactosidase (β-gal) activity closely corresponded to the RNA in situ pattern (Figures 2C, 2F, 2I, and 2L). By both detection methods, higher expression was observed in the liver and stomach region than in the esophagus region (Figures 2A–2C). No expression was detected in the dorsal aortae.

In agreement with previous results, all three Slit genes were expressed in the floor plate of the neural tube at E9.5 and E10.0, while Slit2 and Slit3 were also expressed in the notochord at these stages (Figure 3) (Brose et al., 1999). At E10.0, Slit3 was also high in two domains in the lateral mesentery, between the dorsal aortae and the body cavity (Figure 3L, arrowheads). Considered together, Slit and Robo genes displayed largely complementary expression patterns, with Slit genes predominantly expressed in the neural tube, notochord, and lateral body-wall mesentery, and Robo genes predominantly expressed in the mesothelium, mesenchyme and epithelium of the foregut, as well as the medial body-wall mesentery (Figures 2, 3, 7J, and 7K). We next addressed how disruption of this signaling framework would lead to the observed organ-positioning defects.

**Robo1;2 Mutants Show Defects in Early Foregut Morphogenesis**

To pinpoint the primary defect in the Robo1;2 mutant, we performed two parallel analyses on stage-matched embryos to comprehensively illustrate the tissue context. First, we stained whole embryos or foreguts with anti-Cadherin 1 (CDH1, E-cadherin) antibody to outline the developing gut epithelium (Figure 4, left two columns). Second, we stained Robo1;2 heterozygous
and homozygous mutant embryos for β-gal activity and examined Robo cell distribution on transverse sections (Figure 4, right four columns). Because the double homozygous mutant embryos carry four copies of lacZ transgene compared to two copies in double heterozygotes, β-gal activity is stronger in the mutant embryos. However, the intensity difference does not appear to interfere with detection of β-gal-positive Robo-expressing cells.

The combination of the two approaches allowed us to precisely determine the primary defect in the Robo1;2 mutants. At 18 somites (~E9.0), we observed no discernible difference in foregut morphology and tissue context between control and Robo1;2 embryos (Figures 4A–4D). At 27 somites (~E9.5), we detected the emergence of subtle differences (Figures 4E–4I). Specifically, in control embryos, the distance between the foregut endoderm and notochord was greater at 27 somites than at 18 somites (Figures 4C and 4G). In Robo1;2 mutant embryos, however, the foregut endoderm remained near the notochord at 27 somites, similar to the morphology of embryos at the 18-somite stage (Figures 4D and 4H). This proximity was most apparent at, but not restricted to, the level of a dorsal bulge emerging in the foregut (Figure 4F, arrowhead). The average notochord-foregut distance quantified at multiple representative levels along the anterior-posterior (A-P) axis of the foregut was statistically reduced in mutants compared to controls (Figure 4I). Conversely, along the medial-lateral (M-L) axis where the foregut joins to the body wall, the average M-L distribution of β-gal+ Robo-expressing cells was statistically greater in mutant embryos than in heterozygous controls (Figures 4G–4I). These differences are magnified at E10.5 (~36 somites). In control embryos, the foregut was connected to the body wall by only a thin layer of dorsal mesentery (Figures 4J, 4L, and 4M). The majority of β-gal+ cells were detected in mesenchyme/mesothelium surrounding the foregut, with a few still present in the body wall. In Robo1;2 mutants, however, the foregut remained closely fused to the body wall. A large number of β-gal+ cells remained in the wings of the body wall (Figure 4K, 4M, and 4M'). While most apparent at the stomach, a similar reduced D-V distance between the neural tube and gut tube was observed in the esophagus and liver levels (Figures S1A–S1F) in the mutant. At E10.5, the previously observed bulge was consistently observed in the esophagus, protruding dorsally toward the neural tube (Figure 4K; Figures S1A–S1D).

At E11.5 in control embryos, the foregut in the stomach region had swung to the left and was loosely connected to the body wall by a long and thin band of mesentery (Figures 4N, 4P, and 4P'). However, in the equivalent region in Robo1;2 embryos, the foregut remained midline and closely connected to the body wall by a thick band of dorsal mesentery (Figures 4Q, 4R, and 4Q'). At E13.5 in control embryos, the diaphragm had formed, separating the stomach and the lung (Figures 4R, 4T, and 4T'). However, in Robo1;2 mutants, the stomach was located between the lungs in the thoracic cavity, while the diaphragm had formed around it (Figures 4S, 4U, and 4U'). The dorsal mesentery connecting the stomach to the body wall remained shorter in
Robo1;2 embryos compared to control (Figure 4T and 4U’, arrows). Collectively, these data show that the retarded ventral movement of the foregut tube away from the body wall and the accompanying reduced M-L restriction of Robo-expressing cells are the primary defects in Robo1;2 mutants, and both precede diaphragm malformation by ~3 days.

Previously it was shown that Slit3–/– mutants showed a clear shortening of the dorsal mesentery connecting the stomach to the body wall (Figures S1G–S1J). These data suggest that Slit ligands, like Robo receptors, are required in the early process of foregut morphogenesis away from the body wall.

Altered Localization of Adhesion Molecules in Robo1;2 Mutant Foregut
We reasoned that the delayed foregut morphogenesis in Robo1;2 embryos could be due to a decrease in cell proliferation, an increase in cell death, or a decrease in cell migration. To test these possibilities, we examined embryos at E10.0 shortly after the defect was first observed (Figures 5A and 5B).
We assayed for cell proliferation by labeling cells in S-phase with 5'-ethynyl-2'-deoxyuridine (EdU) (Figures 5C, 5D, and 5G). In the region containing the dorsal mesentery as well as the foregut, we detected a slight but statistically significant increase, rather than a decrease, in the proportion of EdU+ cells in mutants relative to controls (Figure 5G). Next we assayed for cell death using anti-cleaved-Caspase-3 antibody staining (Figures 5E, 5F, and 5H). We found very few cells labeled in either mutant or control and no statistically significant difference between the two genotypes (Figure 5H). Together these data suggest that the delayed separation of the foregut from the body wall is not due to a decrease in cell proliferation or an increase in cell death.

Previous studies have demonstrated that activation of Slit-Robo signaling can interfere with cell adhesion by inhibiting the association of N-Cadherin (also termed Cadherin 2 [CDH2]) with β-catenin (CTNNB1), thereby disrupting the tethering of the actin cytoskeleton to the cell membrane (Rhee et al., 2002, 2007). To address whether this mechanism may mediate Slit-Robo control of foregut cell behavior, we examined the localization of CDH2 and CTNNB1 in control and mutant embryos (Figures 5I–5R). We found that, in agreement with previous reports, CDH2 was localized to the neural tube, mesenchyme, and mesothelium of the foregut tube and body wall in control embryos (Hatta et al., 1987) (Figure 5I). CDH2 was detected in similar groups of cells in Robo1;2 mutants (Figure 5J). In closer examination of the mesenchyme of control embryos, CDH2 showed punctate localization at the membrane, consistent with the pattern observed in cells with activated Slit-Robo signaling (Figures 5M and 5Q, arrows) (Rhee et al., 2002). In Robo1;2 embryos, however, CDH2 was detected in a more uniform distribution along the cell membrane (Figures 5N and 5R). CTNNB1 was detected in control and mutant embryos in similar subcellular patterns (Figures 5K, 5L, 5O, and 5P). Colocalization analyses revealed that a statistically significantly larger proportion of CDH2 immunoreactivity overlapped with CTNNB1 immunoreactivity in Robo1;2 embryos relative to controls (Figures 5Q–5S). Collectively, these data suggest that cells expressing Robo proteins
also express downstream mediators of Slit-Robo signaling, and that disruption of Slit-Robo signaling may interfere with the movement of foregut/body-wall cells by altering the localization of adhesion machinery components.

**Foregut Cells Are Repelled by Slit Protein**

The complementary expression patterns of Slit and Robo genes, coupled with the primary defects in Robo1;2 mutants, suggest that Slit-Robo signaling may promote normal foregut morphogenesis by repelling Robo-expressing foregut cells from the Slit-expressing neural tube and lateral body wall. To test this hypothesis directly, we assayed whether and how foregut cells would respond to Slit protein in vitro using the Boyden chamber assay (Figure 6). We used stomach cells because the mispositioning of this organ is the most striking outcome of the morphogenesis defect and because it offers a rich source of cells. In the first test, we dissociated cells from E10.5 wild-type stomachs and seeded them in the top chamber on a culture insert, above either control COS-7 cells or SLIT2-producing COS-7 cells in the bottom chamber (Figure 6A). After incubation, we scored the number of cells that had migrated to the bottom surface of the insert. We found that approximately 40% fewer stomach cells had migrated when SLIT2-producing cells were in the bottom chamber than when control cells were in the bottom chamber (Figures 6C, 6D, and 6G).

In the second test, we resuspended E10.5 wild-type stomach cells in control- or SLIT2-conditioned media (CM), and seeded them in the top chamber of a culture insert, with control media in the chamber below it (Figure 6B). After incubation, we found that approximately 45% fewer cells from Robo1;2 embryos migrated compared to the number from control embryos (Figure S2). The combined data from the Boyden chamber assays demonstrate that foregut cells are repelled by Slit protein in vitro in a Robo-dependent manner. These findings, together with Slit-Robo gene-expression data, suggest that disruption of a similar process in vivo may contribute to the delayed separation of the foregut from the body wall observed in Robo1;2 mutants.

**Robo Function Is Required in the Mesenchyme and/or Mesothelium for Organ Positioning**

Robo genes are expressed in the foregut in both the endoderm-derived epithelium and the mesoderm-derived mesenchyme/mesothelium. It is unclear whether, for foregut morphogenesis to occur, Robo function is required in both or in only one of these cell lineages. In addition, it remains unclear whether Robo function is required within the lung or extrinsic to the lung for lung inflation to occur at birth. To distinguish among these possibilities, we obtained a Robo2 floxed allele, Robo2\(^{\text{fl}}\) (Lu et al., 2007) and linked it to Robo1\(^{-/-}\) by recombination. We note that Robo1\(^{-/-}\) single mutants show no detectable foregut phenotype in our study. By combining the linked alleles with distinct cre lines, we were able to inactivate the collective function of Robo1;2 in the tissues of interest (Table S1).

We used Shh\(^{\text{cre}}\) to inactivate Robo function in the endoderm-derived epithelium and Dermo1\(^{\text{cre}}\) to inactivate Robo function in the mesoderm-derived mesenchyme and mesothelium (Harfe et al., 2004; Harris et al., 2006; Harris-Johnson et al., 2009; Yu et al., 2003). Shh\(^{\text{cre}}\);Robo1;2 mutants (n = 5) showed normal
organ positioning, diaphragm formation, and lung inflation at birth (Figures S3A–S3D). In contrast, Dermo1cre;Robo1;2 mutants (n = 9) showed a closer attachment of foregut to the body wall, shorter esophagus, protrusion of the abdominal organs into the chest (either stomach or liver is observed in this conditional mutant), aberrant opening of the diaphragm, and inability to inflate the lung at birth (Figure 7; data not shown). These defects are similar to those observed in Robo1;2 global loss-of-function mutants, albeit less severe, likely due to later Robo inactivation by Dermo1cre.

Dermo1cre is active in the mesenchyme/mesothelium of many organs, as well as in the dorsal mesentery (Figure 7C) (Yu et al., 2003). To address whether Robo function is specifically required in the lung mesenchyme, we generated Tbx4cre;Robo1;2 mutants (Figure S3). While Tbx4cre is also active in the hindlimb, external genitalia and a few other posterior mesoderm derivatives, in the context of the foregut, its activity is restricted to the lung mesenchyme. There is no activity in the dorsal mesentery or the diaphragm (Naiche et al., 2011). Tbx4cre;Robo1;2 mutants showed normal lung and diaphragm morphology and lung inflation at birth (n = 6; Figures S3E, S3F, S3H, and S3I).

We confirmed Robo inactivation in the lung by performing quantitative RT-PCR for Robo2 (Figure S3G). Taken together, our data from tissue-specific inactivation show that Robo genes are required in the mesenchyme/mesothelium lineages outside of the lung to control organ positioning, proper closure of the diaphragm, and lung inflation at birth.

**DISCUSSION**

In this study, we report that Robo1;2 mutant mice die at birth with protrusion of the abdominal organs into the thoracic cavity, an affliction that resembles CDH cases in humans (Ackerman and Greer, 2007; Holder et al., 2007; Pober, 2008). In this mouse genetic model of CDH, we traced the morphological defects to a surprisingly early foregut morphogenesis event that is rarely considered. Our findings illustrate that a thorough understanding of the origin of defects, as demonstrated through animal models, can provide important insights into the etiology of human congenital anomalies.

The primary Robo1;2 mutant phenotype led us to focus on the relationship between the foregut and surrounding tissues. After gastrulation, the foregut endoderm is in close apposition to the neural tube and notochord (Li et al., 2007; Que et al., 2006; Teillet...
et al., 1998). As we highlight here, the distance between the endoderm and the notochord increases over time (Figure 4). The observed cell proliferation in this region may contribute to this increase (Figure 5G). Furthermore, our evidence suggests that there is likely a progressive restriction of dorsal body wall cells, as marked by their Robo expression, along the M-L axis, where they are eventually found ventrally surrounding the foregut tube (Figures 4C, 4G, and 4L). Concurrently, the two dorsal aortae that reside just above the body-wall mesenchyme converge and fuse at the midline. These observations suggest that a coordinated cell movement in the medial and ventral directions may contribute to the further increase in distance between the foregut endoderm and notochord (Figures 7J and 7K). As a result, the foregut becomes connected to the body wall only through a thin dorsal mesentery. This loose association would allow the foregut endoderm to elongate freely in relationship to the body-wall tissues.

Our finding that the foregut in Robo1;2 mutants remains closely associated with the body wall indicate that separation of the foregut from the body wall is genetically controlled. Although we have used the stomach as a primary example, this morphogenesis defect is observed at multiple A-P levels along the foregut in the Robo1;2 mutant (Figure 4; Figure S1). All three Slit genes are expressed in the vicinity of the foregut. Additionally, Slit3 mutants show closer foregut-to-body-wall distance compared to controls (Figure S1). Thus, although ligand-independent homophilic interactions among Robo receptors can also modulate cell behavior (Hivert et al., 2002; Jaworski et al., 2010), our data suggest that Robo function in foregut morphogenesis is likely Slit-dependent.

Several lines of evidence lead us to postulate that Slit-Robo signaling controls foregut morphogenesis via cell repulsion. First, we found that Slit/Robo genes are expressed in largely complementary patterns. In a normal embryo, the Robo-expressing foregut moves away from Slit sources. Second, we did not observe a reduction in cell proliferation or an increase in cell death in the dorsal mesentery and foregut of Robo1;2 mutants, suggesting that these are not likely the cause of the reduced distance between foregut and body wall. Third, we observed a wider spread of Robo-expressing body-wall cells and found that the dorsal aortae remain apart in Robo1;2 mutants, suggesting an impairment of concerted medial movement away from the lateral Slit source. Fourth, we showed that in Robo1;2 mutants, the dorsal mesentery and the foregut together facilitate foregut ventral movement away from the neural tube. While Robo genes are expressed in both the endoderm-derived epithelium and mesoderm-derived mesenchyme/mesothelium, data from conditional knockout experiments demonstrate that they are required in the mesenchyme/mesothelium for foregut morphogenesis. At present, we cannot distinguish Slit-Robo function in the mesenchyme versus the mesothelium.

A recent report shows that at the level of the intestine, the emergence of mesothelium progenitors is intrinsic to the gut tube rather than ingressing in as a sheet over the splanchnic mesenchyme (Winters et al., 2012). If this can be generalized to the foregut organs, it is plausible that the mesenchymal and mesothelial cells would coordinate their movement and respond similarly to Slit-Robo signaling.

We speculate that the prolonged close association of the foregut to the body wall may prevent normal foregut elongation and result in retention of the stomach in the chest. Meanwhile, it is important to note that our data do not rule out the possibility that Robo genes may play other essential roles at later steps in the formation of foregut-derived organs. For example, while shortened esophagus is not a primary defect because it is observed later than the reduced foregut separation (Figures 4E, 4F, 4N, and 4O), it could also result from disruption of a direct role of Robo in esophagus elongation. However, it would be difficult to explain a priori why the reduced growth is restricted to the esophagus and not observed in a more posterior region of the foregut (e.g., the stomach), where Robo expression appears to be higher than in the esophagus region (Figures 2A–2C). It is interesting to note that prior to the shortening, the first defect observed within the esophagus is an aberrant bulge toward the dorsal body wall (Figure 4K), suggesting that a morphogenesis defect may be a trigger for the shortening. We considered the possibility that the bulge arose from a disruption of planar cell polarity. However, an examination of planar cell polarity mutants such as Wnt5a−/− embryos failed to reveal a similar defect in the foregut (data not shown). Since the bulge always points toward the dorsal body wall, it is plausible that it may form as a consequence of increased foregut tethering to the body wall.

A critical late phenotype in Robo1;2 mutant mice is the failure of the lungs to inflate at birth. While Robo and Slit genes are expressed in the developing lung (Anselmo et al., 2003; Greenberg et al., 2004), our data suggest that failed inflation is likely secondary to the requirement for Robo outside of the lung. Lungs inflated normally in both Tbx4cre;Robo1;2 and Shhcre;Robo1;2, where Robo genes are inactivated in lung mesenchyme/mesothelium and epithelium, respectively. This is in contrast to Dermo1cre;Robo1;2 mutants, in which failure of the lungs to inflate is preceded by organ misplacement. Thus, we postulate that in Robo1;2 global knockouts, the lungs failed to inflate due to either mechanical compression of the lung by an abdominal organ in the chest and/or ineffective contraction of the malformed diaphragm.

The malformation of the diaphragm in the Robo mutants could be secondary to herniation of the abdominal organs into the chest, or it could be an independent defect due to loss of Robo function in the diaphragm. It is worth noting that while they are separated by 3 days (E10.5–E13.5), the events of foregut morphogenesis and, later, diaphragm formation share some striking similarities. Central to both of these events is the coordinated migration of mesenchymal/mesothelial cells. In this study, we postulate that medial and ventral migration of dorsal body wall mesenchyme/mesothelial cells is important for the separation of the foregut from the body wall. It has been proposed that medial migration of peritoneal buds is important for fusion of multiple mesenchymal/mesothelial populations to form the diaphragm (Yuan et al., 2003). In light of these similarities, it is
plausible that Slit-Robo signaling may play a recurring role in the morphogenesis of multiple mesoderm-derived tissues during development.

Congenital diaphragmatic hernia is a relatively common birth defect, occurring in approximately 1/3000 live births, and is associated with a high rate of neonatal lethality (Ackerman and Greer, 2007; Holder et al., 2007; Pober, 2008). It is generally thought to arise due to malformation of the diaphragm. Here we show that in Robo1;2 mutants, a mouse model of CDH, the foregut morphogenesis defect precedes the diaphragm defects. Whether the foregut defect could serve as a general underlying cause of human CDH remains to be seen. However, it is worth noting that a deletion of the chromosomal region spanning the linked ROBO1 and ROBO2 (del(3)(p12p21)) has been reported in human CDH (Holder et al., 2007; Pfeiffer et al., 1998).

**EXPERIMENTAL PROCEDURES**

**Generation of Mutant Mice**

Embryos were dissected from time-mated mice, counting noon on the day when the vaginal plug was found as embryonic day 0.5 (E). 0.5% Robo1tm1Matl (Robo1), Robo2tm1Matl (Robo2), and Robo1/Robo2 (Robo2) mutant alleles and Dermo1tm1Matl allele have been previously described (Grieshammer et al., 2004; Long et al., 2004; Lu et al., 2007; Yu et al., 2003) (Supplemental Experimental Procedures). Somite-matched littersmates were used as controls. All mouse protocols were approved by the Animal Care and Use Committee of the University of Wisconsin-Madison.

Embryos were fixed in 4% paraformaldehyde at 4°C, then either dehydrated in methanol and stored or equilibrated in 30% sucrose and embedded in either OCT or paraffin for sectioning. Whole-mount in situ hybridization (WISH) was performed as previously described (Abler et al., 2011) (Supplemental Experimental Procedures). Probes have been described previously (Brose et al., 1999).

**β-Galactosidase Staining and Histology**

β-gal activity was assayed by a standard protocol. Embryos at stages E10.5 and younger were fixed and stained prior to being processed for paraffin embedding. Sections were cut at 7 μm and counterstained with 1% eosin. Embryos at stages later than E10.5 were fixed, sectioned at 500 μm thickness with a vibratome, then stained overnight before being dehydrated and processed for paraffin embedding and sectioning.

**Immunofluorescent and Immunohistochemical Staining**

For immunofluorescence, frozen sections (10 μm) were stained using a standard protocol, and mounted in Vectashield (Vector Laboratories). For immunohistochemistry, antigen detection was performed with DAB kit (Vector Laboratories). Colocalization quantification was performed on one to two sections from each of five control and five mutant embryos using JACoP in ImageJ software (Boitè and Cordelierès, 2006). Identical threshold settings were used for all images in the analyses, and overlap was compared using Student’s t test. Results are reported as the mean ± SD, and were considered statistically significant if p ≤ 0.05.

Primary antibodies used were rabbit anti-E-cadherin (Cell Signaling, 1:200), rabbit anti-β-catenin (Invitrogen, 1:200), mouse anti-Cadherin 2 (Invitrogen, 1:200), and rabbit anti-Caspase 9 (Cell Signaling, 1:200). Secondary antibodies were Cy3-conjugated goat anti-mouse, FITC-conjugated goat anti-rabbit, and HRP-conjugated goat anti-rabbit (Jackson Immunoresearches, 1:400 dilution).

**Cell Proliferation Assay**

Pregnant females received an intraperitoneal injection of 100 μg EdU (Sigma) 30 min prior to sacrifice. Samples were frozen in OCT and cryosectioned. EdU detection was performed according to the manufacturer’s protocol (Invitrogen). The percentages of EdU+ nuclei found among the three control (n = 8 sections) and three Robo1/2 (n = 8 sections) embryos were calculated using ImageJ and compared using Student’s t test. Results are reported as the mean ± SD, and were considered statistically significant if p ≤ 0.05.

**Boyden Chamber Cell Migration Assays**

Stomachs from 16 wild-type E10.5 embryos were dissected and incubated for 75 min at 37°C in 1 ml of Dulbecco’s modified Eagle’s medium (DMEM)/F12-K containing 10% fetal bovine serum and penicillin/streptomycin (GIBCO) and 1 mg/mL collagenase/dispose (Roche) to dissociate the cells. For the first set of experiments (Figure 6A), stomach cells were resuspended in DMEM + 0.1% BSA at a density of 5 × 105 cells/mL, and 200 μl aliquots were seeded into the top chamber of 8 μm pore-size cell-culture inserts (BD Biosciences) coated with fibronectin (Sigma). Inserts were placed into a 24-well plate above the transfected COS-7 cells (n = 6 inserts/treatment). For the second set of experiments, stomach cells were washed and resuspended in conditioned media from control or SLIT2-N-transfected COS cells and seeded into chambers as described above. These chambers were placed into wells of a 24-well plate containing 700 μl DMEM + 0.1% BSA. For SLIT2-CM, 48 hr prior to the experiment, COS-7 cells were transfected with plasmid encoding the N-terminal domain of SLIT2 (SLIT2-N) (Nguyen Ba-Charvet et al., 2001; Wang et al., 1999) or empty-vector control using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

For each of the Boyden chamber assays, after 9 hr incubation cells were fixed and stained with crystal violet. Unmigrated cells were removed from the top surface of the cell-culture insert. The membrane was then mounted in Permount (Fisher). Four nonoverlapping 10x fields of view of each membrane were photographed, and the numbers of migrated cells were counted using ImageJ. Results are reported as mean relative ratio of migrated cells ± SEM, and were considered statistically significant if p ≤ 0.05. Each experiment was performed twice.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2012.11.018.

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