PLANT BIOLOGY. For the article “Bacterial volatiles promote growth in *Arabidopsis*,” by Choong-Min Ryu, Mohamed A. Farag, Chia-Hui Hu, Munagala S. Reddy, Han-Xun Wei, Paul W. Paré, and Joseph W. Kloepper, which appeared in issue 8, April 15, 2003, of *Proc. Natl. Acad. Sci. USA* (100, 4927–4932; First Published April 8, 2003; 10.1073/pnas.0730845100), the authors note the following. Due to a printer’s error on page 4930, bars 3 through 7 of histogram A of Fig. 4 were incorrectly labeled. The corrected figure and a corrected legend appear below.

![Fig. 4](image-url)

**Fig. 4.** Growth promotion of *A. thaliana* ecotype Col-0 with exposure to extracted bacterial volatiles from growth-promoting (GB03 and IN937a) and nongrowth-promoting (DH5α) bacteria and synthetic 2,3-butanediol (A) and exposure to volatiles released from *B. subtilis* WT (168) and mutant strains defective in the production of 2,3-butanediol (BSIP1173 and BSIP1174) (B). Different letters indicate significant differences between treatments according to least significant difference at $P = 0.05$. A dose–response curve with synthetic 2,3-butanediol in the presence of *A. thaliana* seedlings confirmed the efficacy of this volatile bacterial metabolite in promoting plant growth. The level of exogenous 2,3-butanediol (2 ng) that was observed to trigger optimal plant growth promotion was lower than those collected from the GB03 or IN937a strains over the 24-h collection interval (1–5 μg), and it may be at least in part due to a high initial release of the synthetic 2,3-butanediol when introduced into the I-plates containing the *Arabidopsis* seedlings, as compared to the more even emissions of VOCs generated from the bacterial strains.

DEVELOPMENTAL BIOLOGY. For the article “A genetic model for a central (septum transversum) congenital diaphragmatic hernia in mice lacking *Slit3*,” by Wenlin Yuan, Yi Rao, Randal P. Babiuk, John Greer, Jane Y. Wu, and David M. Ornitz, which appeared in issue 9, April 29, 2003, of *Proc. Natl. Acad. Sci. USA* (100, 5217–5222; First Published April 17, 2003; 10.1073/pnas.0730709100), the authors note the following correction. The name John Greer should have appeared as John J. Greer. The online version has been corrected. The corrected author list appears below.

Wenlin Yuan, Yi Rao, Randal P. Babiuk, John J. Greer, Jane Y. Wu, and David M. Ornitz

DEVELOPMENTAL BIOLOGY. For the article “Identification of a small molecule inhibitor of the hedgehog signaling pathway: Effects on basal cell carcinoma-like lesions,” by Juliet A. Williams, Oivin M. Guicherit, Beatrice I. Zaharian, Yin Xu, Ling Chai, Hynek Wichterle, Charlene Kon, Christine Gatchalian, Jeffery A. Porter, Lee L. Rubin, and Frank Y. Wang, which appeared in issue 8, April 15, 2003, of *Proc. Natl. Acad. Sci. USA* (100, 4616–4621; First Published April 4, 2003; 10.1073/pnas.0732813100), the authors request that Roel Nusse, Howard Hughes Medical Institute and Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, be added to the list of authors between Christine Gatchalian and Jeffery A. Porter. The corrected author line appears below.

Juliet A. Williams, Oivin M. Guicherit, Beatrice I. Zaharian, Yin Xu, Ling Chai, Hynek Wichterle, Charlene Kon, Christine Gatchalian, Roel Nusse, Jeffery A. Porter, Lee L. Rubin, and Frank Y. Wang
Congenital diaphragmatic hernia (CDH) is a significant cause of pediatric mortality in humans with a heterogeneous and poorly understood etiology. Here we show that mice lacking Slit3 developed a central (septum transversum) CDH. Slit3 encodes a member of the Slit family of guidance molecules and is expressed predominantly in the mesothelium of the diaphragm during embryonic development. In Slit3 null mice, the central tendon region of the diaphragm fails to separate from liver tissue because of abnormalities in morphogenesis. The CDH progresses through continuous growth of the liver into the thoracic cavity. This study establishes the first genetic model for CDH and identifies a previously unsuspected role for Slit3 in regulating the development of the diaphragm.

Congenital diaphragmatic hernia (CDH) was described in 1679 by Riverius (1). CDH occurs in 1–3 in 3,000 newborns and is associated with a 30–60% mortality rate with significant morbidity among survivors (2–4). There are several different types of CDH including Bochdalek, Morgagni, and central (septum transversum) diaphragmatic hernia (5). The Bochdalek CDH accounts for ∼70% of CDH and occurs in a posterolateral region of the diaphragm. Morgagni CDH is less common and forms in the anterior retrosternal diaphragm. Central (septum transversum) CDH occurs in the midline of the septum transversum and accounts for 1–2% of the total cases of CDH. The pathogenesis of CDH results as abdominal contents enter the thoracic cavity. The primary consequence is hypoplasia of the lung due to decreased thoracic volume, resulting in direct compression of the lung and inadequate fetal breathing movements. The resulting compromised pulmonary capacity often results in neonatal death.

Although CDH has been recognized for nearly 350 years, the pathogenesis and etiology of CDH is not clear. Evidence from teratogen-induced rodent models of CDH suggests that defects in diaphragm muscle arise from malformations of the primordial diaphragm, the pleuropitoneal fold (6). Other evidence from rodent models demonstrates that there may be concomitant insults to lung development associated with CDH (7, 8).

The diaphragm is a complex structure that is thought to arise primarily from the septum transversum, the pleuropitoneal membrane, paraxial mesoderm of the body wall, and esophageal mesenchyme (9, 10). Diaphragmatic musculature is thought to originate from condensations within the pleuropitoneal fold (11). The diaphragm is innervated primarily by the phrenic nerve. Previous embryological studies have focused on the ontogeny of the phrenic nerve and muscle. The contribution made by connective tissue to diaphragm development has not been reported.

Slit proteins are large (>200 kDa) molecules with multiple functional domains (12–14). Slit homologs have been identified in multiple organisms (12–14). In *Drosophila*, mutations in the *slit* gene result in collapse of the CNS commissural axon scaffold due to the failure of commissural axons to leave the midline (15–17). In contrast to the single *slit* gene in *Drosophila*, there are three homologues in vertebrates that show both overlapping and distinct expression patterns. Slit proteins function as guidance cues for motor axons, olfactory bulb axons, neuronal cells, myoblasts, and leukocytes and as a branching factor for DRG sensory axons (18–23). These activities have been established for Slit1 and Slit2 in vitro. The Slit1/Slit2 double-null mouse shows an axon-guidance defect in retinal ganglion cells and in several major axonal pathways in the forebrain (24, 25).

The roundabout (robo) gene encodes the receptor for Slit in both *Drosophila* and vertebrates (17–19). Four *robo* genes, including *robo1*, *robo2*, *rig-1* (*robo3*), and magic *robo*, have been identified in vertebrates (26–28). *robo* genes encode large transmembrane proteins in which the extracellular Ig-like domains directly interact with Slit (29, 30). *Dutt1*/*robo1* mutant mice die at birth with defects in lung development (31).

Slit and *robo* genes are expressed in both neuronal and nonneuronal tissues. In contrast to *slit1* and *slit2*, which are expressed prominently in the CNS, *slit3* is expressed weakly in the CNS but strongly in some peripheral tissues (14). No specific function for *slit3* has been identified either in vivo or in vitro. To investigate *in vivo* functions for *slit3*, a null mutation was introduced into *slit3* in mice by targeted mutagenesis in embryonic stem (ES) cells. Interestingly, *slit3* null mice develop a CDH similar to a central (septum transversum) CDH in humans.

Methods

Construction of Slit3 Null Mice. The *slit3* targeting vector was made by introducing a 1-kb deletion encompassing 194 bp of the 3′ half of exon 1, which contains the initiation codon and signal peptide and ∼800 bp of intron 1 (Fig. 1a). This region was replaced by the β-galactosidase (β-gal) gene and a loxP-PGK-neo selection cassette. ES cell colonies were screened by DNA-blot analysis using both 5′ and 3′ probes. Positive clones were injected into *C57BL/6J* blastocysts to obtain chimeric mice, which then were bred with *C57BL/6J* mice to obtain germ-line transmission. In some sublines, the Neo cassette was removed by mating *Slit3* +/− mice with β-actin-cre mice. The *Slit3* −/− mice with the neo cassette removed showed the same phenotype as mice that contained a PGK-neo gene. In this study we primarily analyzed mice containing the neo insert to avoid further mixing of the genetic background. The progeny were genotyped by either DNA-blot analysis or PCR. For DNA-blot analysis, an internal probe was used to detect the changes in size of an 800 bp of intron 1 (Fig. 1). 410-bp fragment (primers a and c) (Fig. 1). PCR conditions were 32 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

Oligonucleotide Sequences. The oligonucleotide sequences used were primer a (5′-GGG AAC AAA CGG-3′), primer b (5′-GGG GAATTCGAGCACCCCAAACTCGCAG-3′, sense), primer c and primer d (5′-CTG ACC CTT GTG GAG GAC AAA AAA-3′, antisense), and primer e (5′-CGG ATT CTC GTG GAC AAA CCG-3′, antisense) (Fig. 1a).

Abbreviations: CDH, congenital diaphragmatic hernia; ES, embryonic stem; β-gal, β-galactosidase; En, embryonic day; Pn, postnatal day.
the BrdUrd-positive nuclei in sections through the anterior mediastinal central tendon were counted. The area of the region counted was measured by using AXIOVISION 3.0 image software (Zeiss). The number of BrdUrd-positive nuclei per unit area was calculated for Slit3–/– and wild-type littermates. At least three sections were counted for each embryo examined. The calculation was based on three Slit3–/– embryos and two control embryos at E13.5.

Results and Discussion

Slit3 gene targeting eliminated 194 bp of the protein-coding region downstream of the initiation ATG in exon 1 and ~800 bp of the first intron (Fig. 1a). This deletion eliminated the amino terminus of Slit3 up to the first leucine-rich repeat. The amino terminus of Slit3 is hypothesized to contain either a signal peptide for secretion or a signal sequence for mitochondrial localization (14, 36). The deletion of this region is predicted to result in a null mutation for Slit3. To follow the fate of cells that normally express Slit3, the β-gal gene was inserted in frame with the initiation ATG (Fig. 1a). Three independently targeted ES cell lines (Fig. 1b) were used to generate chimeric male mice, and all three passed the targeted allele to offspring. Mice heterozygous for the targeted allele (Slit3+/–) were phenotypically normal and bred to produce homozygous offspring. Genotyping by DNA-blot analysis and PCR identified viable homozygous mice (Fig. 1c and d). Because the phenotype of mice derived from all three ES cell lines were similar, only one line was selected for further analysis. RNase protection analysis with a probe covering exons 1–3 did not detect alternative splicing around exon 1 in the wild-type allele and demonstrated that no 5′ Slit3 transcript was produced from the targeted allele (Fig. 1e). β-Gal staining of both whole embryos (Fig. 1f) and tissue sections, when compared with Slit3 expression patterns determined by in situ hybridization (14), showed similar expression patterns. Prominent β-gal staining was observed in the floor plate of the spinal cord, hippocampus, blood vessels in the lung and kidney, outer nuclear layer of the eye, exocrine pancreas, hair follicles, and the anterior region of the limb buds (Figs. 1f and 2g and data not shown).

Slit3–/– mice were maintained on a mixed C57Bl6/J/129/SVJ genetic background. Among 224 genotyped offspring from heterozygous matings, the ratio of wild type to heterozygote to homozygote was 59:116:51 (1:2:2:3:1), which approximated a normal Mendelian ratio. Although Slit3+/– mice were viable, the body weight of homozygous animals was consistently less than that of wild-type and heterozygous animals and became statistically significant (P < 0.05) after postnatal day (P)30 (data not shown). After P30 the morbidity and mortality of homozygous mice increased. Among homozygous animals that died before 9 months of age, >90% presented with a central (septum transversum) CDH in which the liver entered the thoracic cavity (Fig. 2a–f). The cause of death appeared to be lung compression (Fig. 2g) and intestinal obstruction due to later-stage herniation of the transverse colon. Actuarial analysis showed that 24% of the Slit3–/– mice died by 40 days of age, and 58% died by 150 days of age (data not shown). The overall penetrance of the CDH phenotype was 68% and was higher in males (74%) than females (60%) with a ratio of 1:2:1.

Based on the midline central tendon location (Figs. 2b and 3a), the diaphragmatic hernia in Slit3–/– mice is similar to the central (septum transversum) CDH seen in humans. At early postnatal stages up to P14, the liver was the only contents of the hernia sac and was always adherent to the hernia sac (Fig. 2c–e). The sac wall was composed of connective tissue (Fig. 2d–f) and, in some areas, liver tissue (Fig. 2d and e), indicating failure of the two cell types to separate during development. This pathology was similar to several cases reported in humans (37, 38). The herniated liver tissue often appeared vacuolated and in early
Because defective lung growth has been considered as a possible explanation for at least some cases of CDH, the role of Slit3 has been examined in this context. Slit3 was expressed in the developing lung and diaphragm, and it also regulated the innervation of the diaphragm. To determine whether defective innervation could contribute to CDH in Slit3−/− mice, the branching patterns and neuromuscular junction of the phrenic nerve were examined from E14.5 to E18.5. No differences were observed in the overall branching patterns, the density and length of subbranches, or the density and pattern of the diaphragmatic neuromuscular junction in E18.5 Slit3−/− mice and wild-type controls (Fig. 3 d-f).

Fig. 2. CDH and lung pathology in Slit3−/− mice. (a) E17.5 wild-type diaphragm viewed from the top showing the liver underneath. (b) E17.5 Slit3−/− diaphragm showing a CDH sac containing liver tissue (arrow). (c) Sagittal section of a CDH in a P14 Slit3−/− mouse. (d–f) Higher-magnification views of the boxed areas indicated in c. (g) Detection of β-gal in the lung of an E18.5 Slit3−/− embryo by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining. (h) Lung section of a P14 Slit3−/− mouse with CDH. (i and j) Lung sections of a 5-week-old wild-type (i) and Slit3−/− mouse showing pulmonary congestion in the Slit3−/− tissue (j).

Fig. 3. Analysis of the developing diaphragm. (a) Whole-mount immunohistochemistry of an E16.5 Slit3−/− diaphragm using the MF-20 (antilaminin) antibody. The arrow indicates a defect in the central tendon, and the arrowhead indicates a defect in diaphragm muscle. (b and c) Frontal sections of E14.5 wild-type and Slit3−/− embryos showing the thickness of the anterior central tendon (between arrows). (d and e) Whole-mount immunostaining of the phrenic nerve in E18.5 wild-type and Slit3−/− diaphragms using an anti-neurofilament 200 antibody. (f) Merged image showing the phrenic nerve and neuromuscular junction in an E11.5 Slit3−/− diaphragm visualized with anti-neurofilament 200 (green) and rhodamine α-bungarotoxin (red). (g) Pleural mesothelium in E13.5 Slit3−/− mice double-labeled with anti-laminin-γ1 (green) and anti-β-gal (red). (h) Migratory peritoneal mesothelial buds (arrow) in the lateral diaphragm of E13.5 Slit3−/− mice labeled with both anti-laminin-γ1 (for basal lamina, green) and anti-β-gal (red). (i) Developing falciform ligament (FL) in E15.5 Slit3−/− mice labeled with anti-β-gal (green) and anti-desmin (red). (j) Peritoneal mesothelial bud (arrow), defect region in the central tendon (arrowhead) and tendon–muscle junction (asterisk) in an E15.5 Slit3−/− diaphragm labeled with anti-β-gal (green) and anti-desmin (red). D, diaphragm; L, liver.
The observed defects in the anterior muscular part of the diaphragm of some Slit3−/− mice (Fig. 3a) suggested that a defect in myoblast migration or myotube formation could be a possible mechanism. However, frontal sections through E14.5 diaphragm showed that the anterior defect in the central tendon appeared before the formation of muscle bundles in this region (Fig. 3b and c). Additionally, the region of the “tendon–muscle junction” appeared normal (Fig. 3j), which suggested that defects in diaphragm muscle are likely to be secondary to connective tissue dysgenesis of the central tendon.

Anti-laminin-1γ immunohistochemistry showed that the diaphragm at E13.5 consisted of a thin membrane attached directly to the liver, with only pleural mesothelium on top of several layers of mesenchymal cells (Fig. 3g). This part of the liver is the so-called bare area (Fig. 6a). The diaphragm and liver begin to separate due to extension of the peritoneal mesothelial folds (Figs. 3 h and i, 4 a and e, and 6). At E15.5, the mesothelial buds from both sides reach the midline but do not fuse. This results in the formation of a “bridge” of midline connective tissue, called the falciform ligament, attaching the diaphragm and liver (Figs. 3i and 4 a and e).

Several possible mechanisms could lead to central tendon connective-tissue defects in Slit3−/− mice. First, if central tendon connective tissue fails to differentiate within the septum transversum, a congenital opening could occur, allowing liver tissue to enter the thoracic cavity. A second possibility is rupture due to a thinner or more fragile central tendon. A third possibility is stretching of the central tendon connective tissue. The observation of a hernia sac in all cases of Slit3−/− CDH supports the third possibility.

The pathogenesis of the central tendon first appeared as early as E14.5 where diaphragm mesenchyme was significantly thinner in Slit3−/− mice compared with wild-type control mice (Fig. 3b and c). At E15.5, the peritoneal mesothelium of the diaphragm was well developed in normal animals such that the diaphragm was only attached to the liver by the developing falciform ligament (Figs. 3b and 4a and e). In Slit3−/− mice, the central tendon remained adherent to the liver with little mesenchymal tissue and no falciform ligament (Figs. 3j and 4b and f). The diaphragm also began to distend as the liver continued to grow (Fig. 4b and f). However, the pleural mesothelium remained continuous, with no congenital opening or rupture (Fig. 4b). At E18.5, the mesothelium of the hernia sac remained continuous with the pleural mesothelium of the diaphragm (Fig. 4c and d). Therefore, the CDH in Slit3−/− mice formed by stretching of abnormally thin diaphragm tissue, which failed to separate from an expanding liver (Fig. 4f and g). The origin of the hernia sac appeared to arise from the adherent bare area of the diaphragm and liver (Fig. 4).

To establish a direct link between phenotype and gene expression, in situ hybridization and β-gal immunohistochemistry was used to localize sites of Slit and Robo expression in developing diaphragm. Slit3 was expressed predominantly in the mesothelial cells of the developing diaphragm from E12.5 to E18.5 (Fig. 5a and b), which suggested a direct relationship between its expression and CDH. Interestingly, Slit2 was expressed in diaphragm mesenchymal cells (Fig. 5c), suggesting possible functional redundancy. Robo1 was also expressed in diaphragm mesenchyme, and Robo2 was expressed at the interface of the liver and diaphragm (Fig. 5d and e). Slit1 and Robo3 were not detected in developing diaphragm at this stage.

Two related processes, proliferation of mesenchymal cells and extension of peritoneal mesothelial buds, occur during the development of the central tendon. To investigate how Slit3 could function in the morphogenesis of the central tendon, cell
Class 3 semaphorins were found to inhibit branching morphogenesis in the lung, and KAL-1 was involved in epidermal morphogenesis in addition to olfactory axon guidance and neurite branching (43, 44). Our study indicates that Slit3 may also have functions in regulating cell proliferation, synthesis of extracellular matrix molecules, and/or migration of mesothelial cells. The CDH of Slit3−/− mice could also result from the synergistic outcome of multiple factors. Partial penetrance may result from compensation by other signaling molecules and from redundancy with Slit2.

A small percentage of newborn Dutt1/Robo1 mutant mice developed CDH. The phenotype and the mesenchymal expression of Robo1 in the central tendon (Fig. 5d) suggests a role for Slit-Robo signaling in diaphragm pathogenesis. The low penetrance of CDH in the Dutt1/Robo1−/− mice may arise from redundancy with Robo2 and complex ligand–receptor relationships between Slit and Robo. Double or triple knockouts of Slit and/or Robo genes will be important to address this possibility.

Our current knowledge of the signaling pathways downstream of Robo suggests that Slit and Robo interactions are important for cytoskeletal actin dynamics, which are important for axon guidance and cell migration (45, 46). However, it is still difficult to integrate these pathways with functions in morphogenesis of the developing diaphragm. In Drosophila, Abelson (Abl) is one of the molecules acting downstream of Robo and is critical for morphogenesis of epithelial cells (47). Further studies of signaling pathways downstream of Robo will be required to elucidate functional mechanisms regulating development.

Most cases of CDH are sporadic, but familial cases have been described and include autosomal dominant, autosomal recessive, and X-linked inheritance patterns (48). It has been reported that in familial cases males were more commonly affected, whereas in sporadic CDH females were more commonly affected (49). Interestingly, in the Slit3 genetic model the phenotypic penetrance was higher in males than in females (1.2:1).

The Slit3−/− mouse is the first genetic model with a similar phenotype to human central (septum transversum) CDH. The only other rodent model for CDH resembles the Bochdalek-type hernia and is generated by exposure of the embryos to the herbicide nitrofen. In the nitrofen model, the CDH is preceded by lung hypoplasia, which has led many to view the lung abnormality as the primary etiology of the diaphragm pathology. However, based on the Slit3 CDH model, lung pathology is not a prerequisite for a primary CDH to occur. This is consistent with some human cases in which symptoms appeared late in childhood and lung development was normal (50). The Slit3−/− model therefore should provide insight into the pathogenesis of some forms of CDH and to the development of the diaphragm. It will also provide a model to study the secondary effects of lung compression on lung development.

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