Slit–Roundabout Signaling Regulates the Development of the Cardiac Systemic Venous Return and Pericardium


Rationale: The Slit–Roundabout (Robo) signaling pathway has pleiotropic functions during Drosophila heart development. However, its role in mammalian heart development is largely unknown.

Objective: To analyze the role of Slit–Robo signaling in the formation of the pericardium and the systemic venous return in the murine heart.

Methods and Results: Expression of genes encoding Robo1 and Robo2 receptors and their ligands Slit2 and Slit3 was found in or around the systemic venous return and pericardium during development. Analysis of embryos lacking Robo1 revealed partial absence of the pericardium, whereas Robo1/2 double mutants additionally showed severely reduced sinus horn myocardium, hypoplastic caval veins, and a persistent left inferior caval vein. Mice lacking Slit3 recapitulated the defects in the myocardialization, alignment, and morphology of the caval veins. Ligand binding assays confirmed Slit3 as the preferred ligand for the Robo1 receptor, whereas Slit2 showed preference for Robo2. Sinus node development was mostly unaffected in all mutants. In addition, we show absence of cross-regulation with previously identified regulators Tbx18 and Wt1. We provide evidence that pericardial defects are created by abnormal localization of the caval veins combined with ectopic pericardial cavity formation. Local increase in neural crest cell death and impaired neural crest adhesive and migratory properties underlie the ectopic pericardium formation.

Conclusions: A novel Slit–Robo signaling pathway is involved in the development of the pericardium, the sinus horn myocardium, and the alignment of the caval veins. Reduced Slit3 binding in the absence of Robo1, causing impaired cardiac neural crest survival, adhesion, and migration, underlies the pericardial defects. (Circ Res. 2013;112:465-475.)

Key Words: cardiac neural crest ■ heart development ■ pericardium ■ Slit–Robo signaling ■ systemic venous return

Congenital defects of the arterial side of the heart are frequently life threatening and generally diagnosed immediately after birth. Defects of the systemic venous return to the heart often only become symptomatic much later in life. However, these defects can be severe, ranging from congenital malformations, such as misalignment of the connecting veins, to atrial arrhythmias like sick sinus syndrome.1,2 Knowledge about the genes involved in the development of the systemic venous return, the caval veins, and myocardial sinus horns is limited. The T-box transcription factor Tbx18 is required for the differentiation of the sinus horn myocardium from mesenchymal precursors, and its absence causes delayed formation and malformation of the sinus horns.3,4 Recently, a role was shown for Wnt/β-catenin signaling in regulating the differentiation of the sinus horn myocardium.5 Podoplanin,6 Pdgfra,7 and Wt1, as well as its downstream target Raldh2,8 were found essential for normal sinus horn formation. Interestingly, mouse mutants for Tbx18,9 Wt1, and Raldh2/Retinoic acid signaling9 presented with additional defects in the formation of the membrane between the pleural and pericardial cavities, indicating a link between the development of the systemic venous return and the pericardium. Further data on genetic pathways involved in pericardium development are lacking. Absence of part of the pericardium is often symptomless, but it can reveal itself by sudden acute chest pain or dyspnea.10 Recently, expression of genes of the Slit–Roundabout (Robo) signaling pathway was detected at the venous pole of the heart,11 suggesting a possible role in the formation of
This area. Robo receptors are members of the immunoglobulin superfamily of cell adhesion molecules. In mammals, 4 such receptors (Robo1-4) and 3 Slit ligands (Slit1-3) have been identified.\(^1\) In Drosophila and zebrafish, Slit–Robo signaling plays roles in cell adhesion during cardiac cell polarization, morphogenesis, migration, and lumen formation.\(^1\) Furthermore, knowledge about Slit–Robo signaling in the mammalian heart is scant. Slit3 has been shown to be the predominant ligand gene transcribed in the chambers of the early heart.\(^1\) However, knowledge about Slit–Robo signaling in the mammalian heart is scant. Slit3 has been shown to be the predominant ligand gene transcribed in the chambers of the early mouse heart.\(^1\) Furthermore, a role for Slit–Robo signaling has been found during cardiac neural crest migration, where it is indicated that abnormal localization of the caval veins combined with ectopic pericardial cavity formation, caused by increased

**Methods**

Transgenic mice and experimental procedures for in situ hybridization, immunohistochemistry, ligand binding assay, neural crest explants, 3-dimensional reconstruction, and statistical analyses are provided in the Online Data Supplement. All experimental procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and institutional guidelines.

**Results**

**Robo1 Knockout Embryos Lack Part of the Pericardium and Display Systemic Venous Return Defects**

Sections of embryos lacking the Robo1 receptor showed a striking defect in the pericardium (n=11/21). Although most of the pericardium developed normally, the part located between the superior caval veins, the pleuropericardial membrane, was missing. As a result, the expanding lungs were not retained in the pleural cavity, but penetrated into the pericardial cavity and completely enveloped the heart (Figure 1A and 1B). As a result of the absence of the pleuropericardial membrane, the caval veins, which develop in close relation to the pericardium, lacked their medial attachment to the mediastinum (Figure 1A and 1B). Despite the abnormal location of the caval veins, sinus horn myocardium developed relatively normal (Figure 1A and 1B). The pleuropericardial membrane defect was predominantly present on the left side (6/11). However, in several animals, only the right side (2/11) or both sides (3/11) were affected.

Only after embryonic day (E) 11.5, the pleuropericardial membranes start to divide the pericardial cavity from the

**Figure 1. Pericardial defects in the Robo1 mutants.** A–F, In situ hybridization staining for myocardial marker cTnI on Robo1\(^{+/+}\) (A, C, and D) and Robo1\(^{-/-}\) (B, E, and F) embryos. C–F, Sections through cranial (G and E) or caudal (D and F) pericardial cavity in Robo1\(^{+/+}\) and Robo1\(^{-/-}\) mice. Note the normal formation of the pleuropericardial membrane caudal of the entering caval veins in the knockout (red arrowheads). G–N, Three-dimensional reconstructions of an embryonic day (E) 12.5 Robo1\(^{-/-}\) and littermate Robo1\(^{+/+}\) embryo. G and H, A ventral view of the heart and of the dorsal pericardium before and after removal of the heart. Arrowheads indicate the caval vein entrance. I and L, Dorsal views of the myocardium of the heart, sinus horns, and the caval veins. J, K, M, and N, Arrowheads show where the caval veins pass the lungs. Ao indicates aorta; LA, left atrium; LL, left lung; LSCV, left superior caval vein; LSH, left sinus horn; LV, left ventricle; PC, pericardium; PT, pulmonary trunk; PV, pulmonary vein; RA, right atrium; RL, right lung; RSCV, right superior caval vein; RV, right ventricle. Scale bars depict 100 μm.
pleural cavity. In agreement, no morphological defects were observed in Robo1−/− embryos until after E11.5 (data not shown). As the caval veins in the wild-type embryos became progressively located in the pericardial cavity between E11.5 and E12.5, those in the Robo1−/− remained more dorsally inside the body mesenchyme and entered the pericardial cavity much more caudally (Figure 1C–1H, black arrowheads 1G and 1H). As a consequence of their more caudal entrance, the caval veins had to circumvent the developing lungs to gain access to the heart (Figure 1I–1N). Caudal to their entry into the heart, pleuropericardial membrane formation was normal (Figure 1C–1F, red arrowheads).

None of these defects was observed in Robo2−/− embryos, which were indistinguishable from their wild-type littermates (Figure 2A and 2B). However, the combined absence of both Robo1 and Robo2 genes increased the incidence of the pericardial defect (n=5/7; Figure 2C–2F). Furthermore, Robo1−/−;Robo2−/− embryos showed malformed and very thin caval veins (Figure 2C–2H). The connection of the left caval vein to the heart ranged from a small, but relatively normal connection to the right atrium, to only an entrance into the coronary circulation (Figure 2G and 2H, red arrowheads; Online Figure IA–IE). Whereas sinus horn myocardium formation in the Robo1−/− animals was largely unaffected, minimal sinus horn myocardium formed around both caval veins in the double mutants (Figure 2G and 2H) and its size seemed proportional to the extent of the caval vein connection to the heart (Online Figure IA–IE). Furthermore, some of the Robo1−/−;Robo2−/− embryos exhibited a persisting left inferior caval vein, which joined the right inferior caval vein at the entrance into the liver (Figure 2E–2H, black arrowhead). These defects were confined to the systemic venous return, as the pulmonary veins developed normally in all mutants (data not shown).

Although Robo1 seems to be the key Robo receptor in the development of the systemic venous return and pericardium, the additional defects in the double mutants suggest functional redundancy with the Robo2 receptor, which is expressed normally in the absence of Robo1 (Online Figure IF). To determine which Slit ligands and Robo receptors are involved in this region, we next analyzed their expression patterns.

**Robo1 and Robo2 Receptors and Their Ligands, Slit2 and Slit3, Are Expressed During Systemic Venous Return and Pericardium Development**

Of all Robo receptor genes, Robo1 was the most prevalent. Its expression has been described in the outflow tract cushions, and we observed additional signal in the myocardium and cushions of the atrioventricular canal at E9.5 (Figure 3A), which was maintained at E12.5 and E14.5 (Online Figure IIA and IIB). Robo1 expression was not observed in the sinus horn myocardium, but both the pericardium and the mesenchyme directly surrounding the caval veins showed robust expression (Figure 3A; Online Figure IIA and IIB). Robo2 was less broadly expressed and, in contrast to earlier observations, was never seen in the myocardium at any stage. We detected Robo2 signal in the outflow tract cushions and in the atrioventricular cushions at all time-points analyzed (Figure 3A; Online Figure IIA and IIB). Although there was no expression
in the sinus horn myocardium, Robo2 was visible in the mesenchyme surrounding the caval veins and in the body wall at E9.5 (Figure 3A). Robo3 expression was restricted to the central nervous system as described earlier (data not shown). Robo4 expression was found in the vascular endothelium, including that of the aorta, pulmonary trunk, and coronary vasculature, but none was observed in the endocardium. A small number of Robo4 positive cells were observed in or surrounding the caval veins at E9.5 (Figure 3A, arrowheads), but not at later stages.

Slit1 was highly expressed in the floor plate of the neural tube (Figure 3A), but only weakly in the outflow tract vessels at E14.5 (data not shown). In contrast, Slit2 and Slit3 were highly expressed in distinct patterns in and around the heart, and isolated Slit2 signal in the myocardium was confined to the ventricular trabecules (Figure 3A). Furthermore, Slit2 was expressed in the outflow tract vessels as previously observed, in the outflow tract endocardium, the epicardium, and the mesenchyme around the caval veins (Online Figure IIA and IIB). Slit3 was most broadly expressed in the myocardium, with presence in the outflow tract and atrial myocardium, and it was the only gene of the Slit family found in the sinus horn myocardium (Online Figure IIA and IIB, black arrowheads). Slit3 was also detected in the epicardium and all tissues connecting the heart to the body. The high expression of both Slit2 and Slit3 in the systemic venous return and pericardium region prompted us to further investigate these ligands.

**Slit3–Robo1/2 Interaction Is Required for Caval Vein Alignment and Sinus Horn Formation**

Slit2−/− or even Slit1−/−;Slit2−/− embryos did not show any defects in the systemic venous return and pericardium region (Online Figure IIC–IIF). However, Slit3−/− mice showed defects partly resembling those observed in Robo1−/−;Robo2−/− embryos. Slit3−/− caval veins were hypoplastic, although to a lesser extent than observed in the Robo1−/−;Robo2−/− mutants (Figure 3B–3E). As in Robo1−/−;Robo2−/− mice, the left superior caval vein persisted into a left inferior caval vein that connected with the right inferior caval vein at the level of the liver (Figure 3D and 3E, black arrowheads). The left caval vein did only connect via a very small long separate vein to the coronary sinus, and the sinus horn myocardium was very short and severely hypoplastic (Figure 3D and 3E, red arrowheads). The part of the membrane directly surrounding the caval veins was much thicker in Slit3−/− embryos, but no missing parts were observed (Figure 3B and 3C). These data suggest that Slit3 acts as the main ligand required by Robo1 and Robo2 in regulating the formation of the region. However, the absence of pericardial defects in Slit3 mutants indicates involvement of additional factors, most likely the other locally present ligand, Slit2.

To determine specificity of Slit3 and Slit2 for Robo1 and Robo2 receptors in the region, we performed a ligand binding assay on sections. Slit3 binding was observed precisely between the caval veins and the pericardium in and adjacent to the Robo1-expressing region in wild-type embryos (Figure 4A). High-affinity binding of Slit3 was found for the region between the superior caval veins directly cranial to the pericardium, which exhibited high Robo1 expression levels (Online Figure IIIA and IIIB). Slit2 also bound to these regions, albeit at lower levels (Figure 4A and 4B; Online Figure IIIA and IIIB). In contrast to Slit3, affinity of Slit2 was highest for the locally present Robo2 (Online Figure IIIA–IIIC). Differential binding of Slit2 and Slit3 was observed in the outflow tract cushions and surrounding the trachea and esophagus. In the absence of Robo1, binding of Slit3 was largely absent, whereas Slit2 binding was reduced between the caval veins and the pericardium, but still showed binding just cranial of the pericardium (Figure 4A and 4B; Online Figure IIIA and IIIB). In the absence of Robo1, Robo2-expressing areas surrounding the trachea

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**Figure 3. Slit3 is required for caval vein alignment and sinus horn formation.** A, In situ hybridization for Robo1, Robo2, Robo4, Slit1, Slit2, and Slit3 at embryonic day (E) 9.5. B and C, In situ hybridization cTnI staining on Slit3+/+ (B) and Slit3−/− (C) embryos. D and E, Three-dimensional reconstructions of Slit3−/− (D) and Slit3−/− (E) embryos. Black arrowheads Persistent left inferior caval vein in the mutant; red arrowheads the abnormal left venous cardiac connection. ICV indicates inferior caval vein; LA, left atrium; LCV, left caval vein; LL, left lung; LSCV, left superior caval vein; LSH, left sinus horn; LV, left ventricle; PV, pulmonary vein; RA, right atrium; RL, right lung; RSCV, right superior caval vein; RSH, right sinus horn; and RV, right ventricle. Scale bars depict 100 μm.
and oesophagus persisted to bind Slit2 and the ventral body wall persisted to bind both Slit2 and Slit3 (Figure 4B; Online Figure IIIC). In the absence of either receptor, binding of both Slit2 and Slit3 was largely abolished (Figure 4B; Online Figure IIIB). These data confirm Slit3 as the main regional ligand for Robo1, showing strong decrease in binding in the absence of this receptor. Slit2 shows preference for Robo2; however, the absence of pericardial defects in the Slit3 mutant and the regional presence and binding of Slit2 indicate likely functional redundancy of Slit2 and Slit3.

**Sinus Node Development and Lack of Cross-regulation With Previously Identified Systemic Venous Return Regulators Tbx18 and Wilms Tumor 1**

At the border of the right sinus horn with the right atrium, the sinus node develops as part of the sinus horn myocardium.20 As the development of the sinus horns was severely hampered in Robo1−/−;Robo2−/− and Slit3−/− embryos, we next assessed if sinus node development was affected. Of the Slit and Robo genes, only Slit3 was expressed in this structure, identified by the presence of Hcn4 and the absence of Cx40 (Figure 5A, arrowheads). Robo1 as well as Slit2 and Slit3 was expressed in the mesenchyme surrounding the sinus node. Whereas it normally develops in direct contact with the caval vein, we noticed separate development of both structures in Robo1 (Figure 5B), Robo1/2 and in Slit3 mutants at E12.5 (data not shown). The initially malformed sinus node gained a normal structure during later development in Robo1−/− and Slit3−/− (Figure 5C), but part of it was still disconnected from the caval vein in double mutants at E14.5. The size of the sinus node of all Slit and Robo mutants was indistinguishable from control littermates at E14.5 (Figure 5C and 5D).

**Sinus horn, including the sinus node myocardium, is known to differentiate from Tbx18-positive mesenchyme into Tbx18-negative myocardium.**3 Tbx18 was found to be normally expressed in the sinus horns and sinus node of Robo1−/−, Robo1−/−;Robo2−/−, and Slit3−/− mice (Figure 5E and data not shown). Furthermore, the expression of Slit and Robo genes was unaffected in Tbx18 mutants (Figure 5E and data not shown), indicating this pathway is not downstream of the Tbx18-dependent pathway for sinus horn development. As Wt1 and Raldh2 are required for the development of the pleuropericardial membranes and caval veins,8 we next analyzed the expression of these genes in the area of pleuropericardial membrane formation in Robo1−/− embryos. The morphological differences between Robo1−/− and control embryos made exact regional comparison difficult, but expression levels and patterns of both genes were normal in the mesothelium and subcelomic mesenchyme surrounding the pericardial cavity (Figure 6A; Online Figure IVA). Reciprocal analysis in Wt1 mutants showed that the expression of Robo1,
Robo2, Slit2, and Slit3 was unaffected (Figure 5E and data not shown), indicating absence of cross-regulation between the Slit–Robo pathway and Wt1.

**Ectopic Celom Formation Underlies the Partial Absence of the Pericardium**

The caval veins were progressively incorporated into the pericardial cavity in wild types at E12.5, but remained present inside the body mesenchyme in Robo1−/− mice (Figure 1C–1H). Although Wt1 and Raldh2 were normally present between the caval vein and the pericardial cavity in Robo1−/− mice, this expression area was much larger near the cranial pericardium owing to the aberrant location of the veins. Exactly in the larger area of Wt1- and Raldh2-expressing mesenchyme, between the caval veins and pericardium, we observed an area of ectopic celom formation at E12.5 (Figure 6A–6″; Online Figure IV A) that formed directly in contact with the cranial pericardial cavity and immediately became occupied by expanding lung tissue (Figure 6A and 6B). In contrast, expansion of the pleural cavity was only observed caudal to the entrance of the caval veins into the pericardial cavity in wild-type embryos (Figure 6A–6″, black arrowheads). As a result of the ectopic celom formation, the outflow tract connection to the body had to bridge a significantly increased area inside the pericardial cavity to reach the heart, which was quantified by measuring the length of the body connection at outflow tract level, as indicated by the arrows in Figure 6B (Figure 6C). These results suggest that abnormal localization of the caval veins in combination with ectopic celom formation underlies the pericardial defects observed in the absence of Robo1.

**Ectopic Celom Formation Is Caused by Increased Apoptosis in the Neural Crest**

The pleuropericardial membranes are released from the lateral body wall by apoptosis, suggesting ectopic celom formation may be caused by abnormal cell death. Therefore, we analyzed apoptosis in the region where we observed ectopic celom formation at E12.5 (Figure 6A–A″; Online Figure IVA) that formed directly in contact with the cranial pericardial cavity and immediately became occupied by expanding lung tissue (Figure 6A and 6B). In contrast, expansion of the pleural cavity was only observed caudal to the entrance of the caval veins into the pericardial cavity in wild-type embryos (Figure 6A–A″, black arrowheads). As a result of the ectopic celom formation, the outflow tract connection to the body had to bridge a significantly increased area inside the pericardial cavity to reach the heart, which was quantified by measuring the length of the body connection at outflow tract level, as indicated by the arrows in Figure 6B (Figure 6C). These results suggest that abnormal localization of the caval veins in combination with ectopic celom formation underlies the pericardial defects observed in the absence of Robo1.
amount of cell death was still increased in the entire region in between the caval veins and the cranial pericardium in Robo1−/− embryos compared with controls (Figure 6D). At both E11.5 and E12.5, the region of increased apoptosis was condensed around the cranial-most part of the pericardial cavity; further caudal, very few apoptotic cells were observed in both Robo1 mutants and wild-type embryos.

The restricted area of increased apoptosis and its proximity to the cardiac outflow tract suggested a possible relation with the cardiac neural crest. Therefore, we analyzed the contribution of neural crest to the region using Robo1;Wnt1cre;R26REYFP mice at E11.5 (Figure 7B) and E12.5 (data not shown). In both Robo1 mutants and wild-type embryos, the neural crest–derived cells were located in the mesenchyme dorsal to the majority ventral and medial of the caval veins at cranial pericardial cavity level. Below outflow tract level YFP-positive cells between the caval vein and pericardial cavity were found only around the foregut lined the pericardial epithelium in between the caval veins, and in the developing innervation (Figure 7B). At E16.5, neural crest–derived cells were only found around the foregut and in the pericardial cavity at embryonic day (E) 11.5. Dapi indicates 4',6-diamidino-2-phenylindole; LA, left atrium; LL, left lung; LSCV, left superior caval vein; LSH, left sinus horn; OFT, outflow tract; RA, right atrium; RL, right lung; RSCV, right superior caval vein; RSH, right sinus horn; and YFP, yellow fluorescent protein. Scale bars depict 100 μm.

Figure 6. Ectopic celom formation is caused by increased apoptosis in the cardiac neural crest. A and B. In situ hybridization sections stained for Wt1, level of sectioning as indicated in Online Figure IVA. A′ and A″ are magnifications of indicated boxes. Black arrowheads The most cranial area of pleural cavity formation, which is much more cranial in the mutant. Clear arrowheads The further location of the mutant caval vein into the mutant body. Red arrowheads The location of the wild-type sinus horn inside the pericardial cavity, and the mutant sinus horn still mostly inside the body mesenchyme. B, section indicating the location of length measurements shown in C, D, Robo1+/+ and Robo1−/−;Wnt1Cre;R26REYFP immunohistochemistry sections stained for the indicated proteins. Arrowheads The location of increased apoptosis in Robo1−/− mice compared with the wild-type control. Arrows The location of apoptotic cells in the neural crest. Quantification of cleaved Caspase 3 (CC3)+–positive cells shows doubling of the number of apoptotic cells in the mutant compared with controls. Note the presence of neural crest cells between the caval vein and pericardial cavity.

Online Figures VB and VIA). This local Robo2 and high level of Slit3 expression, combined with the adjacent expression of Slit2 in the endoderm, likely accounts for the highly localized increase in apoptosis. Interestingly, we did not observe increased cell death in the cardiac neural crest or surrounding the caval veins in Slit3 mutants, explaining the lack of pericardial defects in the absence of Slit3 (Online Figure IVB).

Absence Migratory Inhibition by Slit3 and Impaired Adhesion of Cardiac Neural Crest Cells in the Absence of Robo1

Despite the increased apoptosis in the neural crest, the overall patterning of the neural crest seemed unaltered in Robo1−/− mice at E11.5 (Figure 7B) and E12.5 (data not shown). In both Robo1−/− and in wild-type controls, YFP-positive cells were in the majority ventral and medial of the caval veins at cranial pericardial cavity level. Below outflow tract level YFP-positive neural crest–derived cells were only found around the foregut and in the developing innervation (Figure 7B). At E16.5, neural crest–derived cells were located in the mesenchyme dorsal to the cranial pericardium, a subset of YFP-positive cells directly lined the pericardial epithelium in between the caval veins, bordering the outflow tract. However, no contribution to the pericardium itself was observed (Figure 7B). In the absence of Robo1, fewer cells lined the pericardium, and ectopic cavity formation was observed in the area dorsal to the pericardium normally filled with neural crest cells (Figure 7B).

To assess whether the defects observed in ectopic celom and pericardial cavity formation are owing to altered cardiac neural crest migration that consequently leads to cell death,
we examined the migratory response of Robo1-deficient and control neural crest cultures to Slit2- and Slit3-conditioned media (Figure 8A). Wild-type neural crest cells showed slightly reduced migration in the presence of Slit3, but not Slit2 (in agreement with previous findings), whereas this response to Slit3 was absent in Robo1−/− cells. Interestingly, the presence of Slit2- or Slit3-conditioned media did not affect the number of apoptotic cells in vitro nor did the absence
of Robo1 in Robo1−/− explants (Figure 8A), underlining the requirement for very local Robo2 expression and exposure to both Slit2 and Slit3. Impaired cell–cell adhesion, as well as altered migration, has also been shown to cause increased cell death.22 To assess this, we performed an adhesion assay using dissociated Robo1-deficient and control neural crest cells and showed reduced adhesion with Robo1-deficient cells compared with control (Figure 8A). These results indicate that the increased local neural crest apoptosis that results in ectopic celom formation is likely owing to impaired cell–cell adhesion, as well as altered neural crest migration.

Discussion

Mice Lacking Slit3 Only Partially Recapitulate the Defects Observed in Robo1−/−;Robo2−/− Mutants

Although most phenotypic features of Robo1/2 and Slit3 mutants are similar, the most striking difference is the intact pericardium in the latter. Interestingly, diaphragmatic hernias have been reported in Slit3−/− mutants,23 providing evidence that Slit3 does play a role in the division of the celomic cavities. In human, diaphragmatic hernias coexist with pericardium defects,10 suggesting a related developmental mechanism or involvement of the same gene pathways. The absence of pericardial defects in Slit3−/− mice suggests phenotypic rescue by binding of locally present Slit2, similar to the rescue of systemic venous return defects by Robo1 in Robo2 mutants, explaining the difference in sinus horn phenotype between the Slit3 and Robo1/2 mutants. Moreover, the discrepancy in phenotype could be explained by requirement of additional interacting partners for the Slit–Robo signaling pathway. For example, Robo1 has been shown to interact with Neurogliin1 to regulate Semaphorin signaling,24 and many of the components of the Semaphorin–Plexin/Neurogliin signaling pathway are known to be involved in heart development.25

In patients, syndromes showing coexistence of all or some of the defects observed in the Robo1/2 and Slit3 mutants have been described,26,27 but no causative genes have yet been identified. Our data suggest Slit3, Robo1, and Robo2 as candidate genes.

Increased Neural Crest Cell Death Underlies Ectopic Celom Formation

Knowledge of both the morphological processes and the molecular pathways underlying pericardial defects is still limited. Different developmental mechanisms have been put forward from premature atrophy of the left common cardinal vein to defective release of the membranes from the body wall.8,10 In Robo1−/− mice, the pericardial defect seems to have a dual cause. First, the caval veins remain inappropriately localized further into the body mesenchyme near the cranial pericardial cavity. Second, as a result, or independently owing to neural crest cell death, the cranial pericardial cavity expands dorsally. Therefore, the lungs are forced to develop ventral to the caval veins resulting in defective closure of the pleuropericardial membranes (Figure 8B).

The cardiac neural crest is well known to contribute to the aorticopulmonary septation complex, the membranous ventricular septum and innervation.25 However, it has not been reported that neural crest cells also fill most of the mesenchyme dorsal to the cranial pericardium, indicating a broader role for neural crest during heart development than previously thought. During normal development, limited controlled cell death is observed in the neural crest contributing to this region, suggesting locally tightly regulated celom expansion. In the absence of Robo1, the increase in localized cell death causes ectopic celom formation. During their migration to the heart, Robo1-positive neural crest cells only locally express Robo2 and encounter very high levels of Slit3, and also Slit2 ligand, directly dorsal to the pericardium, suggesting a vital role, locally, for this signaling pathway. Absent migratory response to Slit3, and significantly reduced adhesive properties in Robo1 mutants, leads to apoptosis of neural crest that is dependent on the presence of correct survival signals. Slit–Robo signaling is a known key player in cell adhesion through regulation of various cadherins and integrins.22 The significant reduction in neural crest adhesion in the absence of Robo1 suggests that the increase in cell death is caused either directly by loosened cell adhesion or by adhesion-dependent protection against apoptosis.22

Slit–Robo Signaling Controls Sinus Horn Development and Acts Independently of Tbx18 and Wt1 Gene Pathways

Tbx18 and Wilms tumor 1 (Wt1) have been identified as key transcription factors involved in systemic venous return formation. Therefore, we analyzed the possibility of regulation of the Slit–Robo signaling pathway by these genes or vice versa. However, no cross-regulation was observed, indicating that the Slit–Robo signaling pathway is a novel independent contributor in the formation of this region. The high expression of Wt1 in the area of ectopic celom formation and previous findings of reduced apoptosis in Wt1 mutants8 indicate a role for Wt1 in the ectopic celom formation. Interestingly, as observed in Slit3−/− mice, diaphragmatic hernias were also found in Wt1 mutants.28

The absence of increased neural crest cell death in Slit3 mutants shows that the caval vein and sinus horn defects develop independently of the pericardial defect caused by neural crest cell death. Both the abnormal location and the hypoplasticity of the caval veins indicate an early role for Slit–Robo signaling in their formation, either by defective angioblast migration or by assembly. The initial caval veins are formed by angioblasts migrating from the somites, which become ensheathed by smooth muscle cells only much later in development.29,30 Slit–Robo is expressed in these somites11 and shown to be involved both in angioblast migration and in lumen formation.13,33 Furthermore, the presence of Robo4, which is required for angiogenesis39 during early caval vein development, can explain the discrepancy in phenotype in the absence of Slit3 in comparison with the Robo1−/−;Robo2−/− mutants. Furthermore, our data suggest a link between the size of the caval vein connection to the heart and the size of the sinus horns.

Like the sinus horn myocardium, the sinus node myocardium forms by differentiation of the surrounding mesenchyme.33 However, the current hypothesis is that the sinus node forms by
a different mechanism than the sinus horn myocardium, in line with its distinct gene program and normal sinus node formation in the Wt1 and Raldh2 mutants. Our results confirm this hypothesis, as loss of Slit3 or Robo1 and Robo2 does not affect the formation of the sinus node, in contrast to the sinus horns. Knowledge of the molecular mechanisms underlying the development of the systemic venous return and pericardium is steadily increasing, and here we found a new signaling pathway that takes part in the formation of this area. The Slit–Robo signaling pathway exerts its function through regulation of a diverse array of processes and, although we shed light on the molecular mechanism underlying the pericardial defects, the precise mechanisms involved in the systemic venous return defects still need to be unraveled. Furthermore, the extensive expression patterns of the Slit–Robo signaling pathway genes in and around the heart point to their likely involvement in other aspects of mammalian heart development.

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Disclosures

None.

References


Novelty and Significance

What Is Known?
• Slit–Roundabout (Robo) signaling is important for Drosophila heart development, but its role in the mammalian heart is largely unknown.
• Congenital malformations of the cardiac venous pole and pericardial defects often present together and share a common pathogenesis.

What New Information Does This Article Contribute?
• Defects in Slit–Robo signaling result in pericardial defects, misalignment of the caval veins, and sinus horn defects.
• Local increase in neural crest cell death and impaired neural crest adhesive and migratory properties underlie the absence of the pericardium.
• There is no cross-regulation between Slit–Robo signaling and known regulators of systemic venous return and pericardium development.

Whereas a whole range of genes are known to underlie congenital defects of the arterial pole of the heart, knowledge about the genetic and cellular mechanisms underlying defects of the venous pole is still limited. Congenital defects of the systemic venous return to the heart often only become symptomatic after childhood. However, these defects can be severe, ranging from congenital malformations, such as misalignment of the connecting veins, to atrial arrhythmias like sick sinus syndrome. Here, we report a novel role for Slit–Robo signaling in the development of this region. We show that the absence of components in Slit–Robo signaling results in misalignment of the caval veins and hypoplastic sinus horn myocardium. Furthermore, defective Slit–Robo signaling results in partial absence of the pericardium. These pericardial defects are caused by increased apoptosis owing to reduced adhesion and migration of the cardiac neural crest. This study is the first to show defects caused by the Slit–Robo signaling pathway in the mammalian heart, implicating a role for the cardiac neural crest in pericardial defects and further elucidating the pathogenesis of congenital defects in this complex region.
Slit–Roundabout Signaling Regulates the Development of the Cardiac Systemic Venous Return and Pericardium
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Detailed Methods

Transgenic mice
All experimental procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and institutional guidelines. Robo1<sup>+/−</sup>, Robo2<sup>+/−</sup> (obtained from William Andrews, UCL, London) Robo1<sup>+/−</sup>;Robo2<sup>+/−</sup>, Slit1<sup>+/−</sup>, Slit2<sup>+/−</sup>, Slit3<sup>−/−</sup> (obtained from Alain Chedotal, Institut de la Vision, Paris, France) Wnt1<sup>Cre</sup>+, R26R<sup>EYFP</sup> (Jackson Lab), Wt1<sup>−/−</sup> and Tbx18<sup>GFP</sup> (obtained from Andreas Kispert, Institut für Molekularbiologie, Hannover, Germany) mice were described previously. Robo1<sup>+/−</sup>, Robo2<sup>+/−</sup>, Slit1<sup>+/−</sup>, Slit2<sup>+/−</sup>, Wnt1<sup>Cre</sup> and R26R<sup>EYFP</sup> mice were maintained on a C57/bl6J background, and back crossed >10 crossings. Robo1<sup>+/−</sup>;Robo2<sup>+/−</sup> and Slit3<sup>−/−</sup> mice were maintained on a mixed C57/bl6J;C3H background (6 back crossings with C57/bl6J) and Wt1<sup>+/−</sup> and Tbx18<sup>GFP</sup> mice were kept on an outbred (NMRI) background. The day the vaginal plug was found was considered as embryonic day (E) 0.5.

In situ hybridization and immunohistochemistry
Probes and methodology of the non-radioactive in situ hybridization<sup>10</sup> and immunohistochemistry<sup>11</sup> were described previously. Embryos were fixed overnight in 4% paraformaldehyde, embedded in paraffin and transversally sectioned at 7-10 μm for immunohistochemistry or 12 μm for in situ hybridization. The following primary antibodies were used: goat polyclonal anti-cardiac Troponin I (cTnl, 1:1000; Hytest Ltd), chicken polyclonal anti-GFP (1:250; Aves), rabbit polyclonal anti-cleaved caspase-3 (CC3, 1:250; Cell Signaling Technology), goat polyclonal anti-Robo1 (1:250; R&D systems) and rabbit polyclonal anti-Robo2<sup>12</sup> (1:250; gift from J.F. Cloutier). Fluorescent secondary antibodies used were Alexa 568 donkey anti-goat, 488 and 568 goat anti-rabbit, 488 goat anti-mouse, and goat anti-chicken (1:250; Molecular Probes). Nuclei were counterstained with Dapi (2.5μg/ml;Sigma).

Apoptosis cell count
Every third 10 μm transverse section was mounted from cranial to the pericardial cavity to caudal of the outflow tract. 16 sections from each embryo were included in the counts. Apoptotic cells were labelled by immunohistochemistry for Cleaved Caspase 3 (CC3). As secondary antibody Alexa 568 donkey anti-rabbit was used. All apoptotic cells in the mesenchyme dorsal of the pericardial cavity and medial and ventral of the caval veins were counted using Metamorph imaging software (Universal Imaging Corporation). As CC3 also non-specifically stained blood cells, sections were counterstained with a 488 goat anti-mouse monoclonal secondary antibody and any co-labelled cells were exclude from the counts. For all experiments n≥3.

Three-dimensional reconstructions and volume quantification
Three-dimensional visualization, geometry reconstruction and volume measurements of protein expression patterns determined by immunohistochemistry were carried out as described previously using Amira 5.4.1 (Visage Imaging).<sup>13</sup> All reconstructions and volume measurements are based on cTnl expression and tissue morphology. Files with reconstructions are available upon request.

Section ligand binding assay
Isolated Robo1<sup>+/−</sup>, Robo1<sup>+/−</sup>, Robo1<sup>+/−</sup>;Robo2<sup>+/−</sup> and Robo1<sup>−/−</sup>;Robo2<sup>−/−</sup> embryos were cryo-protected through a 10-20-30% sucrose gradient (one hour each step) and directly embedded and frozen in Tissue-Tek OCT (Sakura Finetek). 20 μm sections were kept
frozen until 5 minute fixation in cold 100% Methanol. The sections were washed in PBS, blocked with Opti-mem (Gibco) and incubated overnight with recombinant His-tagged mouse Slit2 or Slit3 protein (R&D systems) in Opti-mem (5µg/ml). After washing in PBS the sections were post-fixed for 10 minutes with 4% paraformaldehyde, treated for 45 minutes with 0.3% H$_2$O$_2$ in PBS, incubated for 1 hour with biotinylated mouse monoclonal anti-polyHistidine (R&D systems) and then further processed using the TSA enhancement kit (Perkin Elmer). Control sections were processed identically, but without Slits in the Opti-mem. For double staining procedure, sections were subsequently blocked for peroxide activity using 1% Sodium Azide/0.3% H$_2$O$_2$/PBS and processed using the TSA enhancement kit (for Robo1) or Alexa 488 goat anti-rabbit (for Robo2). Nuclei were counterstained with Dapi (Sigma).

Neural crest explant cultures
Robo1 embryos were collected at E8.5-9, and the yolk sac from each embryo was harvested for genotyping by PCR analysis. The method used has been described previously. The neural tube was roughly isolated from most surrounding tissue before treatment with 0.5 mg/ml of collagenase/dispace (Sigma). The parts of the neural tube containing the cardiac neural crest region (from the otic sulcus to the fourth somite) were cleaned from surrounding mesenchyme and ectoderm. Isolated neural tube parts were washed in PBS and transferred to 15mm wells containing fibronectin coated coverslips and Dulbecco’s modified Eagle’s medium (DMEM) Glutamix (Gibco) with 10% fetal bovine serum and Penicillin/Streptomycin. To obtain Slit2 and Slit3 conditioned medium, COS cells, transfected with Slit2, Slit3 and GFP constructs, were cultured for 3 days in Dulbecco’s modified Eagle’s medium (DMEM) with Glutamix (Gibco), 10% fetal bovine serum and Penicillin/Streptomycin, after which the medium was harvested and filtered. For Slit2, Slit3 and GFP conditioned medium experiments, 1 part DMEM with Glutamix,10% fetal bovine serum and Penicillin/Streptomycin to 1 part of conditioned medium was used. The explants were cultured at 37°C with 5% CO$_2$ for 44h, after which they were fixed for 15 minutes with 4% paraformaldehyde and processed for apoptosis analysis using CC3 (1:1000) and Alexa 568 goat anti-rabbit (1:500) antibodies. Nuclei were counterstained with DAPI. Metamorph imaging software (Universal Imaging Corporation) was used for apoptosis counts, whereas migration analysis was done using Image J (NIH image software). To calculate the outgrowth area, the area of the dense central neural tube tissue was subtracted from the total area. To control for differences in migration area resulting from random variations in the shape of the explant, the outgrowth area (mm$^2$) was divided by the perimeter (mm) of the explant to calculate the migration index, n≥6 per condition.

Neural crest cell adhesion assay
Cardiac neural crest cells were isolated from Robo1$^{+/+}$ and Robo1$^{-/-}$ 44-hour explant cultures cultured in DMEM with Glutamix, 10% fetal bovine serum and Penicillin/Streptomycin. The central neural tube tissue was removed before cells were washed and harvested after treatment for 3 minutes at 37°C with 0.25% Trypsin-EDTA (Gibco). The cells were resuspended in DMEM with Glutamix containing 10% FBS. A sample of the cell suspension containing about 3,000 cells was deposited on 0.7 cm$^2$ wells culture slides (BD Falcon) previously coated with 15g/ml fibronectin and blocked for 1 hour with 1% BSA. The slides were incubated at 37°C for 30 minutes, washed with PBS and fixed with 4% paraformaldehyde for 15 minutes. Attached cells were visualised with DAPI. Per well 2 pictures were taken (5x magnification) and attached cells were counted.

Statistics
Results are expressed as mean ± SEM. Statistical significance was tested with two-tailed, unpaired, unequal variance student’s t-test. (* for P < 0.05, ** for P< 0.01 and *** for P< 0.001).
References


Normal Robo2 expression in absence of Robo1

A-E, *in situ* hybridization showing myocardial cTnl expression in Robo1<sup>+/+</sup>;Robo2<sup>+/+</sup> (A-B), Robo1<sup>-/-</sup>;Robo2<sup>-/-</sup> (C-E) embryos. A-E, the Robo1<sup>+/+</sup>;Robo2<sup>+/+</sup> left sinus horn is myocardial (A) and enters the right atrium (B). The Robo1<sup>-/-</sup>;Robo2<sup>-/-</sup> sinus horn has few myocardial cells (C) and either connects to the right atrium (D) or the coronary circulation (red arrowhead, E). F, *in situ* hybridization and immunohistochemistry for Robo2 on Robo1<sup>+/+</sup> and Robo<sup>-/-</sup> embryos. L/RA, left/right atrium; LSH, left sinus horn; LV, left ventricle; L/RSCV, left/right superior caval vein. Scale bars depict 100μm.
Absence of *Slit1* and/or *Slit2* does not cause pericardium, caval vein alignment or sinus horn defects

A-B, in situ hybridization for Robo1, Robo2, Slit2 and Slit3 at E12.5 (A) and E14.5 (B). Black arrowheads indicate that the sinus horns are negative for all Slit-Robo genes except Slit3. C-F, in situ hybridization showing myocardial cTnI expression in Slit2+/+ (C), Slit2-/- (D), Slit1+/+;Slit2+/+ (E) and Slit1-/-;Slit2-/- (F) embryos. No defects are observed in absence of Slit1 and/or Slit2. PPM, pleuropericardial membrane. L/R, left/right; LA, left atrium; RA, right atrium; L/RV, left/right ventricle; PV, pulmonary vein. Scale bars depict 100μm.
Online Figure III

Strongly reduced Slit binding just cranial of the pericardial cavity in Robo mutants

A, immunohistochemistry for Robo1 on Robo1+/+ and Robo2 on Robo1−/− embryos. A-B, the line though the E12.5 three-dimensional reconstruction indicates the level of section ligand binding assay on Robo1+/+, Robo1−/− and Robo1−/−;Robo2−/− embryos. Robo1 expression is particularly high immediately cranial of the pericardial cavity. Most Slit3 binding in this area is abolished in the Robo1−/−, whereas Slit2 binding is slightly reduced and still present in the Robo2 expressing areas. All binding is abolished in the double mutants. B-C, white arrowheads point to the Robo2 expressing trachea and oesophagus binding Slit2 and 3 in the wild-type and Slit2 in the Robo1−/−, showing that remaining Slit2 binding is to the Robo2 receptor in absence of Robo1. CPW, cranial pericardial wall; FG, foregut; Tr, trachea. For other abbreviations, see the legend of Figure 1. Scale bars depict 100μm.
Abnormal localization of the caval veins and ectopic coelom formation in Robo1<sup>−/−</sup> embryos

A, the line though the E12.5 three-dimensional reconstruction (lateral view) indicates the level of in situ hybridization sections stained for Raldh2. Black arrowhead indicates the most cranial area of pleural cavity formation, which is much more cranial in the mutant. Clear arrowheads point out the further location of the mutant caval veins into the mutant body. Red arrowhead indicates the location of the wild-type sinus horn inside the pericardial cavity, and the mutant sinus horn still mostly inside the body mesenchyme B, quantification of CC3-positive cells shows comparable CC3-positive cell numbers in Slit3<sup>−/−</sup> and control littermates. L/RA, left/right atrium; LSH, left sinus horn; LL, left lung; L/RSCV, left/right superior caval vein. Scale bars depict 100μm.
Cardiac neural crest cells express Robo1 and Slit3

A, confocal imaging of a Robo1<sup>−/−</sup>;Wnt1<sup>Cre</sup>;R26R<sup>EYFP</sup> immunohistochemistry section stained for YFP and CC3. Most apoptotic cells express YFP. B, double in situ hybridization/immunohistochemistry for YFP and the indicated genes. Magnification of the region between the right caval vein and pericardium shown in Figure 7A. RSCV, right superior caval vein; FG, foregut; OFT, outflow tract. Scale bars depict 100μm.
Slit2 and Slit3 expression is highest immediately dorsal to the heart
A, *in situ* hybridization for YFP, Slit2 and Slit3 on a Wnt1\textsuperscript{Cre};R26R\textsuperscript{EYFP} embryo. Black arrowheads indicate higher levels of Slit3 in the neural crest and Slit2 surrounding it immediately dorsal to the heart compared to the pharyngeal arches. PA, pharyngeal arch; OFT, outflow tract. Scale bars depict 100μm.