Slit–Robo signalling in heart development

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Abstract

The Slit ligands and their Robo receptors are well-known for their roles during axon guidance in the central nervous system but are still relatively unknown in the cardiac field. However, data from different animal models suggest a broad involvement of the pathway in many aspects of heart development, from cardiac cell migration and alignment, lumen formation, chamber formation, to the formation of the ventricular septum, semilunar and atrioventricular valves, caval veins, and pericardium. Absence of one or more of the genes in the pathway results in defects ranging from bicuspid aortic valves to ventricular septal defects and abnormal venous connections to the heart. Congenital heart defects are the most common congenital malformations found in life new-born babies and progress in methods for large scale human genetic testing has significantly enhanced the identification of new causative genes involved in human congenital heart disease. Recently, loss of function variants in ROBO1 have also been linked to ventricular septal defects and tetralogy of Fallot in patients. Here, we will give an overview of the role of the Slit–Robo signalling pathway in Drosophila, zebrafish, and mouse heart development. The extent of these data warrant further attention on the SLIT–ROBO signalling pathway as a candidate for an array of human congenital heart defects.

Keywords

Congenital heart defects • Heart development • Slit–Robo signalling • Ventricular septal defects • Bicuspid aortic valves

1. Congenital heart disease and the role of the Slit–Robo signalling pathway

Congenital heart disease affects ~1% of all live births and includes a wide range of conditions such as ventricular septal defects, atrial septal defects, bicuspid aortic valves, and tetralogy of Fallot. Advances in cardiac developmental biology have significantly improved our understanding of the signalling pathways and transcriptional networks underlying heart development. Additionally, improved human genetic testing on a large scale using SNP arrays and whole exome sequencing has opened up the possibility of searching for new causative genes, while targeted resequencing using SNP arrays and whole exome sequencing has opened up the possibility of searching for new causative genes, while targeted resequencing

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2. The Slit–Robo signalling pathway: structure and interactions of the ligands and receptors

Slit proteins are large secreted extracellular molecules, which were initially discovered in a genetic screen in Drosophila melanogaster and subsequently found to be midline axon repellents during the development of the central nervous system. During development, most axons cross over the midline to innervate the contralateral side of the body. Slit was found to be expressed at the midline and a mutation in the gene was shown to cause axons to enter but never to leave the midline, indicating Slit acts as a midline repellent. The receptor for Slit was identified as the single-pass transmembrane roundabout (Robo) receptor. The first robo gene was named after the phenotype of Drosophila mutants in which axons were observed to inappropriately cross and re-cross the midline, resembling the circular traffic junction (ROUndaBOut). The growth cones of the axons that will cross the midline to the contralateral side initially do not express the Robo receptor and, therefore, are able to cross the Slit-expressing midline. As soon as the axons have crossed to the contralateral side, their growth cones start to express Robo, preventing them from re-crossing.

A single slit and three robo genes have been identified in Drosophila. In mammals, there are three Slit genes (Slit-1, -2, -3), all of which are expressed in the nervous system as well as in a broad range of other organs. All Slits have a similar protein structure: four stretches of leucine-rich repeat (LRR) domains; seven to nine epidermal growth factor (EGF) repeats; an Agrin-Perlecain-Laminin-Slit/Laminin-G-like domain; and a C-terminal cysteine knot (Figure 1A). Slits are able to homodimerize through their fourth LRR domain, as well as able to bind to other extracellular matrix molecules, such as Netrin1, Glypican, Syndecan, Type IV Collagen, and Dystroglycan. Furthermore, Slits are able to bind to Dscam1 (down syndrome cell adhesion Molecule 1) and the Eva1C receptor. Slit2 protein can be cleaved into a long N-terminal and a short C-terminal fragment at the proteolytic site between the fifth and sixth EGF domain. The N-terminal fragment stays associated with the cell surface and, like full-length Slit, binds Robo to induce chemorepulsion, whereas the C-terminal fragment enters the extracellular space and binds the basement membrane scaffolding protein Dystroglycan and the Plexin A1 receptor. In most vertebrates, there are three Robo receptors Robo1 (Dutt1), Robo2, and Robo3 (Rig1). However, in zebrafish and mammals, a fourth Robo receptor was discovered, Robo4, also known as Magic Roundabout. The Robo receptors contain five immunoglobulin-like domains (two in Robo4), three fibronectin repeats (two in Robo4), a transmembrane domain as well as four conserved cytoplasmic domains (Figure 1A). The cytoplasmic domains do not have autocatalytic or enzymatic activity but interact with downstream signalling molecules. The receptors can be alternatively spliced and undergo ectodomain shedding by Adam proteases, a process required for recruiting intracellular signalling molecules. Further cleavage by γ-secretase results in a C-terminal fragment that translocates to the nucleus, although function of this fragment is still unknown. Robos bind to the concave face of the Slit LRR2 domains through their Ig1 domain. The interaction between Slits and Robos can be stabilized by complex formation with heparan sulfate proteoglycans (HSPGs) (Figure 1A).

In Drosophila, Robo was found to be able to bind directly, in trans, to Robo2 on neighbouring cells, inhibiting Slit-mediated repulsion. Whereas in mammals, Robo1 and Robo2 have been identified to function as (cis) homo and heterodimers. The Robo receptors have been shown to interact with a number of other membrane receptors, including Netrin receptor deleted in colorectal carcinoma (DCC) and Cxcr4. Upon binding of Slit ligand, signalling through the Robo receptor can be propagated intracellular to regulate axon guidance, cytoskeletal dynamics, cell adhesion, angiogenesis, and proliferation (Figure 1B–F).

Although Slits and Robos were initially characterized as repulsive guidance cues for neuronal axons, further studies using different animal models have since found important roles for the pathway during many aspects of heart development, from lumen formation to valve development, which will be discussed in detail.

3. Slit–Robo signalling is required for heart cell migration, alignment, and lumen formation in Drosophila

The fruit fly, D. melanogaster, has an open circulatory system, with a simple linear tube-like heart that pumps the haemolymph from posterior in the body towards the anterior region. Although this anatomy is very different from the adult vertebrate heart, the tube-like heart resembles the initial heart tube of the developing vertebrate heart. Seventy-five percentage of all human disease genes have homologues in Drosophila and several genes causing human congenital heart disease have similar roles during Drosophila heart development. The cardiac progenitors originate from mesodermal cells that undergo a mesenchymo-to-epithelial transition and migrate towards the midline as two bilateral sheets of cells (Figure 2A and B). Both sheets are composed of an inner row of contractile cardioblasts and an outer row of pericardial cells. The two sheets meet each other at the midline, where the cardioblasts make adherens junctions and start forming a lumen that enlarges during the late stages of embryogenesis (Figure 2E and F).

The earliest expression of slit mRNA is observed in the lateral mesoderm. As soon as the cells are migrating towards the midline, both Slit and Robo (the homologue of vertebrate Robo1) protein are uniformly expressed on the cardioblasts. By the time the rows fuse, Slit protein has shifted location to uniquely localize on the apical side of the cardioblasts, where the ligand accumulates between the two rows of cells (Figure 2C). Robo now mainly concentrates on the apical surface of the cardioblasts, whereas robob seems to remain limited to the surface of pericardial cells. Although cardioblast mRNA expression has been reported, absence of slit, robob, or myocardial overexpression of robo2 results in delayed cardioblast and pericardial cell migration. Furthermore, absence of slit, both robob and robob, or overexpression of robob in mesodermal cells causes the cardioblasts and pericardial cells to fail to align properly in the midline. The normally highly regular row of cardioblasts is already perturbed before they reach the midline, resulting in gaps between the cells and cardioblasts to become inappropriately interspersed within the pericardial cells (Figure 2D). Slit and Robo expression specifically within cardioblasts is sufficient to rescue the slit and robob/robob knock out phenotype. These results indicate the importance of Slit–Robo signalling for heart cell migration and adhesion.

After the two rows of cardioblasts have aligned in the midline, lumen formation starts. Contralateral pairs of cardioblasts make specific dorsal and ventral contacts between their opposing apical sides to form the lumen. During this process, both Slit and Robo become localized on the part of the cardioblast membrane that will form the lumen (Figure 2G).
Figure 1 Schematic representation of the Slit ligands and Robo receptors.19,21,22,32,35–44 A, Slit binds to the Robo Ig1 domain with their LRR D2 domain. The Robo4 receptor only has two immunoglobulin-like domains and two fibronectin repeats. Interaction between Slits and Robos can be stabilized by HSPGs. B–E, Examples of interactions between Robo and other membrane receptors, as well as downstream signalling cascades that are known to be involved in heart development or that have been identified in other organ systems and might also play a role during heart formation. B, The Robo1 intracellular domain interacts with the intracellular domain of the Dcc receptor, inhibiting Netrin-induced cellular attraction.48 C, Robo1/2, together with Vegfr2, promote endothelial cell polarity during sprouting angiogenesis.54 Robo1 also promotes auto-phosphorylation of Vegfr2 to regulate endocardial migration.53 D, Tgfbr2 interacts with Robo1 to elevate Robo1 expression, resulting in inhibition of transcription of Wnt-responsive genes.52 E, Signalling through Robo can be propagated intracellular to GTPases of the Rho family such as RhoA, Rac1, and Cdc42, which are small guanosine-5′-triphosphate (GTP)-binding proteins that regulate cytoskeletal dynamics to control cell motion.55 The adaptor protein Dreadlocks (Dock) links Robo to the GTPases in both Drosophila and mammals.56,57 E, Cytoplasmic kinase Abelson (Abl) phosphates the Robo CC1 domain to antagonize Robo activity.65 Additionally, Abl and its substrate cables link Robo to N-cadherin, which leads to the detachment of β-catenin from N-cadherin. Both inhibit cell adhesion.92
Figure 2 Schematic representation of the role of Slit–Robo signalling during Drosophila heart development based on62–67. A, 14 h post-fertilization Drosophila, during migration of the two rows of cardioblasts (red) and pericardial cells (yellow) to the midline. Main cardioblasts are depicted slightly larger than the ostia cardioblasts. B, 16 h post-fertilization Drosophila, during cardioblast and pericardial cell alignment at the midline. C, Expression of Slit, Robo, and Robo2 during these stages. D, The wild-type phenotype compared with the different mutant phenotypes during migration and alignment. E, 17 h post-fertilization Drosophila, during cardiac lumen formation. Dotted line indicates the line of sectioning to obtain the transversal view through the two contralateral cardioblasts as shown under Number 5. F, The process of lumen formation. The two cardioblasts first join on their dorsal sides, followed by the ventral side. The middle section of the membrane never meets, resulting in the lumen being formed. G, Slit and Robo expression becomes restricted to the luminal side, whereas cell adhesion molecules become localized where the cardioblasts join on their dorsal and ventral side. H, Phenotypes as observed in mutants for Slit or Robo.
During normal development, cell adhesion proteins, such as E-cadherin are specifically located at the dorsal and ventral side of the membrane of the cardioblast, exactly where the two contralateral cardioblasts make cell contact (figure 2G), resulting in strong adhesion between the two cells. However, the presence of Slit and Robo on the medial part of the cell membrane and in between the cells, ensures that the cell membranes of the two contralateral cells are repulsed and a lumen is formed. Embryos overexpressing slit show ectopic lumen formation, whereas robo overexpression causes a larger lumen. In mutant embryos lacking either slit or both robo and robo2, the cardioblasts do not become triangular but remain rounded. As a result, the contralateral cells come into contact with each other along most of their apposing surfaces, blocking lumen formation (figure 2H). These results indicate the important role of Slit–Robo mediated local repulsion in creating the lumen of the heart. Additionally, Slit–Robo signalling is important for outflow tract formation of the Drosophila heart. In slit and double robo/robo2 mutant embryos, heart-anchoring cell (cells that share similarities to cardiac neural crest cells in vertebrates) migration is delayed or disrupted, and cardiac outflow tract muscles do not attach to the tip of the heart.

4. Cardiac progenitor migration and lumen formation are also disturbed in slit or robo knock-down zebrafish

More evidence on the function of the Slit–Robo signalling pathway during heart development has come from zebrafish. In the zebrafish, after bilateral cardiac progenitor formation, endocardial cells start migrating towards the midline, slightly later followed by myocardial cells. When the bilateral endocardial cells and myocardial cells fuse at the midline, a cardiac disc is formed, which subsequently is transformed into a linear heart tube. The atrium and ventricle start to balloon out from the heart tube, to form the single atrium and ventricle of the adult fish heart. During cardiac progenitor cell migration (19 h post-fertilization), slit2 is mainly expressed in endocardial cells, whereas slit3 and robo1 are observed more broadly in the myocardial and endocardial/endothelial cells. Robo4 expression is sparsely detected in the endocardium, while robo2 levels are very low in all these tissues (figure 3A). Both slit2 and slit3 are still strongly expressed in the heart around the time of chamber formation.

**Figure 3** Expression patterns of the Slit and Robo genes during zebrafish and mouse heart development. A, Expression of the Slit and Robo genes in the endocardium and myocardium at 19 h post-fertilization in zebrafish. B, Table of expression of the Slit and Robo genes in the different tissues at E9.5 in the mouse. C, Expression of the Slit and Robo genes at E12.5 in the mouse. The left section focuses on the outflow tract region, while the right section shows the atrioventricular region. Ao, aorta; PT, pulmonary trunk; RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle.
Using morpholinos, the different ligands and receptors were knocked down, revealing a similar requirement for the pathway in controlling cardiac cell migration and lumen formation as found in Drosophila. Slit2 knock-down embryos show normal myocardial cell migration towards the midline. However, endocardial cell migration is disrupted; individual cells migrate faster with loss of directionality. Additionally, collective cell migration is disturbed, as individual migrating endocardial cells do not contact neighbouring cells and they extend numerous filopodia in multiple directions. Slit2 morphant hearts develop multiple lumens, whereas in slit2 gain-of-function embryos, endocardial cells do not form a disc but are located more diffusely, less densely packed at the midline. Endocardial cells also appear larger and less rounded than in control embryos. In contrast to slit2 knock-down, rob1 knock-down inhibits both endocardial and myocardial cell migration, resulting in unfused heart fields. Interestingly, also rob1 gain-of-function results in incomplete heart field fusion. Endocardial cells in rob1 morphants exhibit a rounded, non-migratory morphology. Migration of the heart fields to the midline is not delayed in rob4 morphants, however, endocardial cell morphology and directionality are affected. At 48 hpf, fish with knockdown of slit2, rob1, and rob4 show pericardial edema and circulation defects, without clear vascular patterning defects. The role of Slit3 seems more confined to the vasculature, as slit3 morphants display highly penetrant vascular defects, including missing or detached intersomitic vessels and/or dorsal longitudinal anastomotic vessels. Slit3 morphants do not show pericardial edema at 48 h post-fertilization but have not yet been analysed at earlier stages. In agreement with the very low levels of robo2, robo2 homozygous knockout fish do not show defects in the cardiovascular system. These data suggest that Slit2, Robo1, and Robo4 are important for heart development and function in zebrafish, whereas Slit3 is mainly essential for vascular development. Intriguingly, both slit2 and slit3 have a miRNA encoded within an intron, mir218-1, and mir218-2, respectively. This miRNA is very well-conserved across species, from human and mouse to zebrafish and Xenopus, and has been shown to regulate Tbx5 expression in the heart. mir-218 is able to repress expression of both robo1 and robo2 miRNAs. mir-218 knock-down in zebrafish results in a phenotype similar to rob1 overexpression, suggesting functional regulation of Slit-Robo signalling by mir-218. Additionally, cross-talk was found between Robo1, Vegfa, and the Vegfr2 receptor to control heart field migration, indicating a Slit/miR-218/Vegfr feedback regulatory loop regulating heart field migration.

5. Disruption of Slit–Robo signalling in the mammalian heart affects multiple processes during heart development

Just as in Drosophila and zebrafish, formation of the mammalian heart starts by the migration of bilateral strips of cardiac progenitor cells to the midline, which happens around embryonic day (E) 7.5 in the mouse. The bilateral strips meet in the midline, where they fuse to form the heart tube. Cells are added to both poles of the heart to elongate the heart tube. Endocardial cells undergo endocardial-to-mesenchymal transformation to form the outflow tract and atriocaventricular cushions. Subsequent expansion of the chambers, atrial, and ventricular septation and remodelling of the cardiac cushions into the valves and membranous septa will result in the septated four-chambered heart.

Three Slit and the Robo1, Robo2, and Robo4 genes display very distinct spatial and temporal expression patterns during mouse heart development. Robo3 expression is mainly confined to the central nervous system and has not been detected in the heart at any stage during murine development, while Robo4 is selectively expressed by coronary vessel, caval vein, aorta, and pulmonary trunk endothelial cells rather than endocardium. Slit1 expression is not observed at any stage in the developing heart after E9.5, although till E9.5 expression in the atria has been reported. Slit2 and Slit3 on the other hand show distinct expression patterns in the heart all throughout development. From E8.5 to E9.5, Slit2 is not observed in the myocardium but is strongly expressed in the pharyngeal region which is essential for the pharyngeal arch artery formation. Around E9.5–E10.5, strongest Slit2 expression is detected in the ventricular trabecular myocardium but the ligand is also observed in parts of the second heart field, and the endocardium lining the outflow tract and atriocaventricular cushions. Later, Slit2 also becomes expressed in the epicardium, the aortic semilunar valves, and the mesenchyme surrounding the cavitary veins (Figure 3B). Of the Slit genes, Slit3 is the earliest to be expressed in the developing heart, with detection at E7.5 in the cardiac crescent. At E8.5, Slit3 is expressed in the ventral wall of the heart tube. By E9.5–E10.5, Slit3 starts to show a similar expression pattern in the trabecular region of the ventricles to Slit2, albeit with much lower expression levels. In contrast to Slit2, Slit3 is highly expressed in the myocardium, with presence in the outflow tract, atrial, and sinus horn myocardium including the sinus node. Furthermore, it is expressed in the cardiac neural crest, the second heart field, the tissues connecting the heart to the body, and later, the epicardium (Figure 3B). Slit3 has been reported to still be expressed in the adult ventricle.

Of the two Robo receptors expressed in the developing mouse heart, Robo1 is more broadly distributed than Robo2. Both receptors are expressed in the venous pole of the linear heart tube at E8.5. At E9.5, Robo1 expression is detected in the cardiac neural crest, second heart field, outflow tract and atriocaventricular canal cushions, the myocardium of the atriocaventricular canal and robustly in the mesenchyme surrounding the venous pole of the heart (Figure 3B). When the cushions start maturing into the valves, Robo1 expression is maintained in the atriocaventricular and pulmonary semilunar valves, however, disappears from the aortic semilunar valves just before birth. Furthermore, Robo1 remains strongly expressed in the atriocaventricular canal myocardium, which can now be recognized as the ventricular conduction system, including the atriocaventricular node, the His bundle, and bundle branches. At E9.5–E10.5, Robo2 expression is seen in a limited number of cardiac neural crest cells, and strongly in both the outflow tract and atriocaventricular cushions (Figure 3B). Robo2 expression has been reported to be expressed in the atria at E10.5 but was later not detected in the myocardium at any stage during heart development. Robo2 is still highly expressed in the pulmonary and aortic semilunar valves just before birth. These specific and partially overlapping expression patterns of the Slit and Robo genes in the developing heart indicate a large array of functions during heart development.

5.1 Slit–Robo signalling regulates cardiac neural crest and second heart field contribution to the heart

The initially formed primary heart tube is derived from progenitors called the ‘first heart field’, which will eventually form the left ventricle of the adult heart. After primary heart tube formation, cells are added to both the arterial and venous poles of the heart tube from a second heart
field of progenitors. The second heart field, together with the neighbouring cardiac neural crest, are the main sources of cells contributing to the heart after primary heart tube formation. The second heart field contributes most of the cells to the right ventricle and atria, while contribution of the cardiac neural crest cells is important for septation of the aorta and pulmonary trunk as well as for proper development of the membranous ventricular septum. In the formation of the arterial pole of the heart, there is intricate signalling between the cardiac neural crest, the second heart field, and the neighbouring pharyngeal endoderm and ectoderm. Defects in this interplay often result in congenital heart disease. Intriguingly, both the cardiac neural crest and the second heart field cell populations express low levels of Robo2 but high levels of Slit3 and Robo1. Slit2 is highly expressed in the surrounding pharyngeal endoderm and ectoderm, where it overlaps with Slit3 expression. The components of the Slit–Robo pathway have been found to be regulated by a number of transcription factors in this region. Haploinsufficiency of TBX1 causes DiGeorge Syndrome, which includes aortic arch patterning defects, conotruncal heart defects, and malformations of the thymus gland, parathyroid gland, and craniofacial structures. Homeobox-containing transcription factor Gbx2 acts downstream of T-box transcription factor Tbx1 to navigate cardiac neural crest cell migration. In both Tbx1-mutant and Gbx2-mutant, Slit2 expression is diminished in the pharyngeal endoderm, whereas the number of Robo1-positive cardiac neural crest cells is reduced. These results suggest that the Robo1-expressing neural crest cells require Slit2 signalling from the surrounding tissues for normal development. Interestingly, both Tbx1 and Gbx2 mutants show abnormally organized endothelial cells, possibly linking back to the defects observed in Drosophila and zebrafish. As in Drosophila and zebrafish, in mouse, Robo1 and Robo4 have found to be involved in endothelial cell filopodia formation and cell motility. These data indicate a role for the Slit–Robo pathway in the developmental processes regulated by Tbx1 and Gbx2, suggesting its involvement downstream of TBX1 and GBX2 in DiGeorge syndrome.

5.2 Ventricular septal defects in absence of Slit–Robo signalling

The membranous ventricular septum closes the communication between the right and left ventricles by fusion of the outflow tract cushions with the atrioventricular cushions and is normally completely closed in the mouse at E14.5. Loss of Robo1 or both Robo1 and Robo2 results in membranous ventricular septum defects at birth, a defect also found in Slit3, but not in Slit2 mutants (Figure 4A and B). Mice with an ENU-induced mutation in Robo1 furthermore show double outlet right ventricle with membranous ventricular septal defects, muscular ventricular septal defects, and atrioventricular septal defects. Why the mice with ENU-induced Robo1 mutation show a more severe phenotype than the full Robo1 or Robo1/2 mutants is not yet understood. The cause of the membranous septal defect in the Slit and Robo mutant mice is still not completely clear. Membranous ventricular septal defects can be caused by reduced contribution of cardiac neural crest or second heart field cells to the outflow tract or defects in endothelial-to-mesenchymal transformation and maturation of the cardiac cushions. All these processes may play functional roles in both Slit and Robo mutant mice, with first, decreased outflow tract cushion closure in the part of the cushions that is neural crest derived. Second, there is delayed maturation of the cardiac cushions. Third, the strong expression of Robo1 as well as Slit2 and 3 in the second heart field also suggests a role in the second heart field, although this will still need to be further examined. The aorta and pulmonary trunk are normally separated in all mutants. However, in both the Robo1 and double Robo mutant, the outflow tract vessels are slightly less rotated than normal, with the aorta slightly more to the right of the pulmonary trunk.

5.3 Early cardiac chamber formation requires repression of Slit3 by Nkx2-5 and Tbx2

The cardiac chambers locally balloon out from the primitive heart tube. Transcription factors Gata4, Nkx2-5, Tbx2, and transcriptional activator Tbx5 are expressed throughout most of the heart tube, and interact to activate the chamber formation program. However, in the atrioventricular canal, where transcriptional repressors Tbx2 and Tbx3 are present and can bind instead of Tbx5, chamber formation is repressed. This area will largely form the cardiac conduction system. These transcription factors are some of the genes most frequently screened for mutations in congenital heart disease, causing ventricular, atrial, and atrioventricular septal defects, as well as a range of outflow tract defects. Although data on Slit2 is largely lacking, Slit3 expression seems tightly interlinked with these genes. In absence of Nkx2-5, Slit3 expression expands throughout the entire heart tube at E8.5, whereas Robo2 expression is absent. In contrast, when Tbx20 is absent, Slit3 expression completely disappears from the heart. The same phenotype is observed in hearts overexpressing Tbx2, whereas when Tbx2 is knocked out, Slit3 expression is activated in the atrioventricular canal. In vitro assays indicate that Tbx2 can directly bind to Slit3, together suggesting that Slit3 expression is restricted to the ventral wall of the E8.5 heart tube by Nkx2-5 and excluded from the atrioventricular canal by Tbx2. These data indicate a role for the Slit–Robo pathway during early heart patterning.

CDH7 mutations are linked to CHARGE syndrome, which is characterized by a specific pattern of defects, including ocular coloboma, heart malformations, atresia of the choanae, growth retardation, genital hypoplasia, and ear abnormalities. Both Slit2 and Robo2 expression is reduced in heart-specific mutants for Cdh7, suggesting possible involvement of the pathway in the development of CHARGE syndrome.

5.4 Slit and Robo mutants display a spectrum of valve malformations

The mesenchymal cushions lining the early heart tube will remodel to form the semilunar aortic and pulmonary outflow tract valves as well as the mitral and tricuspid atrioventricular valves. Robo1/Robo2 double mutants have thickened immature semilunar and atrioventricular valves as well as highly penetrant bicuspid aortic valves (Figure 4A, C, and D). Bicuspid aortic valves only have two complete leaflets, while the third leaflet is either absent or incomplete. Bicuspid aortic valves are among the most common of congenital defects, affecting around 1–2% of the population. Although Slit2 mutants have normal atrioventricular valves, these mutants do display bicuspid aortic valves, albeit with lower penetrance than in the Robo1/Robo2 double mutants. In contrast, Slit3 mutants have clearly thickened atrioventricular valves. The posterior non-coronary aortic valve is hypoplastic in Slit3 mutants but never absent. NOTCH1 is one of the few transcriptional regulators linked to bicuspid aortic valve disease in humans to date. The different genes of the Slit–Robo and Notch–Hey/Hey pathways have very similar overlapping expression patterns during heart development. As the expression of Notch- and downstream Hey and Hes genes is down-regulated in Robo1 mutants, reduced Notch signalling might
Figure 4. Heart defects found in patients and mouse mutants for the Slit and Robo genes, adapted from 5–7, 20, 90. A, Table showing the range of congenital heart defects identified in patients and mouse mutants. +, defect; −, no defect; ±, party affected; NA, not analysed. B, E14.5 wild-type and Robo1/Robo2 knockout heart showing the absence of the MVS in the knockout (arrow), which is also observed in the single Robo1 and Slit3 mutants. cTnI, cardiac troponin I, which labels the myocardium in red. Nuclear 4',6-diamidino-2-phenylindole (DAPI) staining in green. MVS, membranous ventricular septum; VS, ventricular septum. C, 3D reconstruction of the semilunar valves of a wild-type and Robo1/Robo2 mutant at E15.5. R, right valve; L, left valve; P, posterior valve. The posterior aortic valve is missing in Robo1/Robo2 as well as Slit2 knockouts. D, Thickened immature atrioventricular valves (arrow) in the Robo1/2 double mutants at E15.5 and Slit3 mutants compared with wild-type littermates. E, Partial absence of the pericardium in Robo1 and Robo1/2 mutants. Red arrow indicates the location of the pericardium. In the mutant, the lungs completely envelope the heart (black arrows). LSH, left sinus horn; RSH, right sinus horn. F, dorsal view on the heart (grey) and caval veins (red) surrounded by sinus horn myocardium (blue) or only the caval veins of an E15.5 Robo1/2 double mutant and littermate control. Black arrow indicates the persistent left ICV in the mutant, which is also found in Slit3 mutants. The mutant shows an abnormally small connection to the right atrium (red arrow). ICV, inferior caval vein; SCV, superior caval vein. Scale bars are 100 μm.
Although the caval veins develop too far dorsally in the pericardial cavity, they are relatively normal and enter the pericardial cavity more caudally, they are relatively normal and enter the pericardial cavity more caudally, they are relatively normal. In Drosophila, this leads to obstruction of blood flow through the heart. Fifty percentage of mice lacking the Robo1 receptor and 70% of mice lacking both the Robo1 and Robo2 receptor show partial absence of the pericardial, in particular the part of the membrane between the superior caval veins is consistently missing (Figure 4E). As a result, the lungs penetrate through the hole into the pericardial cavity and completely envelop the heart. This defect is not recapitulated in Slit2 and Slit3 mutants, suggesting functional redundancy of the ligands in this process. However, congenital diaphragmatic hernias have been identified in absence of Slit3, indicating that Slit3 is important for the division of the coelomic cavities. One of the patients with a loss of function variant in ROBO1 was diagnosed with congenital diaphragmatic hernia. In human, diaphragmatic hernias coexist with pericardial defects, suggesting a related developmental mechanism or involvement of the same gene pathways. Associated cardiac anomalies are seen in 30% of all pericardial defect cases, including sepal defects, patent ductus arteriosus, bicuspid aortic valves, and anomalous inferior caval veins. Although no causative genes have been identified yet in patients, the combination of defects points to the Slit3, ROBO1, and ROBO2 genes as likely candidates. Very little is known on how the pericardial defects develop and which molecular pathways are involved. In Robo1 mutants, this seems to be caused by the fact that the cranial pericardial cavity expands too far dorsally and the caval veins do not become localized normally within the cavity. The neural crest cells present dorsally of the cranial pericardial cavity show reduced adhesion and increased cell death, likely allowing the pericardial cavity to extend into the region normally occupied by neural crest cells. As a result, the lungs are forced to develop ventral to the caval veins, indirectly blocking the closure of the pericardial membranes.

5.5 Loss of Robo1 results in partial absence of the pericardium

Pericardial (pleuropericardial membrane) defects can involve the entire pericardium, or be partial, with the majority affecting the left side of the pericardium rather than the right. This congenital defect is mostly asymptomatic and is often discovered incidental. In case of partial absence, herniation of the heart through the defect can lead to obstruction of blood flow through the heart. Fifty percentage of mice lacking the Robo1 receptor and 70% of mice lacking both the Robo1 and Robo2 receptor show partial absence of the pericardial, in particular the part of the membrane between the superior caval veins is consistently missing (Figure 4E). As a result, the lungs penetrate through the hole into the pericardial cavity and completely envelop the heart. This defect is not recapitulated in Slit2 and Slit3 mutants, suggesting functional redundancy of the ligands in this process. However, congenital diaphragmatic hernias have been identified in absence of Slit3, indicating that Slit3 is important for the division of the coelomic cavities. One of the patients with a loss of function variant in ROBO1 was diagnosed with congenital diaphragmatic hernia. In human, diaphragmatic hernias coexist with pericardial defects, suggesting a related developmental mechanism or involvement of the same gene pathways. Associated cardiac anomalies are seen in 30% of all pericardial defect cases, including sepal defects, patent ductus arteriosus, bicuspid aortic valves, and anomalous inferior caval veins. Although no causative genes have been identified yet in patients, the combination of defects points to the Slit3, ROBO1, and ROBO2 genes as likely candidates. Very little is known on how the pericardial defects develop and which molecular pathways are involved. In Robo1 mutants, this seems to be caused by the fact that the cranial pericardial cavity expands too far dorsally and the caval veins do not become localized normally within the cavity. The neural crest cells present dorsally of the cranial pericardial cavity show reduced adhesion and increased cell death, likely allowing the pericardial cavity to extend into the region normally occupied by neural crest cells. As a result, the lungs are forced to develop ventral to the caval veins, indirectly blocking the closure of the pericardial membranes.

5.6 Persistent left inferior caval veins in Slit3 and Robo1 Robo2 double mutants

One of the main roles of the Slit–Robo pathway during Drosophila and zebrafish heart development is its function during cardiac lumen formation. Although this has not been investigated sufficiently, so far, similar defects in cardiac lumen formation have not yet been identified in mouse mutants for the Slit and Robo genes. However, lumen formation of the caval veins is affected by varying degrees in these mutants. Although the caval veins develop too far dorsally in Robo1 mutants, and enter the pericardial cavity more caudally, they are relatively normal and surrounded by sinus horn myocardium near the entrance to the atrium. However, additional absence of Robo2 or absence of Slit3 results in severely malformed and thin caval veins (Figure 4F). If this strongly reduced vein lumen develops through a similar mechanism as during Drosophila and zebrafish heart development is yet to be determined. The connection of the left caval vein to the right atrium is abnormally small or only connecting to the coronary circulation, with minimal sinus horn myocardium development. The sinus node, which largely forms within the sinus horn myocardium, has a slightly different morphology but has a normal molecular signature. Additionally, the double Robo and Slit3 mutants display persistent left inferior caval veins, which join the right inferior caval vein at liver level. These defects are not present in Slit2 mutants. Although the left superior caval veins always persists in mouse, it normally regresses in human to become the coronary sinus, however, a left superior caval vein was identified in a patient with a loss of function ROBO variant. Based on our knowledge from Drosophila and zebrafish, a role for the pathway in the endothelium is expected during early caval vein lumen formation. However, this has not been studied yet in detail and our knowledge on how these caval vein defects develop is limited. Endothelial cells require Slit2–Robo4 interaction for stabilizing the vasculature during angiogenesis, indicating that further research is necessary at much earlier stages of caval vein development.

6. Conclusions and future directions

The range of different defects observed between the various Slit and Robo mutants indicates the requirement of several specific combinations of ligand–receptor interactions during different stages of development and in different parts of the mammalian heart. For example, Slit3–Robo1 interaction seems most important during development of the membranous ventricular septum, while Slit2 binding to both Robo1 and Robo2 is most important for the presence of all three aortic semilunar valve leaflets. The fact that, for example, pericardial defects are not observed in any of the Slit mutants, suggests functional redundancy of these ligands. The presence of three Slit ligands and four Robo receptors in mammals might also explain the less severe phenotype observed in single Slit mouse mutants, compared with the severe early hearts defects observed in Drosophila that only has one Slit ligand, eventhough triple Slit mutants survive till at least E12.5. Although we now have an extensive overview of the role of Slit–Robo signalling during the many different aspects of heart development, our knowledge on how the different cell types interact during this process is very limited. Especially in mouse, all research so far has been performed using full constitutive knock outs, and further research using tissue-specific mutants will allow to dissect out the different source and responsive tissues in the different parts of the heart. The role of the pathway during Drosophila heart development has been studied on a more detailed cellular level and it will be important to extrapolate these findings to mouse and human. In particular, the aetiology of the caval vein defects requires studying earlier stages of vessel development. Further study of the identified range of valve defects, and in particular the already early in development recognisable bicuspid aortic valves, might help understand the aetiology of common congenital valve defects found in patients. The pathway has been studied most extensively during axon guidance and it will be interesting to study a role of the pathway during the development of the innervation of the heart.

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