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DEVELOPMENT AT A GLANCE

Slit-Robo signaling

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ABSTRACT

Slits are secreted proteins that bind to Roundabout (Robo) receptors. Slit-Robo signaling is best known for mediating axon repulsion in the developing nervous system. However, in recent years the functional repertoire of Slits and Robo has expanded tremendously and Slit-Robo signaling has been linked to roles in neurogenesis, angiogenesis and cancer progression among other processes. Likewise, our mechanistic understanding of Slit-Robo signaling has progressed enormously. Here, we summarize new insights into Slit-Robo evolutionary and system-dependent diversity, receptor-ligand interactions, signaling crosstalk and receptor activation.

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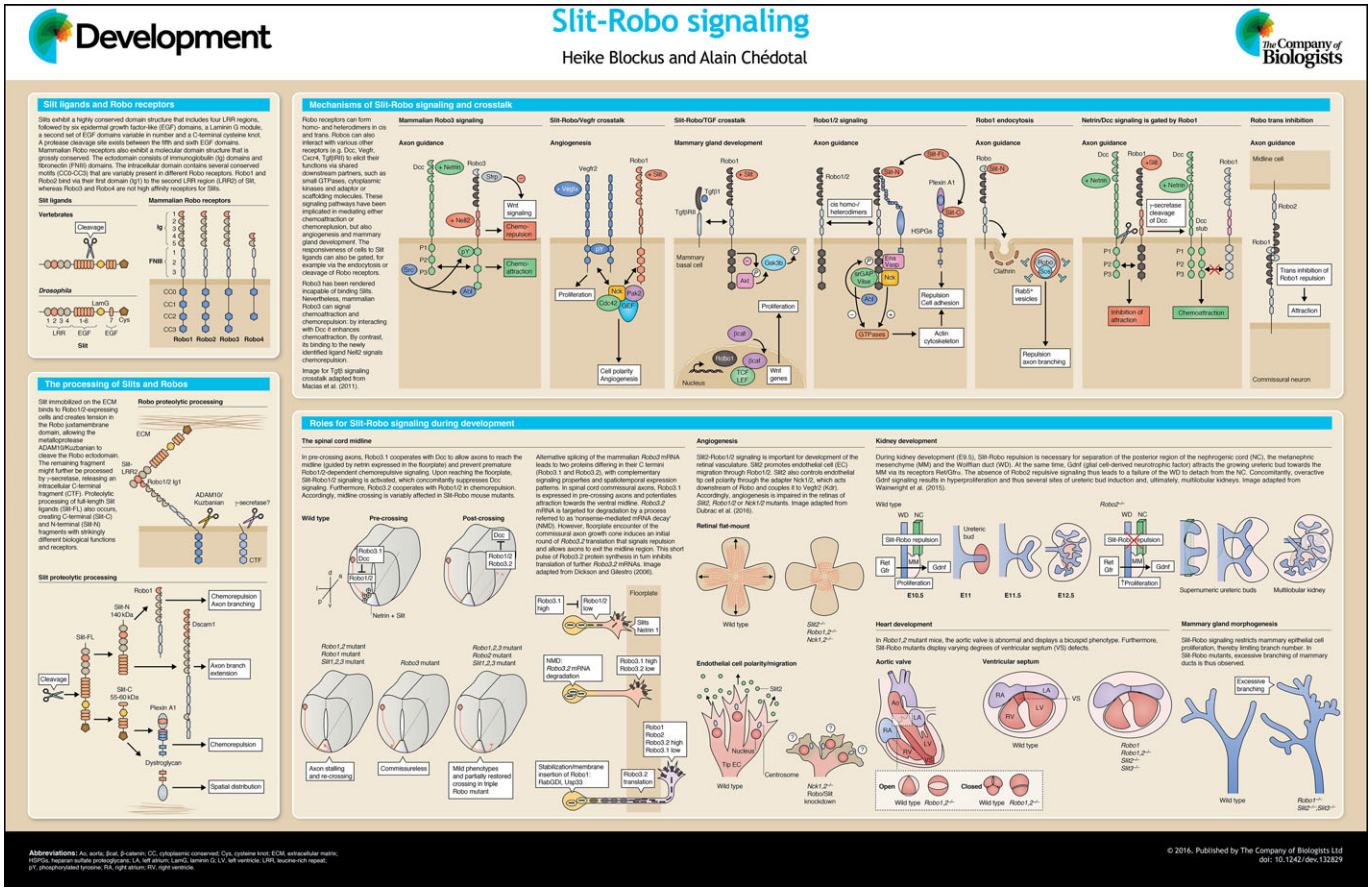
Introduction

In all organisms with bilateral symmetry, axon tracts connect the left and the right half of the central nervous system (CNS) during development, allowing communication between both sides of the body. These connections, called commissures, exist along the entire neuraxis of the organism (Lartillot and Philippe, 2008). How commissural axons find and follow the right trajectory from one (ipsilateral) side of the CNS to the other (contralateral) had long been a daunting question. However, in the 1990s, studies of the *Drosophila* embryo CNS revealed that the secreted protein Slit acts as a chemorepulsive midline cue that binds to the transmembrane receptor Robo, which is expressed on axonal growth cones, to control midline crossing (Hummel et al., 1999; Kidd et al., 1998; Rothberg et al., 1988, 1990; Seeger et al., 1993). The *Drosophila* CNS midline has since served as a model for understanding Slit-Robo signaling. Nonetheless, it has become increasingly clear that axon guidance mechanisms, including those mediated by Slits and Robos, are not as evolutionarily conserved as previously assumed; evolution has led to



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the molecular diversification of axon guidance genes, thereby increasing crosstalk between different ligands and receptors (Dascenco et al., 2015; Delloye-Bourgeois et al., 2014; Wang et al., 2015; Zelina et al., 2014). Furthermore, it has emerged that the outcome of Slit-Robo interactions is highly context dependent, creating a multifunctional platform for cell-cell or cell-matrix interactions impacting multiple physiological and pathological processes. Indeed, this interactive Slit-Robo hub contributes to cell proliferation, stem cell regulation, angiogenesis and organ development, as well as to tumorigenesis and other diseases. Here, and in the accompanying poster, we provide an overview of Slit-Robo signaling mechanisms and highlight how these translate to the pleiotropic functions of these versatile molecules.

Slit ligands and Robo receptors

Slits are highly conserved across species (Chédotal, 2007). They are generally secreted, but often associate with cell membranes and the extracellular matrix (ECM). Slit, the founding member, was identified in *Drosophila* through a genetic screen for patterning and lethality (Nüsslein-Volhard et al., 1984). Three Slit genes (*Slit1-3*) exist in most vertebrates and encode proteins of ∼200 kDa. These proteins each contain four stretches of leucine-rich repeat (LRR) domains (also called D1-D4), seven to nine epidermal growth factor (EGF) repeats, an Agrin-Perlecan-Laminin-Slit (ALPS)/Laminin-G-like domain, and a C-terminal cysteine knot.

Slits are the main ligands of Roundabout (Robo) receptors. The first three Robo genes were also discovered in *Drosophila* (Kidd et al., 1998; Rajagopalan et al., 2000; Seeger et al., 1993; Simpson et al., 2000). The name 'Robo' stems from the phenotype of *Drosophila* mutants in which commissural axons stalled at the midline of the ventral nerve cord, creating an axonal 'ROundaBOut' or 'Robo' (Tear et al., 1993). One *Robo* ortholog (*SAX-3*) exists in *Caenorhabditis elegans*, three in chick and *Xenopus*, and four in mammals and zebrafish (Kidd et al., 1999). Robo receptors are single-pass transmembrane proteins with no autocatalytic or enzymatic activity, suggesting that they depend on downstream signaling and scaffolding molecules to mediate their function. The extracellular domain of Robos – except that of mammalian Robo4 – contains five immunoglobulin-like domains (Ig1-5) and three fibronectin repeats (FNIII 1-3). The transmembrane domain (TM) is followed by a cytoplasmic tail containing several conserved regions termed CC ('cytoplasmic conserved') domains, although it should be noted that these CC domains are quite variable between species (Dickson and Gilestro, 2006). In addition to alternative splice isoforms (Chen et al., 2008; Clark et al., 2002; Dalkic et al., 2006; Yuan et al., 1999), post-translational modifications, such as ectodomain shedding, further expand the repertoire of Robo proteins (Barak et al., 2014; Colak et al., 2013; Coleman et al., 2010; Ito et al., 2006; Seki et al., 2010).

Slits bind through their LRR2 domain to the first Ig domain (Ig1) of Robo proteins (Morlot et al., 2007). Heparan sulfate proteoglycans can also bind to both Slit and Robo, forming a ternary complex and stabilizing their interaction at the membrane (Hohenester, 2008; Morlot et al., 2007; Zelina et al., 2014; Zhang et al., 2013). Slits can also homodimerize; the LRR4 domain is required for this dimerization (Seiradake et al., 2009). Importantly, Slits show additional heterophilic binding to other ECM molecules, including Neurexins, Type IV Collagens (Xiao et al., 2011), Netrin 1 (Brose et al., 1999), dystroglycan (Wright et al., 2012), Glypican (Liang et al., 1999) and Syndecan (Johnson et al., 2004; Steigemann et al., 2004).

The proteolytic processing of Robo and Slits

Both Robos and Slits undergo proteolytic processing, and this processing appears to be important for signaling. The most recent model (Barak et al., 2014), based on structural studies of the Robo1 extracellular domain, suggests that the binding of ECM-immobilized Slit creates molecular tension in the Robo receptor. This leads to exposure of a metalloproteinase cleavage site in the juxtamembrane region of Robo that is conserved between human (Seki et al., 2010) and *Drosophila* (Coleman et al., 2010) Robo1. In *Drosophila*, Robo ectodomain shedding involves the metalloprotease Kuzbanian (ADAM10 in mammals), and cleavage of Robo is required for the recruitment of downstream signaling molecules (Coleman et al., 2010); accordingly, an uncleavable form of Robo is not able to rescue midline-dependent repulsion in Robo mutants. It was also shown that, in human cancer cells, the ROBO stub remaining after ectodomain shedding is further cleaved by the action of a γ-secretase. The resulting C-terminal fragment can then translocate to the nucleus, although the function of this fragment is yet to be determined (Seki et al., 2010). However, whether ectodomain shedding occurs in all vertebrate Robos and plays a role in developmental processes in vertebrates remain open questions.

Slit ligands are also subjected to proteolytic processing. Full length Slit (Slit-FL) can be cleaved into large N-terminal (Slit-N) and short C-terminal (Slit-C) fragments by an unknown protease. The cleavage site itself is conserved in most Slits and between *Drosophila* and vertebrate Slits (Brose et al., 1999). These various forms of Slit have different activities and functions. For example, mammalian Slit-FL and Slit-N are more tightly associated with the cell surface, whereas Slit-C is mostly shed into the extracellular space (Brose et al., 1999). Both Slit-FL and Slit-N bind Robo receptors and induce chemorepulsion, although Slit-FL antagonizes the Slit-N-dependent induction of branching in sensory axons (Nguyen Ba-Charvet et al., 2001; Wang et al., 1999). Slit-C was recently shown to bind plexin A1 and to induce growth cone collapse of mouse spinal cord commissural axons (Delloye-Bourgeois et al., 2014). In addition, Slit-C binds to the basement membrane scaffolding protein dystroglycan (Wright et al., 2012).

The function of Slit fragments in *Drosophila* is still unclear as an uncleavable form of Slit is able to rescue axon midline-crossing defects (Coleman et al., 2010). By contrast, another study identified Slit-N as the only stable form of *Drosophila* Slit protein (Ordan et al., 2015). This fragment localizes on tendon cells and functions as a short-range chemorepellent to guide muscle cell migration during development (Ordan et al., 2015), whereas the C-terminal fragment is quickly degraded. Based on these findings, it was argued that the major role of Robo2 expressed in tendon cells is to fix cleaved Slit-N on tendon cells hence rendering it stable (Ordan and Volk, 2015). A recent study also showed that Slit-N functions via Dscam1 (Down syndrome cell adhesion molecule 1), and independently of Robos, to regulate axon collateral extension of *Drosophila* mechanosensory neurons (Dascenco et al., 2015). In this context, the binding of Slit to Dscam1 leads to intracellular dephosphorylation of the receptor via the phosphatase RPTP69D (Ptp69D).

Vertebrate Robo3: a divergent member of the Robo family

The first evidence that mammalian *Robo3* was a divergent member of the Robo family came from a phenotypic comparison of midline-crossing defects in Robo mouse mutants. Whereas commissural axons stall at the midline in *Robo1;2* double mutants, all commissural axons fail to cross the midline in *Robo3* knockout mice (Marillat et al., 2004; Sabatier et al., 2004). Phylogenetic

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analyses of the four mammalian Robo genes show closer homology between *Robo1* and *Robo2* compared with *Robo3* and *Robo4*, which differ in their domain structure. *Robo3* (also known as *Rig-1*) was identified as a gene upregulated in a mouse retinoblastoma-deficient cell line (Yuan et al., 1999). It was also found that mutations in *ROBO3* cause a rare autosomal recessive human disorder called horizontal gaze palsy with progressive scoliosis (HGPPS) (Jen et al., 2004). HGPPS patients suffer from severe scoliosis, are unable to perform conjugate lateral eye movements, and exhibit major commissural axon crossing defects (Haller et al., 2008; Jen et al., 2002, 2004). Together, these findings suggested that Robo3, expression of which is downregulated in commissural axons after crossing, might inhibit Slit-Robo repulsion thereby allowing axons to approach the midline.

More recently, it was shown that, due to a few amino acid changes in its Ig1 domain, mammalian Robo3 has lost the ability to bind Slits (Zelina et al., 2014). Concomitantly, Robo3 has acquired new signaling properties, exhibiting interactions with the netrin 1/Dcc chemoattractive signaling pathway. Hence, Robo3 in mammals might rather promote midline attraction instead of counteracting repulsion. Interestingly, it was also shown that neural epidermal growth factor-like-like 2 (Nell2), which is expressed by motoneurons in the mouse ventral spinal cord, is a repulsive ligand for Robo3 that prevents commissural axons from entering the motor columns (Jaworski et al., 2015). However, commissural axon midline crossing is not affected in *Nell2* knockout mice and it is only upon additional loss of one *Robo3* allele that commissural axons aberrantly divert into the ventral horn. How Robo3 functions in other vertebrates is still unknown, but commissural guidance defects were also reported in Zebrafish *robo3* mutants (Burgess et al., 2009).

Several Robo3 splice variants have been characterized: Robo3A and Robo3B, which differ at their N termini, and Robo3.1 and Robo3.2, which have distinct C termini. Robo3.1 is only detectable in pre-crossing commissural axons, whereas Robo3.2 is expressed in post-crossing axons. Robo3.1 acts to either counteract Slit-mediated repulsion or enhance attraction, whereas Robo3.2 repels post-crossing axons from the midline (Chen et al., 2008). Furthermore, *Robo3.2* mRNA is usually targeted for degradation via nonsense-mediated mRNA decay (NMD) (Colak et al., 2013), but an unknown floor plate factor helps to overcome this and allows for temporary expression of Robo3.2 only in post-crossing axons. The initial round of Robo3.2 translation triggers a negative feedback loop that degrades the remaining *Robo3.2* mRNAs. This results in a transient short pulse of Robo3.2 protein expression exclusively upon midline crossing, thereby controlling mediolateral distribution of postcrossing axon tracts.

The spatiotemporal gating of Slit responsiveness in Robo-expressing cells

The initial *Drosophila* mutagenesis screen (Seeger et al., 1993) for factors that affect midline guidance identified not only Robo, but also Commissureless (Comm) (Georgiou and Tear, 2002, 2003; Gilestro, 2008; Keleman et al., 2002, 2005). Comm is a transmembrane protein that is required in commissural axons to promote midline crossing by sorting Robo from the Golgi to the endosomal pathway for degradation, thus preventing its membrane insertion (Keleman et al., 2002, 2005). Comm expression is subsequently downregulated after crossing to allow contralateral axons to move away from the midline. Comm functions similarly in dengue and yellow fever mosquitoes (Sarro et al., 2013), although non-Dipterian insects seem to lack Comm (Evans and Bashaw, 2012). In addition, no vertebrate homolog of Comm has been identified to date, suggesting that other mechanisms must regulate Slit-Robo repulsion in commissural axons in higher organisms. In *Drosophila*, another mechanism also regulates chemorepulsive signaling in pre-crossing axons: Robo2 expressed in midline cells at the time of crossing binds in trans to Robo1 on commissural axons, thereby inhibiting Slit-mediated repulsion (Evans et al., 2015). Interesting in this context is the finding that Robo1 and Robo2 show homophilic binding properties in mammalian systems and interaction in trans can promote axon outgrowth of retinal neurons (Hivert et al., 2002).

Surprisingly, the sorting of Robo to endosomes does not only restrict signaling from the cell surface. Instead, early endosome translocation of the receptor after Slit binding is a prerequisite for the recruitment of adaptor molecules such as Sos and, therefore, downstream signaling (Chance and Bashaw, 2015). These findings show that precise control over Robo receptor surface levels can function to both restrict and initiate signaling, depending on the context.

How Slit-Robo repulsion is gated in vertebrates is still largely unknown. In chick spinal commissural neurons, Robo1 trafficking to the cell surface is controlled by Rab GDP dissociation inhibitor (RabGDI) together with the cadherin superfamily member calsyntenin1 (Alther et al., 2016; Philipp et al., 2012). Calsyntenin1 and RabGDI ensure membrane fusion of Robo1-containing Rab11-positive vesicles to the surface upon midline crossing. Studies using chick and mouse neurons also show that Robo1 is deubiquitylated by Usp33, leading to its stabilization on the cell surface and preventing its degradation on growing axons at appropriate time points (Yuasa-Kawada et al., 2009).

Additional evidence suggesting an intersection between Slit-Robo signaling and other signaling pathways comes from the discovery of Slit2 inhibitory activity on chemokine-induced migration of leukocytes and breast cancer cells (Marlow et al., 2008; Prasad et al., 2004, 2007; Wu et al., 2001). This effect was Robo dependent since an N-terminal Robo receptor fragment was able to release the inhibitory effect of Slit on these cells. It was also shown that Robo1 interacts with the major Sdf-1 (Cxcl12) receptor Cxcr4 through its CC3 domain and Slit2 was found to antagonize several components that act downstream of Cxcr4 signaling, including FAK (Ptk2), Akt, Rac and the Src family kinases Src and Lck, but not Lyn (Prasad et al., 2007). A variety of studies on chemokine signaling in the nervous system using *Cxcr4* and *Sdf-1* knockout mice followed. For example, it was shown that presence of Sdf-1 was able to reduce the repulsive function of Slit2, Sema3A and Sema3C on E7 dosal root ganglion explant cultures, possibly via elevated intracellular cAMP concentrations triggered downstream of Cxcr4 (Chalasani et al., 2003). The SDF-dependent attenuation of Robo signaling seems to be conserved across species, as zebrafish retinal ganglion cell axon pathfinding defects in the tectum can be rescued by reducing SDF-1 in a Robo2-heterozygote background (Chalasani et al., 2007).

Signaling downstream of Slit-Robo

Major players in the propagation of Robo-triggered signaling responses are cytoplasmic kinases and the actin and microtubule cytoskeleton. Notably, the cytoplasmic kinase Abelson (Abl) plays a key role and, through its effectors, influences both the actin and microtubule cytoskeleton. In *Drosophila*, Abl has been shown to inhibit Robo activity by phosphorylating its CC1 domain (Bashaw et al., 2000). However, another study on the Abl-interacting protein Capulet (Wills et al., 2002) argues that Abl functions to promote, not oppose, repulsive midline guidance. More recently, it was

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shown that different domains of *Drosophila* Abl control repulsive and attractive axon guidance, respectively (O'Donnell and Bashaw, 2013). In chick retinal cells *in vitro*, yet another Abl effector, Cables, provides a scaffold that allows the formation of a multimeric protein complex between Robo1 and N-cadherin; Slit engagement with this complex ultimately leads to the loss of N-cadherin-mediated cell adhesion via phosphorylation of β-catenin by Abl (Rhee et al., 2007, 2002).

Many studies, in particular related to different types of cancer, implicate Slit and Robo in regulating E-cadherin (cadherin 1)-dependent adhesion via the Wnt downstream signaling axis, including GSK3β and β-catenin (Prasad et al., 2008; Tseng et al., 2010; Zhou et al., 2011). However, controversy remains whether this regulation favors or attenuates malignant transformation.

Slit-Robo signaling also involves GTPases, which are small GTP-binding proteins that rearrange the cytoskeleton thereby modulating cell polarity and motion. An important link between GTPases and upstream signaling is the scaffolding protein Dock/Nck, which binds to Robos in *Drosophila* and mammals, triggering axon branching and outgrowth in cortical neurons *in vitro* (Fan et al., 2003; Round and Sun, 2011; Yang and Bashaw, 2006). GTPase activating proteins (GAPs), which control the activity of GTPases, also function in the Slit-Robo pathway. These so-called Slit-Robo-GTPase activating proteins (srGAPs) were identified in a yeast-two-hybrid screen with Robo1 as bait (Wong et al., 2001). How this interaction can affect directional cell migration has recently been investigated in NIH3T3 mouse fibroblasts (Fritz et al., 2015). In this context, srGAP2 downstream of Slit-Robo signaling controls Rac1 activity specifically in cell protrusions where two cells spatially overlap. This induces repolarization of the cell, resulting in directed migration at the leading edge, a process termed contact inhibition of locomotion (CIL). Robo1-srGAP1 signaling also stimulates the infiltration of peripheral immune cells into the brain in a rat model of neuroinflammation, an effect that could be alleviated by application of Slit2 (Sherchan et al., 2016), which suggests promising potential for therapeutic approaches. Recently, another RhoA-GAP, Myo9b, was shown to bind directly to the intracellular domain of Robo1 (Kong et al., 2015), leading to Myo9b inhibition and, in this context, the inhibition of lung cancer cell migration and invasion.

Roles for Slit-Robo signaling during development

Given the finely tuned molecular control over Slit-Robo signaling as described above, it comes as no surprise that this ligand-receptor pair functions in hugely different processes during the development of an organism. Way beyond its most well-studied function in axon guidance in the developing nervous system, it has become obvious that Slit-Robo signaling additionally plays a role in cell proliferation (Borrell et al., 2012), organogenesis, and reproductive tract (Li et al., 2015) and mammary (Mommersteeg et al., 2013, 2015) development. Without pretense for completeness, this following section provides an overview of how Slit-Robo signaling functions in a variety of developmental processes.

Slit-Robo signaling at the spinal cord midline

As touched on above, Slit-Robo signaling plays crucial roles during axon pathfinding in the spinal cord. In the mouse spinal cord, commissural interneurons are born in the dorsal part of the neural tube and their axon crosses the ventral midline, or floor plate, a specialized structure of glial cells, by embryonic day (E) 10.5-11. All three vertebrate Slits are expressed in the floor plate, and *Slit1;2;3* triple knockouts exhibit re-crossing and axon stalling phenotypes at the midline (Long et al., 2004). Robo2 alone does not seem to contribute to floor plate crossing in the spinal cord, and only the *Robo1*$^{-/-}$ mutant partially phenocopies the triple Slit mutants. *Robo1;2* double mutants display similar, but less severe, axon stalling phenotypes as *Slit1;2;3* triple mutants (Jaworski et al., 2010), suggesting that Slits may also act independently of Robos in commissural axons and that additional Slit receptors exist. Indeed, a candidate Slit receptor, Eva1C, first identified in *C. elegans* (Fujisawa et al., 2007), is expressed in Slit-responsive axons in the mouse (James et al., 2013). As mentioned above, Slit fragments can also bind to the semaphorin receptor plexin A1 to to induce commissural axon chemorepulsion (Delloye-Bourgeois et al., 2014) and to dystroglycan, which is also expressed in the floor plate (Wright et al., 2012) suggesting that dystroglycan captures Slit-C at its sites of action; accordingly, dystroglycan mutant mice phenocopy the spinal cord midline defects seen in *Robo1;2* double mutants. As in *Drosophila* (Rajagopalan et al., 2000; Simpson et al., 2000), Slit-Robo repulsion might also influence the lateral positioning of axons extending parallel to the CNS midline axis (Spitzweck et al., 2010).

Evidence also suggests that Slit-Robo signaling intersects with the Netrin-1/DCC axis. In the presence of Slit, Robo1 and DCC interact in *Xenopus* spinal cord commissural axons, in turn silencing attraction to Netrin-1 (Stein and Tessier-Lavigne, 2001). However, Slit-Robo chemorepulsion is an active process that can occur in the absence of DCC signaling, at least in *Drosophila* (Garbe and Bashaw, 2007), arguing against the hypothesis that the sole function of Robo1/2 is to silence attraction. Mammalian motoneurons differ from commissural neurons in that they co-express Robo1/2, Dcc and Slit2. In this context, Slit2 seems to have an autocrine function that controls motor axon fasciculation towards their muscle targets (Jaworski and Tessier-Lavigne, 2012). Within the spinal cord, Slit binding to Robo1/2 on motor axons silences Dcc function and prevents these axons from being attracted by netrin 1 at the floor plate (Bai et al., 2011).

Slit-Robo signaling in angiogenesis

A number of studies have highlighted roles for Slit-Robo signaling during angiogenesis – the formation of blood vessels from existing vessels. Vertebrate Robo4 is selectively expressed by endothelial cells and has been reported to control angiogenesis and blood vessel permeability (Bedell et al., 2005; Huminiecki et al., 2002; Jones et al., 2009). Many studies have linked Robo4 function to Slit (Enomoto et al., 2016; Fritz et al., 2015; Jones et al., 2009; Yu et al., 2014), although it was subsequently proposed that Robo4 does not bind Slits (Koch et al., 2011), with genetic evidence in knockout mice also failing to support a direct link between Robo4 and Slits (Rama et al., 2015). Endothelial cells also express Robo1/2 receptors and Slits, in particular Slit2, which favors angiogenesis by promoting endothelial cell motility and polarity (Dubrac et al., 2016; Rama et al., 2015). Given the overlapping expression pattern of Robo1/2 and Robo4, it is possible that Slit functions through receptor heterodimers without direct binding to Robo4. This has indeed been shown in human endothelial cells *in vitro*, where a ROBO1/ROBO4 heterodimer promotes cell migration (Sheldon et al., 2009). In blood vessels, Slit-Robo cooperates with vascular endothelial growth factor A (Vegfa) to induce neovascularization, and this involves the scaffolding proteins Nck1/2 and Rac1 (Dubrac et al., 2015; Rama et al., 2015). Interestingly, a splice isoform of Slit2 (generated by splicing at exon 15) has a stronger effect on vessel tube formation and angiogenesis than its canonical counterpart, possibly in a Robo-independent manner (Yang et al., 2015).

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Slit-Robo signaling in organogenesis

Slits and Robos are also involved in the development of many organs. All three Slits and Robo1/2 are expressed in the developing kidneys of mice (Piper et al., 2000). In *Slit2*- or *Robo2*-deficient mice, ureteric bud formation fails to be restricted to a single site, leading to multilobular kidneys (Grieshammer et al., 2004; Yu et al., 2004), resembling the defects observed in human patients carrying mutations in *ROBO2* (Lu et al., 2007). Robo2 is also important for the separation of the nephrogenic cord from the Wolffian duct, which prevents hyperproliferation of the cord (Wainwright et al., 2015). Even after development, Robo2 is necessary for podocyte stability in the mouse kidney by inhibiting nephrin-induced actin polymerization in podocyte feet via its binding partner Nck (Fan et al., 2012). Slit-Robo signaling has also been implicated in kidney development in humans. Whole exome-sequencing of patients suffering from congenital abnormalities of the kidney and urinary tract (CAKUT) identified mutations in *SRGAP1* and *SLIT2* (Hwang et al., 2015); the *SRGAP1* mutation results in a gain-of-function phenotype with increased RAC1 inhibition, whereas the *SLIT2* mutations all cluster in the regions encoding the LRR domains and hence may interfere with ROBO binding.

Slits and Robos also play versatile roles during heart development in both *Drosophila* and mammals, and have been implicated in congenital cardiac malformations in humans (Vogler and Bodmer, 2015). Phenotypic analyses of knockout mice show that Robo1/2 and Slit2/3 contribute differentially to heart development: the ventricular septum is absent in *Robo1;2* and *Slit3* mutants, whereas septum defects were less severe in *Slit2* mutants (Mommersteeg et al., 2015). By contrast, Slit2 is more important for proper aortic valve formation (Mommersteeg et al., 2015). *Robo1* knockout mice also lack parts of the pericardium, a phenotype probably caused by the ectopic formation of a pericardial cavity (celom) at E12.5 due to increased apoptosis of cardiac neural crest cells (Mommersteeg et al., 2013). Cardiac vein morphogenesis is also disturbed to varying degrees in these mutants (Mommersteeg et al., 2013), in line with the aforementioned role of Slit-Robo signaling in the developing vasculature.

Another remarkable example of Slit-Robo signaling during organogenesis is its role in mammary gland development. It was first shown that the two cell layers making up mammary ducts (outer myoepithelial and inner luminal epithelial cells) were separated in *Slit2*$^{-/-}$ mutants and that the lumen of the ducts was decreased (Strickland et al., 2006). Later on, it was shown that Slit-Robo signaling also functions in regulating branching morphogenesis in the mammary gland, and *Robo1* as well as *Slit2,3* mutants show increased tubular branching (Macias et al., 2011). The signaling pathway underlying this phenotype involves TgfβRII signaling-mediated induction of *Robo1* transcription. Robo1 signaling in turn inhibits Akt, leading to activation (i.e. dephosphorylation) of GSK3β. When Slit-Robo signaling is active, therefore, less β-catenin is present in the nucleus, and the transcription of pro-proliferative genes is thus inhibited.

Lastly, Slit-Robo signaling is important for proper inner organ localization and diaphragm development (Domyan et al., 2013; Liu et al., 2003). Importantly, the analysis of Slit-Robo mutant mice, in which the stomach is misplaced from the abdomen through the diaphragm to the thoracic cavity, has provided important insights into the mechanisms underlying human congenital diaphragmatic hernia (CDH) (Ackerman and Greer, 2007). During development, gut tube reorganization gives rise to digestive and respiratory systems and its proper morphogenesis requires separation from the body wall, a process disturbed in *Robo1;2* mutants (Domyan et al., 2013).

Slit-Robo signaling in cell proliferation and stem cell regulation

Many studies on Slit-Robo signaling in the nervous system have focused on non-proliferating mature neurons. However, Robo1 and Robo2 are also expressed in many neurogenic niches of the developing brain (Borrell et al., 2012). In line with this expression pattern, cortical volume and the density of active mitotic cells is widely reduced at early embryonic stages in *Robo1/2* knockout mice. This is linked to a shift in the balance between neural progenitor self-renewal and intermediate progenitor cell (IPC) production, giving rise to an increased pool of IPCs. Cells also divide more slowly due to a failure of these cells to detach from the ventricular surface, supporting a role for Slit-Robo in percursor cell adhesion. Furthermore, this study identified Robo as an upstream regulator of the transcription factor *Hes1*, which is also a component of the Notch signaling pathway. Interestingly, however, Robo can activate *Hes1* independently of Notch.

Slit-Robo signaling also controls the division mode of mammary stem cells: in the absence of Robo1, symmetric divisions are favored rather than asymmetric ones (Ballard et al., 2015). This is due to an increase in the spindle apparatus protein Insc. A higher amount of symmetric division in turn leads to increased self-renewal of the stem cell population and mammary outgrowth. Although Robo2 does not play a role in this context, it has been shown to regulate the senescence of mammary stem cells by inhibiting Wnt signaling (Harburg et al., 2014). Interestingly, these two studies highlight different signaling outputs via different receptors by a single ligand, Slit2.

Robo4 and Cxcr4 are both important for anchoring hematopoietic stem cells to bone marrow niches, an adhesive effect that needs to be overcome for effective recruitment of these cells into the bloodstream (Smith-Berdan et al., 2011). A physical interaction between the receptors however, was not shown in this study, even though Cxcr4 is compensatorily upregulated in *Robo4*$^{-/-}$ mutant mice.

In the *Drosophila* testis, cells compete for occupancy of the stem cell niche, a process regulated by cell adhesion. Recently, it was shown that, yet again, Abl kinase functions downstream of Slit-Robo in this context and inhibits E-Cadherin (Shotgun)-mediated adhesion (Stine et al., 2014). Interestingly, activation of JAK-STAT chemokine signaling can induce *robo2* expression in this system.

Finally, Slit-Robo signaling has been shown to be important for lineage specification in adult enteric stem cells in *Drosophila* (Biteau and Jasper, 2014). Enteroendocrine cells that are responsible for lineage specification secrete Slit, which binds to Robo2 expressed on intestinal stem cells, thereby inhibiting their endocrine potential. In this context, Robo2 acts upstream of the transcription factor Prospero expressed in these cells, forming a signaling loop that functions to maintain the enteroendocrine lineage.

Conclusions and perspectives

The initial discoveries of Slit-Robo-dependent axon guidance phenotypes in *Drosophila* and vertebrates represented important milestones in the molecular understanding of Slit-Robo signaling in space and time. Recently, this understanding has been largely inspired by studies of Slit-Robo signaling outside the nervous system. Furthermore, the investigation of Slit-Robo signaling mechanisms in a species-specific manner has highlighted the evolutionary diversity of Slit-Robo signaling. In mammals, Robo3 and Robo4 do not bind Slits and have acquired new ligands and co-receptors, strikingly setting them apart functionally from their classical Robo1 and Robo2 counterparts. Likewise, the interaction

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between Slit and Robo is not exclusive, and a number of other Slit receptors, such as Eva1C, plexin A1, Dscam1 and dystroglycan, have been identified. Endocytic trafficking, proteolytic processing and heterodimeric receptor interactions at the membrane have also emerged as key regulatory mechanisms that gate if and how cells respond to cues in their environment.

Despite the high diversity of functional processes governed by Slit-Robo signaling, how these different outcomes are regulated via different downstream signaling pathways remains obscure. Therefore, in order to further tease apart how Slit-Robo responses shape different systems, future studies should address context- and cell-dependent downstream signaling pathways that can steer cellular responses in different environments. At the other end of the scale from molecules to organisms, data obtained from mouse models of diseases or human genetic studies clearly indicate therapeutic potential of understanding Slit and Robo signaling, and this is likely to remain an active area of investigation. Even though the road to Slits and Robos as potential drug targets or biomarkers of neurodevelopmental disorders is still long, ideally the molecular understanding that has been hard-earned through decades of research can soon enhance and unify translational studies from bench to bedside.

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Competing interests

The authors declare no competing or financial interests.

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Development at a Glance

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