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Moving away from the midline: new developments for Slit and Robo

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Summary

In most tissues, the precise control of cell migration and cell-cell interaction is of paramount importance to the development of a functional structure. Several families of secreted molecules have been implicated in regulating these aspects of development, including the Slits and their Robo receptors. These proteins have well described roles in axon guidance but by influencing cell polarity and adhesion, they participate in many developmental processes in diverse cell types. We review recent progress in understanding both the molecular mechanisms that modulate Slit/Robo expression and their functions in neural and non-neural tissue.

Key words: Axon guidance, Cell migration, Cell-cell interaction

Introduction

In most organisms, the central nervous system (CNS) develops along a bilateral axis of symmetry located at the midline (Placzek and Briscoe, 2005). During development, the ventral midline or floor plate acts as an organiser through the secretion of diffusible proteins (Placzek and Briscoe, 2005; Gore et al., 2008), which control the growth of axons and dendrites and the migration of neurons across the midline. In the forebrain, glial or neuronal cells delineate the midline (see Glossary, Box 1) and also control axon guidance. For more than two decades, many developmental neurobiologists have tried to understand the mechanisms that control midline crossing in the CNS and to answer some key questions. Firstly, how are commissural axons (see Glossary, Box 1) attracted to the midline and how do their growth cones (see Glossary, Box1) receive and integrate the multiple and contrasting signals released by midline cells? Secondly, what are the molecular and signalling changes that enable commissural axons to leave the midline and often to switch to a longitudinal growth mode? All midline-derived axon guidance factors are expressed at other locations in many developing and mature tissues, where they control a wide range of biological processes.

Roundabout receptors (Robo) and their Slit ligands form one of the most crucial ligand-receptor pairings among the axon guidance molecules. Robos were identified in *Drosophila* in a mutant screen for genes that control the midline crossing of commissural axons (Kidd et al., 1998; Seeger et al., 1993). Similarly, Slit was discovered in *Drosophila* as a protein secreted by midline glia (Rothberg et al., 1988; Rothberg et al., 1990). Homologues of both proteins have since been discovered in many species (for a review, see Chédotal, 2007). However, the Slit/Robo couple not only functions in axon guidance but also in a variety of developmental

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Box 1. Glossary

Choroid plexus Specialised ependymal cells located inside the ventricles of the brain that secrete cerebrospinal fluid.

Commissural axons Axons with cell bodies (somata) located on one side of the central nervous system (CNS) that project axons across the midline to contact target cells on the opposite side.

Growth cone A specialised bulbous enlargement at the end of growing axons that is characterised by dynamic filamentous extensions, known as filopodia. They sense the environment and respond to adhesion and guidance molecules.

Inferior olive (IO) A characteristic olive-shaped nucleus of the medulla oblongata (a region of the brainstem located above the spinal cord) involved in motor learning. Neurons of the IO project to Purkinje cells in the cerebellar cortex. The terminal axonal arborisation of IO axons is called a climbing fibre.

Midline An imaginary line that runs along the longitudinal axis of the CNS and delineates the axis of symmetry. In the midbrain, hindbrain and spinal cord, the ventral midline is called the floor plate and the dorsal midline, the roof plate.

Mossy fibers Axons originating from several hindbrain and spinal cord nuclei that synapse onto granule cell dendrites in the cerebellar cortex.

Myotopic map A particular type of topographic map in which muscle fibers project to neurons of the peripheral nervous system in an ordered manner to create a structured projection pattern.

Netrin/Dcc Netrins are axon guidance proteins best known for their role in the development of commissural tracts. Deleted in colorectal cancer (Dcc) is a transmembrane receptor for netrin 1 that mediates its chemoattractive action.

Neuroblast A neuronal progenitor cell that originates in the subventricular zone and that migrates along the rostral migratory stream towards the olfactory bulb, where it differentiates into olfactory interneurons.

Olfactory bulb An anterior protrusion of the brain to which olfactory and vomeronasal sensory neurons project, and from which the olfactory tract originates.

Overlay assay A technique in which immobilised proteins or cells are incubated with a protein of interest that is labelled to identify binding sites and measure binding affinity.

Pontine nucleus (PN) neurons A major source of mossy fiber axons. The cell bodies of PN neurons are located in the basilar pons, a structure of the ventral midbrain.

Radial migration A type of neuronal migration during which neurons migrate perpendicular to the brain surface and follow radial glia processes.

Septum A region of the forebrain comprising several nuclei that have afferent and cholinergic efferent connections with various forebrain and brainstem areas. It secretes Slit proteins, which might contribute to repelling neuroblasts towards the olfactory bulb.

Subventricular zone A brain structure located along the walls of the lateral ventricles. It is one of two regions in the adult mammalian brain where neurogenesis occurs.

Tangential migration A type of migration during which neurons migrate parallel to the surface of and independently of radial glia.

processes outside of the midline and the CNS. As such, it is insightful to explore the roles of Slit/Robo in different systems and to contrast their modes of action. Moreover, in this review, we delve into some of the mechanisms of Robo regulation and signalling that vary tremendously from one region to another. Although we focus on recent findings discovered in vertebrates, we also mention relevant studies in invertebrates where appropriate.

Robo and Slit structure

Robo receptors belong to the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs) and, as with other IgCAMs, they can mediate homophilic and heterophilic interactions (Hivert et al., 2002). In most vertebrates, there are three Robo receptors expressed in brain cells (Chédotal, 2007). In zebrafish and mammals, a fourth Robo receptor, Robo4, is expressed by endothelial cells and functions in angiogenesis (Bedell et al., 2005; Jones et al., 2008); this receptor will not be discussed further here. The standard/archetypical Robo receptor contains five Ig motifs, three fibronectin type III domains and four conserved cytoplasmic domains, expressed in different combinations within the Robo receptor family (Fig. 1).

All Robo receptors can be alternatively spliced to generate various isoforms. Alternative splicing in the 5′ coding sequence can generate two distinct receptor isoforms, A and B, which differ at their N-terminal end. Type A isoforms are longer (16-40 residues) than type B isoforms (Camurri et al., 2005; Kidd et al., 1998; Sundaresan et al., 1998; Yue et al., 2006). In the case of Robo3, alternative splicing by the retention or excision of one intron in the 3′ coding sequence generates two isoforms that differ in their C-terminal domain, with Robo3.1 being longer than Robo3.2 (Chen et al., 2008a). For more information on the structure of Robo receptors, we refer readers to a recent review by Chédotal (Chédotal, 2007).

Slits are the principal ligands for the Robo receptors (Kidd et al., 1999). Slit was originally discovered in *Drosophila* (Rothberg et al., 1988; Rothberg et al., 1990), and its homologues have since been discovered in several species, including worms, teleosts and mammals (Chédotal, 2007). In mammals, there are three Slit genes, all of which are expressed in the nervous system and in most organs (Marillat et al., 2002). Slits are secreted glycoproteins that possess the following general structure: an N-terminal signal peptide, four domains (D1-D4) containing leucine-rich repeats (LRR), several EGF-like sequences, a laminin-G domain and a C-terminal cysteine-rich knot (see Fig. 1). Slits can be cleaved to yield a short C-terminus fragment of unknown function and a long N-terminus fragment that is active and mediates binding to Robos (Nguyen Ba-Charvet et al., 2001; Wang et al., 1999).

Analysis of the Slit LRR regions has demonstrated that *Drosophila* Robo receptors bind to a common site located within the D2 region of Slit (Howitt et al., 2004). Moreover, Robos bind

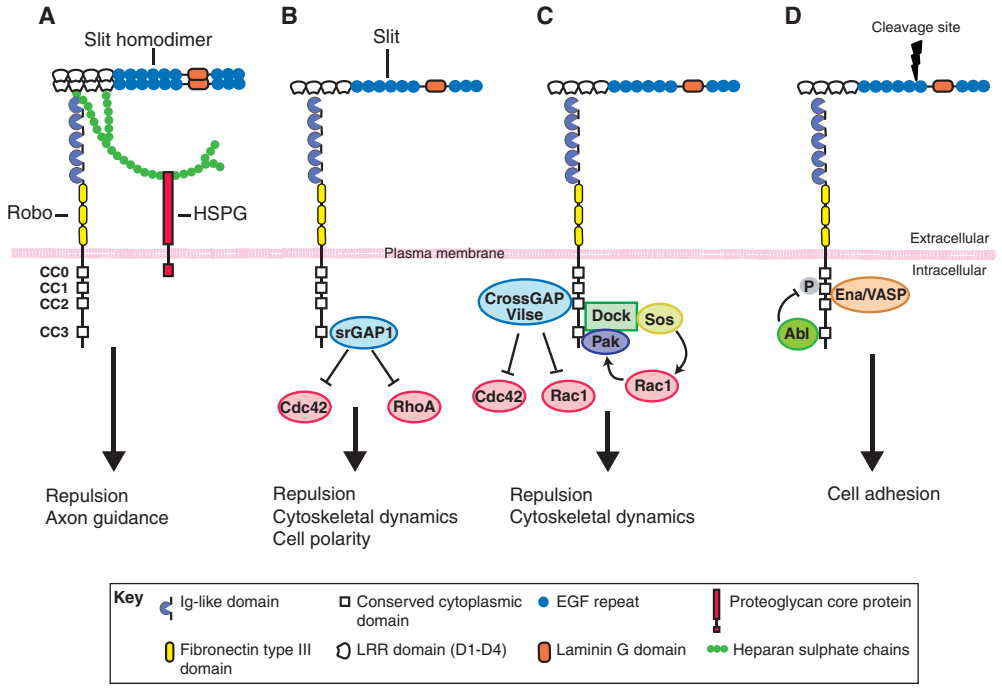

Fig. 1. Slit-Robo signalling. A schematic of Slit-Robo signalling. (**A**) Slits bind to the immunoglobulin (Ig) 1 domain of Robo receptors through its second (D2) domain, which contains leucine-rich repeats (LRRs). Heparan sulphate proteoglycans (HSPGs) such as syndecan (Sdc), which consist of a proteoglycan core protein and heparan sulphate (HS) chains, are co-receptors for Robo and Slit. HSPGs stabilise Robo and Slit binding by forming ternary complexes with the Ig1 domain of Robo receptors and the D2 domain of Slit through their HS polymers. (**B**) The Rho GTPases and their regulators (GAPs and GEFs) are key components of the Slit-Robo signalling pathway. In the presence of Slit, Slit-RoboGAP1 (srGAP1) binds to the CC3 domain of Robo and inactivates RhoA and Cdc42. These effector proteins are able to mediate, among other outcomes, repulsion, control of cytoskeletal dynamics and cell polarity. (**C**) In the presence of Slit, Vilse/CrossGAP can also bind to the CC2 domain of Robo and inhibit Rac1 and Cdc42. Rac1 is also activated by the recruitment of the GEF protein Son of sevenless (Sos) via the adaptor protein Dreadlocks (Dock), which binds to the CC2-3 domain of Robo. This activates the downstream target of Rac1 and p21-activated kinase (Pak), which also binds to Robo CC2-3 domains. These downstream signalling partners of Robo control repulsion and cytoskeletal dynamics. (**D**) The tyrosine kinase Abelson (Abl) binds Robo CC3 domain and antagonises Robo signalling through phosphorylation of the CC1 domain and mediates cell adhesion. Enabled (Ena) a substrate of Abl also binds Robo CC1 and CC2 domains. The slit cleavage site is also shown.

to Slits through their Ig1 and Ig2 domains (Liu et al., 2004). The resolution of the structure of a minimal Slit-Robo complex revealed that the Ig1 domain of Robo1 is bound by the concave face of Slit-D2, and that the binding residues of the Slit LRR2 domain are highly conserved among all Slits across species, as are those of the Robo Ig1 domain (Morlot et al., 2007). This conservation could explain the promiscuous binding of Slit ligands to Robo receptors that has been previously described using overlay assays (see Glossary, Box 1) (Brose et al., 1999; Sabatier et al., 2004). Although Slit does not always bind to Robo as a dimer, heparan sulphate proteoglycans (HSPGs) have been shown to stabilise the Slit homodimer through the interaction of heparan sulphates (HSs) with the D4 domain of Slit, which furthermore seems to potentiate Slit activity (Seiradake et al., 2009).

However, Slit is not the only ligand for Robo and vice versa. In *Caenorhabditis elegans*, SLT-1/Slit also binds to the EVA-1 transmembrane protein that has homologues in mammals (Fujisawa et al., 2007). Likewise, biochemical and genetic data demonstrate that an interaction occurs between the LRR tendon-specific protein (LRT) and Robo, highlighting a mechanism by which LRT can modulate Robo-Slit interactions and influence muscle cell migration (Wayburn and Volk, 2009). Moreover, as we discuss below, several studies have identified HSPGs as co-receptors for Slits that are necessary for Slit-Robo signalling (Hu, 2001; Steigemann et al., 2004). Recent structural analysis has revealed that Slit/Robo form a ternary complex with a heparin/HS that stabilises and strengthens the Slit-Robo interaction (Fukuhara et al., 2008; Hussain et al., 2006). And, as mentioned above, structural studies have also shown that the Slit D4 region binds HS and promotes Slit homodimerisation (Seiradake et al., 2009).

A ménage à trois with heparan sulphates

HSPGs are proteoglycan core proteins attached to HS chains, which are either membrane bound, like syndecans (Sdc) and glypicans, or secreted, like perlecan and agrin. Modifications of the heparan polymers by epimerisation or sulphation generate many distinct HS isoforms. HSPGs have a dual role: as regulators of cell adhesion, as they can bind to other components in the extracellular space; and as signalling molecules, because they can act as co-receptors (Johnson et al., 2006; Lee and Chien, 2004; Van Vactor et al., 2006).

HSPGs have been implicated in Slit/Robo signalling in various studies over the years. For example, the enzymatic removal of HS along the optic tract of developing *Xenopus* embryos induces guidance defects in retinal axons (Walz et al., 1997). HSPG removal also decreases the affinity of Slit2 for Robo and abolishes the repulsive activity of Slit2 on rat olfactory axons in vitro (Hu, 2001). Slit2 also binds to glypican 1 (Ronca et al., 2001). In addition, when heparan sulphates are disrupted, Slit2 fails to repel *Xenopus* retinal axons (Piper et al., 2006). In zebrafish, dopaminergic axons are surrounded by HS, and their removal enhances the mis-positioning of this longitudinal tract in *Robo2/astray* mutants (Kastenhuber et al., 2009). In addition, the ablation of exostosin 1 (*Ext1*), which encodes a glycosyltransferase involved in HSPG synthesis, induces defects at the mouse optic chiasm, which resemble those observed in *Slit1/Slit2* double-knockout mice (Inatani et al., 2003; Plump et al., 2002). Furthermore, Slit and Ext1 have been shown genetically to interact (Inatani et al., 2003). In *Drosophila*, the transmembrane HSPG Sdc interacts with Robo and Slit, and contributes to the Slit-dependent repulsion of axons at the CNS midline (Johnson et al., 2004; Steigemann et al., 2004). The extracellular domain of Sdc can be proteolytically cleaved and shed as a soluble proteoglycan (Fitzgerald et al., 2000), which is necessary and sufficient to mediate Slit signalling in target cells (Chanana et al., 2009). The enzymatic modification of HS is also important for Slit activity, and *O*-sulphation of glypican 1 significantly potentiates Slit2 binding in human embryonic kidney 293 cells (Ronca et al., 2001). Recent genetic data in *C. elegans* strongly suggest that HS *O*-sulphation of LON-2/glypican regulates motor axon guidance, probably by modulating SLT-1/Slit binding to EVA-1, and its interaction with SAX-3/Robo (Bulow et al., 2008; Fujisawa et al., 2007). So far, HSPGs have been shown to mostly affect the functions of Slit/Robo in axon guidance. As discussed in the following sections, other molecules also interact with Robos and Slits and their downstream effectors to influence cell migration.

Slit and Robo in cell migration

Repulsion and cell polarity

In most developing tissues, cells migrate in a precise direction, following stereotyped pathways under the influence of repulsive and attractive factors. In vertebrates, two main types of neuronal migration have been described: radial migration (see Glossary, Box 1) and tangential migration (see Glossary, Box 1). As discussed below, Slit/Robo repulsion influences tangential migration in several systems (Andrews et al., 2008; Metin et al., 2008).

Slit/Robo function in tangential neuronal migration was first shown in the mouse forebrain, where Slit1 and Slit2, secreted from the choroid plexus and septum (see Glossary, Box 1), repel neuroblasts (see Glossary, Box 1) derived from the subventricular zone (see Glossary, Box 1), thus guiding them towards the olfactory bulb (see Glossary, Box 1) (Hu, 1999; Nguyen-Ba-Charvet et al., 2004; Sawamoto et al., 2006; Wu et al., 1999).

Slit/Robo repulsion also orchestrates tangential migration in the mouse hindbrain. Hindbrain precerebellar nuclei (see Glossary, Box 1) contain neurons that project to the cerebellum. These neurons are born in the dorsal edges of the neuroepithelium that lines the fourth ventricle (the so-called rhombic lip) and migrate tangentially towards the floor plate under the attractive action of netrin 1 (see Fig. 2) (Bloch-Gallego et al., 1999; Marcos et al., 2009; Sotelo and Chédotal, 2005; Yee et al., 1999). Axons that originate from neurons of the inferior olive (IO; see Glossary, Box 1), the climbing fibers, synapse onto Purkinje cells. All other hindbrain precerebellar neurons, from the pontine nucleus (PN; see Glossary, Box 1), the lateral reticular nucleus (LRN) and the external cuneatus nucleus (ECN), project mossy fibers (see Glossary, Box 1) that synapse onto granule cell dendrites. All hindbrain precerebellar neurons migrate towards the floor plate, but whereas the cell bodies of LRN and ECN neurons cross it, the soma of IO neurons (IONs) and of most PN neurons stop at the midline and only their axons cross the midline (see Fig. 2D).

Migrating precerebellar neurons express all Robo receptors (except Robo4) (Marillat et al., 2002; Marillat et al., 2004; Di Meglio et al., 2008; Geisen et al., 2008). Slits are expressed in the floor plate, the rhombic lip and in several cranial motor nuclei, such as the facial nucleus (Geisen et al., 2008; Gilthorpe et al., 2002; Hammond et al., 2005). In vitro assays had previously shown that Slits can repel migrating precerebellar neurons (Causeret et al., 2002; Causeret et al., 2004; Gilthorpe et al., 2002). Recent studies of Slit and Robo mutant mice have confirmed that Slit/Robo signalling controls the migration of precerebellar neurons in vivo.

In *Robo1/Robo2* double knockouts and to a lesser extent in *Slit1/Slit2* double knockouts, a significant proportion of the somata of IONs do not stop at the floor plate but migrate to the

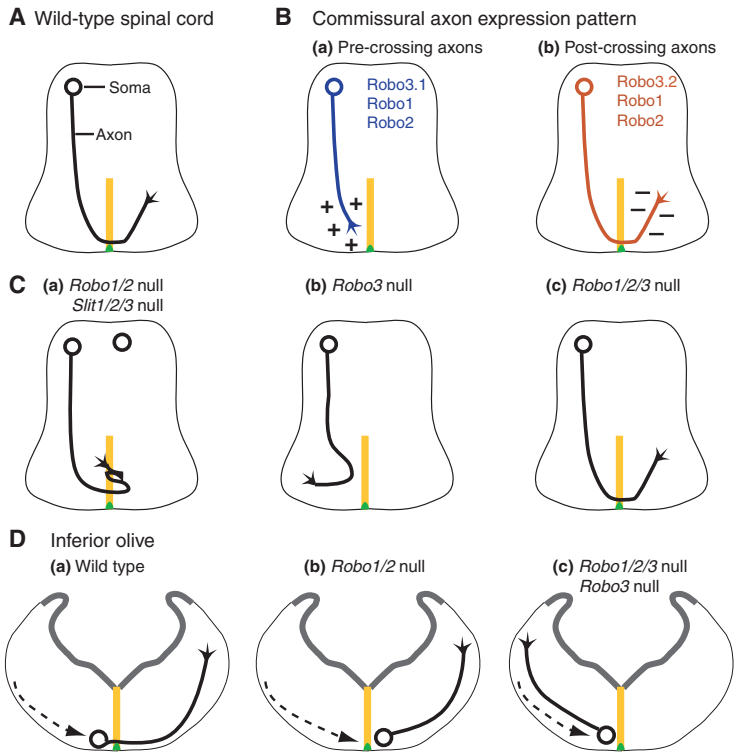

Fig. 2. Role and mechanism of Slit/Robo action in mouse commissural axons. Schematics of cross sections of the mouse embryonic spinal cord (A-C) and hindbrain (D), showing the midline (yellow) and floor plate (green). In these schematics, the dorsal region is facing up. (**A**) Commissural axon (black) in the mouse spinal cord of a wild-type adult mouse. (**B**) Spinal commissural axons (blue) grow towards the floor plate (green) under the attractive action (+) of netrin 1 and Shh. (**a**) Before crossing the midline, these axons express Robo1, Robo2 and Robo3.1; Robo3.1 blocks the repulsive activity of Slit. (**b**) After midline crossing, the axons (red) express Robo3.2, a second isoform of Robo3, in place of Robo3.1, which cooperates with Robo1 and Robo2 to repel axons (–) away from the midline. (**C**) Commissural axons in *Slit* and *Robo* knockouts. (**a**) In *Slit* triple and *Robo1/Robo2* double-knockout mice, axons stall at the floor plate and make targeting errors. (**b**) In *Robo3* knockout mice, they are prematurely repelled and fail to cross. (**c**) In *Robo1/Robo2/Robo3* triple knockouts, crossing is restored. (**D**) Development of inferior olive (IO) neurons in the hindbrain of Robo knockouts. (**a**) In wild-type mice, IO neurons migrate from the rhombic lip dorsally (where the arrow begins) towards the midline. Their axons cross the midline but their somata do not. (**b**) In *Robo1/Robo2* double knockouts, IO neurons initially migrate as in wild-type mice but many IO neurons fail to stop prior to reaching the midline and migrate across the midline. (**c**) In *Robo3* knockout mice, both IO neurons and their axons fail to cross the midline, as in the spinal cord. However, unlike in the spinal cord, IO axon midline crossing is not restored in *Robo* triple-knockout mice, indicating that Robo3 does not modulate Robo-dependent repulsion in these neurons.

contralateral side (Fig. 2D). Thus, in these mice the olivary projection is bilateral and not exclusively contralateral, as in wild-type mice. These findings are in agreement with Slit/Robo having a gate-keeper role at the midline, selecting neurons and axons that are allowed to cross it. Interestingly, the migration of PN neurons is also perturbed in these mutants but in a different way. Upon leaving the rhombic lip, PN neurons migrate rostrally across several rhombomeres in a compact stream before turning ventrally towards the midline (Geisen et al., 2008). In *Robo1/Robo2* and *Slit1/Slit2* double knockouts, the stream of PN neurons splits and many neurons prematurely migrate towards the floor plate (Geisen et al., 2008). In this system, the major source of Slit1 and Slit2 seems to be the facial nuclei, rather than the floor plate (Geisen et al., 2008).

At a subcellular level, novel data suggest that Slits control neuronal migration by influencing cell polarity. Calcium (Ca^{2+}) signalling and Rho GTPases are known to modulate the radial migration of cortical neurons and cerebellar granule cells (Gomez and Zheng, 2006; Komuro and Rakic, 1996). In granule cell/radial glia co-cultures, the focal application of Slit2 to one pole of the migrating neuron induces an elevation of intracellular Ca^{2+} concentration (Xu et al., 2004), accompanied by a reversal of the direction of migration. Although the signalling pathway activated by Slit2 during repolarisation is still obscure, Slit2 controls the orientation of tangentially migrating rat cerebellar granule cells by inducing a local burst of Ca^{2+} in their leading process and soma. This Ca^{2+} burst redistributes active RhoA from the front of the migrating granule cell to the opposite side, and reverses the direction of migration (see below) (Guan et al., 2007). The forward movement of migrating cells also correlates with the positioning of the centrosome or microtubule organising center (MTOC) at the rostral pole of the cell (Higginbotham and Gleeson, 2007). In migrating mouse SVZ neuroblasts in vitro, a Slit gradient influences cell polarity by controlling the positioning of the centrosome (Higginbotham et al., 2006). In a breast cancer cell line, Slit also seems to primarily act upon cell motility by influencing centrosome movement and cell polarity (Yuasa-Kawada et al., 2009a). Slits also regulate the polarity of murine retinal ganglion cell (RGC) axons (Thompson et al., 2006). During development, axons extend from RGC neurons in a highly polarised fashion, projecting radially towards the optic disc. In *Slit1/Slit2* double-knockout mice, as well as in *Robo2* knockout mice, the initial polarity of RGC axons is abnormal with many axons forming loops in the retina (Thompson et al., 2009; Thompson et al., 2006).

Cytoskeletal dynamics

The cytoplasmic domains of Robo receptors do not possess autonomous catalytic activities and they must therefore interact with different signalling molecules to exert their specific effects, such as controlling cytoskeletal rearrangements (for reviews, see Chédotal, 2007; O'Donnell et al., 2009). Robo signalling is mediated by a range of secondary molecules and/or co-receptors, such as the netrin 1 receptor Dcc, which binds to the Robo1 CC1 domain (Stein and Tessier-Lavigne, 2001) (see Box 2).

Small GTPases of the Rho family (Heasman and Ridley, 2008), such as RhoA, Rac1 and Cdc42, are key regulators of actin cytoskeletal dynamics. These proteins switch from an inactive GDP-bound state to an active GTP-bound state and are regulated by the GTPase-activating proteins (GAPs) and the guanine nucleotide-exchange factors (GEFs). Downstream of Robo receptors, GAP proteins, such as CrossGAP/Vilse (Hu et al., 2005; Lundstrom et al., 2004) and Slit-RoboGAPs (srGAPs), control cytoskeletal dynamics (Wong et al., 2001), as do GEF proteins like

Box 2. The netrin/Dcc axon guidance signalling pathway

Netrins are a family of extracellular guidance molecules that share sequence homology with laminin and that control axon guidance and cell migration. Netrin is also involved in other functional roles, such as tissue morphogenesis, vascular development, cancer and cell survival (Rajasekharan and Kennedy, 2009). In vertebrates, the netrins consist of three secreted proteins, netrin 1, netrin 3 and netrin 4, and two glycosylphosphatidylinositol (GPI)-anchored membrane proteins, netrin G1 and netrin G2 (Cirulli and Yebra, 2007). The secreted netrins can function as either a chemoattractant or a chemorepellent. This dual activity is dependent upon the presence of distinct receptors, cell types and cellular context. Netrin 1 produces attractive effects via receptors of the deleted in colorectal cancer (Dcc) family, which includes the vertebrate-associated receptors Dcc and neogenin (Keino-Masu et al., 1996), the *C. elegans* receptor UNC-40 (Chan et al., 1996) and the Frazzled protein of *Drosophila* (Kolodziej et al., 1996). Netrin 1 mediates repulsive effects through UNC-5 receptors alone or in combination with Dcc (Hong et al., 1999; Keleman and Dickson, 2001). Recently, the Down syndrome cell adhesion molecule (Dscam) has been also identified as a netrin 1 receptor (Ly et al., 2008). Netrins signal via several different transduction pathways, including the small GTPases and various kinases [such as mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK), Src family kinase and phosphatidylinositol 3-kinase (PI3K)], and also through calcium, cyclic nucleotides, transcription factors, nuclear factor of activated T-cells (NFAT), and Elk1, which contributes to the reorganisation of the cytoskeleton (Barallobre et al., 2005). Although the mechanism remains unclear, Netrin/Dcc activity can also be influenced by the Slit/Robo signalling pathway through the direct interaction of their intracellular domains, which results in the inhibition of netrin attraction (Stein and Tessier-Lavigne, 2001).

Son of sevenless (Sos) (Yang and Bashaw, 2006) (see Fig. 1). Slit-Robo GAP1, 2 and 3 (srGAP1, 2 and 3) were identified in a yeast two-hybrid screen for binding partners of the CC3 domain of Robo1 (Wong et al., 2001). These proteins contain a GAP domain, a SH3 domain (which binds the proline-rich region of Robo1) and a Fes/CIP4 (FCH) homology domain (Wong et al., 2001; Li et al., 2006). SrGAPs are widely expressed in the mouse nervous system (Bacon et al., 2009; Endris et al., 2002; Guerrier et al., 2009; Madura et al., 2004; Waltereit et al., 2008; Yao et al., 2008), and bind to WASP/WAVE proteins, which are involved in actin reorganisation (Soderling et al., 2007; Wong et al., 2001). SrGAP1 mediates the repulsive effect of Slit on migrating subventricular zone-derived neural progenitors, through its GAP domain, by inactivating Cdc42 and RhoA but not Rac1 (Wong et al., 2001). By contrast, SrGAP3 (also known as MEGAP and WRP), mutations in which have been associated with severe mental retardation in humans (Endris et al., 2002) (see also Hamdan et al., 2009), regulates Rac activity and plays a role in neuronal morphogenesis, synaptic plasticity and cell migration (Endris et al., 2002; Soderling et al., 2002; Soderling et al., 2007; Yang et al., 2006). A recent study showed that srGAP2, through its F-BAR (Fes-Cip4–Bin/Amphiphysin/Rsv) domain, is a key regulator of neuronal migration (Guerrier et al., 2009). SrGAP2 promotes neurite extension and branching during mouse cortical development in vivo by generating outward protrusions of the neuronal membrane, thereby inhibiting neuronal migration as observed in vitro in cortical slices. However, it will be important to determine to what extent srGAP functions are associated with Slit/Robo signalling.

Regulation of cell adhesion

Increasing and recent evidence suggests that Slit and Robo are important regulators of cell adhesion, especially during migration.

Neural crest cells (NCCs) and placodal cells delaminating from the neural tube and epibranchial ectoderm, respectively, migrate along precise pathways in the embryo to generate a variety of structures, such as sensory ganglia and parts of the cardiovascular system (Shiau et al., 2008; Stoller and Epstein, 2005). It had been reported that Slits influence the motility and patterning of NCCs, through a classic repulsive mechanism (De Bellard et al., 2003). Therefore, Slit2 appears to prevent Robo1-expressing trunk NCCs from entering the gut region (De Bellard et al., 2003) and might repel them from the dorsal part of the embryo (Jia et al., 2005). Robo1/Slit2 signalling could also control the migration of cardiac NCCs, as suggested by the fact that Robo1 is expressed in the NCC lineage and by the fact that Slit2 is selectively downregulated in mice deficient for the T-box transcription factor Tbx1. Slit is also downregulated in *Gbx2* (gastrulation brain homeobox 2) knockout mice, and both Tbx1 and Gbx2 control cardiac NCC migration during fourth pharyngeal arch artery formation (Calmont et al., 2009). Some cranial ganglia, such as the trigeminal ganglion, have a mixed origin, containing both placodal cells and NCCs. It was recently shown that during gangliogenesis cross talk occurs between these two cell populations, and that Slit1, secreted by NCCs, promotes the aggregation of Robo2-expressing placodal cells (Shiau et al., 2008). Interestingly, Robo2 function involves the calcium-dependent cell-adhesion molecule N-cadherin, which is also expressed by placodal cells (Shiau and Bronner-Fraser, 2009). N-cadherin is expressed at the surface of placodal cells, and the aggregation of these cells increases upon Slit1 binding to Robo2, although the exact molecular mechanism is unknown (Shiau and Bronner-Fraser, 2009). A similar process could participate in the development of several neuronal cell types, including precerebellar neurons and cerebellar granule cells, which express Robo2, and the migration of which requires N-cadherin (Rieger et al., 2009; Taniguchi et al., 2006).

In vitro studies had previously linked Slit/Robo signalling to N-cadherin and cell adhesion (Rhee et al., 2007; Rhee et al., 2002). These studies reported that the activation of Robo2 by Slit triggers the formation of a quaternary complex, consisting of Robo, Abelson tyrosine kinase (Abl), Cables (a Cdk5 and Abl substrate) and N-cadherin, which leads to the phosphorylation and detachment of β-catenin from N-cadherin to result in the inhibition of cell adhesion. In breast cancer cells, Slit2 overexpression increases proteasomal degradation of β-catenin, but also seems to facilitate/promote its association with E-cadherin (Prasad et al., 2008). Although these studies link Slit/Robo signalling to cell adhesion, this can cause either inhibition or enhancement of cell adhesion depending on the system, and, possibly, on the presence of co-receptors.

An anti-adhesive activity of Slit/Robo signalling has also been reported to function during cardiac tube formation in *Drosophila* (Qian et al., 2005; Santiago-Martinez et al., 2006). In this system, the cardiac lumen forms after the preferential attachment of the dorsal and ventral edges of pairs of cardioblasts, which are aligned in two symmetrical rows along the dorsal midline. It was previously shown that the migration and alignment of cardiac cells is regulated by Slit/Robo repulsion (Qian et al., 2005; Santiago-Martinez et al., 2006; Zmojdzian et al., 2008). More importantly, the lack of contact between the membrane of bilateral pairs of cardioblasts at the lumenal surface involves Slit/Robo signalling,

which, via an autocrine or paracrine action, leads to the selected inhibition of E-cadherin adhesion in this central/luminal domain (Medioni et al., 2008; Santiago-Martinez et al., 2008). In vertebrates, Slit/Robo function was also proposed to contribute to the development of the cardiovascular system (Calmont et al., 2009; Liu et al., 2003), although it is still unknown whether this involves a classic, repulsive, mechanism or cadherin-dependent cell adhesion.

Although, Robo receptors share many structural features and molecular partners, mounting evidence suggests that distinct Robo receptors exert some unique functions. Vertebrate Robo3 provides the best example.

Vertebrate Robo3: the black sheep of the Robo family?

Vertebrate Robo3 differs from other Robo receptors for several reasons. First, Robo3 shows greater structural heterogeneity; it possesses at least four splice variants and possibly a secreted form (Chen et al., 2008b; Yuan et al., 1999), and in mammals it lacks the CC1 domain (Yuan et al., 1999). Second, mammalian Robo3 has only been detected in the nervous system (Barber et al., 2009; Jen et al., 2004; Marillat et al., 2004; Sabatier et al., 2004). Third, its ability to bind Slits is debated (Camurri et al., 2005; Mambetisaeva et al., 2005), and there is to date no biological response associated with the direct binding of Slit to Robo3. Last, although it primarily controls axon guidance at the midline, Robo3 function is unusual when compared with the functions of the other Robos (Fig. 2).

In the developing spinal cord and hindbrain, Robo3 is expressed at high levels on commissural axons until they have crossed the floor plate (Marillat et al., 2004; Sabatier et al., 2004; Tamada et al., 2008). In *Robo3* knockout mice, most commissural axons fail to cross the midline and neuronal migration across the floor plate is prevented (Marillat et al., 2004; Sabatier et al., 2004; Tamada et al., 2008) (see Fig. 2C). These findings have led to a model in which, before midline crossing, the presence of Robo3 in commissural axons interferes with Slit/Robo repulsion, allowing commissural axons and neurons to progress towards the ventral midline under the action of chemoattractants, such as netrin 1 (Fig. 2A-C). After crossing, Robo3 expression is downregulated, allowing the activation of Robo1 and Robo2 by Slit, which triggers axon repulsion. This model is further supported by the fact that there is axon stalling and errors at the midline in Slit and Robo mutants (Chen et al., 2008b; Long et al., 2004). In addition, crossing is 'partially' restored for commissural axons of the spinal cord in *Robo1/Robo3* double knockouts and in *Robo1/Robo2/Robo3* triple knockouts (Chen et al., 2008b; Sabatier et al., 2004). Likewise, the migration of LRN neurons across the midline is also rescued when Robo1 and Robo2 are deleted in a *Robo3* null background (Di Meglio et al., 2008). Interestingly, in spinal cord commissural axons, two splice variants of Robo3, Robo3.1 and Robo3.2, have opposite actions on commissural axons (Chen et al., 2008b). Robo3.1 is expressed before crossing and seems to inhibit Robo1/Robo2 repulsion, whereas Robo3.2 is expressed after midline crossing and appears to cooperate with Robo1 and Robo2 to repel commissural axons away from the floor plate (see Fig. 2A). However, the mechanism by which Robo3 blocks Slit/Robo signalling is still unknown (Chen et al., 2008b; Sabatier et al., 2004). In addition, the axons of IONs are still unable to cross the midline in *Robo1/Robo2/Robo3* triple knockouts (Di Meglio et al., 2008), indicating that in this system Robo3 functions independently of other Robos (Fig. 2). An alternative model supports a chemoattractive function for Robo3 in commissural axons rather than an anti-repulsive activity (Sabatier et al., 2004); in this model, in the absence of Robo3, commissural axons would fail to be attracted to the floor plate.

Although it is expressed in the forebrain, the function of Robo3 in this region remains mysterious as the main forebrain commissures develop normally in mice and humans deficient for *Robo3* (Haller et al., 2008; Jen et al., 2004; Marillat et al., 2004; Renier et al., 2010). To date, the only evidence that Robo3 possesses any function in this region comes from a study of cortical interneuron migration (Barber et al., 2009). In *Robo3* null mice, but not in *Robo1* or *Robo2* nulls, fewer cortical interneurons are present in the marginal zone of the early cortex, a region in which Slit1 and Slit3 are strongly expressed, indicating that the absence of Robo3 decreases Slit repulsion and allows some neurons to enter the marginal zone. By contrast, forebrain commissures are disorganised in *Slit1/Slit2* and *Robo1/Robo2* simple and double knockouts (Andrews et al., 2006; Bagri et al., 2002; Lopez-Bendito et al., 2007). This suggests that, in mammals, Robo3-independent molecular mechanisms regulate Slit/Robo function, at least in some commissural axons.

Whatever its mechanism of action is, Robo3 deficiency has some profound effects on motor and sensory motor behaviours in mice, zebrafish and humans (Amoiridis et al., 2006; Burgess et al., 2009; Jen, 2008; Jen et al., 2004; Renier et al., 2010). Humans suffering from a rare autosomal recessive disease named 'horizontal gaze palsy with progressive scoliosis' (or HGPPS) carry mutations in *ROBO3*. In HGPPS patients, both the descending corticospinal tract motor projections and the ascending lemniscal sensory projections are abnormally uncrossed (Haller et al., 2008; Jen, 2008). In addition, patients are unable to perform conjugate lateral eye movements (Jen, 2008). Likewise, *robo3/twitch twice* double-mutant zebrafish have defects in eye movements and balance (Burgess et al., 2009). *Robo3* null mice die shortly after birth, precluding behavioural studies. However, recently, a unique genetic tool designed to probe the function of selected hindbrain commissures was generated by crossing a novel *Robo3* conditional-knockout line with transgenic mice expressing Cre recombinase in specific subsets of hindbrain commissural neurons (Renier et al., 2010). In Robo3-deficient lines, commissural axons project ipsilaterally, and this is associated with profound motor, sensory and sensory-motor deficits.

Apart from controlling commissural axon guidance and cell migration, there is evidence that Robo/Slit signalling also controls later aspects of axonal development, as we discuss below.

A role in axonal targeting

Once they have reached their terminal territory, axons have to find their target cells and form synaptic contacts. In most neuronal systems, axons do not project randomly on target cells but follow a precise spatial pattern that often reflects their respective origins in the brain, and defines so-called topographic maps.

A role for Slits and Robos in axonal targeting has best been described in the mouse olfactory system (Cho et al., 2009). Briefly, in the principal olfactory system, olfactory sensory neurons (OSNs) extend from the olfactory epithelium to the olfactory bulb (OB) where they connect with the major OB output neurons, mitral cells and tufted cells. A distinct feature of the mammalian olfactory system is that all OSNs that express the same odorant receptor converge within each OB on a single glomerulus (Ressler, 1994; Vassar, 1994). In many mammals, an accessory olfactory system exists, which plays an important role in pheromone sensing and

social behaviour (Dulac and Wagner, 2006). In this system, vomeronasal sensory neurons (VSN) of the vomeronasal organ (VNO), project to glomeruli located in the accessory olfactory bulb (AOB). VSNs project in a topographical manner. As such, VSNs that originate in the apical and basal regions of the VNO, project to the anterior and posterior portions of the AOB, respectively (Dulac and Wagner, 2006) (Fig. 3). Slits and Robos have been implicated in controlling the proper targeting of axons in both olfactory systems.

Slits are expressed in a gradient in the AOB, with high expression in the anterior region and low expression posteriorly (Knoll et al., 2003). In addition, Slit1 controls the targeting of basal VSN axons, which show abnormal extension into the anterior AOB in *Slit1* knockouts (Cloutier et al., 2004) (Fig. 3B). Basal VSN axons are also misrouted in *Slit1/Slit2* double-heterozygous mice, but not in *Slit3* knockouts, thus establishing a role for Slit2, in addition to Slit1, in the proper targeting of VSN axons to the AOB (Prince et al., 2009). Although VSNs express both Robo1 and Robo2 receptors, only Robo2 appears to be essential for maintaining proper segregation of VSN axons in the AOB. Indeed, when Robo2 is specifically deleted from VSNs, some of the basal VSN neurons are misrouted into the anterior AOB, indicating that Slit1 function is primarily mediated by Robo2 (Prince et al., 2009).

In mice, OSNs from the dorsomedial epithelium project to dorsally located glomeruli in the OB, and OSNs from the ventrolateral region of the olfactory epithelium (OE) extend to glomeruli more ventrally in the OB (Miyamichi et al., 2005) (Fig. 3C). Robo2 has a graded expression profile in the OE, with highest levels in the dorsomedial region (Cho et al., 2007). In *Robo2* and *Slit1* knockout mice, a subset of OSN axons that normally segregate in a glomerulus located in the dorsal region of the OB form a glomerulus in the ventral region, which indicates that Robo2 and Slit1 are necessary for the proper targeting of OSN axons to their appropriate location within the OB (Cho et al., 2007). In *robo2* mutant zebrafish, glomerular spatial arrangements in the main olfactory system are also impaired (Miyasaka et al., 2005). The mistargeting of OSN axons is even more obvious in *Robo1/Robo2* and *Slit1/Slit2* double-null mice, in which OSNs that express the same odorant receptors project on to multiple glomeruli (Nguyen-Ba-Charvet et al., 2008) (Fig. 3C). Interestingly, Slits and Robos also act on other aspects of the development of the olfactory system, in controlling the migration of OB interneurons (see references above) and in the pathfinding of the axons of OB output neurons (Fouquet et al., 2007; Nguyen-Ba-Charvet et al., 2002).

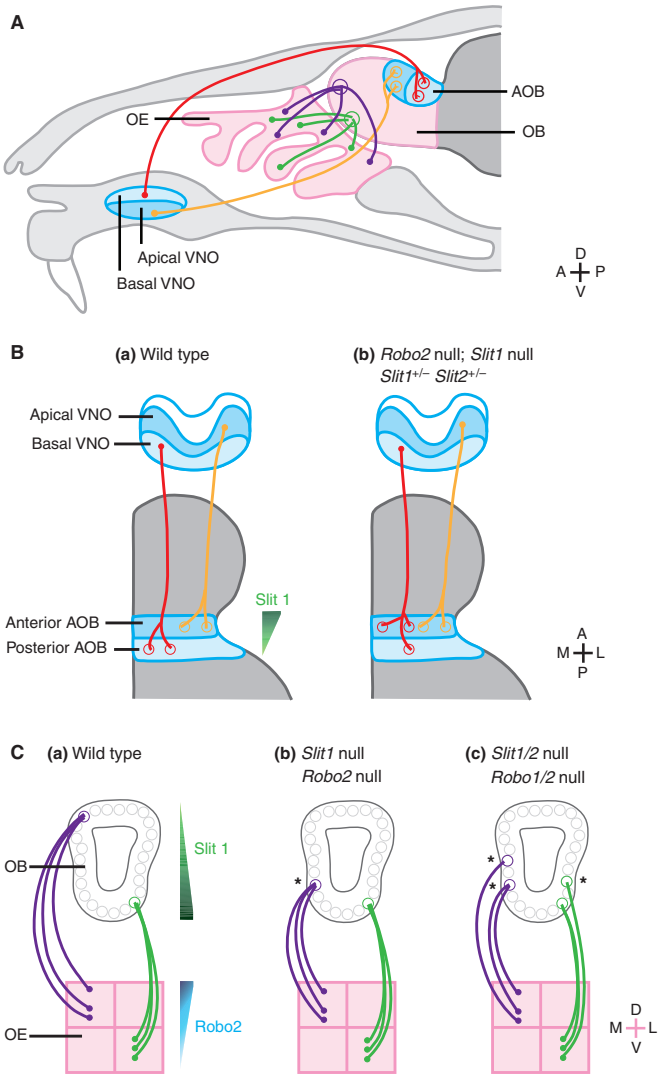

Fig. 3. The main and accessory olfactory systems in wild-type and *Slit/Robo* knockout mice. (**A**) A sagittal section through a rodent head, depicting the types of axonal segregation that occur in the main and vomeronasal olfactory systems. In the main olfactory system [shown in pink, consisting of the olfactory bulb (OB) and olfactory epithelium (OE)], olfactory sensory neurons (OSNs) located in the OE that express the same olfactory receptors (ORs, depicted here in purple and green) project to specific glomeruli in the OB. In the accessory olfactory system (shown in blue), vomeronasal sensory neurons (VSNs) located in the apical vomeronasal organ (VNO) (depicted in orange) project to the anterior portion of the accessory olfactory bulb (AOB), and those located in the basal VNO (depicted in red) project to the posterior AOB. (**B**) Depiction of the VNO and the AOB in the horizontal plane. In the AOB, there is a Slit1 (and Slit3) gradient, with high levels in the anterior AOB and lower levels in the posterior AOB. (**a**) In wild-type mice, Robo2-expressing VSNs (depicted here in red and orange) from the basal and apical VNO project to the posterior and anterior AOB, respectively. (**b**) In *Robo2* knockout mice, *Slit1* knockout mice and *Slit1/Slit2* double heterozygous mice, basal VSNs misproject to glomeruli located in the posterior AOB. (**C**) A coronal depiction of the main olfactory system. OSNs (purple and green) from the OE (depicted as a pink box) project to glomeruli (shown as pale grey circles) located within the olfactory bulb. (**a**) In wild type, a Robo2 gradient exists in the main olfactory system, with highest levels in the dorsomedial OE. A Slit1 gradient also exists in the OB, with high levels ventrally and lower levels dorsally. OSNs that express the same OR and that are located in the dorsomedial OE normally project to glomeruli in the dorsal OB. (**b**) In *Robo2* and *Slit1* knockout mice, certain dorsal OSNs project aberrantly to a ventrally located glomerulus (highlighted with an asterisk). (**c**) In *Robo1/Robo2* and *Slit1/Slit2* knockout mice, dorsal and ventral OSNs expressing the same receptor aberrantly project to multiple glomeruli (highlighted with asterisks). A, anterior; D, dorsal; l, lateral; M, medial; P, posterior; V, ventral.

Interestingly, in *Drosophila*, two recent papers have uncovered a similar role for Slit/Robo outside of the nervous system, in the development of a myotopic map (see Glossary, Box 1), in which leg motoneuron dendrites are targeted to their respective muscle field by the combinatorial signalling of Slit/Robo and netrin 1/Frazzled (Brierley et al., 2009; Mauss et al., 2009). It is unclear whether Slit/Robo play a similar role in the targeting of motoneuron dendrites in vertebrates.

Molecular mechanisms regulating Slit/Robo function

Migrating cells and growing axons often change direction during their journey, and the repertoire of receptors they express at their surface is tightly controlled by the environmental factors they encounter. Accordingly, during mouse development, the expression of Robo receptors is known to be precisely regulated in time and space. By contrast, Slits appear to be expressed in a more stable way in the embryo. Below, we review some of the ways in which transcriptional regulation, receptor transport to the membrane and alternative splicing control the expression of Robo receptors.

Transcriptional regulation

The transcriptional regulation of Robo receptor expression at the midline contributes to the modulation of axon guidance decisions. The molecular mechanisms that underpin this regulation have remained elusive, but recent studies have identified some of the transcription factors involved.

In *Drosophila,* the protein Midline, which belongs to the T-box family of transcription factors (Stennard and Harvey, 2005), controls the transcription of Robo and Slit along the midline of the central and peripheral nervous systems (Liu et al., 2009). Interestingly, Midline has orthologues in other species, such as Tbx20 in the mouse (Liu et al., 2009). Moreover, this transcription factor is evolutionarily conserved from flies to humans, suggesting that its transcriptional regulation of Robos and Slits is a conserved mechanism (Takeuchi et al., 2005). Accordingly, both *Tbx20* and *Slit1/Slit2* knockout mice have defects in the development of hindbrain motoneurons (Hammond et al., 2005; Song et al., 2006). In the mouse, Tbx1 regulates a second transcription factor, Gbx2, which functions in hindbrain and pharyngeal arch artery (PAA) development (Calmont et al., 2009). In further support of this, the expression of Slit2 and Robo1 is downregulated in the pharyngeal region of *Tbx1* and *Gbx2* null mouse mutants (Calmont et al., 2009). This suggests that these transcription factors directly regulate Slit2 and Robo1 expression, and that Slits and Robos play a role in controlling PAA development.

In the case of Robo3, several transcription factors that could control its expression have been identified. Robo3 was discovered as a gene that is upregulated in retinoblastoma (*Rb*)-deficient mice (Yuan et al., 1999). It was later confirmed that Rb represses *Robo3* promoter activity in vitro but also that Pax2 activates *Robo3* transcription by binding to Rb and thereby inhibiting its repressor function on *Robo3* (Yuan et al., 2002).

Robo3 expression is also upregulated in a subset of mammillary neurons of the hypothalamus of mouse embryos deficient for the basic helix-loop-helix (bHLH) transcription factors Sim1 and Sim2 (Marion et al., 2005). Interestingly, mammillary axons are rerouted toward the midline in *Sim1/Sim2* null mice, possibly because of abnormal Robo3 expression (Marion et al., 2005).

Different combinations of transcription factors from the LIM homeodomain (LIM-HD) family have been found to confer subtype identity to neurons in which they are expressed; they are also able to control the migration of these neurons (Avraham et al., 2009; Polleux et al., 2007). In the dorsal spinal cord of the mouse, a subset of interneurons, the dI1 neurons, express the two LIM-HD transcription factors, Lhx2 and Lhx9. However, the level of expression of these two factors differs between dI1 interneurons that project ipsilaterally and those that project contralaterally (Wilson et al., 2008). The commissural contingent expresses higher levels of Lhx2 and lower levels of Lhx9 than does the ipsilateral group. In *Lhx2*/*Lhx9* double-null mutants, the fate of dI1 interneurons is not affected, but commissural dI1 neurons fail to express *Robo3*, and, as a result, their axons do not project across the floor plate. In gel shift assays, Lhx2 directly interacts with consensus LIM binding sites in *Robo3*, which indicates that LIM-HD proteins are activators of *Robo3* transcription (Wilson et al., 2008).

The *Hox* genes encode homeodomain-containing transcription factors that control the patterning of the body axis, as well as neuronal specification and connectivity (Dasen et al., 2008). They are highly expressed in the hindbrain in a rhombomere-specific code (Kiecker and Lumsden, 2005). The analysis of pontine neuron development in *Hoxa2* and *Hoxb2* null mice has revealed that their migration is perturbed and that cohorts of neurons abandon the main stream prematurely (Geisen et al., 2008). This phenotype is highly reminiscent of the defects observed in *Robo1/Robo2*, *Slit1/Slit2* and *Robo2/Slit2* double-null mice. This result together with the presence of Hox binding sites in the *Robo2* locus, suggests that Robo genes could be direct targets of Hox transcription factors. This hypothesis is further supported by chromatin immunoprecipitation experiments and the downregulation of *Robo2* expression in *Hoxa2/Hoxb2*-deficient pontine neurons (Geisen et al., 2008). The presence of Hox binding sites in the *Drosophila Robo2* gene suggests that Hox transcription factors might control Robo gene expression in many species and tissues (Kraut and Zinn, 2004).

Robo receptor transport regulation

In the ventral nerve cord of *Drosophila*, Robo-expressing commissural axons are repelled by Slits secreted at the midline. As pre-crossing commissural axons grow towards the midline, Robo is negatively regulated by the transmembrane protein Commissureless (Comm), which diverts Robo to the endosomes for degradation (Keleman et al., 2002). Upon entering the midline, an unknown mechanism diminishes Comm expression, resulting in increased amounts of Robo at the membrane and, consequently, in the axon being repelled from the midline to prevent it from re-crossing (Keleman et al., 2005). Comm expression can also be regulated in other ways. For instance, a recent study has revealed that Frazzled/Dcc (Fra), in addition to being a receptor for Netrin, can also influence the expression of *comm* mRNA (Yang et al., 2009). In this context, Frazzled concurrently regulates the attraction of commissural axons in response to Netrin and the silencing of repulsive cues by negatively regulating the presence of Robo at the membrane via *comm* expression. This latter function occurs independently of the presence of Netrin. Moreover, other data obtained in *Drosophila* show that the RNA-binding protein Elav (embryonic lethal, abnormal vision) interacts with Robo and Slit in a dose-dependent manner and regulates *comm* mRNA (Simionato et al., 2007).

As a Comm homologue has not been found in vertebrates, it was not clear until recently whether receptor sorting also contributed to the regulation of Robo expression in vertebrates. However, new yeast two-hybrid experiments have identified the ubiquitin-specific protease 33 (Usp33) as a Robo1 partner and have provided in vitro

and in vivo evidence that these two proteins interact (Yuasa-Kawada et al., 2009a; Yuasa-Kawada et al., 2009b). The role of ubiquitin-mediated modification in regulating protein stability and function has been well established (Hershko and Ciechanover, 1998). Upon examining the effect of Usp33 interaction with Robo, it was demonstrated that this de-ubiquitylating enzyme is required for the Slit-responsiveness of commissural neurons and of breast cancer cells (Yuasa-Kawada et al., 2009a; Yuasa-Kawada et al., 2009b). Usp33 mediates both of these functions by either preventing Robo1 from being targeted for degradation or by facilitating its endosomal recycling.

Slit/Robo and netrin 1/Dcc inter-regulation

Slit/Robo and netrin 1/Dcc (see Box 2) are key components in midline crossing, and there is increasing evidence that their signalling pathways are intertwined and regulate one another at different level. As mentioned above, netrin 1 attracts commissural axons at the midline upon binding to Dcc, whereas Slit repels them after binding Robo1 or Robo2 (Fig. 4A). A genetic study in *Drosophila* using Robo/Dcc chimeras demonstrated that in commissural axons the cytosolic domains of these receptors are the effectors of their attractive or repulsive responses (Bashaw and Goodman, 1999). Moreover, in the presence of Slit, Robo1 binds to Dcc to silence netrin 1 attraction (Stein and Tessier-Lavigne, 2001). Whether Dcc can interact directly with the other Robo receptors to modulate attraction or repulsion is currently unknown. In addition, experiments in *C. elegans* have shown that UNC-40/Dcc participates in SLT-1/SAX-3 signalling in a netrin-independent manner and that this modulation involves UNC-34/Ena. It had been previously shown that UNC-40/Dcc potentiates the netrin 1 repulsion mediated by the receptor UNC-5 (Colavita and Culotti, 1998; Hedgecock et al., 1990; Hong et al., 1999). In

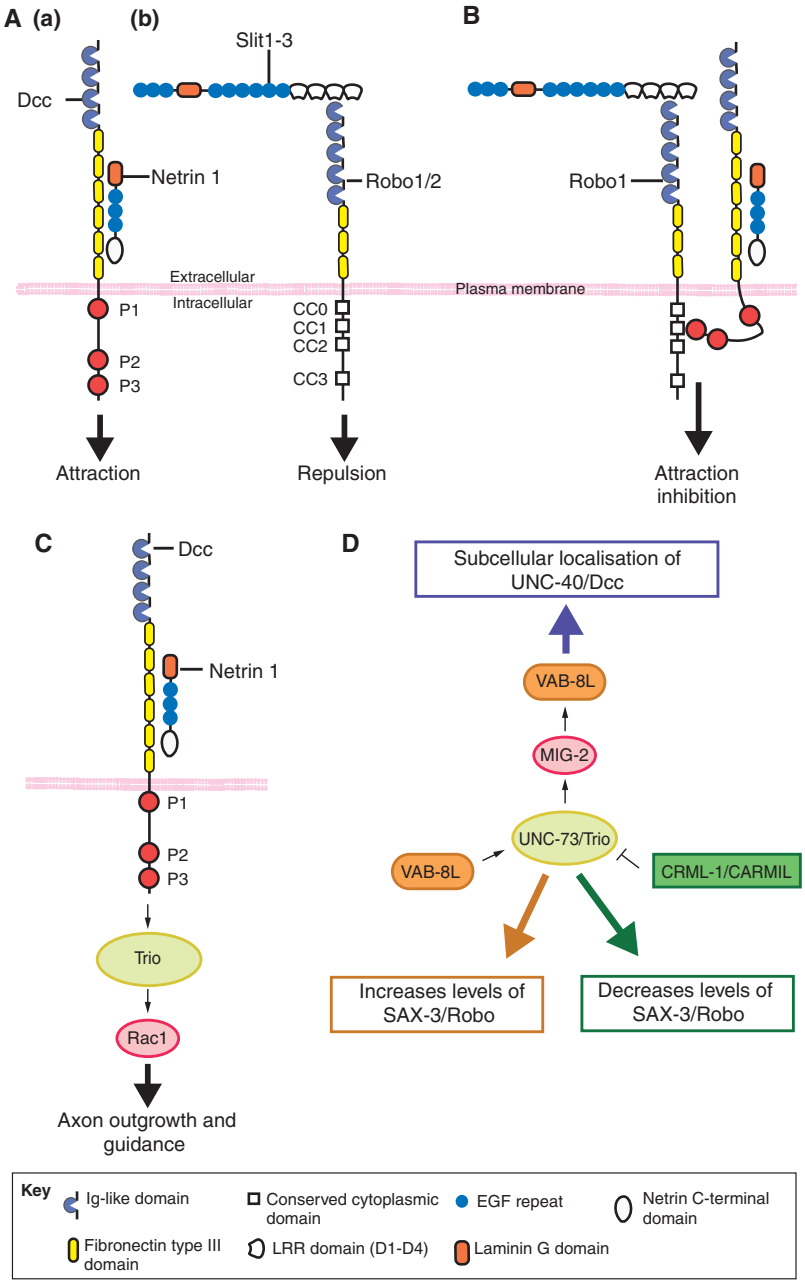

Fig. 4. Netrin/Dcc and Slit/Robo pathway interactions. Schematic of the interactions between Netrin/Dcc and Slit/Robo pathways. (**A**, **a**) At the midline, netrin 1 attracts commissural axons through Dcc. (**b**) Conversely, Slit mediates commissural axon repulsion upon binding to Robo1 or Robo2. (**B**) In the presence of Slit and netrin 1, the intracellular domains of Dcc (P3) and Robo1 (CC1) interact, silencing netrin 1 attraction. (**C**) Trio is a GEF that is required during netrin/Dcc signalling to activate Rac1 in mice. (**D**) In *C. elegans*, UNC-73/Trio modulates both UNC-40/Dcc and SAX-3/Robo signalling pathways. UNC-73/Trio targets the Rho GTPase MIG-2 and the kinesin-like protein VAB-8L, which then act as upstream regulators of UNC-40/Dcc to specify its subcellular localisation. However, VAB-8L and CRML-1/CARMIL actively and negatively regulate UNC-73/Trio to induce the up- and downregulation, respectively, of SAX-3/Robo levels at the membrane.

mouse neurons, netrin 1 binding to Dcc induces Rac1 activation in the presence of the RhoGEF Trio protein (Briancon-Marjollet et al., 2008) (Fig. 4C). Moreover, in *C. elegans*, UNC-73, a homologue of Trio, modulates netrin and Slit signalling. Indeed, UNC-73/Trio influences UNC-40/Dcc subcellular localisation by acting through MIG-2, a Rho GTPase, and VAB-8L, a kinesin-like protein (Levy-Strumpf and Culotti, 2007) (Fig. 4D). Likewise, genetic evidence suggests that VAB-8L modulates UNC-73/Trio, which then increases SAX-3/Robo levels at the membrane (Watari-Goshima et al., 2007) (Fig. 4D). By contrast, recent work indicates that UNC-73/Trio can also be negatively regulated by the CRML-1/CARMIL (Capping, Arp2/3, Myosin I Linker) protein. This decreases the levels of SAX-3/Robo at the membrane (Vanderzalm et al., 2009) (Fig. 4D).

Finally, although the effect of netrins and Slits are diametrically opposed, they act together to specify the lateral positioning of dopaminergic axons in the diencephalon (Kastenhuber et al., 2009). In this system, attraction mediated by Dcc/netrin 1 appears to be counterbalanced by Slit/Robo2 signalling (Kastenhuber et al., 2009). Similarly, Robo and Frazzled are required cell autonomously in motoneurons for the appropriate targeting of their neurites to their respective muscle fields; this action is controlled by the combinatorial and opposing actions of Robo and Frazzled (Brierley et al., 2009; Mauss et al., 2009).

Conclusion

As illustrated here, the range of biological functions that involve the Slits and Robos is much broader than was originally thought. Thus, a major question in this field concerns how different Slits are able to mediate their disparate actions. Slits have been shown to bind with similar affinity to all of the Robo receptors using evolutionarily conserved binding residues (Brose et al., 1999; Morlot et al., 2007). However, in different systems, Slits mediate differing effects; for instance OSN axonal targeting is modulated by Slit1 and Slit2, but not by Slit3 (Prince et al., 2009). It will therefore be important to identify the molecules, such as co-receptors, that mediate such ligand specificity.

Another remaining enigma is how increasing or decreasing levels of Robo receptors and/or different combinations of Robo receptors mediate varying effects within a given cell or between neighbouring cells.

In *Drosophila*, a combinatorial Robo code specifies the lateral position of longitudinal axons; in this system, the expression of only one Robo receptor (Robo1) specifies a medial positioning of these axons, whereas expression of all three Robo receptors drives axons laterally. By contrast, the expression of Robo1 and Robo3 causes an intermediate positioning (Rajagopalan et al., 2000; Simpson et al., 2000). One possible interpretation of these results is that the higher the levels are of Robo receptors at the surface of longitudinal axons, the more these axons will respond to the repulsive effect of Slits that are present at the midline. Similarly, there are phenotypical differences between *Robo1* null and hypomorphic mutant mice, which indicate that different levels of Robo receptors at the membrane within the same cell mediate subtly different effects (Andrews et al., 2006; Lopez-Bendito et al., 2007).

However, more recent studies suggest that, in *Drosophila*, the lateral position of longitudinal axons is not determined by a particular combination of Robo proteins [the so-called 'Robo code' (Rajagopalan et al., 2000; Simpson et al., 2000)], but is rather driven by differential Robo gene expression (Evans et al., 2010; Spitzweck et al., 2010). The different responses of Robos to Slits would reflect intrinsic structural differences in the extracellular Ig domains of Robos. For instance, Robo2 Ig1 and Ig3 domains specify lateral positioning of axons, the Ig2 domain promotes midline crossing and the Robo1 cytoplasmic domain prevents midline crossing (Spitzweck et al., 2010). There are also cases in which the identity of the Robo receptors or of their combination could be the determining factor in the type of response generated. This could for instance contribute to specifying the position of longitudinal axons in the *Drosophila* nerve cord. Likewise, in the mouse spinal cord, Robo2 rather than Robo1, controls the positioning of a longitudinal axonal tract, the lateral funiculus (Long et al., 2004). Moreover, chick commissural axons that prematurely misexpress Robo2 fail to enter the floor plate and preferentially adopt a longitudinal phenotype (Reeber et al., 2008). Finally, in the *Drosophila* tergotrochanteral motoneuron (TTMn), the dendrites of which contact the giant fiber, the overexpression of Robo repels TTMn dendrites from the midline. By contrast, the overexpression of Robo2 or Robo3 at the surface of TTMn dendrites has no noticeable effect (Godenschwege et al., 2002). Further studies need to be conducted on Robo receptor subtypes to elucidate the specificity of each isoform in mediating particular effects in different systems.

Research into the signalling mechanisms of Slit/Robo needs to advance in order to resolve these outstanding questions. Such advances will require adopting a cross-disciplinary approach for studying Slits and Robos in order to integrate information from such disparate fields as CNS and heart development, and immune cell migration and tumourigenesis.

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Competing interests statement

The authors declare no competing financial interests.

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