Chapter 9
Structure and Function of Roundabout Receptors

Francesco Bisiak and Andrew A. McCarthy

Abstract The creation of complex neuronal networks relies on ligand-receptor interactions that mediate attraction or repulsion towards specific targets. Roundabouts comprise a family of single-pass transmembrane receptors facilitating this process upon interaction with the soluble extracellular ligand Slit protein family emanating from the midline. Due to the complexity and flexible nature of Robo receptors, their overall structure has remained elusive until now. Recent structural studies of the Robo1 and Robo2 ectodomains have provided the basis for a better understanding of their signalling mechanism. These structures reveal how Robo receptors adopt an auto-inhibited conformation on the cell surface that can be further stabilised by *cis* and/or *trans* oligmerisation arrays. Upon Slit-N binding Robo receptors must undergo a conformational change for Ig4 mediated dimerisation and signaling, probably via endocytosis. Furthermore, it's become clear that Robo receptors do not only act alone, but as large and more complex cell surface receptor assemblies to manifest directional and growth effects in a concerted fashion. These context dependent assemblies provide a mechanism to fine tune attractive and repulsive signals in a combinatorial manner required during neuronal development. While a mechanistic understanding of Slit mediated Robo signaling has advanced significantly further structural studies on larger assemblies are required for the design of new experiments to elucidate their role in cell surface receptor complexes. These will be necessary to understand the role of Slit-Robo signaling in neurogenesis, angiogenesis, organ development and cancer progression. In this chapter, we provide a review of the current knowledge in the field with a particular focus on the Roundabout receptor family.

Keywords Neurons · Guidance cues · Receptors · Signalling · Robo · Slit

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Introduction

Neurons are a highly differentiated cell type that can receive, process, and transmit nerve impulses over long distances. They are characterised by a cell body (soma) surrounded by short branched extensions (dendrites) and a long extension (axon). Neurons typically make multiple connections to form complex circuits such as the central nervous system (CNS). During development the coordinated extension of neurons to their target destination, essential for proper CNS wiring, is called axon guidance (Chédotal and Richards 2010). In Bilateria, the midline forms an important division between the two symmetric halves of the CNS, and therefore acts as an important guidance checkpoint during neuronal development. Critical for proper CNS function is a connection of the two bilateral sides, requiring a crossing of the midline in a strictly regulated manner (Placzek and Briscoe 2005). Here, a subset of commissural neurons cross the midline for connection, while another, ipsilateral neurons, remain on the same side (Fig. 9.1). In order to achieve this remarkable feat a navigational pathway is delineated by a series of attractive and repulsive cues.

Neuronal development cues bind their cognate cell surface receptors to trigger attractive or repulsive cellular responses. There are four major families of axon guidance cues that can interact with one (or more) receptors (Kolodkin and Tessier-Lavigne 2011; Seiradake et al. 2016): ephrins and Eph receptors (Kania and Klein 2016); semaphorins and plexin receptors, including neurophilin co-receptors

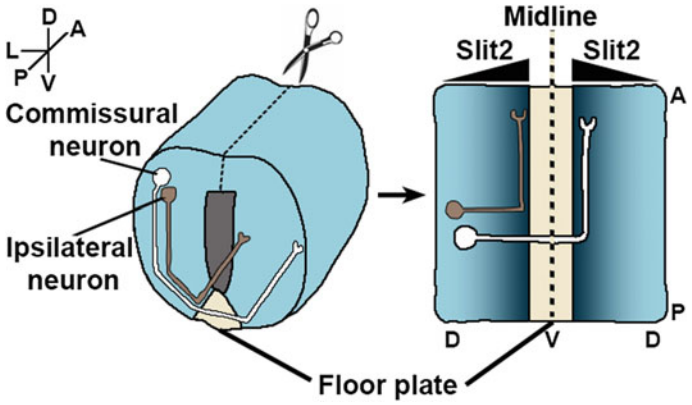

Fig. 9.1 Schematic of classical commissural and ipsilateral axon pathfinding in the spinal cord of mouse embryos. On the left is a spinal cord section from a mouse embryo showing commissural and ipsilateral axons, and midline floor plate. Commissural neurons express Robo receptors only upon crossing the midline to prevent their re-crossing, while ipsilateral maintain Robo receptor expression to always remain on the same side. The more classic 'open-book' flat format, as dissected along the spinal cord, is shown on the right to illustrate how a Slit gradient emanating from the midline is used for axon repulsion. Commissural and ipsilateral neurons are white and grey, respectively. A, anterior; P, posterior; D, dorsal; V, ventral; L, lateral

(Koropouli and Kolodkin 2014; Kolodkin and Tessier-Lavigne 2011); netrins with multiple receptors (DCC/neogenin, UNC5, Dscam and NGL1) (Seiradake et al. 2016); and Slits with Robo receptors (Blockus and Chedotal 2016). However, more recently Slit has been shown to interact with multiple partners, including Eva1C (Fujisawa et al. 2007), plexin A1 (Delloye-Bourgeois et al. 2015), dystroglycan (Wright et al. 2012), and Dscam (Dascenco et al. 2015; Alavi et al. 2016). Several other morphogen-receptor interactions are also known to contribute to axon guidance, including the Wnt and Sonic hedgehog (Shh) families that signal via interaction with multiple receptors, fibronectin leucine-rich repeat transmembrane proteins (FLRT) and some members of the Cadherin superfamily (Kolodkin and Tessier-Lavigne 2011; Seiradake et al. 2016).

The *roundabout* gene, *Robo*, was first identified in *Drosophila* genetic screens for commissural axons midline crossing defects (Kidd et al. 1998; Seeger et al. 1993). The 'Robo' name originates from stalled commissural axons at the midline creating a 'ROundaBOut' or Robo phenotype (Tear et al. 1993). Many homologs have since been identified in other species (Chédotal 2007). Following their discovery the Robo receptors were shown to act as the cognate receptors for the secreted guidance factor Slit (Brose et al. 1999; Kidd et al. 1999). Subsequently, the Slit-Robo signalling pathway has been shown to be important for many other developmental processes, including organogenesis of the kidney (Grieshammer et al. 2004), lungs (Domyan et al. 2013), and heart (Mommersteeg et al. 2013). Given their importance during development the Robo receptors have also been implicated in several types of cancer (Seth et al. 2005; Legg et al. 2008; Ballard and Hinck 2012; Huang et al. 2015), and chronic diseases such as kidney disease (Hwang et al. 2015) and liver fibrosis (Chang et al. 2015). As such they are now considered as attractive therapeutic targets. Here, we provide an overview of the Robo receptors from a structural perspective and how these insights have shed light on their activation mechanism.

The Robo Receptor Family

Robo receptors are evolutionarily conserved across bilateral anatomical species. Three *robo* genes have been identified in *Drosophila* (Seeger et al. 1993; Simpson et al. 2000; Rajagopalan et al. 2000), Zebrafish (Yuan et al. 1999b; Lee et al. 2001), and chick (Vargesson et al. 2001) while *C. elegans* contains a single *robo* ortholog, SAX-3 (Hao et al. 2001). In mammals four Robo receptors (Robo1, Robo2, Robo3 and Robo4) have been identified (Kidd et al. 1998; Yuan et al. 1999a; Huminiecki et al. 2002). Robo1, Robo3 and Robo4 are sometimes referred to as Deleted in U twenty twenty (DUTT1), Retinoblastoma-inhibiting gene 1 (Rig-1) and magic Robo, respectively, in older manuscripts. The *ROBO1* and *ROBO2* genes are located on human chromosome 3, while the *ROBO3* and *ROBO4* genes are located on chromosome 11, both in a similar head to head configuration. This closely linked chromosomal organization suggests the Robo receptors emerged from a single

ancestral vertebrate *ROBO* gene by a tandem duplication that was followed by two whole genome duplications and losses before vertebrate radiation (Zelina et al. 2014). Two tandem duplication copies exist in most vertebrates' genomes today. To add further complexity alternative splicing of mammalian *ROBO* genes with varied expression patterns and distinct guidance responses have also been reported (Clark et al. 2002; Dalkic et al. 2006; Chen et al. 2008). This mechanism was shown to be particularly important for Robo3 function (Chen et al. 2008), as discussed later, but whether this is valid for Robo1 and Robo2 function remains to be shown.

The Robo receptors are single-pass type I membrane proteins that belong to the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs). Robo receptors typically contain five C2-type Ig domains, three fibronectin (FN) type III domains, a transmembrane helix and a large unstructured intracellular region incorporating four conserved cytoplasmic (CC) motifs (Fig. 9.2). All vertebrate Robo receptors are expressed by CNS neurons, except Robo4, which is vascular specific (Huminiecki et al. 2002; Park et al. 2003). In addition, Robo4 is much smaller than the other family members, containing only two Ig and FN3 domains (Fig. 9.2). The structures of several individual and tandem Robo receptor domains, alone or in complex with interaction partners, have been determined. In particular hRobo1 Ig1–Ig2 (PDB 2V9R, 2V9Q) (Morlot et al. 2007), dRobo Ig1–2–heparin complex (PDB 2VRA) (Fukuhara et al. 2008), hRobo1 FN2-3 juxta-membrane (PDB 4HLJ) (Barak et al. 2014), and hRobo1 FN3 in complex with antigen-binding fragment (Fab) B2212A (PDB 3WIH) (Nakayama et al. 2015) (Fig. 9.3). These were further complemented by the hRobo2 Ig4-5 (PDB 5NOI) (Yom-Tov et al. 2017), hRobo1 Ig5 (5O5I) and hRobo1 Ig1–4 (PDB 5OPE, 5O5G) crystal structures (Fig. 9.3), as well as a 20 Å 3D negative stain electron microscopy (EM) reconstruction of the whole hRobo1 ectodomain (Aleksandrova et al. 2018). But the most detailed structural insights on Robo receptors to date comes from a crystal structure of the complete hRobo2 ectodomain at 3.6 Å resolution (PDB 6IAA) (Barak et al. 2019) (Fig. 9.3).

Robo1 and Robo2 can form homophilic adhesion interactions (Hivert et al. 2002) that are dependent on the whole ectodomain when coated on fluorescent beads (Liu et al. 2004), with no single domain being responsible, implying the entire intact Robo ectodomain is required for homophilic binding. Similar results were obtained at the cell surface using Fluorescence Resonance Energy Transfer (FRET) and Spatial Intensity Distribution Analysis (SpIDA) imaging techniques (Zakrys et al. 2014). Here, the whole ectodomain is again required for dimer formation, with the Ig domains essential for homophilic interactions. Interestingly, this predominant Robo1 dimeric assembly was not altered upon Slit binding (Zakrys et al. 2014). However, a recombinantly produced Robo1 ectodomain from baculovirus was also shown to be monomeric in solution (Barak et al. 2014). This may indicate that other factors, such as high local concentrations, may be required for dimerisation.

These results are all consistent with the low resolution reconstruction of hRobo1 (Aleksandrova et al. 2018). Here, tetrameric Robo1 assemblies from recombinant mammalian cells stabilised using the GraFix cross-linking technique before

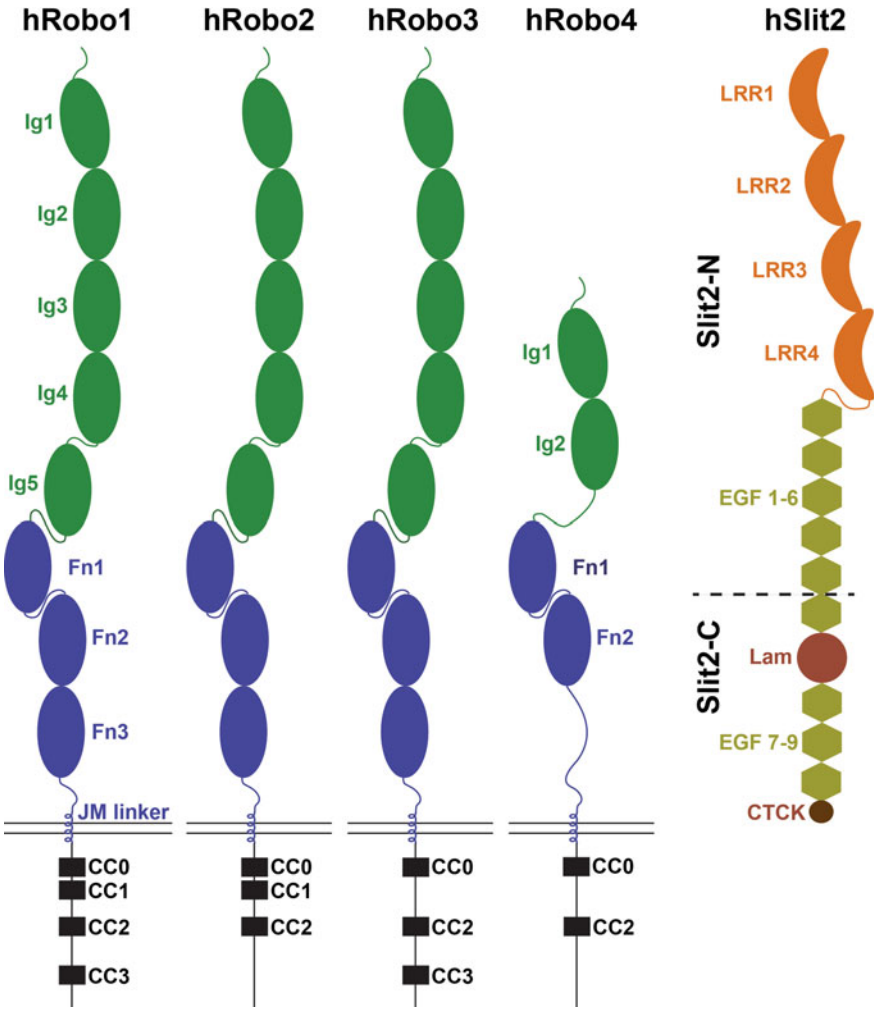

Fig. 9.2 Domain composition of human Robo receptors and Slit2 ligand. The Robo receptors domains are coloured as follows: Ig domains in green; FN in blue; JM linker in blue; and CC0-3 in black. Slit2 domains are coloured as follows: LRR in orange, EGF in lime, Lam in light brown, and CTCK in dark brown

negative staining were observed (Fig. 9.4). The available hRobo1 crystal structures (hRobo1 Ig1–4, Ig5 and FN2–3) were combined with homology and SAXS models to fit a Robo1 ectodomain 'dimer-of-dimers' tetrameric assembly. In this model the hRobo1 ectodomain monomers adopt a domain arrangement that folds back on itself, before turning and extending towards the membrane. This rather compact association is facilitated by the longer linker regions between the Ig4–5 (7 amino acids), Ig5-FN1 (11 amino acids), and FN1–2 (9 amino acids), whose length are

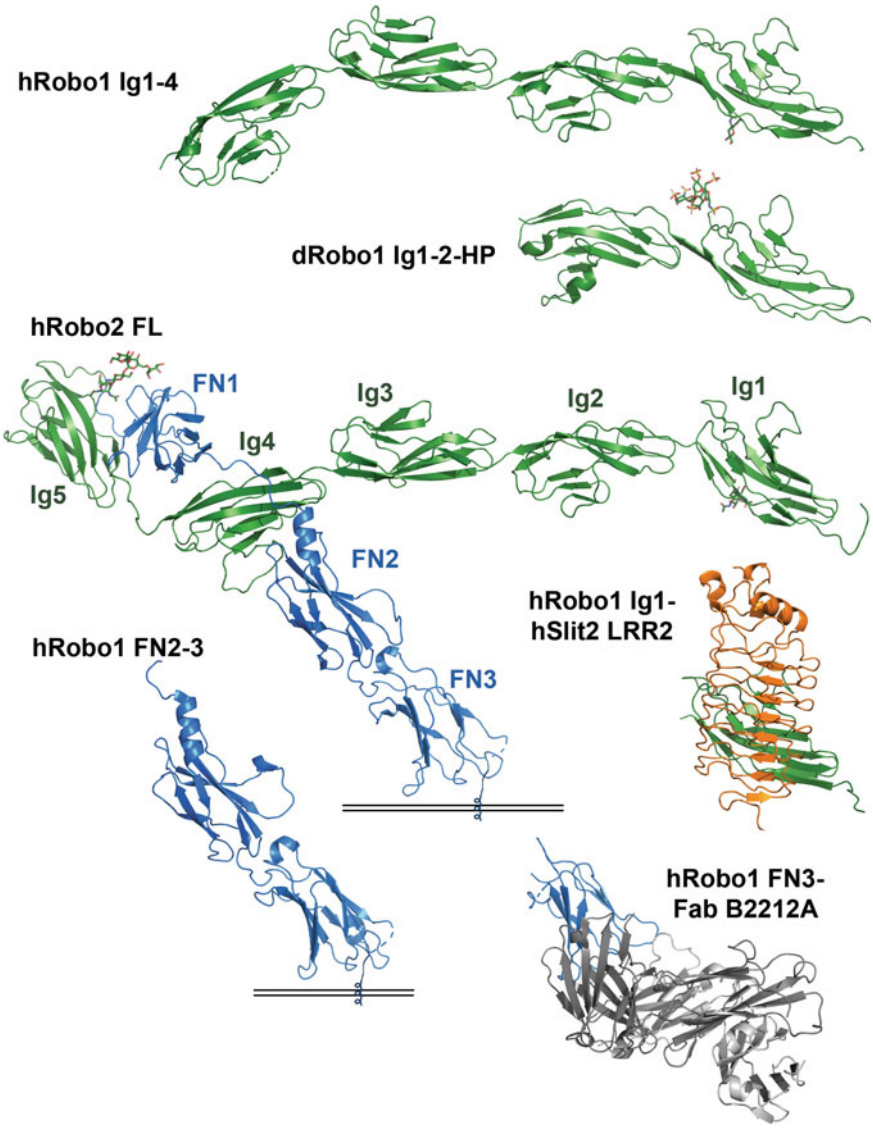

Fig. 9.3 Full length, domain and complex structures for various human (h) and *Drosophila* (d) Robo receptors as determined by X-ray crystallography and currently available in the PDB. The full length Robo2 ecotodomain (PDB 6IAA) is shown in the center with other Robo1 domain and complex structures shown in a similar orientation. A schematic of the membrane is shown to help orientation for Robo1 FN2-3 and Robo2 FL. Domain structures shown include hRobo1 Ig1-4 (PDB 5O5G) and hRobo1 FN2-3-JM (PDB 4HLJ); Complex structures include dRobo Ig1-2-heparin (HP) (PDB 2VRA), hRobo1 Ig1-hSlit2 LRR2 (PDB 2V9T), and hRobo1 FN3-Fab B2212A (PDB 3WIH). Robo Ig and FN domains are coloured green and blue respectively, hSlit2 is coloured orange, and Fab B2212A is coloured gray. Glycosylation and HP moieties are shown in a stick format

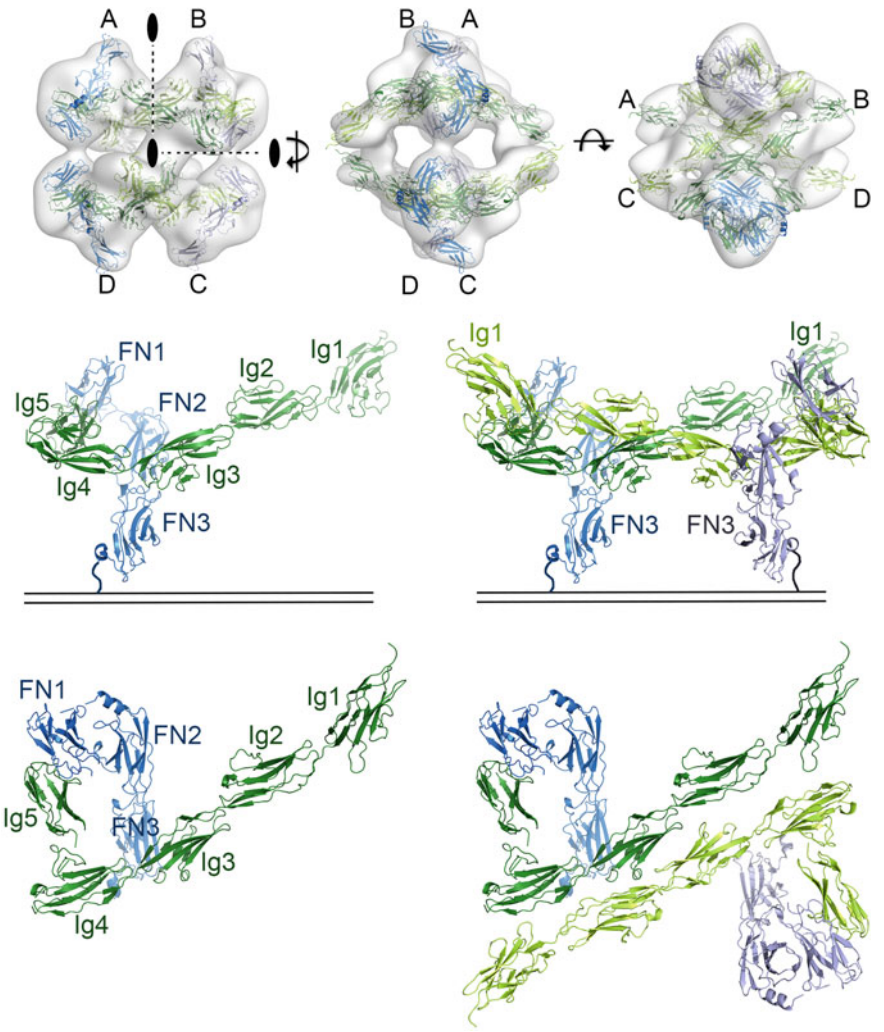

Fig. 9.4 Robo1 forms a 'dimer-of-dimers' tetrameric assembly. Three orientations of the Robo1 domains modelled into the low resolution 3D reconstruction are shown on top to illustrate the 'dimer-of-dimers' (A-B/C-D) configuration. The overall domain organisation is shown below in two orientations (side and top) to illustrate how monomers are arranged as dimers on the cell surface. For illustration purposes the membrane insertion region was included in the middle panel. The Ig and FN domains are labelled and coloured dark or light green and marine or deep blue respectively for each Robo1 monomer

conserved across all Robo receptors except Robo4. The major, and presumably most biologically relevant, dimeric interaction is mediated by the Ig2–4 domains. This is consistent with the role of Robo2 Ig3 in the dimerization and lateral positioning of axons in *Drosophila* (Evans and Bashaw 2010). These dimers at the cell

surface can further interact in a 'back-to-back' conformation to complete the tetrameric Robo1 assembly observed (Fig. 9.4).

The hRobo2 ectodomain crystal structure adopts a similar conformation overall to the low resolution hRobo1 EM reconstruction (Figs. 9.3 and 9.4). However, the higher resolution hRobo2 structure provides a much more detailed molecular insight into the unique hairpin domain arrangement observed. The hRobo2 structure determined represents an auto-inhibited Robo receptor conformation, with the Robo Ig4 interface shown to be important for dimerization and signalling being sequestered by the FN2 domain (Barak et al. 2019) (Fig. 9.5). While a similar hRobo1 dimer interaction was not observed in hRobo2, it does form an extended interaction array in the crystallographic lattice. This is mostly mediated by hRobo2 Ig4–Ig5–FN1 domains, with some contributions from Ig3, in a *cis* direction. But a more extensive interaction interface is observed in *trans* from two opposing *cis* layers. This is reminiscent of a cell-cell interaction as the transmembrane proximal domains are oriented in opposite directions. These interactions are primarily mediated by direct contacts between Ig5 and an Ig5 linked glycan branch from one layer with Ig1–2 from an opposing layer. A larger array is assembled by the Ig1–2

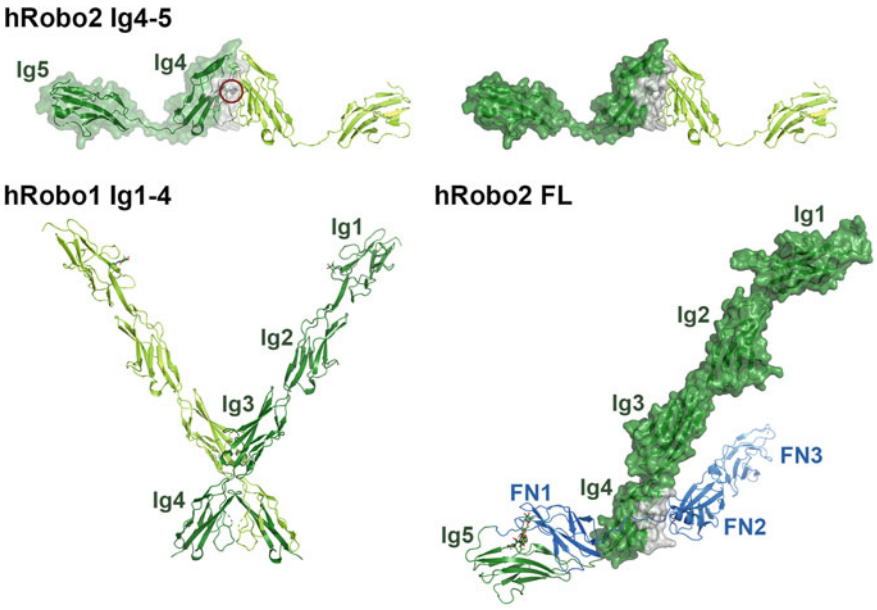

Fig. 9.5 Robo receptors dimerise using a sequence conserved interface sequestered in the full length ectodomain context. hRobo2 Ig4-5 (PDB 5NOI) and hRobo1 Ig1-4 (PDB 5O5G) dimers are shown in a similar orientation to the full Robo2 ectodomain (PDB 6IAA) for easy comparison. The Phe-357 shown to be important for hRobo2 Ig4-5 dimerisation is shown in stick format and circled. The hRobo2 Ig4 dimerisation interface is coloured grey and predominantly precluded from interaction by FN2 in the full hRobo2 ectodomain. The Ig and FN domains are labelled and coloured green and blue, respectively, and glycosylation is shown in stick format

domains from the first molecule interacting with Ig5 and an Ig5 linked glycan of another opposing molecule. The hRobo2 N426 glycosylation site on Ig5, and the residues important for its interaction on Ig1–2, R99 and R132, are sequence conserved across species, suggesting this interaction may be functionally relevant. This was validated by in vitro cellular assays and genetic *C. elegans* experiments showing the importance of Ig5 in fastening an inhibited form of Robo receptors on the cell surface (Barak et al. 2019).

On the cytoplasmic side, the four CC motifs are scaffolding elements responsible for the recruitment of specific proteins, or formation of protein complexes. These adapter proteins are probably shared between various Robo receptors, although not all have been verified. However, the final downstream signalling pathway is largely dependent on the combination of CC motifs present, and the cell type in question. In *Drosophila* CC0 and CC1 are both tyrosine phosphorylation targets of the cytoplasmic Abelson kinase (Abl), which results in Robo inhibition (Bashaw et al. 2000). In the presence of Slit2 and netrin-1, Robo1 CC1 is reported to mediate a cytoplasmic interaction with Deleted in Colorectal Cancer (DCC) that is required to silence netrin-1 attraction (Stein and Tessier-Lavigne 2001). CC2 was shown to interact with Enabled (Ena), an Abl substrate and actin binding protein, which is required for Robo mediated repulsion (Bashaw et al. 2000). In Robo4, CC2 was shown to mediate an interaction with Mena, the mammalian homolog of Ena (Park et al. 2003; Jones et al. 2009). Abl and Ena are long known to interact and regulate reorganization of the actin cytoskeleton (Comer et al. 1998), and in the case of endothelial cells, influence cell migration by promoting filopodia formation. Lastly, CC3 is a polyproline stretch (Kidd et al. 1998) involved in the recruitment of Slit/Robo GTPase activating proteins (srGAP) (Wong et al. 2001; Li et al. 2006) using a two-component molecular mechanism to achieve tighter binding (Guez-Haddad et al. 2015). Another GAP protein, CrossGAP/Vilse, binds the CC2 motif to mediate Robo repulsion (Lundström et al. 2004; Hu et al. 2005). Both GAPs act on small GTPases of the Rho family, RhoA and Cdc42 in the case of SrGAPs, Rac1 and Cdc42 in the case CrossGAP/Vilse, which are known to regulate cytoskeletal dynamics (Chédotal 2007; Ypsilanti et al. 2010).

Slit Proteins Are Robo1/Robo2 Receptor Ligands

Upon midline crossing commissural neurons become sensitive to Slit repulsive cues to ensure they are correctly expelled and prevented from recrossing aberrantly (Long et al. 2004; Zou et al. 2000). Three slit genes (*Slit1*, *Slit2*, *Slit3*) have been identified in mammals (Brose et al. 1999; Holmes et al. 1998; Itoh et al. 1998). Slit1 and Slit2 are known to function as chemorepellents (Nguyen Ba-Charvet et al. 1999; Ringstedt et al. 2000; Hammond et al. 2005). Slit3 is presumed to act similarly but as it exhibits a different neuronal expression pattern (Ringstedt et al. 2000; Hammond et al. 2005) and was shown to be non-repulsive for motor axons (Hammond et al. 2005) this remains to be determined. Slits are large glycosylated proteins secreted by

midline glial cells, but often found associated with the extracellular matrix (Brose et al. 1999). Slits are generally composed of four N-terminal leucine rich repeat (LRR) domains (LRR1–4) followed by six epidermal growth factor (EGF)-like domains (EGF1–6), a laminin G–like domain (Lam), another three EGF domains (EGF7–9), and a C-terminal cysteine knot domain (CTCK) (Fig. 9.2).

Slit2 can be cleaved between EGF5 and 6 by an unknown protease to generate N- and C-terminal fragments, Slit2-N and Slit2-C respectively, with distinct properties (Nguyen Ba-Charvet et al. 2001). Most Slits probably undergo proteolysis as the cleavage site is conserved from *Drosophila* to vertebrates (Brose et al. 1999). Slit2-N binds Robo1 and Robo2 to mediate neuronal repulsion (Nguyen Ba-Charvet et al. 2001) while Slit2-C binds PlexinA2 to signal independently (Delloye-Bourgeois et al. 2015). Slit2-C was shown to induce growth cone collapse, and because PlexinA1 expression is upregulated on growth cones at the midline, it might act as a second chemorepellent to reinforce crossing and post-crossing (Delloye-Bourgeois et al. 2015). Slits can homodimerise through their LRR4 domains (Howitt et al. 2004; Seiradake et al. 2009), but LRR1–4 has also been reported to be monomeric (Hohenester 2008).

An analysis of the *Drosophila* Slit LRR domains identified the LRR2 domain as the binding site for Robo receptors (Howitt et al. 2004), while the first two Ig domains of Robo1 were similarly shown to be important (Liu et al. 2004). The structure of the minimal hRobo1-Ig1-hSlit2-LRR2 complex (PDB 2V9T) confirmed that Robo1 Ig1 interacts with the highly conserved concave face of Slit2 LRR2 (Morlot et al. 2007). Their binding buries a 1380 $Å^2$ solvent accessible area in two distinct regions (Fig. 9.6). The first is electrostatic in nature and mediated by salt bridges, hydrogen bonds, and bridging water molecules with the side-chains undergoing conformational changes upon complex formation. The second is largely hydrophobic in composition and most of this surface is buried by crystallographic contacts in the hRobo1 Ig1–2, hRobo1 Ig1–4 and dRobo Ig1–2–heparin complex crystal structures.

Heparan Binding

Heparan sulphate proteoglycans (HSPGs) are extracellular matrix associated core proteins containing one or more heparan sulphate (HS) glycosaminoglycan chains. HSPGs are known to play a role in neural patterning, and can be divided into three major classes, membrane bound syndecans and glypicans, and secreted molecules such as perlecan, collagen and agrin (Saied-Santiago and Bulow 2018). Full length Slit2 and Slit2-N are long known to tightly associate with cell membranes and can be disassociated by treatment with either high salt (1 M NaCl) or heparin (Brose et al. 1999). A HS dependent binding of Slit to glypican-1 was reported soon after (Liang et al. 1999; Ronca et al. 2001), and enzymatic removal of HS from the cell surface not only decreased the affinity of Slit2 for Robo1, but also abolished its repulsive activity on olfactory neurons (Hu 2001).

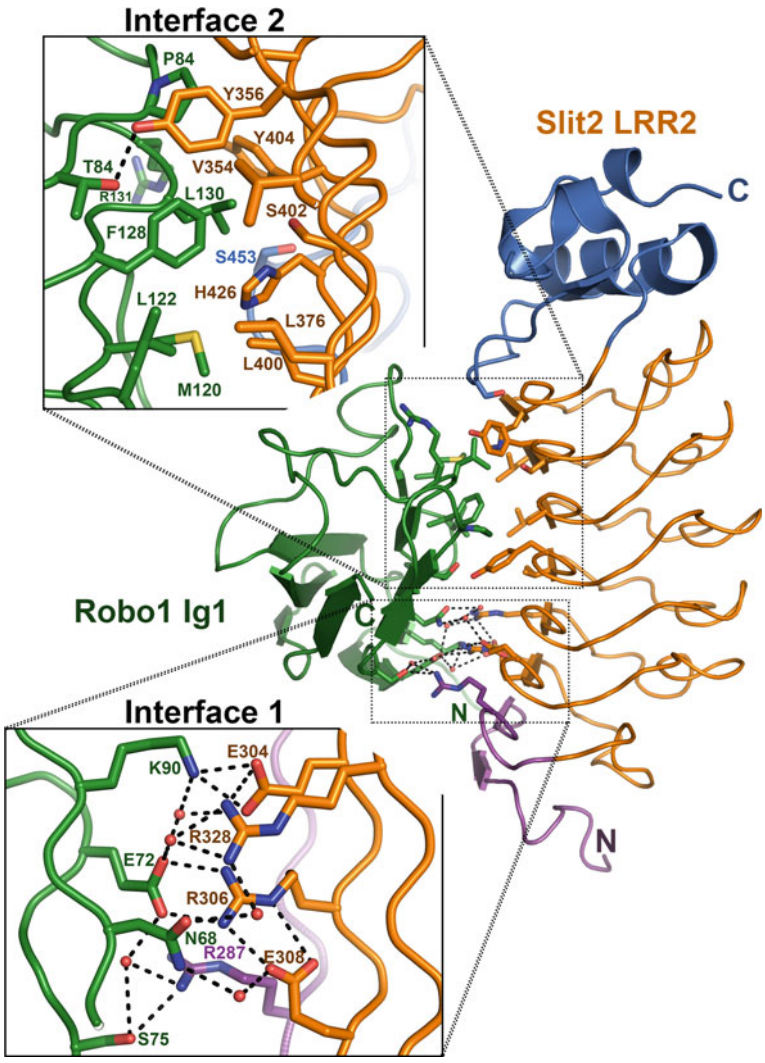

Fig. 9.6 The human Slit2 LRR2-Robo1 Ig1 minimal complex (PDB 2V9T). hRobo1 Ig1 is coloured green, and hSlit2 LRR2 N- and C-terminal caps are coloured magenta and blue, respectively, while the LRRs are coloured orange. Interacting residues are shown in stick representation with water molecules coloured red

This important role in Slit/Robo signalling was further supported when a genetic interaction between Slit and exostosin 1 (*Ext1*), a glycosyltransferase involved in HS biosynthesis, in Slit–mediated retinal axon guidance was observed (Inatani et al. 2003). Syndecan was subsequently shown to be a critical genetic component of Slit-Robo signalling to stabilise Slit-Robo interactions (Steigemann et al. 2004; Johnson et al. 2004). Interestingly, while Slit was still present in midline glia cells it was

absent on axon fascicles in *sdc* mutants, indicating syndecan might play an additional regulatory role in the extracellular localisation of Slit on axons (Johnson et al. 2004). The role of HS was further strengthened in genetic *C. elegans* studies, where it was shown to be not only important, but that particular HS sulphation patterns played a key role in certain cellular contexts (Bülow and Hobert 2004). Furthermore, this is likely to be conserved because a genetic link between *Slit1* and/or *Slit* and HS sulphation modifications was also shown in mice (Conway et al. 2011).

Heparin, a highly sulphated form of HS, was observed to bind between Ig1 and 2 of Robo1, and at the C-terminal cap region of Slit2 LRR2 (Hussain et al. 2006; Fukuhara et al. 2008). This interaction can enhance Slit binding to Robo (Hussain et al. 2006), which together form a continuous HS binding interface to increase the stability of the Slit-Robo complex for signalling (Fig. 9.7). The ability of Slit2 conditioned media or Slit2 LRR2 to induce collapse of *Xenopus* retinal growth cones in a heparin/HS dependent manner provides direct evidence for the importance of this interaction in Slit-Robo signalling (Piper et al. 2006; Hussain et al. 2006). Additional HS binding sites have also been identified at the hRobo1 N–terminus (Li et al. 2015); *Drosophila* Slit LRR1 and C–terminus (Hussain et al. 2006); hSlit2 C-terminus (Ronca et al. 2001) and LRR4 (Seiradake et al. 2009). These additional HS binding sites together with LRR2 on Slit have been proposed

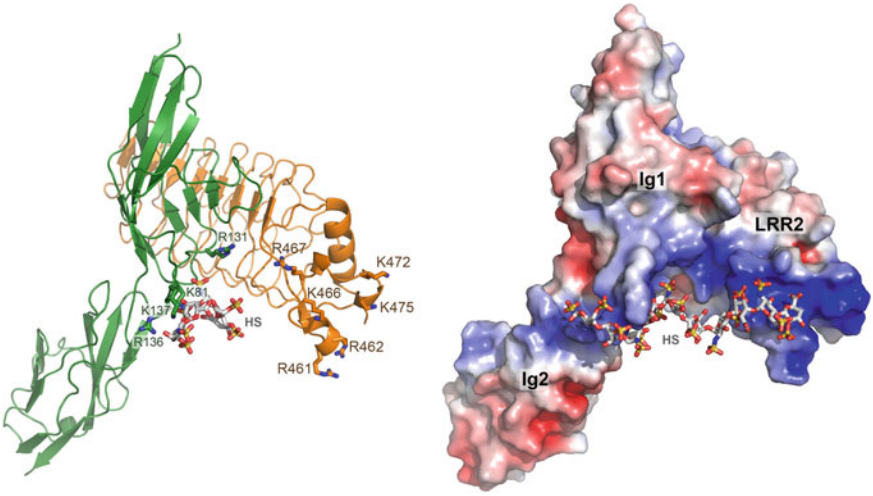

Fig. 9.7 hRobo1 Ig1-2-hSlit2 LRR2 form a composite heparin (HP) binding site. On the left is a model of a tripartite hRobo1 Ig1-2-hSlit2 LRR2-HP (dp10) complex obtained by superimposing the hRobo1 Ig1-hSlit2 LRR2 (PDB 2V9T) and dRobo Ig1-2-HP (PDB 2VRA) complexes onto hRobo1 Ig1-2 (PDB 2V9R) and extending the HP molecule. On the right is an electrostatic surface representation of the same complex (*red*, negative potential; *blue*, positive potential). The positions of HP binding residues in hRobo1 Ig1-2 and hSlit2 LRR2 are shown as sticks and labelled. The hRobo1 Ig and hSlit2 LRR2 domains are coloured green and orange, respectively, and the modelled HP moiety is shown in a grey stick representation

to play a secondary role by concentrating Slit on target cell surfaces, perhaps in a sulphation dependent pattern, or by modulating its diffusion properties (Hussain et al. 2006).

Robo3

Commissureless (Comm) is a transmembrane protein expressed in the commissural neurons of flies that directs Robo receptors to the endocytic pathway to allow midline attraction (Keleman et al. 2002; Georgiou and Tear 2002). After midline crossing Comm is downregulated to ensure newly synthesized Robo receptors can reach the cell surface for interaction with Slit to mediate repulsion. However, no functional Comm homologue was ever identified in vertebrates until the recent discovery of PRRG4 (Justice et al. 2017). But Robo3 was shown to be important for commissural axon midline attraction by blocking Slit mediated Robo1 repulsion (Marillat et al. 2004), suggesting Robo3 might play an attractive rather than repulsive role in midline crossing (Domyan et al. 2013).

To add further complexity four vertebrate Robo3 alternative mRNA splicing isoforms with distinct properties have been identified (Camurri et al. 2005; Chen et al. 2008). Robo3A and Robo3B are evolutionarily conserved and differ by 26 amino acids at the N-terminus (Camurri et al. 2005). Interestingly, in biochemical pull-down assays the shorter Robo3B isoform was shown to bind Slit2 while the longer Robo3A was unable to. In addition, Robo3A was shown to interact homophilically and heterophilically with Robo1. At the C-terminus Robo3 alternative splicing produces Robo3.1 and Robo3.2, which have sequential and opposing roles (Chen et al. 2008). Robo3.1 is observed on pre-crossing axons to suppress Slit-mediated repulsion while Robo3.2 is upregulated upon midline crossing and contributes to repulsion. *Robo3.2* mRNA differs from *Robo3.1* by intron retention that results in a premature stop codon, making it target for nonsense–mediated mRNA decay (NMD) (Colak et al. 2013). Furthermore, while *Robo3.2* mRNA is transported to the axon, possibly due a localisation element in the retained intron, *Robo3.1* mRNA remains in the cell body. This difference in RNA transcript location is believed to facilitate midline crossing by ensuring a sharp and transient spike in Robo3.2 to prevent re-crossing of commissural axons.

More recently it was conclusively shown that mammalian Robo3 receptors do not bind Slit (Zelina et al. 2014). This is primarily due to three mutations (N84P, K86R, and in particular P126L) acquired in the Slit binding Ig1 region of Robo3 during evolution. Interestingly, these mutations were not acquired by non-mammalian vertebrates, which maintain Slit binding. These results support a functional change for Robo3 in mammalian and non-mammalian species. Furthermore, mammalian Robo3 gained the ability to interact with DCC via its cytoplasmic region (Fig. 9.8), possibly via an adaptor protein, and that Netrin-1 binding to DCC can induce Robo3 phosphorylation to function as a chemoattractive receptor complex (Zelina et al. 2014). The subtle changes that occurred in Robo3 to

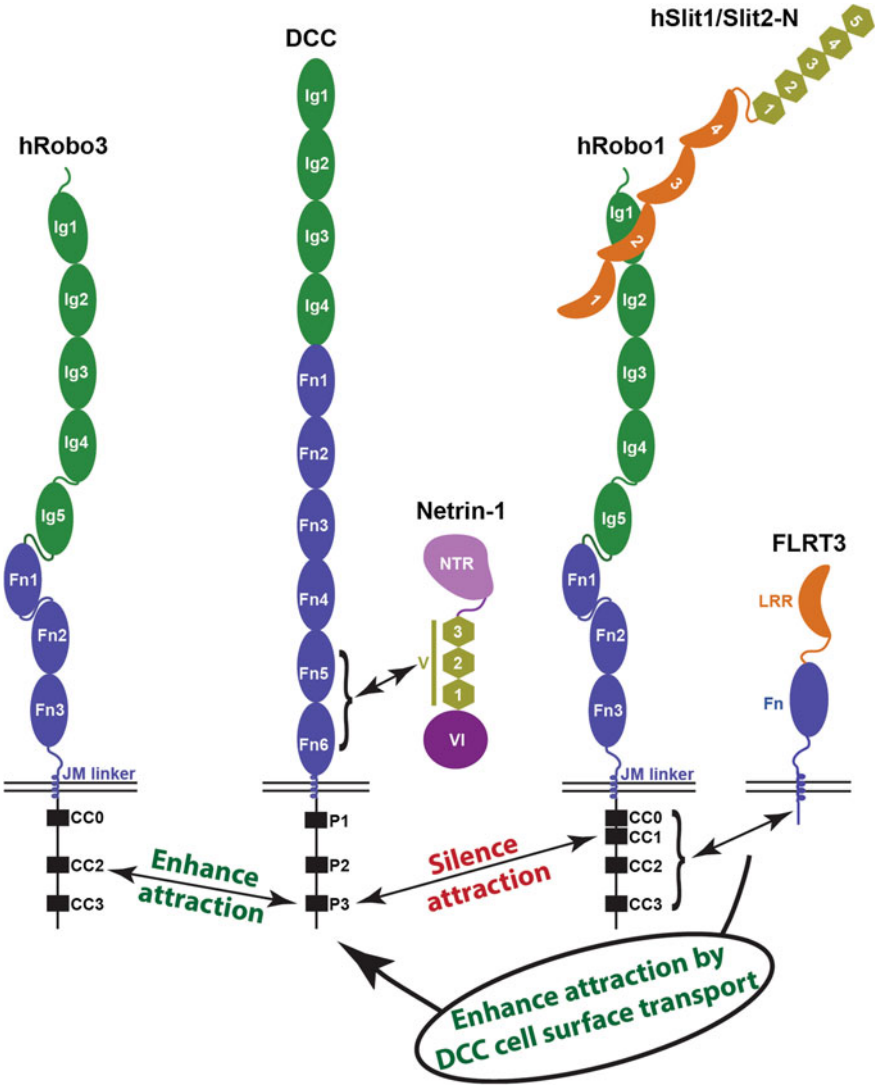

Fig. 9.8 Intracellular Robo co-receptor interactions. Robo3 and Robo1 interact with DCC in a netrin-1 dependent manner using different CC domains to enhance or silence axonal attraction signals. The Robo and DCC receptor Ig and Fn domains are coloured green and blue, respectively. The cytosolic Robo (CC0-3) and DCC (P1-3) motifs are shown as black boxes and labelled. Netrin-1 N-terminal laminin VI, EGF 1-3 (or V domain), and C-terminal netrin-like (NTR) domains are coloured dark purple, green and light purple, respectively. Slit LRR and EGF domains are coloured orange and green, respectively. *Note* Slit is shown as a monomer but likely forms dimers via LRR4. FLRT3 LRR and Fn domains are coloured orange and blue, respectively

switch from a repulsive cue via Slit to an attractive cue via Netrin-1/DCC suggest that this family member may have made a significant contribution to the evolution of mammalian neuronal development.

Intriguingly, an extracellular protein microarray screen for Robo3 binding partners identified an interaction with Neural epidermal growth factor-like-like 2 (NELL2) (Jaworski et al. 2015). NELL2 is the human ortholog of chick Neural EGF-like (Nel), which has been shown to inhibit retinal axon outgrowth and induce growth cone collapse and axon retraction (Jiang et al. 2009). NELL2 is a large secreted glycoprotein containing a laminin G-like domain, six EGF-like domains, and five von Willebrand factor (VF) C domains. The Robo3 FN and NELL2 EGF-like domains were shown to mediate this interaction. Furthermore, NELL2 was shown to repel mouse spinal cord commissural axons in a Robo3 dependent manner while acting as a midline attractive cue in vivo (Jaworski et al. 2015). These findings show how Robo3 can perform a multifunctional receptor axon guidance role to inhibit Slit repulsion, facilitate Netrin attraction, and mediate NELL2 repulsion at the midline. Together all these studies underline the complex roles Robo3 plays in neuronal development. Structural information on the Robo3-NELL2 interaction would likely provide some valuable insights on these mechanisms.

Robo4

Unlike other members of its family, Robo4, is primarily expressed by endothelial cells at sites of active angiogenesis (Park et al. 2003), proliferating cell types such as hematopoietic stem cells and vascular smooth muscle cells (Shibata et al. 2009; Smith-Berdan et al. 2011; Liu et al. 2006), and is involved in cell migration processes (Park et al. 2003; Sheldon et al. 2009; Yadav and Narayan 2014). The knockdown of Robo4 in Zebrafish lethally disrupts vessel sprouting during early embryonic development, underlying its important role in angiogenesis (Bedell et al. 2005). However, Robo4 was also shown to be important for the migration of neuronal cells in mice during development (Zheng et al. 2012). If this is the result of Robo4 alone, or is dependent on interaction with other Robo receptors or partners, remains unclear.

Robo4 expression is regulated by an upstream promoter region that contains binding sites for specificity protein 1 (SP1) (Okada et al. 2007), GA-binding protein (GABP) (Okada et al. 2007, 2008), Sry-related high mobility box (SOX) (Samant et al. 2011), and activator protein-1 (AP-1) transcription factors. This region is demethylated when induced pluripotent stem (iPS) cells undergo differentiation into endothelial cells following a recently described regulatory mechanism, which promotes transcription of downstream genes (Tanaka et al. 2018).

As mentioned previously, the structural organisation of Robo4 is distinctly different from the other Robo receptors. Both its shorter extracellular region (composed of only two Ig and two FN3 domains), and cytoplasmic region

(containing only CC0 and CC2 motifs) influence its ability to interact with canonical Slit ligands and their downstream signalling pathways (Fig. 9.2). For instance, Robo1 and Robo2 are unable to rescue Robo4 activity (Bedell et al. 2005), and many of the Slit2 binding residues identified in Robo1 are different (specifically: E72V, N88S, K90Q, F128L and R131Q) (Morlot et al. 2007). Furthermore, Robo4 was shown to be monomeric (Bisiak 2018), lacking the third and fourth Ig domains, which are important for homophilic interactions (Yom-Tov et al. 2017; Aleksandrova et al. 2018).

Despite what is already known of the common extracellular and intracellular Robo receptor partners, the interaction network of Robo4 is quite different, and still poorly mapped. For instance, while Slit2 mediated effects through Robo4 have been observed despite a lack of key interaction residues (Jones et al. 2009; Park et al. 2003), some studies report a complete absence of direct interaction with Slit2 (Suchting et al. 2006; Koch et al. 2011). This is still debated, where, to cite just one example, one study determined that Slit2 acts as a strong angiogenic inducer through Robo4 in primary human endothelial cells (Park et al. 2003). While in a second study, the authors showed Robo4 inhibiting a Slit2-Robo1 mediated primary human umbilical vein endothelial (HUVEC) cell migration through an unknown intracellular mechanism (Enomoto et al. 2016). As the expression of different Robo receptors can overlap in some cell types, most modern models tend to suggest that Slit functions on Robo4 are mediated through receptor heterodimers. To sustain this line of thought, Robo1/Robo4 heterodimers where shown to promote cell migration in vitro by the Robo4 sequestering of Robo1 in intracellular vesicles, although the specific domains involved were not identified (Sheldon et al. 2009).

UNC5B is a member of the UNC5 receptor family that plays a major role in neuronal guidance via Netrin ligand binding (Seiradake et al. 2016). However, UNC5B is especially involved in angiogenetic processes, being highly expressed in developing blood vessels (Lu et al. 2004; Navankasattusas et al. 2008; Tai-Nagara et al. 2017). UNC5B acts as a repulsive receptor upon netrin-1 binding in vascular morphogenesis (Lu et al. 2004) or FLRT2 binding in the placental labyrinth (Tai-Nagara et al. 2017). UNC5B is also activated upon Robo4 binding, which inhibits angiogenesis to maintain vascular integrity (Koch et al. 2011), and does not require the Robo4 cytoplasmic region (Zhang et al. 2016). UNC5 receptors are type I transmembrane proteins, containing two Ig and two thrombospondin (TSP)-like ectodomains (Seiradake et al. 2014; Bisiak 2018), while the intracellular region is composed of a death and regulatory domain (Wang et al. 2009). The UNC5 Ig1 domain and Ig1–2 domains are required for interaction with FLRT (Seiradake et al. 2014) and netrin-1 (Grandin et al. 2016), respectively. Deletion studies showed that the UNC5B TSP domains are sufficient for interaction with the Robo4 Ig1–2 domains (Koch et al. 2011).

At the cytoplasmic level, Robo4 encodes variants of the CC0 and CC2 motifs (Legg et al. 2008). As such Robo4 can recruit other proteins apart from the classical Abl and Mena (in mammals), or Enabled (in *Drosophila*), commonly observed throughout the Robo family. The WASP family proteins, WASP and NWASP, interact with Robo4 through their own polyproline stretches (Sheldon et al. 2009),

and are directly responsible for filipodia formation induced by recruitment in a larger complex of the regulatory proteins WIP and Syndapin (Martinez-Quiles et al. 2001; Kaur et al. 2006). Yet another mechanism triggered in the presence of Slit2, includes the recruitment of the cytoplasmic protein Paxillin by Robo4, which inhibits protrusive activity (Jones et al. 2009; Sherchan et al. 2017). Paxillin is a well-known cell matrix adhesion protein found at focal adhesion points, which through regulation of the GTPase Arf6 activity, increases vascular stability (Turner et al. 1990; Deakin and Turner 2008; Jones et al. 2009).

Robo Co-receptors

Slit proteins are long established Robo1 and 2 receptor ligands, while NELL2 was recently shown to act as a Robo3 receptor ligand for midline repulsion (Jaworski et al. 2015). However, other direct and indirect Robo co-receptors have been identified (Fig. 9.8 and 9). Early studies with *Xenopus* spinal axons, complemented by cellular assays, showed how an interaction between the cytoplasmic domains of DCC and Robo upon Slit activation is necessary to silence netrin-1 attractive signalling during midline crossing (Stein and Tessier-Lavigne 2001). Later, this same interaction was shown to attenuate Slit2 mediated repulsion in neocortical axons (Fothergill et al. 2014). Conversely, an intracellular interaction between Robo3 and DCC was shown to enhance netrin-1 attractive signalling (Zelina et al. 2014). Moreover, while the same cytosolic P3 domain of DCC is required for Robo receptor binding, Robo1 and Robo3 utilize different intracellular regions. The Robo1 interaction was mapped to the CC1 region missing in Robo3 (Stein and Tessier-Lavigne 2001), and the Robo3 interaction was mapped to CC2, or between CC2 and CC3 (Zelina et al. 2014), which may account for their different responses to netrin-1 (Fig. 9.8). Both of these interactions are hierarchical signalling events, where one cue can suppress or potentiate the effect of another. Away from the midline, a fine–tuned axonal guidance can occur via a new context–dependent signalling mechanism, as reported for rostral thalamocortical axons (rTCAs) (Leyva-Diaz et al. 2014). Here, a *cis* interaction between the FLRT3 and Robo1 cytoplasmic domains was shown to be necessary in the presence of netrin-1 and Slit2 guidance cues (Fig. 9.8). In this case FLTR3 modifies a Slit1 mediated Robo1 signalling by activating protein kinase A to promote vesicular transport of DCC to the growth cone surface for netrin-1 mediated attraction.

The co-receptor interactions discussed above involve intracellular *cis* interactions, presumably mediated by cytosolic adaptor proteins binding to the CC domains of Robo receptors. But extracellular co-receptor interactions have also been reported (Fig. 9.9). The migration of cortical interneurons through the developing striatum is known to be mediated by chemorepulsive class 3 semaphorin ligands (Sema3A and Sema3F) acting on cell surface neuropilin (Nrp1 and Nrp2) receptors (Marin et al. 2001). It was later shown that cortical interneurons lacking Robo1 were less responsive to semaphorins due to a reduction in Nrp1 and

PlexinA1 receptor levels (Hernandez-Miranda et al. 2011). This is not Slit1/Slit2 dependent, and careful biochemical studies identified a direct interaction between the Robo1 Ig1–2 domains and Nrp1 (Fig. 9.9). This extracellular *cis* interaction shows how Robo1 can modulate the response of interneurons to semaphorin signalling.

Slit was shown to interact with Down syndrome cell adhesion molecule 1 (Dscam1) on mechanosensory neurons for specific axon collateral branch formation in a Robo independent manner (Dascenco et al. 2015). Later it was reported that Dscam1 forms a co-receptor complex with Robo1 that is dependent on Slit-N to promote longitudinal axonal growth in *Drosophila* (Alavi et al. 2016). Immunoprecipitation assays showed Slit cleavage was necessary for Dscam1-Robo1 complex formation as Dscam1 only binds Slit-N. Further biochemical assays identified that the EGF1–3 domains of Slit-N were required to bind two sites

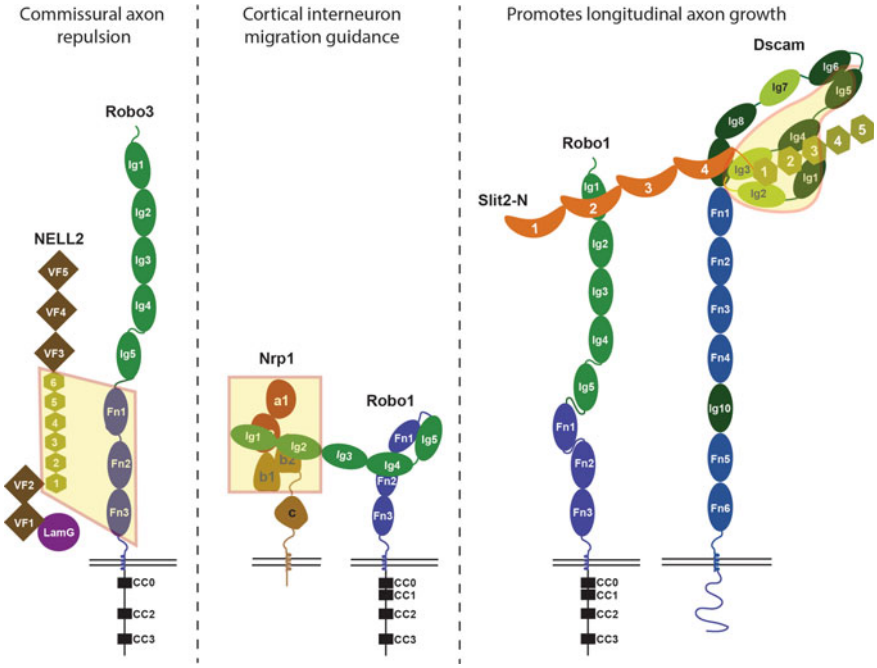

Fig. 9.9 Extracellular Robo co-receptor interactions. NELL2 interacts with Robo3 to mediate axonal repulsion (left). Nrp1 and Robo1 form a co-receptor complex to mediate axon migration (middle). Slit2-N enables a Robo1-Dscam co-receptor complex to promote axon growth (right). The minimal interaction regions determined are highlighted in yellow. NELL2 Laminin G (LamG), von Willebrand factor C (VF), and EGF domains are coloured magenta, brown and green, respectively. Nrp1 a1, a2, b1, b2, and c domains are coloured brown. Robo and Dscam receptor Ig and Fn domains are coloured green and blue, respectively. The cytosolic Robo (CC0-3) motifs are shown as black boxes and labelled. The constant and variable Dscam1 Ig domains are coloured dark and light green, respectively. *Note* Slit is shown as a monomer but likely forms dimers via LRR4

on the N-terminal region of Dscam1 somewhere between Ig1–5 (Fig. 9.9). All these recent examples illustrate how Robo co-receptor interactions can modify cell signals in a hierarchical or context dependent manner. Structural information on these newly identified signalling complexes would likely provide invaluable insights on how multiple guidance effects during neuronal development are integrated.

Robo Signalling Mechanism

Although Slit–Robo signalling has been intensely studied for over three decades there was, until recently, a clear lack of knowledge on how their interaction is relayed across the membrane to mediate intracellular signalling. A major insight into Robo activation was provided by elucidation of the auto-inhibited Robo2 ectodomain structure (Barak et al. 2019). Complementary cellular and in vivo genetic studies further showed the importance of a highly conserved Robo Ig4 domain dimerization interface for Robo signalling. Taken together with previous studies (Zakrys et al. 2014; Aleksandrova et al. 2018) there is now a consensus that Robo receptors on the cell surface probably undergo a conformation change upon Slit binding that is required for dimerisation (or oligomerisation) and subsequent intracellular signalling (Fig. 9.10).

Early studies reported Robo1 shedding as a potential heptocellular carcinoma marker for liver cancer (Ito et al. 2006). Later it was shown that Robo can undergo proteolytic processing by the Kuzbanian (Kuz) Adam family metalloprotease (ADAM10 in mammals) in *Drosophila* (Coleman et al. 2010). Because an uncleavable form of Robo is unable to maintain midline repulsion, this suggested that Kuz mediated ectodomain shedding may play an important role in signalling (Fig. 9.10). For this a cytoskeletal rearrangement upon cleavage by the Slit dependent recruitment of the downstream signalling molecule son of sevenless (Sos) was proposed as a possible mechanism (Coleman et al. 2010). This mechanism is supported by structural studies on the Robo1 juxtamembrane domain region, showing an enhanced ectodomain shedding upon the exposure, or relief, of this structured region (Barak et al. 2014). In this case immobilized Slit on one extracellular cell surface is proposed to create a tension upon Robo1 binding on an approaching axon, leading to cleavage site exposure and intracellular signalling (Barak et al. 2014). The Robo1 cleavage site was located between Q888 and Q889 at juxta-membrane (JM) domain region using mass spectroscopy in human cancer cells (Seki et al. 2010). In addition, following ectodomain shedding Robo1 was observed to be further processed by γ-secretase, with the resulting C-terminal fragment translocated to the nucleus (Seki et al. 2010). However, the function of this C-terminal fragment, and whether ectodomain shedding plays a role in vertebrate neuronal development remain open questions.

Commissural neurons are known to accumulate high levels of Robo only after midline crossing in *Drosophila* (Kidd et al. 1998). Further studies showed how Comm plays an important role by intercepting Robo in the endoplasmic reticulum/

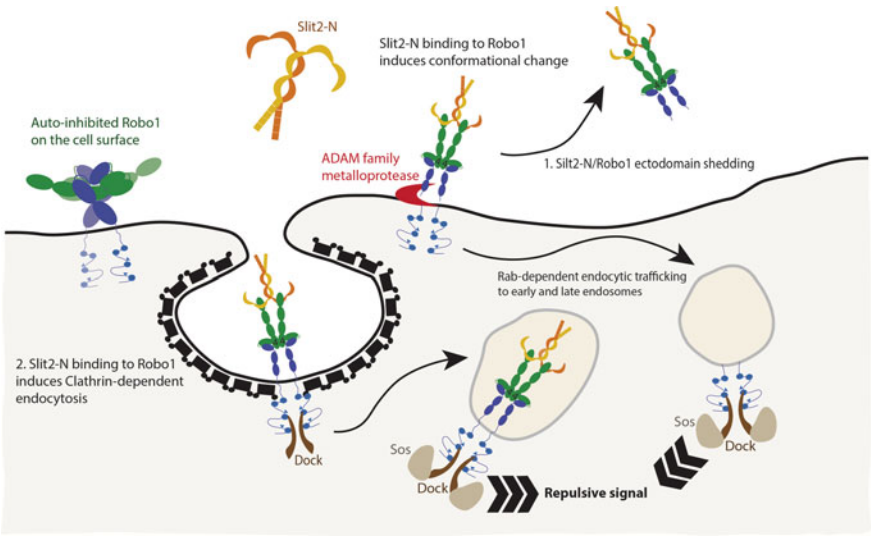

Fig. 9.10 Possible mechanism for Slit2-N mediated Robo1 signalling. Robo1 adopts a compact auto-inhibited dimeric (or oligomeric) assembly on the cell surface. Slit2-N binding induces a conformational rearrangement of Robo1 without dramatically changing its' oligomerization state. This can either induce (1) Slit2-N/Robo1 ectodomain shedding followed by endocytosis of intracellular domains, or (2) Slit2-N mediated endocytosis of the entire Slit2-N/Robo1 complex to the late endosome for Sos recruitment and subsequent cell signalling

Golgi for trafficking away from the growth cone surface to the late endosomes, thus enabling midline crossing (Keleman et al. 2002, 2005). Once this was achieved, the downregulation of Comm results in a surge of cell surface Robo for Slit binding to prevent the axons from re-crossing. While Slit-Robo signalling is conserved in bilateral species, and endocytosis was shown to be important for Slit2 mediated collapse of *Xenopus* retinal growth cones (Piper et al. 2006), no Comm proteins were identified in vertebrates. However, a proline rich and Gla domain protein, PRRG4, containing the L/PPxY motifs found in Comm was recently shown to affect a hRobo1 expression phenotype in flies (Justice et al. 2017). In addition, PRRG4 is able to re-localise hRobo1 from cell surfaces, which taken together strongly suggest it's a functional homologue of Comm (Justice et al. 2017). The role of endocytosis in Slit-Robo signalling is further supported by *Drosophila* genetic experiments and complementary in vitro data (Chance and Bashaw 2015). Two putative YXXϕ sequence motifs, YLQY and YQAGL, known to be important for interaction with the Clathrin adaptor complex, were shown to be important for Robo1 endocytosis (Fig. 9.10). Furthermore, dRobo trafficking to late endosomes is required for recruitment of Sos, mediated by Dock binding to CC2 and CC3, and repulsive signalling. From sequence analyses the endosomal trafficking of Robo1 upon interaction with Slit2 is likely conserved in vertebrates. Only Robo1 homologues have this particular sequence so it will be important to investigate if other

Robo receptors can also undergo endocytosis or require a heterophilic interaction with a Robo1 homologue.

Concluding Remarks

Recent Robo receptor structures have provided valuable insights for the design of complementary biochemical, genetic, and cell based assays to probe Slit mediated Robo signaling (Aleksandrova et al. 2018; Barak et al. 2019). Taken together with previous studies these provide important mechanistic details on Robo mediated signaling pathways. Robo receptors on the cell surface primarily exist in an auto-inhibited conformation as dimers, or higher oligomers, that can be further stabilized in *trans*. Once exposed to Slit emanating from the midline Robo receptors auto-inhibition is relaxed and they must undergo a conformational change to allow Robo Ig4 mediated dimerisation for subsequent intracellular signaling (Fig. 9.10). While the field is still lacking structural details on a Slit-N-Robo ectodomain receptor complex, it is likely that Slit-N mediated endocytosis of Robo1 receptors is required for internal cell signalling (Chance and Bashaw 2015). However, many open questions still remain unresolved. For example, is Robo ectodomain shedding required for signalling? Can hRobo2 (and 3) also undergo endocytosis, or are heterophilic interactions with Robo1 required, because it alone contains endocytic sequence motifs? Lastly, what is the precise role of PRRG4 in hRobo1 cell surface localisation (Justice et al. 2017)?

More recent studies have shown that Slit mediated Robo receptor signalling during neuronal development is not always a simple ligand-receptor mediated event. Here, the ability of Robo receptors to modify axonal growth, as well as attractive or repulsive neuronal signals is often dependent on their interaction with other cell surface receptors and ligands (Stein and Tessier-Lavigne 2001; Hernandez-Miranda et al. 2011) (Figs. 9.8 and 9.9). These, and newer interaction networks (Leyva-Diaz et al. 2014; Jaworski et al. 2015) are continually being discovered, and how they can all coordinate in such a highly cooperative manner is fascinating. In addition, the role of proteoglycans in Slit-Robo and development signaling pathways adds yet another layer of complexity (Townley and Bülow 2018). The interplay and communication between the major neuronal receptor classes is fast becoming an important research focus in the neurobiology field, and many examples now exist, such as the recently reported Robo1-Dscam1 co-receptor complex that is mediated by Slit2-N (Alavi et al. 2016). Structural studies on these complex signalling hubs can provide molecular details that will enable the design of complementary biochemical, genetic, and cell based assays to probe their signaling roles. A collaborative research effort is therefore necessary to provide further insights into the assembly, and signaling, of larger complexes on the cell surface for neuronal guidance. These will also help elucidate the role of Slit-Robo signaling in angiogenesis, organ development and cancer progression. Indeed, once enough experimental results are available on these signalling pathways one could envisage

the design of computer algorithms to model such developmental and disease processes.

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