Structural Principles in Robo Activation and Auto-Inhibition

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Short title: Structure-Function of Robo

Abstract

Proper brain function requires high precision neuronal expansion and wiring, processes controlled by the transmembrane Roundabout (Robo) receptor family and their Slit ligands. Despite their great importance, the molecular mechanism by which Robos' switch from "off" to "on" states remains unclear. Here, we report a 3.6 Å crystal structure of the intact human Robo2 ectodomain (domains D1-8). We demonstrate that Robo *cis* dimerization via D4 is conserved through hRobo1, 2, and 3, and the *C. elegans* homolog SAX-3, and is essential for SAX-3 function *in-vivo*. The structure reveals two levels of auto-inhibition that prevent premature activation: 1) *cis* blocking of the D4 dimerization interface, and 2) *trans* interactions between opposing Robo receptors that fasten the D4-blocked conformation. Complementary experiments in mouse primary neurons and *C. elegans* support the auto-inhibition model. These results suggest that Slit stimulation primarily drives the release of Robo auto-inhibition required for dimerization and activation.

In Brief

Robo receptors are auto-inhibited for dimerization by intra- and inter-molecular contacts, which are critical for axon guidance and signaling.

Highlights

- Crystal structure of the intact hRobo2 ectodomain at 3.6 Å.
- Dimerization through the extracellular domain 4 (D4) is required for Robo axon guidance.
- Robo receptors are maintained in an auto-inhibited conformation in which D4 is blocked.
- We suggest that Slit dissociates *trans*-interacting Robos, leading to auto-inhibition relief, which is followed by dimerization and signaling.

Acknowledgments:

We thank Prof. Abdussalam Azem from Tel-Aviv University for AUC measurements and analysis. We thank Drs. Avi Jacob and Irit Shoval from the Bar Ilan University scientific equipment center for help in light microscopy image acquisition and ImageStream FACS analysis, and Jennifer Israel Cohen Benichou for *C. elegans* data statistical analysis. We thank members of the Opatowsky and Henis-Korenblit laboratories for technical assistance, to Dr. Einav Gross of the Hebrew University Faculty of Medicine and Dr. Moshe Dessau of Bar-Ilan University Faculty of Medicine for useful advice, to Prof. Cornelia Bargmann of the Chan Zuckerberg Initiative, for the gift of *sax-3* template DNA, and to Dr. David Sprinzak of Tel Aviv

University for the gift of Notch-Delta expression vectors. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center. We thank the staff of the ESRF and EMBL-Grenoble ID30B, ID23, and ID29 of ESRF, and the staff of BESSY II for assistance in using beamlines BL14.1 and 14.2. This work was supported by funds from ICRF to Y.O. and ISF grant nos. 182/10 and 1425/15 to Y.O.

AUTHOR CONTRIBUTIONS

Y.O. conceived the project. R.B. cloned, purified and crystallized hRobo2^{D2-3} and hRobo2^{D1-8}. R.B. and Y.O. collected X-ray diffraction data. M.N.I., R.B. and Y.O. solved the structures and performed model building and refinement. Y.O analyzed the structures to identify key structural elements for biochemical and biological studies. J.G.H. and Y.O. developed the Robo-cKIT chimera phosphorylation assay, carried out by J.G.H. G.Y.T. performed all *C. elegans* experiments, with technical instructions from S.H.K., and assistance from M.C.B. G.Y.T. performed the Robo cell aggregation assays; and J.G.H. - the Notch-Delta control. E.P. and R.M. performed the neurite expansion assay, with lentivirus vectors constructed by L.G.P. Y.O. wrote the manuscript, edited by A.A.M. and S.H.K. with input from all authors.

Declaration of interests:

The authors declare no competing interests. Patent applications concerning Robo activation and inhibition are currently pending.

Introduction

The Robo family of transmembrane receptors mediate a variety of neuronal responses, including neurogenesis, migration, branching, dendritic patterning, synaptogenesis and axonal guidance. Outside the nervous system they are involved in angiogenesis, and organogenesis of kidney, diaphragm, lungs, heart, and bone (Blockus and Chedotal, 2016). Robo receptors are considered to be attractive drug targets for the treatment of several types of cancer (Ballard and Hinck, 2012; Gara et al., 2015; Gohrig et al., 2014; Gu et al., 2015; Huang et al., 2015; Mehlen et al., 2011) and various developmental and chronic diseases, such as ocular neovascular diseases (Rama et al., 2015), loss of bone mass (Xu et al., 2018), kidney diseases and proteinuria (Fan et al., 2012; Hwang et al., 2015), and liver fibrosis (Chang et al., 2015). However, there is currently no Robo directed therapeutic approach available because of an insufficient structural and mechanistic understanding of Robo activation and signaling.

Robo receptors, and their Slit ligands, are evolutionary conserved and can be identified across animals with bilateral anatomical symmetry, from *Caenorhabditis elegans* nematodes to humans. Indeed, similar aspects of biological activity were demonstrated in Robo receptors from *Drosophila* (Brose et al., 1999; Kidd et al., 1998), *C. elegans* (Hao et al., 2001), beetles (Evans and Bashaw, 2012), chicken (Hammond et al., 2005), *Xenopus* (Chen et al., 2000), zebrafish (Challa et al., 2001) and mouse (Yuan et al., 1999). In several animal lineages, gene duplication events resulted in multiple active Robo paralogs, with humans and other mammals having four Robos (1-4), along with three Slits (1-3), while *Drosophila* has three Robos (1-3) and one Slit. However, *C. elegans* has maintained a single cognate Robo/SAX-3 receptor and Slit/SLT-1 ligand. Following duplication, adaptive mutagenesis and alternative splicing led to a divergence in certain Robo functions. For example, mammalian Robo3 became insensitive for Slit, and instead of eliciting axon repulsion at the CNS midline, as Robo1/2 do, it facilitates attraction (Zelina et al., 2014). However, other functions overlap, such as Robo1 and Robo2 response to Slit2 in mouse retinal neovascularization (Rama et al., 2015). Complementation between paralogs, as in the formation of Robo hetero-dimers, is yet another form of specialization that was shown in the inhibitory trans-interaction between *Drosophila* Robo1 (presented on crossing axons) and Robo2 (presented on midline cells) that allow midline crossing of commissural axons (Evans et al., 2015) and between *Drosophila* muscle and tendon cells (Ordan and Volk, 2015). Interestingly, although Robo paralogs in insects and vertebrates are thought to have emerged from independent duplication events (Zelina et al., 2014), similar *trans* hetero interactions are also reported in promoting axon outgrowth of mouse retinal neurons (Hivert et al., 2002).

With the exception of the divergent mammalian Robo4, Robo homologs share a similar architecture, containing five Ig-like and three FnIII extracellular domains (D1–8), a single-pass

transmembrane segment, and an unstructured cytosolic region to which intracellular effectors bind (Fig. 1A, S1) (Blockus and Chedotal, 2016). The basic architecture of Slits is also conserved, and includes four leucine-rich repeat domains (LRR 1-4), 7-9 EGF repeats, a laminin-G domain, and a carboxy-terminal cysteine knot (Fig. 1A). Slit-Robo interaction involves the Slit LRR2 (Howitt et al., 2004), and Robo D1 (Liu et al., 2004; Morlot et al., 2007). Heparan sulfate proteoglycans (HSPGs) tighten Slit-Robo interactions by forming a Slit-Robo-HSPG tertiary complex (Hu, 2001; Hussain et al., 2006). Slits are cleaved into N' and C' terminal products (Wang et al., 1999), and whereas the full-length Slit and the large N' product, which harbors the LRR2 domain, stimulate Robo-mediated responses (Nguyen Ba-Charvet et al., 2001), the shorter C' product is a ligand for the Plexin1A receptor (Delloye-Bourgeois et al., 2015). Full-length Slit was also shown to induce axon extension in *Drosophila* through interaction with another cell surface receptor - Dscam1 (Alavi et al., 2016; Dascenco et al., 2015), demonstrating non-monogamous Slit-Robo relations. Crystal and NMR structures of several Robo domains have been determined, notably the hRobo1^{D1}-hSlit2^{LRR2} complex (PDB 2V9T) (Morlot et al., 2007), the dRobo^{D1-2}-heparin complex (PDB 2VRA) (Fukuhara et al., 2008), and the hRobo1^{D7-8} juxtamembrane (PDB 4HLJ) (Barak et al., 2014; Barak and Opatowsky, 2013). These structures provided important information about Robo binding to Slit and HSPGs, and hRobo1 ectodomain proteolysis. More recently, our crystal structure of hRobo2^{D4-5} (PDB 5NOI) (Yom-Tov et al., 2017) identified D4 as a dimerization domain *in vitro*, and the crystal structures of hRobo1^{D1-4} (PDB 5OPE, 5O5G) that, as will be discussed here, reveals an identical dimerization interface. Also insightful is the low-resolution electron microscopy single particle structure of hRobo1^{D1-8} (Aleksandrova et al., 2018), showing a head-to-head dimer of dimers *trans* arrangement of the receptor. However, none of these provide the mechanistic insights needed to address how Robo receptors become stimulated and avoid premature activation.

In this report, we first describe the hairpin-like architecture of intact hRobo2 ectodomain (domains D1-8). In this structure, D4 is intramolecularly blocked and unable to mediate dimerization. We show that D4 mediated dimerization is required for Robo signaling, leading to the conclusion that the hRobo2^{D1-8} structure displays an auto-inhibited conformation. The structure also reveals how intermolecular *trans* interactions can impose a tighter auto-inhibition, and cluster Robo molecules close together. Our cell assays support the importance of these intra and intermolecular contacts on Robo signaling, while genetic experiments in *C. elegans* and mouse neuronal outgrowth assays further substantiate the conservation of these observations. Based on the results presented here, we describe a mechanism for Robo auto-inhibition and activation, in which D4-mediated dimerization is key for Robo activation, and D4 occlusion - for maintaining Robo inactive. Accordingly, we suggest that stimulation by Slit

involves dissociation of trans-interacting Robos, leading to relief of auto-inhibition that is followed by dimerization and signaling.

Results

Overall structure of an intact hRobo2 ectodomain monomer

The entire ectodomain of hRobo2 (^{24}LRQ…KQP^{859}) is composed of eight domains, designated D1-8 (Fig. 1, S1), and was expressed in insect cells using the baculovirus expression system. Purified hRobo2^{D1-8} was subjected to extensive crystallization screening and optimization, followed by structural determination to a resolution of 3.6 Å (Table S1). For structure determination, a hRobo2^{D2-3} crystal structure (Table S1) was determined and used as a search model in molecular replacement to provide the first accurately-located domains. This was followed by the positioning of individual D4, D5, D1, D6, D7 and D8 domains (by that order) available from crystal and NMR structures of hRobo1 and 2 (PDB codes 2V9Q, 5NOI, 2EO9, 1UEM, 4HLJ).

hRobo2^{D1-8} shows an intricate hairpin-like domain arrangement with approximate dimensions of 130 × 80 × 165 Å (Fig. 1B), and bears no resemblance to any other known protein structure in the PDB. The D1, D2, D3, D4, and D5 domains have a typical Ig-like C2 fold, and domains D6, D7 and D8 have a Fibronectin type III (FnIII) fold (Fig. S2). In D3 and D4, a 3_{10} helix appears at the loop connecting strands E and F. A 9-residue alpha helix is located at the N-terminus of D7, extending out from the main FnIII fold.

There are three molecules of hRobo2^{D1-8} in the crystal asymmetric unit, baring only small cross-differences, resulting in R.M.S.D values of 1.9 - 2.6 Å between all atoms of the three copies. The overall high quality of the electron density maps, combined with the availability of high resolution crystal structures of individual Robo domains, and the unbiased locations of Selenomethionine (SeMet) substructures (calculated from the anomalous signal of SeMet - derivatized hRobo2^{D1-8} crystals) allowed us to trace ~85% of the residues with high confidence (Fig. 1D). The exception is D8, in which the atomic side chain details are not clearly observed due to high B-factor values.

The special domain organization of hRobo2^{D1-8} requires that the loops linking individual domains are of a particular length. In this way, short one-residue linkers between D1-2, D2-3, D3-4, and D7-8 enforce compact rod-like arrangements, while the 11-residue long D5-6 linker enables the antiparallel head-to-tail arrangement of the two domains, and the 9-residue long D6-7 linker facilitates the flanking of D4 on two opposing faces. Notably, while the sequence identities of the D5-6 and D6-7 linkers are not well conserved between Robo orthologs and paralogs (Fig. S1), their lengths are no shorter than those of hRobo2, demonstrating the key role these linkers play in facilitating a correct domain organization.

Preclusion of D4 dimerization interface in the hRobo2^{D1-8} structure

D4 is located at the center of the hRobo2^{D1-8} structure, flanked by D3 on its N' terminal side, D5 and D6 on one domain face, and by D7 on the other (Fig. 1B, 2C). Remarkably, we have previously identified the same surface of D4 that is sequestered by D7 as a direct mediator of dimeric hRobo2 interactions (Yom-Tov et al., 2017). Specifically, we showed that a predominantly hydrophobic surface on D4 mediates close homotypic contacts with a reciprocal D4 in the crystal lattice of hRobo2^{D4-5} (PDB 5NOI) (Fig. 2A, S1, S3).

The identification of D4 as a Robo dimerization module is supported by the recently published structure of hRobo1^{D1-4} (PDB 5O5G and 5OPE) (Aleksandrova et al., 2018), which forms identical crystal contacts with those observed in the hRobo2^{D4-5} structure (Fig. 2A, B, S3). Given the differences between the two Robo paralogs, in sequence (hRobo1 vs. hRobo2), constructs used (D1-4 vs. D4-5), and crystallization conditions in these two studies, we consider the overlapping crystal contacts to genuinely reflect a conserved role and functionality for D4 dimerization in Robo signaling.

To study Robo extracellular (EC) dimerization at cell membranes, we developed a protein chimera system, replacing the catalytically inactive Robo cytosolic portion with the intracellular (IC) tyrosine kinase domain of the stem cell factor (SCF) receptor cKIT (Fig. 3). The chimera readout system is based on the principle that cKIT cannot phosphorylate itself or a distant molecule. Rather, following cKIT dimerization by SCF, one cKIT phosphorylates in *trans* the now-juxtapositioned second cKIT molecule (Fig. 3A) (Lemmon et al., 1997; Lev et al., 1992; Opatowsky et al., 2014). In our Robo^{EC}-cKIT^{IC} chimera system, the tyrosine phosphorylation intensity is indicative of the dimerization strength mediated by the extracellular Robo portion.

First, we tested three hRobo2 constructs: hRobo2$^{D1–8}$-cKIT^{IC}, and the truncated hRobo2$^{D1–4}$-cKIT^{IC} and hRobo2$^{D1–3}$-cKIT^{IC}. As a control, we measured the tyrosine phosphorylation of full-length cKIT^{FL}, before and after addition of the SCF dimerizing ligand (Fig. 3B). To confirm the surface expression of all protein constructs in this experiment, we used FITC-linked immunolabeling (Fig. 3E). The results show a strong dimerization of hRobo2$^{D1–4}$-cKIT^{IC}, and weaker, but still considerable responses of hRobo2$^{D1–8}$-cKIT^{IC} and hRobo2$^{D1–3}$-cKIT^{IC}, indicating that the latter two are not strictly monomeric.

Next, we examined whether auto-inhibition of D4-mediated dimerization is conserved in other Robo orthologs and paralogs, and compared the dimerization of D1–8 and D1-4 constructs from hRobo1, hRobo3, and SAX-3 using the cKIT chimera phosphorylation assay. In all cases, the D1-8 constructs show different levels of weaker dimerization when compared with their equivalent D1-4 based constructs (Fig. 3C). To support these results, we conducted sedimentation equilibrium analytical ultracentrifugation (SE-AUC) measurements to determine the oligomeric state of hRobo2^{D1-8} and hRobo2$^{D4–5}$ in solution. We found that hRobo2^{D1-8} is predominantly monomeric, with a weak estimated dimerization constant K_D of 100-300 μM

(Fig. S4). In comparison, our previous AUC measurements of isolated hRobo2^{D4-5} (Yom-Tov et al., 2017) showed a significant dimeric fraction, with a calculated dimerization dissociation constants of 16.9 μM.

Finally, because D7 directly sequesters the D4 dimerization interface in the hRobo2^{D1-8} structure, we postulated that removal of D7 should release the autoinhibitory constraints for D4 dimerization. Indeed, a hRobo2$^{D1-8\Delta7}$-cKIT^{IC} truncation mutant shows increased dimerization in comparison with intact hRobo2^{D1-8}-cKIT^{IC}, and hRobo2$^{D1-8\Delta5}$-cKIT^{IC} and hRobo2$^{D1-8\Delta6}$-cKIT^{IC} deletion mutants (Fig. 3D). Based on these results, we conclude that D4 makes a dominant contribution to hRobo2 dimerization, and that D4 is predominantly sequestered from *cis*-dimerization in the context of an intact Robo ectodomain.

Importance of D4-mediated dimerization *in vivo*

To investigate the functional role of Robo D4-mediated dimerization, and other aspects of Robo activation *in vivo*, we used the *C. elegans* AVM neuron model system. Specifically, we investigated how putative loss- and gain-of-function mutations in *sax-3*, designed based on the Robo structure, affect AVM migration pattern. Notably, unlike *Drosophila* and mouse Slit-Robo model systems with several Robo paralogs, *C. elegans* has maintained a single cognate Robo/SAX-3 and Slit/SLT-1, offering a simple and well characterized *in vivo* model to probe mechanistic elements of Robo activation.

In this model, the knockout of *sax-3* has severe consequences on *C. elegans* physiology (Zallen et al., 1998), including reduced viability, and defects in animal morphology, movement and egg-laying. Defects were also identified and characterized in several neurons, most notably in the mechanosensory AVM. In w.t animals, the AVM's cell body is positioned on the lateral hypodermis and projects an axon ventrally to the ventral midline, which then turns anteriorly, forming a distinctive inverse "L" shape, that we refer to here as the "turn" phenotype (Fig. 4A). Two signaling systems, repulsive and attractive, act in parallel in AVM ventral guidance: SLT-1 is secreted from dorsal muscles and acts through SAX-3 to repel the AVM axon (Hao et al., 2001); and UNC-6 (Netrin), which is secreted from ventral cord axons, attracts the AVM axon through its cognate receptor UNC-40 (DCC) (Yu et al., 2002). Mutations in either component of the SLT-1/SAX-3 and UNC-6/UNC-40 signaling systems result in defective AVM ventral guidance, failing to grow ventrally, and instead directly extending anteriorly (Fig. 4A). Hereafter, we refer to this AVM phenotype as "straight".

In our experiments, we used a *sax-3(-/-)* (ky123) mutant strain, carrying a *Pmec-4::gfp* transcriptional reporter expressed in six neurons, including AVM (Hao et al., 2001). In this strain, 70% of the animals (n=71) showed a "straight" AVM growth, and 24% had a "turn" phenotype. Contrary to that, w.t. *Pmec-4::gfp* animals (n=89) showed an inverse ratio of 15% "straight" and 77% "turn". Clearly, both phenotypes are naturally occurring, but the "turn" is

much more prevalent in w.t animals, and the "straight" in *sax-3(-/-)* ones. We have also observed a few other AVM phenotypes that are neither "turn" nor "straight", and include indirect ventral-anterior routes, and ventral positioning of the AVM cell body. These phenotypes, which we refer to as "other", are rare in w.t and *sax-3(-/-)* animals, but constitute a significant portion in some of the rescue mutant strains (Fig. S5). We consider that these "other" phenotypes reflect a miss-regulated SAX-3 function, and not necessarily loss- or gain-of-function. To avoid unnecessary interpretations, we have therefore based most of our statistical analyses on the "straight" phenotype percentage, which is clearly associated with loss-of-function.

On the *sax-3(-/-)* (ky123) background we established several *Pmec-7* transgene strains to drive expression of mCherry-fused SAX-3 and SAX-3 mutants in AVM (and 13 other neurons). In a *Pmec-7::sax-3*$^{w.t}$ rescue transgene, we found that 69% of the animals (n=93) showed a "turn" AVM phenotype, while 24% were "straight", and 7% had "other" phenotypes. These proportions are not significantly different than the w.t animals, showing a *sax-3* rescue by the transgene in the AVM neuron. However, a rescue transgene with a D4 deletion *Pmec-7::sax-3*$^{\Delta D4}$ had only 31% of the total animals (n=91) showing the "turn" phenotype, 53% "straight" and 16% "other", demonstrating a near-complete loss of SAX-3 function. To directly address the role of dimerization of D4 in SAX-3 activity, we introduced a F360R point mutation, directed to specifically interfere with D4 dimerization. The D4-D4 dimerization interface, as revealed in the crystal structures of hRobo2^{D4-5} and hRobo1^{D1-4}, includes several conserved residues, one of which is hRobo2 F357 / hRobo1 F394 (Fig. S1, S3). Previously, we found that a F357R substitution eliminates hRobo2^{D4-5} dimer formation in AUC measurements (Yom-Tov et al., 2017), and therefore targeted the homologous SAX-3 F360 in the *C. elegans* AVM guidance model. As anticipated, the *Pmec-7::sax-3*^{F360R} transgene showed a loss-of-function phenotype, with only 30% of the total animals (n=91) with a "turn" phenotype, 53% "straight", and 16% other phenotypes.

Taken together, these results establish the critical role of D4 in SAX-3 function, and link D4-mediated dimerization, which we have demonstrated to be important *in vitro* and in cell culture assays, with SAX-3 function *in vivo*.

D5 is important for hRobo1-hRobo2 *trans* interactions

Inhibitory Robo *trans* interactions are documented in both *Drosophila* and mammalian systems. In *Drosophila*, dRobo2 expressed in midline cells acts to inhibit dRobo1-mediated repulsion of commissural axons (Evans et al., 2015). Rat Robo1 and Robo2 interact in *trans* to promote axon outgrowth of retinal neurons (Hivert et al., 2002), and mouse Robo2 is engaged in homotypic interactions to control repulsive responses in dendritic self-avoidance of cerebellar Purkinje cells (Gibson et al., 2014).

Crystal contacts in the hRobo2^{D1-8} lattice provide molecular details on how Robo receptors might interact in *trans* when presented on neighboring cells or cell processes (Fig. 5A-C, Fig. S6). The *trans* interactions are made between two opposing layers of Robo molecules, where the membrane proximal side (the carboxy-terminal end of D8) of Robos point in opposite directions, resulting in a distance of 186 Å from each other, consistent with a role in mediating cell-cell contacts. The *trans* interactions are not restricted to a 1-on-1 stoichiometry, but rather form a continuous array, where each Robo molecule from one layer simultaneously binds two Robo molecules from the opposing layer (Fig. S6). The interactions involve direct contacts between D5 and a D5-linked glycan branch from the first Robo molecule presented on one layer, to D1 and D2 of an opposing Robo. While the D1 and D2 of the first Robo interact with D5 and a D5-linked glycan branch of another opposing Robo molecule, and so on. The contact surface of trans interacting Robo molecules is mostly water mediated, with few direct amino acid side chain and backbone contacts. The most significant interacting surfaces are the A-B and E-F loops (and to a lesser extent the carboxy-terminal residues of strands C and G) of D5, with the E-F and C-D loops (and to a lesser extent the A-B loop) of D1 (Fig. S1, S2). The N-linked glycan branch on the sequence conserved Asn426 of D5's mediates a secondary interaction with the highly conserved Arg99 and Arg132 of D1 and D2 respectively.

To validate the role of D5 in mediating Robo *trans* interactions between neighboring cells, we performed a cell aggregation assay, an approach widely used to study various cell adhesion molecules (e.g. Protocadherin (Goodman et al., 2017)) (Fig. 5D). Based on previous data that hRobo1 and hRobo2 mediate hetero *trans* interactions, measurable in a bead aggregation assay (Hivert et al., 2002), we replaced the intracellular portion of hRobo1 and hRobo2 with mCherry and CFP fluorescent proteins, respectively. hRobo1^{D1-8}-mCherry^{IC} and hRobo2^{D1-8}-CFP^{IC} were individually expressed for 4 hours in two separate HEK293F suspension cultures. The two cultures were then mixed and left to grow in suspension for 44 hours. The same procedure was undertaken with hRobo2 truncated in D5 (hRobo2$^{\Delta D5}$-CFP^{IC}), an unrelated control Ig-like receptor cKIT^{EC}-CFP^{IC} (there is no indication, or record, for Robo-cKIT interactions), and Notch^{EC}-Citrine Delta^{EC}-mCherry^{IC} as a positive control, a well-characterized cell aggregation trans-interaction system (e.g. Pandey and Jafar-Nejad, 2018). Our first observation was that, unlike classical cell adhesion molecules (CAMs), no large aggregates were formed in individual or mixed Robo cultures. However, small aggregates of 2-5 cells were present in all cultures, which we sorted and recorded using an imaging flow-cytometer device. We found that on average, 12.1% of aggregates in the hRobo1-hRobo2 mixed cultures included both hRobo1 and hRobo2 expressing-cells, while only 3.2% and 5.1% were observed in the hRobo1-hRobo2$^{\Delta D5}$ and hRobo1-cKIT mixtures, respectively (Fig. 5E). The positive control had 22.4% mixed-cell aggregates, demonstrating stronger Notch-Delta *trans* interactions relative to Robo. We than plated the sorted aggregates and inspected these

cultures under a high magnification microscope after an overnight incubation and fixation (HEK293F cells settle and grow on lysine-coated slides when not stirred). In contrast to the smooth cell-cell interfaces observed in the hRobo1-hRobo2$^{\Delta D5}$ and hRobo1-cKIT mixtures, the hRobo1-hRobo2 (and Delta-Notch) mixed cultures featured large contact area membrane extensions, indicative of tight heterotypic interactions between hRobo1 and hRobo2 that depends on the presence of D5 in hRobo2 (Fig. 5E).

Slit-independent activity in a Robo gain-of-function mutant

Our results implicate D5 as a potential Robo *trans* inhibitory interaction domain. We therefore investigated the consequence of D5 truncation on Robo biological activity. For this, we followed two experimental approaches: the AVM *in vivo* model of *C. elegans* (Fig. 4); and a neurite outgrowth assay of mouse spinal cord dissociated neuronal cultures infected with Robo-expressing lentiviruses (Fig. 6, Fig S7). In *C. elegans*, we introduced a rescue transgene with a D5 deletion *Pmec-7::sax-3*$^{\Delta D5}$ to the *sax-3* (ky123) mutant carrying *Pmec-4::gfp.* Of the total animals (n=80), 54% had the "turn" AVM phenotype, 24% were "straight", and 22% "other". Compared with the *sax-3*$^{w.t}$ rescue, *sax-3*$^{\Delta D5}$ exhibits a similar percentage of the "straight" anterior phenotype (24%), indicating that D5 deletion does not involve a loss of function.

We next considered that, if indeed *sax-3*$^{\Delta D5}$ brings about a release of auto-inhibitory constraints, its activity would be independent of SLT-1. In order to test this hypothesis, we silenced SLT-1 expression using *slt-1* RNAi (Fig. 4B, C). Treatment with *slt-1* RNAi did not have a substantial impact on w.t *Pmec-4::gfp* animals, reducing the "turn" phenotype by 8%, and elevating the "straight" phenotype by 11%. However, the *sax-3(-/-)* (ky123) mutant that is rescued by the *sax-3*$^{w.t}$ transgene proved to be much more sensitive to the RNAi treatment, showing a dramatic 33% reduction in the ventral AVM "turn" phenotype, from 69% (in the untreated experiment) to 36%, with a matching increase in the "straight" phenotype, from 24% to 55%. Control RNAi treatment with pAD12 (Dillin et al., 2002) did not have significant consequences on the *sax-3*$^{w.t}$ transgene, with 54% of the animals (n=82) showing the "turn" phenotype, 26% were "straight", and 20% "other". Not surprisingly, it seems that the *sax-3*$^{w.t}$ rescue strain is more sensitive to SLT-1 silencing than the w.t animals.

Contrary to the *sax-3*$^{w.t}$ transgene, the *sax-3*$^{\Delta D5}$ rescued animals have maintained a similar AVM phenotype distribution, with- and without *slt-1* RNAi (54% and 53% "turn"; 24% and 30% "straight") respectively. From these *C. elegans* experiments we conclude that SAX-3$^{\Delta D5}$ has, at least in the context of AVM ventral guidance, a constitutive, SLT-1 independent activity. Similarly, in *sax-3*$^{\Delta D7}$ rescued animals, removal of D7, which directly blocks D4 in *cis*, as seen in the hRobo2^{D1-8} crystal structure (Fig. 2), and as demonstrated in the Robo-cKIT chimera phosphorylation assay (Fig. 3D), has a SLT-1 independent activity effect, with a low percentage of "straight" (36%) phenotype, which is kept under *slt-1* RNAi (28%) treatment.

Next, to investigate the impact of D5 truncation in mammalian Robos, we infected dissociated mouse spinal cord neurons with lentiviruses expressing either intact or D5-truncated hRobo1 and 2 compared to a GFP control (Fig. 6, Fig. S7). Normally, cultured neurons in this assay project neurites that connect neighboring cells. The well documented repulsive effects of Robo1/2 in axon guidance and dendritic avoidance (Blockus and Chedotal, 2016), led us to hypothesize that Robo 1/2 activity would reduce the number, and/or length of neurites in this assay. Indeed, hRobo1$^{\Delta D5}$ had a dramatic effect, eliminating most neurite extensions after five days, while hRobo1^{FL} and hRobo1$^{\Delta D4}$ had no significant response, thereby demonstrating the activating effect of a D5 truncation, and consistent with the *C. elegans sax-3*$^{\Delta D5}$ transgene phenotype. hRobo2$^{\Delta D5}$ also had a significant response, which was similar to that of hRobo2^{FL}. Unlike hRobo1^{FL}, hRobo2^{FL} has some basal activity, which may be due to a difference in auto-inhibition levels. Interestingly, when compared with hRobo2^{FL}, hRobo2$^{\Delta D4}$ is not active, providing further evidence for the importance of D4 in Robo signaling, and concordant with the *C. elegans sax-3*$^{\Delta D4}$ transgene phenotype.

Discussion

D4-mediated dimerization is important for Robo signaling

Dimerization of cell surface receptors drives the activation of many cytokine and growth-factor signaling systems (Atanasova and Whitty, 2012). Although it's not known how dimerization might affect Robo, it was for many years considered important for activation and signaling (Blockus and Chedotal, 2016; Seiradake et al., 2016).

The identification of D4 as a dimerization domain of hRobo2 *in vitro* and cell-size assays (Yom-Tov et al., 2017), together with identical D4 dimerization interfaces observed in hRobo2^{D4-5} (PDB 5NOI) and hRobo1^{D1-4} (PDB 5OPE/5O5G) (Fig. 2, S3), have further focused attention on Robo D4-mediated dimerization. A high level of evolutionary sequence conservation is observed in the D4 dimerization interface (Yom-Tov et al., 2017), including those of hRobo3 and the *C. elegans* SAX-3 (Fig. S1). Here, we further demonstrate homo-dimerization of hRobo1, 2, 3 and SAX-3 using a Robo-cKIT chimera assay (Fig. 3). After confirming that Robo dimerization is conserved in *C. elegans,* we utilized the well-established role of *sax-3* in guiding the navigation of the AVM neuron to investigate the functionality of D4-mediated dimerization *in vivo* (Fig. 4). Our results show that removal of D4 eliminates most SAX-3 activity, and furthermore that substitution of a single critical residue in the dimerization interface (F360R) significantly reduces activity. Under *slt-1* RNAi silencing conditions, the loss of function in D4 mutants' phenotype is not aggravated, indicating that their residual SAX-3 activity is *slt-1* independent. The importance of D4 in mediating Robo activity is further supported by our experiments on dissociated spinal cord neurons infected with Robo-expressing lentivirus constructs, where unlike the intact hRobo2^{FL} that reduces neurite outgrowth, hRobo2$^{\Delta D4}$ showed no significant response (Fig. 6).

Taken together, several lines of evidence establish D4 dimerization as a critical and conserved component in Robo function. But are additional dimerization interfaces present? We think this is quite likely. Previously, Evans and Bashaw have demonstrated an important functional role for D3 in *Drosophila* Robo receptors (Evans and Bashaw, 2010), and here, in the hRobo2^{D1-3}-cKIT^{IC} chimera, a dimerization signal stronger than background is also observed (Fig. 3B). Dimerization through the membrane proximal domains is also conceivable, however our experiments show no evidence for this so far. Purified hRobo2^{D5-8} appears as a strict monomer in AUC (Fig. S4), and no crystal contacts in the previously determined hRobo1^{D7-8} crystal structure (PDB 4HLJ) are indicative of homotypic dimers (Barak et al., 2014). Still, very weak interactions can evade detection by AUC and crystallography. Another open question is whether D4 is restricted to homo-dimerization, as we have demonstrated throughout our investigations, or to additionally mediate functional *cis*-hetero interactions.

Auto-regulation of Robo signaling by D4-dimerization interface sequestering

After establishing a critical role for D4-mediated dimerization in the activation of Robo receptors, it became obvious from the hRobo2^{D1-8} crystal structure that auto-inhibition of the D4 dimerization interface restriction could occur (Fig. 2E), which was confirmed using Robo-cKIT chimera assays (Fig. 3). This form of auto-regulation is found in other signaling systems, and was intensively investigated in members of the epidermal growth factor receptor (EGFR/ErbB) family, where ligand binding promotes (or rather stabilizes) dramatic conformational changes in the receptor's ectodomain to reveal hitherto buried dimerization interfaces (Lemmon and Schlessinger, 2010). In the hRobo2^{D1-8} crystal structure, the D4 dimerization interface is occluded by D7 and the D6-7 linker from the same molecule (auto-inhibition in *cis*). This inhibitory conformation can be further fastened by an opposing Robo molecule presented on a neighboring cell, or cell process, (auto-inhibition in *trans*) (Figs. 5, S6).

How tight are these two levels of auto-inhibition? There are several indications that *cis* inhibition, by itself, is insufficient. FRET measurements (Zakrys et al., 2014) of recombinant mouse Robo1 expressed in adherent HEK293 cells identified the presence of both monomeric and oligomeric species, and showed that domains D1-5 are critical for oligomerization. This study is consistent with our Robo-cKIT chimera results, also conducted in adherent cell culture, where a basal level of dimerization is observed (Fig. 3). Under these experimental conditions, surface presented Robo receptors are exposed to extracellular space, and most do not interact with reciprocal Robo receptors on neighboring cells. However, when *trans* interactions do occur, the auto-inhibited conformation is less likely to open and expose D4 for dimerization.

Releasing auto-inhibition: proposed mechanisms for Slit-induced Robo activation

Following the realization that active Robo has an "open" conformation, and that auto-inhibited Robo is "closed", we consider that Slit ligand stimulation would invoke a shift in favor of the "open" conformation (Fig 7). We further suggest that Slit achieves this differently, depending on how Robo is presented on the cell surface. There are two basic scenarios for Robo presentation i) exposed to extracellular space and free from *trans* interactions; or ii) interacting in *trans* with reciprocal Robo molecules presented on neighboring cells or cell processes.

In the first scenario, as discussed previously, we conclude that auto-inhibition is not stringent. We base this on our Robo-cKIT chimera phosphorylation assays, showing higher phosphorylation levels of hRobo 1,2,3 and SAX-3 compared to non-liganded cKIT (Fig. 3), and previous FRET measurements of mouse Robo1 (Zakrys et al., 2014) reporting a monomer-oligomer mixed population. In both assays, Robo was recombinantly over-expressed in a cell culture monolayer, and therefore unlikely to be engaged in *trans*

interactions. If indeed Robo receptors are presented in this way *in vivo* (that is, without being engaged in *trans* interactions), how might Slit induce Robo activation? In this scenario, we suggest that Slit affects Robo conformation and clustering. In a previous structural study of the juxtamembrane hRobo1 D7-8 domains (Barak et al., 2014) we proposed that binding of Robo to Slit, which is conjugated by several extracellular matrix components to cells lining the path of Robo-exhibiting migrating cells or cell processes, inflicts a mechanical tension that extends Robo's ectodomain to expose a juxtamembrane proteolytic site. The same concept is relevant with the conformational change required to release Robo *cis* auto-inhibition and expose D4 for dimerization. Slit binding might also promote Robo clustering, because Slit LRR4 domains can form dimers (Howitt et al., 2004; Seiradake et al., 2009), through which Robo can also dimerize. But it was also reported that a larger Slit segment was monomeric (Hohenester, 2008), so further structural studies of Slit are clearly warranted.

The second scenario, where Robo receptors are interacting in *trans* with neighboring Robo molecules, is substantiated in several previous studies (Evans et al., 2015; Gibson et al., 2014; Hivert et al., 2002). Our results support these reports, and highlight the critical role of D5 in mediating *trans* interactions. Consistent with the notion that Robo *trans* interactions are functionally inhibitory, the crystal packing of hRobo2^{D1-8} displays how tight auto-inhibition on individual Robo molecules (Fig. 5) can prevent the disengagement of D4 from D7, and subsequent exposure of D4 for *cis* dimerization and signaling. Based on this analysis, and on prior structural knowledge, we propose how Slit binding might activate Robo in the context of *trans* interacting receptors.

Superimposing the Slit LRR2 domain on the hRobo2^{D1-8} structure using the hRobo1^{D1}-hSlit2^{LRR2} complex structure (PDB 2V9T) (Morlot et al., 2007) results in a clash with the *trans* interacting Robo's D5 (Fig 7B, C). Specifically, Slit LRR2 overlaps with the C-D loop and D strand of D5. In this way, the tight binding of Slit to D1 of one Robo molecule would displace D5 of the reciprocal Robo molecule, thereby relaxing the imposed *trans* confinement. Interestingly, to achieve this, even the minimal Robo-interacting domain of Slit (LRR2 domain) suffices, consistent with previous functional reports in dendritic self-avoidance mediated by Slit2/Robo2 (Gibson et al., 2014), and growth cone collapse assays (Hussain et al., 2006; Seiradake et al., 2009).

In summary, our analyses show that auto-inhibition and receptor dimerization are key elements in Robo signaling at cell surfaces, following three principles: i) Robo activity requires D4-mediated dimerization; ii) Pre-stimulated Robo is auto-inhibited by adopting a conformation in which D4 is sequestered from mediating dimerization and; iii) How stimulation by Slit can release Robo auto-inhibition. This work further highlights some fundamental differences between Robo receptors on the cell surface, when either presented and exposed

to extracellular space (as in most monolayer cell cultures), or interacting with reciprocal Robos on neighboring cells, or cell processes.

Figure legends

Figure 1. Crystal structure of hRobo2 full-length ectodomain. A) Color-coded organization and nomenclature of Robo extracellular Ig and FnIII domains and intracellular conserved motifs. Novel and previously established domain functionalities are indicated. Domain organization of Slit is shown in gray, with the Robo-binding LRR2 domain, darkened. B) Crystal structure of hRobo2^{D1-8} in surface representation. The juxtamembrane, transmembrane, and intracellular portions appear, for orientation purposes, as schematic representation. C) Simplified representation of the hRobo2^{D1-8} structure. D) $2F_o$-F_c electron density map (blue mesh) contoured at 1.8 σ level showing a close-up view of the D5 core region. Anomalous difference map (red) contoured at 4 σ level marks the selenium substructure locations at methionine residues 594 and 609 (marked). The backbone and side chains are represented as sticks.

Supplementary figure S1. Structure-based sequence alignment of D1-8 to the human Robo1, 2 and 3, and the *C. elegans* homolog SAX-3. Color coded symbols and Uniprot protein accession numbers are listed below.

Supplementary figure S2. Crystal structure of hRobo2 extracellular domains. Domains are individually displayed in cartoon representation, with secondary structure elements labeled, according to the same color scheme as in Fig. 1. Oligosaccharide (Glycan) molecules bound to D1 and D5 are represented as sticks.

Figure 2. D4-mediated dimerization is inaccessible in the hRobo2^{D1-8} structure.
A) Finding the same needle in two different haystacks. In the crystal lattice packings of hRobo1^{D1-4} (PDB 5OPE) and hRobo2^{D4-5} (PDB 5NOI), two protomers are highlighted – following the same domains color code as in Fig. 1. The domains of one protomer are labeled, and those of the second protomer are labeled and underscored. In both cases, the two highlighted protomers dimerize through D4. B) Superimposition of the D4 dimers from the hRobo1 (pale yellow) and hRobo2 (dark yellow) lattices reveal an identical D4-mediated dimerization interface (area boxed in A). C) A zoom-in view of the D4 dimerization interface in the hRobo2^{D1-8} crystal structure. D4 is presented as surface, while the rest of the molecule – as cartoon. The D4 dimerization interface is colored in white, and is blocked by D7.

Supplementary figure S3. Related to figure 2. Robo D4 dimerization in two independent crystal lattices.
Crystallographically related dimers of hRobo1^{D1-4} (A) and hRobo2^{D4-5} (B) are presented, with one protomer displayed as a cartoon and the second with surface representation. Below, an

open-book representation of the D4 dimerization interface (white) highlights the conserved and critical hRobo1 Phe394 / hRobo2 Phe357 residue, for which the homologous residue in SAX-3 is Phe360. Note that we have used the hRobo1 isoform 5 in this work, in which the critical phenylalanine position is Phe358 (Fig. S1).

Figure 3. Robo-cKIT chimera trans-phosphorylation dimerization assay.
As Robo receptors lack intrinsic catalytic activity, the intracellular (IC) portion of the receptor tyrosine kinase cKIT (in green) was fused to Robo/SAX3 ectodomain constructs and dimerization monitored through anti phospho-tyrosine immunoblotting in HEK293 cells.
(A) Schematic representations of Robo and cKIT. SCF (magenta) brings about cKIT dimerization and trans-phosphorylation, and was used for control.
B) Strongest hRobo2 dimerization are seen in the hRobo2^{D1-4}-cKIT^{IC} construct, and weaker dimerization levels (yet stronger than non-stimulated cKIT) for the hRobo2^{D1-8}-cKIT^{IC} and hRobo2^{D1-3}-cKIT^{IC}. Quantification is based on cross-referenced w.b. intensity densitometry from three repeats' mean ± SEM.
C) In the same way, strong and weak dimerization levels are also demonstrated for the chimera constructs D1-4 and D1-8 of hRobo1 and SAX-3, and a moderate difference in the case of hRobo3.
D) Consistent with the hRobo2^{D1-8} crystal structure, in which D7 directly blocks the D4 dimerization interface (Fig. 2), removal of D7 (but less so D5 or D6) elevates dimerization of hRobo2.
E) Surface expression on COS-7 cells is confirmed by adherence of secondary FITC-labeled antibodies to the N-terminal FLAG-tags of the various protein constructs, visualized in fluorescence microscopy (scale bar - 10 μm).

F) We conclude that removal of D5-8 exposes D4 and drives Robo to dimerization, while a smaller fraction of the intact Robo ectodomain allows dimerization.

Supplementary figure S4. Sedimentation equilibrium analytical ultracentrifugation (SE-AUC) of soluble hRobo2 constructs. SE-AUC data shows much weaker dimerization of hRobo2^{D1-8} than hRobo2^{D4-5}, consistent with the data presented in Fig. 3. Under these experimental conditions, hRobo2^{D5-8} does not show any dimerization. The hRobo2^{D4-5} measurement was reported previously in (Yom-Tov et al., 2017). Data fitting was performed with Sedphat (Gabrielson et al., 2007) yielding clear results with small random residuals.

Figure 4. SAX-3/SLT-1 in *C. elegans*. A) Schematic diagram of the AVM axon guidance system in *C. elegans*. Middle panel: Dorsal muscles express the repellent SLT-1/Slit (red).

The attractant UNC-6/netrin (blue) is expressed ventrally. In *sax-3* (-/-) mutants, the AVM axon fails to grow ventrally and instead directly extends anteriorly. Lower panel: confocal images of w.t (left) and *sax-3(ky123)* (-/-) mutant, where the AVM is visualized by a *Pmec-4::gfp* and arrowheads point at the extending AVM axon. B, C) Graphical and numerical representation of AVM axon guidance in *sax3* rescue lines and under *slt-1* RNAi silencing. AVM guidance phenotypes were classified into three categories: "turn" (blue), which appears in most w.t and w.t-rescued animals; "straight" (red), which appears in most *sax3* knockout animals, and is associated with *sax3* and/or *slt1* loss-of-function; "other" (green), which includes several other AVM axon trajectories (Fig. S5). n - total number of animals that were scored. C) Each cell includes the values for w.oRNAi / wRNAi. D) Representative images of *sax-3(ky123)* (-/-) animals, rescued with mCherry-fused *sax3* w.t and mutant transgenes. Arrowheads point at the AVM's cell body.

In the statistical analysis, phenotype proportions in different experiments were compared using the Chi-squared test of independence. Biological replicates (two lines, three replicate groups for each) were merged after initial Chi-squared tests were performed to ensure there were no significant difference ($P>0.05$) between them. A post-hoc analysis was performed to compare the proportion of the straight phenotype. Finally, all p-values were corrected for multiple comparisons with the FDR procedure, ***$P<0.005$, ns: no significance.

Supplementary figure S5. AVM "other" morphologies in *C. elegans*. A) Schematic diagrams of the AVM axon "turn" morphology - associated with normal *sax-3* and *slt-1* function, and of the "straight" morphology - associated with *sax-3* and *slt-1* loss of function. B-F) Representative images and diagrams of aberrant AVM tracks. These phenotypes that collectively referred to as "other", are unusual to find in w.t and *sax-3(-/-)* animals, but are more common in rescue mutant strains.

Figure 5. Asymmetric trans-interactions between D5 and D1 from opposing Robo molecules. A) Crystal contacts in the hRobo2^{D1-8} lattice reveal *trans* interactions between two opposing arrays of Robo molecules, with only one pair presented here. The analogous continuous array is shown in Fig. S6. The trans-interactions involve direct contacts between D5 and a D5-linked glycan branch from the upper Robo molecule, with D1 and D2 of the opposing Robo. B) Back view of (A). C) Close-up view of the N-linked glycan branch extending from N426 of D5, with conserved arginine sidechains (in blue) on D2 from an opposing Robo molecule (see details in figure S1). D) D5 trans interactions in cell aggregation assay. Robo1^{mCherry} and Robo2^{CFP} were separately expressed in HEK293F suspension cells, mixed together and then sorted through a FACS imaging device. A positive cell aggregation control Notch^{Citrine} - Delta^{mCherry}

were treated similarly. E) Sorted mixed-population cell aggregates were plated and cultured for confocal high-resolution microscopy. Robo1-Robo2 interacting cells have large contact area with lamellipodia extensions (marked by white arrows), unlike the non-specific interactions seen in Robo1-cKIT expressing cells. Scale bars: 10 μm. F) Quantification of co-aggregation FACS sorting shows that cell mixtures of Robo1^{mCherry} and Robo2^{CFP} expressing cells have higher percentage of mixed population aggregates, compared to Robo1^{mCherry} mixed with control cKIT^{CFP}, or hRobo2$^{\Delta D5\text{-}CFP}$ truncation mutant (mean of three repeats ± SEM).

Supplementary figure S6. hRobo2$^{D1\text{-}8}$ *trans* interactions are not restricted to a 1-on-1 stoichiometry, but are rather continuous, forming an array, where each Robo molecule from one layer binds two Robo molecules from the opposing layer simultaneously. A) Crystal lattice packing. B) Two-dimensional cartoon of the trans-interacting lattice.

Figure 6. hRobo1&2 and truncation mutants' effects on mouse SC neurons (A) Upper panel - Representative Images of E12.5 primary SC neurons culture. The cells were infected with Robo LV's 6 hr after plating, as indicated. Neurite length was measured using the incuCyte imaging system after 5 DIV. Lower Panel - Representative images with incuCyte mask output for neurite length analysis. Scale bar: 50 μm (B) Neurite length quantification revels a significant decrease in the Robo1$^{\Delta D5}$, Robo2^{FL} and Robo2$^{\Delta D5}$ in compare to GFP control and Robo2$^{\Delta D4}$, demonstrating gain-of-function of ΔD5 and loss-of-function of ΔD4 Robo truncation mutants on the SC neurons (3 biological repeats, 45 fields per each repeat, Student t.test; ***p<0.001, **P<0.01, *P<0.05). (C) High magnification representative image of SC neuron expressing GFP after LV infection demonstrating its expression. Lower Panel – Inset shows SC neuron axon demonstrating GFP expression also in this compartment. Scale bar: 10 μm

Supplementary figure S7. Expression of different LV's constructs in dissociated SC neurons culture. High magnification representative images of SC neuron expressing GFP after LV's infection demonstrating expression in all the different constructs, as indicated. Blue indicates DAPI staining, Gray indicates alpha tubulin staining and green indicate GFP expression. Scale bar: 10 μm.

Figure 7. A model for Slit-induced Robo activation by dissociation of Robo *trans*-inhibition A) In *trans* interacting Robos, D1 (in pink) of one molecule interacts with D5 (in green) of a reciprocal Robo. B, C) Crystallographic analysis of Slit LRR2 binding vis-à-vis trans-interacting Robo shows that Slit binding and Robo *trans* interactions are mutually exclusive, due to a clash between Slit LRR2 and the reciprocal Robo D5. D) According to this model, trans-interacting Robo molecules are tightly auto-inhibited. Slit binding inflicts Robo trans

dissociation, reducing Robo auto-inhibition to allow an “open” active conformation, which facilitates the formation of D4-mediated Robo dimers. Dimer formation, presumably, activates the intracellular portion of the receptors (by an unknown mechanism), allowing the recruitment of intracellular effectors and signaling.

Experimental procedures

Protein expression and purification – Constructs of hRobo2 used for crystallography and analytical ultracentrifugation were prepared by PCR amplification from the complete cDNA clone (ImaGenes) of human Robo2 (Acc. no. BC146772.1/Q9HCK4). Robo2^{D2-3} (spanning residues 129-311), Robo2^{D5-8} (spanning residues 415-859), and Robo2^{D1-8} (spanning residues 24-859) were amplified and ligated into a modified pFastBac (pK503-9) insect cell expression vector containing an N-terminal FLAG tag and a C-terminal hexahistidine tag, followed by a stop codon. To produce baculoviruses expressing hRobo2 D2-3, D5-8, and D1-8, recombinant bacmids were extracted and transfected into Sf9 cells using Cellfectin® II Reagent (ThermoFisher) and according to procedures described in the Bac-to-Bac instruction manual (Invitrogen). Insect Sf9 cells were grown at 27°c in 4 L culture of protein-free ESF 921 insect cell culture medium (Expression Systems) in spinner flasks, and incubated for 5 days post-infection. For selenomethionine (SeMet) incorporation in hRobo2^{D1-8}, cells were transferred to roller-bottles at a cell density of 0.5x10^6/ml for 24 h. Medium was than exchanged to Methionine-deficient ESF 921 Delta Series media (Expression Systems) for 30 h following BaculoVirus infection and further 15 h culture. We next added 50 mg/L of L-(+)-Selenomethionine, Anagrade (ANATRACE) to the medium. After an additional five days incubation, the medium was collected for purification. Growth media with secreted proteins were concentrated and buffer-exchanged to 50 mM phosphate buffer, pH 7.6, 0.3 M NaCl and 10% glycerol using the QuixStand benchtop system. The concentrated and buffer-exchanged medium was then loaded onto a metal-chelate column (HisTrap, GE Healthcare) pre-equilibrated with buffer A (50 mM phosphate buffer, pH 7.4, 0.3 M NaCl, 10% glycerol) at a flow rate of 3 ml/min. The column was washed with buffer A until a stable baseline was achieved. After applying a 70–200 mM imidazole gradient elution, protein-containing fractions were selected for further purification.

hRobo2^{D1-8} and SeMet-hRobo2^{D1-8} fractions were pooled and diluted 1:10 (v:v) with 20 mM Bis-Tris pH 5.8 and 5% glycerol for cation exchange chromatography using pre-equilibrated Mono S (GE Healthcare). Proteins were than eluted with gradient of 20 mM Bis-Tris pH 5.8, 5% glycerol and 1M NaCl. Protein containing fractions (hRobo2^{D1-8} and SeMet-hRobo2^{D1-8} from the MonoS elution, and hRobo2^{D2-3} and hRobo2^{D5-8} from the metal-chelate elution) were pooled and loaded onto pre-equilibrated Superdex 200 HiLoad 26/60 (GE Healthcare) for size exclusion chromatography and elution was performed with 20 mM HEPES pH 7.4 and 150 mM NaCl. Protein-containing fractions were pooled and concentrated using a spin concentrator: hRobo2^{D2-3} to 19.5 mg/ml, hRobo2^{D1-8} and SeMet-hRobo2^{D1-8} to 13.5 mg/ml, and hRobo2^{D5-8} to 4 mg/ml. The concentrated proteins were split into aliquots and flash-frozen in liquid N_2.

Crystallization, data collection, and structure determination - Samples of hRobo2^{D2-3}, hRobo2^{D1-8} and SeMet-hRobo2^{D1-8} were screened for crystal growth conditions with the commercial crystallization screens Crystal screen, PegRX, PEG/Ion, and SaltRX (Hampton Research) at 277K and 293K in 96-well hanging-drop clear polystyrene microplates (TTP LabTech) using the mosquito robot for crystallography (TTP LabTech) as in (Sporny et al., 2016). A 1:1 sample:reservoir ratio was used with a drops size of 0.2 µl.

In the case of hRobo2^{D2-3}, a single crystal that was formed under PEG/Ion screen condition G4 (consisting 0.2M Sodium format pH 7 and 20% PEG 3350 at 277K) was harvested directly from the screen plate and flash-frozen in liquid N_2 four days after appearance using 7.5% glycerol, 30% PEG 3350 and 0.2M sodium format as a cryo- protectant solution. Diffraction data was measured at 100K on beamline ID14.2 (Gerlach, 2016) at Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung (BESSY) II (Berlin, Germany) using MAR-225 detector, and was processed and scaled using the XDSAPP software package (Krug, 2012). The crystal belonging to the $P2_12_12_1$ space group, with unit cell dimensions of a=62.224 b=70.524 c=97.274 α=γ=β=90, has two molecules in the asymmetric unit, and diffracted to a maximal resolution of 2.46 Å. The structure was solved by molecular replacement using the online BALBES molecular replacement server (Long et al., 2008). Molecular replacement was followed by electron density modification procedures and cycles of model refinement and re-building using COOT (Emsley et al., 2010), PHENIX refine (Afonine et al., 2012), and the ReDo server (Joosten RP, 2012). Data collection and model statistics are summarized in Table 1. In the case of hRobo2^{D1-8}, hits from several crystallization conditions with different morphologies were obtained and reproduced in 24-well hanging drop vapor-diffusion plates in 2µl drop size. Diffraction was assessed with a X-ray Bruker X8 Proteum rotating anode generator operating at 45 kV and 60 mA with PLATINUM135 CCD detector at 100K. Only one crystal form (obtained at condition H1 from Crystal Screen HT, composed of 8% Ethylene glycol, 10% PEG 8000, 0.1M HEPES pH 7.5, at 293K) showed ordered diffraction and was further pursued. Crystallization conditions were refined by changing protein concentrations, pH values, PEG concentrations, growth temperatures (277K, 285K, 293K), and additive supplements. Refined crystallization condition consisted of a protein concentrated to 13.5 mg/ml and precipitant mixture containing 10% PEG 8000, 0.1M HEPES pH 7 - 7.125, 9.5% Ethylene glycol and 2% MPD at 293K. Crystals appeared three to five days after setup, dehydrated by replacing reservoir contents to 15% PEG 8000, 0.1 M HEPES pH 7.125 and 10% Ethylene glycol 24 h - three weeks prior to harvesting directly from the mother liquor and flash-frozen liquid N2. SeMet-hRobo2^{D1-8} crystallization conditions followed those of native hRobo2^{D1-8} and were optimized at 293K in 24 wells plate using hanging-drop vapor-diffusion with 2µl drop size. Best diffracting crystals grew at 10.5-11.5%

PEG 8000, 0.1M HEPES pH 7-7.5, 9.5% Ethylene Glycol. Crystals were dehydrated at least 24 h before harvesting by replacing the reservoir contents with 15% PEG 8000, 11% Ethylene Glycol, and 0.1M HEPES pH 7.25, and were harvested directly from mother liquor four weeks after setup.

Diffraction data for hRobo2^{D1-8} and SeMet-hRobo2^{D1-8} crystals were measured at 100K in the ESRF (Grenoble, France) on beamlines: ID23-1 with ADSC Q315R detector, ID29 with Pilatus 6M detector, and at BESSY II (Berlin, Germany) on beamline BL14.1 with Pilatus 6M detector. Data were processed and scaled using the XSDSAPP (Kabsch, 2010; Krug, 2012) software package in the space group C2 with unit cell parameters a=290.767, b=81.569, c=158.656 Å, α=γ=90, β=94.0°. Data were subjected to anisotropic ellipsoidal truncation using the STARANISO server (Tickle et al., 2018). Strong non-origin native Patterson function peaks (0.3-0.45 of the origin height) at (u,v,w)=(0, 0, 0.32) and (0.01, 0, 0.68) indicated presence of three hRobo2^{D1-8} molecules in similar orientations in the asymmetric unit. Probably, this pseudo-translation hindered attempts to get experimental phases. The hRobo2^{D1-8} structure was solved by molecular replacement using single domain search models as detailed in the results section. The models of hRobo1^{D1} and hRobo1^{D8} were modified to match the hRobo2 sequence using MOLREP (Vagin and Teplyakov, 2010). hRobo2^{D1-7} in the three monomers were positioned consequently starting with hRobo2^{D2} using Phaser (McCoy et al., 2007), three copies of poorly ordered hRobo2^{D8} were positioned using phased MR as implemented in MOLREP (Vagin and Isupov, 2001). SeMet-hRobo2^{D1-8} data were chosen for model refinement as being the least anisotropic. The model was refined with REFMAC5 (Murshudov et al., 2011) using external phases from multi-crystal averaging by DMMULTI (Cowtan, 2010). X-ray data from several native and derivative hRobo2^{D1-8} crystals were used in averaging alongside native data from hRobo2^{D2-3} and hRobo2D$^{4-5}$ crystals. The model rebuilding was performed in COOT (Emsley et al., 2010). CC2/1 is defined in (Karplus and Diederichs, 2012) and^{d} Wilson B-factor was estimated by SFCHECK (Vaguine et al., 1999).

Robo-cKIT chimera tyrosine phosphorylation assay - Cell culture and transfection - HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 100 units/mL penicillin, and 100 µg/ml streptomycin, at 37°C, under 5% CO_2. For transfection, cells were seeded at 5×10^4 cells per well in a 6-well plate, cultured for 24 h, and transiently transfected with 5 µg DNA using the calcium phosphate-mediated transfection protocol (Kingston et al., 2003). Transfected cells were incubated at 37°C, 5%CO_2 for 24 h.

Cells were then serum-starved for 16 h, washed twice with PBS, solubilized in lysis buffer containing 120 mM NaCl, 25 mM Hepes (pH 7.4), 1 mM EGTA, 0.75mM $MgCl_2$, 10% glycerine, 1% triton, 1 mM NaF, 2 mM sodium orthovanadate, protease inhibitor cocktail (Complete Mini

EDTA-free, Roche) and incubated on ice for 10 min. For phosphorylation of intact cKIT, 2.5 µg/ml of the SCF ligand (produced as in (Langley et al., 1994) using the NEB® 5-alpha E. coli strain for plasmid propagation and the T7 Express (NEB) E. coli strain for expression (these E. Coli strains were used thoughout the work)) were added for 10 min before wash and lysis. Lysates were centrifuged at 15000*g for 10 min at 4°C and then the supernatants were incubated with ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich) for 2h at 4°C. After incubation, immunopellets were washed twice with 120 mM NaCl, 25 mM Hepes (pH 7.4), 1 mM EGTA, 0.75mM $MgCl_2$, 10% glycerol, 0.1% triton, 2 mM sodium orthovanadate and analyzed by SDS/PAGE followed by immunoblotting with anti-flag M2 (Sigma-Aldrich) and anti-phosphotyrosine (Bio-Rad) antibodies.

Protein visualization by chemiluminescence and quantification were performed using an ImageQuant LAS 4000 imager (GE Healthcare).

cKIT and Robo-cKIT chimeras DNA subcloning - All constructs were prepared by PCR amplification from human cDNA or assembled *sax-3* cDNA. The human cDNAs include Robo1 (Acc. no. Q9Y6N7-5), Robo2 (Acc. no. Q9HCK4), Robo3 (Acc. no. Q96MS0) and cKIT (Acc. no. X06182). The *sax-3* cDNA (Acc. no. G5EBF1) was generated by assembly PCR reactions for intron deletions from the *Pmec-7::sax-3* transgene (Zallen et al., 1998).

First, the PCR product of the full cKIT (residues 33-976) and the intracellular cKIT (KIT^{IC}, residues 514-976, including the transmembrane segment) were cloned into p3XFLAG-CMV™-25 Expression Vector (Sigma-Aldrich). For the Robo-cKIT chimeras, $hRobo1^{D1-4}$ (residues 20-416), $hRobo1^{D1-8}$ (residues 20-845), $hRobo2^{D1-3}$ (residues 22-312), $hRobo2^{D1-4}$ (residues 22-413), $hRobo2^{D1-8}$ (residues 22-838), $hRobo3^{D1-4}$ (residues 55-448), $hRobo3^{D1-8}$ (residues 55-866), $SAX\text{-}3^{D1-4}$ (residues 24-412), $SAX\text{-}3^{D1-8}$ (residues 24-849) were amplified. The PCR amplifications were then cloned into $cKIT^{IC}$ -p3XFLAG-CMV™-25. Domain deletions on $hRobo2^{D1-8}$-$cKIT^{IC}$ chimeras were performed by assembly PCR reactions as described: $hRobo2^{D1-8\Delta D5}$ (del aa417-507), $hRobo2^{D1-8\Delta D6}$ (del aa520-617), $hRobo2^{D1-8\Delta D7}$ (del aa627-732).

For surface expression validation, Immuno-Staining was applied. COS7 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 100 units/mL penicillin and 100 µg/ml streptomycin, at 37°C under 5% CO_2. Cells were seeded at 5×10^4 cells in a 24-well plate with a bottom coverslip, cultured for 24 h and transiently transfected with 500 ng DNA using TurboFect Transfection Reagent™ (Thermo Fisher Scientific). Transfected cells were incubated at 37°C for 20 h, washed twice with phosphate-buffered saline (DPBS), fixed with 4% PFA (Paraformaldehyde solution 4% in PBS) and blocked with 1% BSA in PBS. Cells were probed with primary antibody to the N-terminal 3xFLAG (Genscript) and conjugated secondary anti-mouse FITC antibody (Genscript) was then applied for detection. For Nuclei staining, cells were stained with Hoechst

33258 (Sigma-Aldrich). Coverslips were mounted on glass slides using HIGHDEF IHC fluoromount (Enzo Life Sciences). Confocal images were recorded using Leica SP8 Confocal live microscope.

Analytical ultracentrifugation - All the sedimentation equilibrium experiments with hRobo2 constructs were performed using an XL-I analytical ultracentrifuge (Beckman-Coulter Inc.), with a UV-visible optics detection system, using an An60Ti rotor and 12-mm double sector centerpieces. Sedimentation curves were recorded and analyzed at 280 nm while spinning at 20°C in 10, 12, 14, and 20 h time points, to assure that equilibrium was reached. Proteins were each measured and analyzed in 20 μM. The proteins include an N' terminal FLAG and C' terminal 6XHis tags. Taking into account the glycosylation component, we have set the partial specific volume ($\bar{v}$) of the glycoprotein to 0.722, as suggested in (Lewis and Junghans, 2000). The sedimentation coefficient distributions were calculated using SEDFIT and SEDPHAT (Gabrielson et al., 2007; Schuck et al., 2014).

C. elegans - All constructs were made by PCR amplification from the complete clone of *C. elegans Pmec-7::sax-3* (Zallen et al., 1998). The *Pmec-7::*sax-3 transgene contains ~850bp of the *mec-7* promoter and a partial sax-3 cDNA (nucleotides 1-1343) followed by the remaining sax-3 genomic region containing the rest of the sax-3 open reading frame (altogether encoding for residues 1-1273 of the SAX-3 protein, Acc. G5EBF1). The entire *Pmec-7::sax-3* fragment was amplified and ligated into pCFJ104 mCherry-N1 plasmid, giving rise to a SAX-3::mCherry fusion protein. The *mec-7* promoter drives expression in AVM and 13 other neurons (ALML, ALMR, ALNL, ALNR, BDUL, BDUR, FLPL, FLPR, PLML, PLMR, PVDL, PVDR, PVM). For preparation of the F360R point mutant, and truncation mutants, we used “round-the-horn” PCR procedure (based on https://openwetware.org). The domain truncation boundaries are as follows: ΔD4 (residues Δ313- 413); ΔD5 (residues Δ423-514); ΔD7 (residues Δ642-750). The *C. elegans* Bristol strain N2 was used as wild-type animals. Worms were grown at 20°c and maintained using standard methods (Brenner, 1974). Strains used in this study include CX3198 [sax-3(ky123) X] (in which the signal sequence and the first exon of *sax-3* are deleted), CX5067 [*zdIs5 (Pmec-4::GFP)*] (which was used for the visualization of the AVM neuron). CX3198 [*sax-3(ky123) X*] was crossed with CX5067 [*zdIs5 (Pmec-4::GFP)]* to create SHK524 *zdIs5(Pmec-4::GFP); sax-3(ky123)*.
Transgenic strains expressing *sax-3* and *sax-3* mutants were established by injecting the respective pCFJ104 mCherry-N1-based plasmids (10 ng/μl) along with the pRF4 *rol-6(su1006)* plasmid (70 ng/μl) as a co-injection marker into N2 worms (service of KNUDRA

Transgenics, UT. USA). Then, animals expressing the transient array were crossed into *zdIs5(Pmec-4::GFP); sax-3(ky123)*.

Day-1 of adulthood animals were mounted on 2% agarose pads containing 2 mM levamisol. Images were taken with a CCD digital camera using a Nikon 90i fluorescence microscope. Exposure time was kept constant through the experiments. The axon guidance defect was scored as failure of the AVM axon to extend ventrally from the cell body. We further distinguished between defective axons that directly extended anteriorly ("straight" phenotype) and other aberrant phenotypes, which included indirect ventral-anterior routes, and improper positioning of the AVM cell body ("other" phenotype).

For *slt-1* silencing, we used the *slt-1* RNAi clone (F40E10.4) from the Vidal library (Rual et al., 2004) after it was verified by sequencing. Bacteria expressing the *slt-1* dsRNA were cultured overnight in LB supplemented with tetracycline and ampicillin. Prior to seeding, 10mM IPTG was added to the liquid culture, which was then seeded on NGM plates containing 2mM IPTG and 0.05mg/ml carbenicillin. Eggs were placed on the NGM plates, and synchronized worms were analyzed at Day-1 of adulthood.

HEK293F cell aggregation assay - For the Notch-Delta experiment, we used previously described protein expression constructs. In human Notch1, Citrine ectodomain is inserted between two EGF-like repeats (between G1435 and A1436) and the intracellular domain was replaced with a transcriptional activator Gal4 (to avoid activation of endogenous Notch targets) (Shaya et al., 2017). In human Delta (Dll1), the intracellular segment was replaced with mCherry fusion, as described in (Sprinzak et al., 2010). All the other constructs were made by PCR amplification from complete cDNA clones as used for "Robo-cKIT chimera tyrosine phosphorylation assay". The intracellular segments of hRobo1 and hRobo2 were removed (designated hereafter "ΔIC" constructs) and replaced by a RTLEVLFQGP linker. Robo2$^{\Delta IC}$ (residues 1-882) was cloned into a pECFP-N1 plasmid (Clontech). Robo1$^{\Delta IC}$ (residues 1-902) was cloned into mCherry-N1 plasmid (Clontech). cKIT$^{\Delta IC}$ (residues 32-555) was cloned into a pECFP-N1 modified plasmid that includes the leader sequence and FLAG tag cassette as in the commercial p3XFLAG-CMV™-25 Expression Vector (Sigma-Aldrich). hRobo2$^{D1\text{-}8\Delta D5\ CFP}$ (deletion of aa417-507) was generated by assembly PCR from the hRobo2$^{D1\text{-}8\ CFP}$ clone. Cell culture and transfection - HEK293F cells (Thermo Fisher Scientific) were maintained in FreeStyle™ 293 expression medium in an orbital shaker incubator at 37°C, 120 rpm, 8% CO_2 according to the manufacturer guidelines. For transfection, cells were seeded at 0.2×10^6 cells/ml in a 30 ml culture. After 24 h, individual plasmids were transfected using linear polyethylenimine (PEI) (3 ug PEI for 1 ug plasmid DNA for $0.4x10^6$ cells). Individual cultures were grown for 4 hours post transfection, and then mixed in a 1:1 volume ratio with reciprocal cultures, for 44 h, before analyzed for cellular cross-interactions.

Imaging flow cytometry analysis: To remove large cells aggregates, 1ml of each culture mixture was filtered through a 35 µm nylon mesh strainer (FALCON) before analyzed using a multispectral imaging flow cytometry (ImageStreamX mark II imaging flow-cytometer; Amnis Corp, Seattle, WA, Part of EMD Millipore). Images of cells were collected in the following channels: channel 1 (bright filed), channel 4 (mCherry) and channel 7 (CFP). A minimum of 1×10^4 cells were collected from each sample, and the data was analyzed using the image analysis software (IDEAS 6.2; Amnis Corp). The serial gating strategy to identify CFP-mCherry small aggregates in the mix culture was as follow: All cells were first gated for small aggregates, using the area and aspect ratio features on the bright-field image (aspect ratio is the minor axis divided by the major axis, and describes how round or elongated an object is). This first gate helped us to select only the small aggregates population (2-5 cells), eliminating single cells and larger aggregates. Next, we eliminated the Cropped cells by plotting the cell area of the bright field image against the Centroid-X feature (the number of pixels in the horizontal axis from the upper left corner of the image to the center of the cell mask). Focused cells were gated using the Gradient RMS (root mean square for image sharpness) histogram, when cells with better focus have higher gradient RMS values. For the final step, we gated the cells population with high intensity of both channel 4 (mCherry) and channel 7 (CFP) in order to identify CFP-mCherry small aggregates.

Spinal cord neuron culture - Primary spinal cord (SC) neurons of E12.5 mouse embryos of either sex was cultured. Briefly, SCs were excised, trypsinized, and triturated. Supernatant was collected and centrifuged through a 4% BSA cushion. The pellet was resuspended and centrifuged through an OptiPrep gradient (10.4% OptiPrep, Sigma-Aldrich; 10 mM Tricine, 4% glucose) for 20 min at 760x*g* with the brake turned off. Cells were collected from the interface, washed once in complete medium, and then plated in coated growth chambers. Cells were maintained in Complete Neurobasal Medium (Invitrogen) containing B27 (Invitrogen), 10% (v/v) horse serum (Biological Industries), 25nM β-mercaptoethanol, 1% penicillin-streptomycin (PS; Biological Industries), and 1% GlutaMAX (Invitrogen) supplemented with 1 ng/ml GDNF, 0.5 ng/ml CNTF, and 1 ng/ml BDNF (Alomone Labs). Before plating, the growth plates were coated with 1.5 g/ml poly-DL-ornithine (Sigma-Aldrich) overnight at 37°C and 3 g/ml laminin (Sigma-Aldrich) for 2 h at 37 c. Neurons were treated with lentiviruses (LV's) 6 hr after plating. Neurite lengths were examined and recorded using the incuCyte imaging system at 5 DIV.

Lentiviral constructs for expression of human hRobo1 and hRobo2 were prepared by PCR amplification from the complete cDNA clone as used for “Robo-cKIT chimera tyrosine phosphorylation assay”. hRobo1 (residues 22–1606, LRQE-ETES, supplemented with an N-terminal FLAG tag and a C-terminal c-Myc tag, followed by a stop codon) and hRobo2

(residues 22–1378, SRLR-TGEL, supplemented with an N-terminal FLAG tag and a C-terminal hexahistidine tag, followed by a stop codon) were ligated into a pHAGE IRES GFP vector (addgene). Assembly PCR mutagenesis was used (based on https://openwetware.org) to generate each one of the Robo's deletion constructs. hRobo1; ΔD4 (deletion of 314-416, PPHF-IADR), ΔD5 (deletion of 417-510, PPPV-QEFG). hRobo2; ΔD4 (deletion of 310-410, VRAP-EVTD), ΔD5 (deletion of 418-507, PPII-DVTE).

The lentiviral plasmids were mixed with the packaging plasmid (PsPax) and the envelope plasmid (pMD2.G) in a ratio of 2:1:1. HEK293T cells were used as the packaging cell line and were seeded 1 day before transfection. Cells were kept for 3 days after transfection without changing medium. The conditioned medium, containing virus particles, was briefly centrifuged to remove cell debris and was then filtered through a 0.45 µm pore size.

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