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Report

Functional Diversity of Robo Receptor Immunoglobulin Domains Promotes Distinct Axon Guidance Decisions

Timothy A. Evans[1] and Greg J. Bashaw[1,*]
[1]Department of Neuroscience, University of Pennsylvania School of Medicine, 421 Curie Boulevard, Philadelphia, PA 19104, USA

Summary

Recognition molecules of the immunoglobulin (Ig) superfamily control axon guidance in the developing nervous system. Ig-like domains are among the most widely represented protein domains in the human genome, and the number of Ig superfamily proteins is strongly correlated with cellular complexity [1]. In *Drosophila*, three Roundabout (Robo) Ig superfamily receptors respond to their common Slit ligand to regulate axon guidance at the midline: Robo and Robo2 mediate midline repulsion, Robo2 and Robo3 control longitudinal pathway selection, and Robo2 can promote midline crossing [2–5]. How these closely related receptors mediate distinct guidance functions is not understood. We report that the differential functions of Robo2 and Robo3 are specified by their ectodomains and do not reflect differences in cytoplasmic signaling. Functional modularity of Robo2's ectodomain facilitates multiple guidance decisions: Ig1 and Ig3 of Robo2 confer lateral positioning activity, whereas Ig2 confers promidline crossing activity. Robo2's distinct functions are not dependent on greater Slit affinity but are instead due in part to differences in multimerization and receptor-ligand stoichiometry conferred by Robo2's Ig domains. Together, our findings suggest that diverse responses to the Slit guidance cue are imparted by intrinsic structural differences encoded in the extracellular Ig domains of the Robo receptors.

Results and Discussion

Longitudinal Pathway Choice Is Dictated by Individual Robo Receptors

In the *Drosophila* embryonic central nervous system (CNS), Robo receptors are expressed in overlapping domains that divide the longitudinal axon connectives into three broad zones: axons occupying the medial zone express Robo, axons in the intermediate zone express Robo and Robo3, and axons in the most lateral zone express Robo, Robo2, and Robo3. Loss of *robo2* shifts lateral axons to intermediate positions, whereas loss of *robo3* shifts intermediate axons to medial positions. Conversely, ectopic expression of Robo2 or Robo3 in medial axons forces them to select more lateral pathways, whereas increased levels of Robo do not. The "Robo code" model posits that a combinatorial code of Robo receptor expression determines the lateral position of CNS axons [3, 4]. To test whether a combinatorial code is necessary, we assayed the ability of Robo2 and Robo3 to shift apterous axons in embryos deficient for various combinations of *robo* genes and found that removing endogenous *robo* or *robo3* did not affect Robo2's ability to shift apterous axons laterally (Figure 1). Indeed, *UAS-Robo2* was sufficient to direct the apterous axons to the lateral edge of the connectives even in *robo3, robo* double mutant embryos. Similarly, removal of *robo2* or *robo* had little or no effect on the ability of *UAS-Robo3* to redirect the apterous axons to more lateral pathways (see Figure S1 available online). Thus, it is the individual expression of Robo2 and Robo3 that dictates lateral positions of CNS axons, not a combinatorial Robo code.

An Unexpected Role for Robo Extracellular Domains

Robo2 and Robo3 dictate the lateral position of axons in the *Drosophila* CNS, a role that is not shared by Robo (Figure S2). What is the basis for this differential activity? All three receptors have similar ectodomains with five immunoglobulin (Ig) domains and three fibronectin (Fn) III repeats, whereas their cytoplasmic domains are more divergent. In particular, Robo2 and Robo3 both lack two conserved motifs (CC2 and CC3) that mediate interactions with several downstream effectors and are required for Robo's midline repulsive function [6], leading to the speculation that distinct Robo functions are directed by their cytoplasmic domains [4, 7]. To determine whether the functional difference between Robo2-Robo3 and Robo is due to a qualitative difference in cytoplasmic signaling, we assayed a set of chimeric receptors for their ability to induce lateral shifting in the medial apterous axons.

First, the cytoplasmic domain of Robo was replaced with that of Robo2 or Robo3 (Robo1:2 and Robo1:3). Neither of these receptor variants was able to reposition the apterous axons (Figure S3). In contrast, when the cytoplasmic domains of Robo2 or Robo3 were replaced by that of Robo, the resulting chimeric receptors (Robo2:1 and Robo3:1) exhibited lateral positioning activity similar to full-length Robo2 and Robo3 (Figure S3). These results reveal that the lateral positioning activities of Robo2 and Robo3 are specified by their ectodomains. Importantly, the cytoplasmic domains of Robo2 and Robo3 are not dispensable for lateral positioning activity, because receptors without any cytodomains are unable to redirect the apterous axons laterally (data not shown). Because Robo cytoplasmic domains are functionally interchangeable for longitudinal pathway selection, any required intracellular events must be mediated by cytoplasmic sequences that are common to Robo, Robo2, and Robo3.

Robo2 Ig Domains Specify Lateral Positioning Activity

To dissect the structural basis underlying the differential activities of Robo receptor extracellular domains, we examined the relative contributions of Robo2's Ig and Fn domains by generating a more restricted set of domain swaps between Robo and Robo2 (Figure 2). Exchanging all five Ig domains between Robo and Robo2 completely swapped their lateral positioning activities (Figures 2B–2E; Figure S4). These results reveal that Robo2's ability to position axons is specified entirely by its Ig domains. However, the Fn repeats are not completely dispensable for lateral positioning activity because Robo2 variants lacking these elements displayed reduced activity (data not shown). Thus, when combined with Robo2's five Ig domains,

*Correspondence: gbashaw@mail.med.upenn.edu

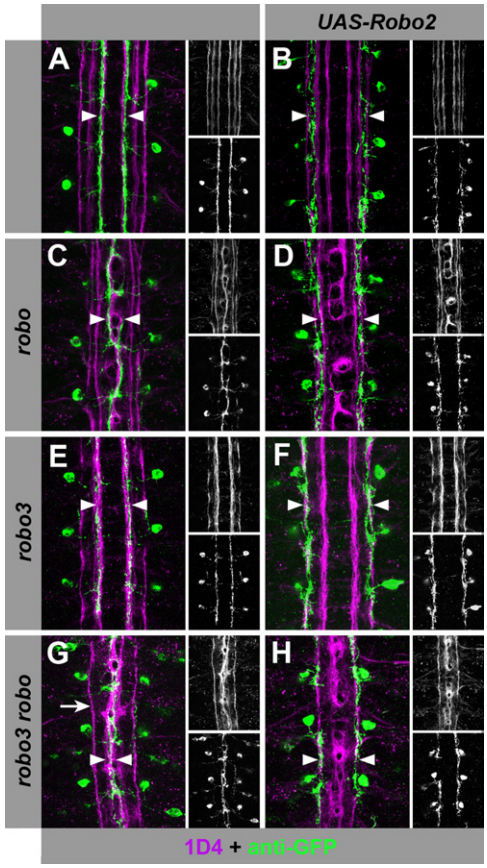

Figure 1. Robo2 Dictates Lateral Position in the Absence of Robo and Robo3

Stage 16–17 *apGAL4::UAS-TauMycGFP* embryos stained with mAb 1D4 (anti-FasII, magenta) and anti-GFP (green; labels the cell bodies and axons of the apterous neurons). Small panels show 1D4 (top) and anti-GFP (bottom); large panels show merged images. Anterior is up.

(A) In wild-type embryos, the apterous axons (arrowheads) select a longitudinal pathway that lies directly adjacent to the medial FasII-positive axon tract.

(B) Ectopic expression of Robo2 in the apterous neurons of wild-type embryos causes their axons to select a longitudinal pathway farther from the midline (arrowheads).

(C) In *robo* mutants, the medial FasII pathway ectopically crosses and recrosses the midline. The apterous axons also collapse along the midline and follow the medial FasII tract (arrowheads).

(D) Loss of endogenous *robo* does not affect the ability of ectopic Robo2 to direct the apterous axons to lateral pathways (arrowheads).

(E) In *robo3* mutants, the intermediate FasII tract shifts toward the midline and joins the medial pathway. Thus, only two distinct FasII-positive bundles (medial and lateral) are visible in these embryos. The apterous axons are found in their wild-type position directly adjacent to the medial FasII tract (arrowheads).

(F) Loss of endogenous *robo3* does not affect the ability of ectopic Robo2 to force the apterous axons to extreme lateral positions (arrowheads).

(G) In *robo3, robo* double mutants, the intermediate FasII tract fuses with the medial tract (because of loss of *robo3*), and this thick fascicle crosses the midline (because of loss of *robo*). As in *robo* mutants, the apterous axons follow this fascicle across the midline (arrowheads). Note that the lateral FasII-positive tract remains in *robo3, robo* double mutants, confirming that Robo2 alone is sufficient to specify the correct position of this pathway (arrow).

(H) Ectopic Robo2 is able to direct the apterous axons to extreme lateral positions even in the absence of both *robo* and *robo3*.

the Fn repeats and cytoplasmic domain of Robo can act permissively to facilitate lateral pathway choice.

The five Ig domains of Robo2 are necessary and sufficient to functionally distinguish it from Robo in the context of longitudinal pathway choice. To subdivide the ectodomains of Robo and Robo2 further, we targeted the presumptive Slit-binding region (Ig1). We initially swapped Ig1 and Ig2 together, because some evidence suggested that Ig2 could contribute to Slit binding of human Robo receptors [8]. Robo variants possessing the first and second Ig domains of Robo2 (Robo1^{R2I1+2}) displayed activity comparable to full-length Robo2 (Figure 2F; Figure S4). However, the converse swap revealed that Robo2 still retained its activity even when its Ig1+2 was replaced with those of Robo (Robo2^{R1I+2}) (Figure 2F; Figure S4). These results reveal a bipartite contribution to Robo2's lateral positioning activity from (at least) two genetically separable elements located within Ig1+2 and Ig3–5, respectively.

We next tested whether Ig1 and Ig3 together could be responsible for dictating the lateral positioning activity of Robo2. Replacing Ig1 or Ig3 of Robo with those of Robo2, alone (Robo1^{R2I1} and Robo1^{R2I3}) or in combination (Robo1^{R2I1+3}), was sufficient to confer Robo2-equivalent activity to Robo (Figures 2G and 2H). Importantly, replacing Ig1–3 of Robo2 with the corresponding domains of Robo eliminated its lateral positioning activity, demonstrating that the Ig1–3 region is both necessary and sufficient to functionally distinguish Robo1 and Robo2 in the context of longitudinal pathway choice (Figure 2G).

We have shown that Ig1 and Ig3 of Robo2 can independently specify its ability to redirect medial axons to more lateral pathways. Further, the lateral positioning activities of chimeric receptors containing Ig1 or Ig3 of Robo2 were indistinguishable in our apterous neuron assay. To determine whether these receptors could also influence longitudinal pathway choice in a broader context, we examined the effects of pan-neuronal misexpression of selected chimeric receptors on lateral positioning of FasII-positive axon pathways (Figure 3).

In wild-type embryos or *elavGAL4;UAS-Robo* embryos, three major FasII-positive tracts were detectable on either side of the midline (Figures 3A and 3D). Pan-neuronal misexpression of Robo2, in contrast, disrupted longitudinal pathway formation such that the intermediate FasII pathway was absent in nearly all segments (Figure 3B). Notably, this effect appeared to depend solely on Ig3 of Robo2, because it was recapitulated by *UAS-Robo2*$^{R1I1+2}$ and *UAS-Robo1*^{R2I3}, but not by *UAS-Robo1*$^{R2I1+2}$ or *UAS-Robo2*$^{R1I1-3}$ (Figure 3; data not shown). These observations draw a functional distinction between the activities of Ig1 and Ig3 of Robo2 and suggest that these two domains regulate longitudinal pathway choice via distinct mechanisms.

The Lateral Positioning Activity of Robo2 Is Slit Dependent

Because the Slit-binding Ig1 contributes to Robo2's lateral positioning activity, it is possible that Robo2 regulates longitudinal pathway selection in response to Slit. If so, then removing *slit* or disrupting its interaction with Robo2 should reduce or eliminate Robo2's lateral positioning activity. Therefore, we examined the effects of Robo2 misexpression in apterous axons in a *slit* mutant background. In the absence of Slit, the entire axon scaffold collapsed at the midline, and even high levels of ectopic Robo2 could not force the apterous axons laterally (Figures 4A and 4B). This may indicate a direct requirement for Slit or instead reflect the inability of

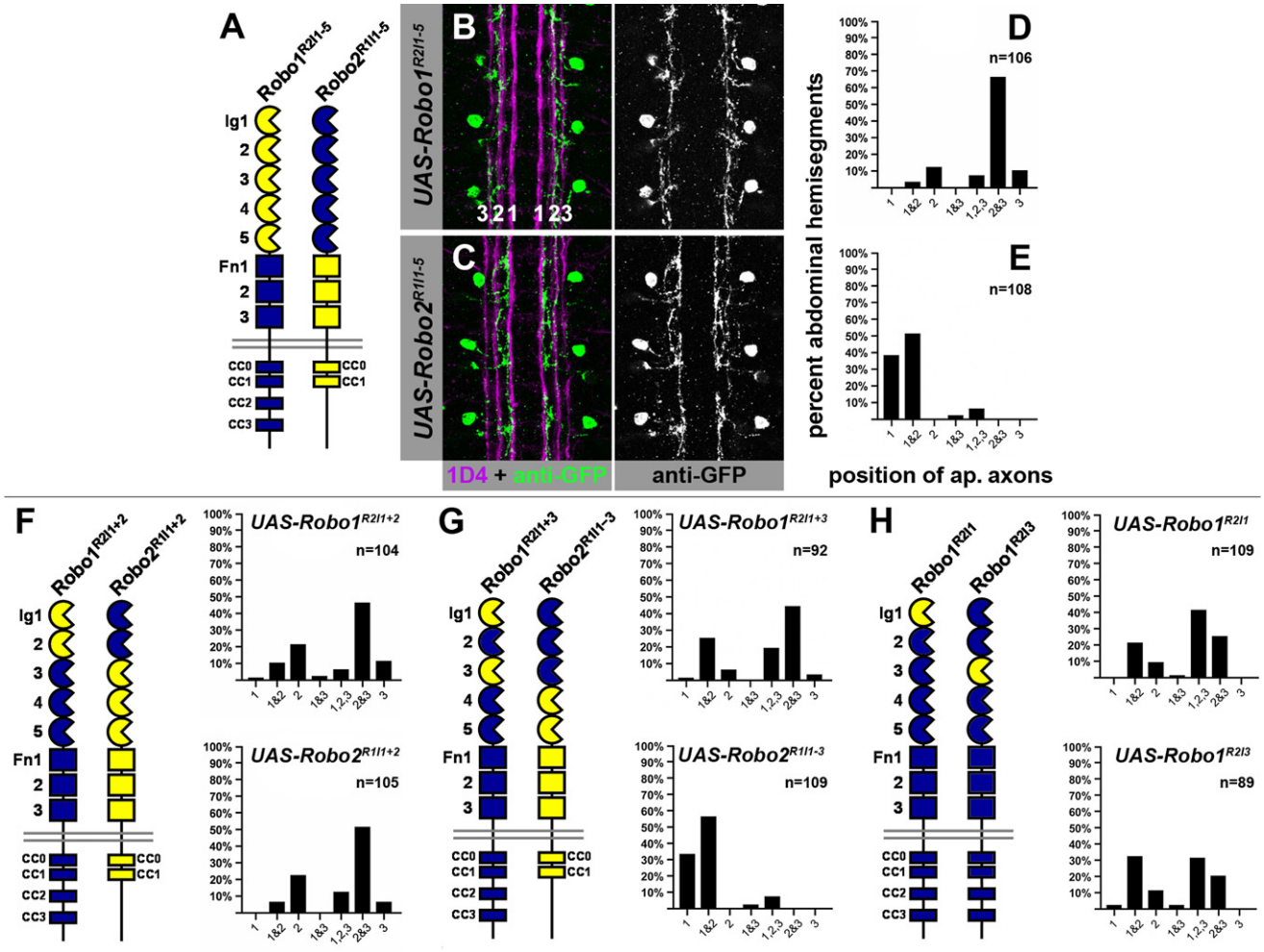

Figure 2. Multiple Robo2 Ig Domains Independently Dictate Its Lateral Positioning Activity

(A and F–H) Schematic representation of domain swap constructs targeting all five Ig domains (A), the first two Ig domains (F), or the first and third Ig domains (G and H) of Robo and Robo2. Sequence elements derived from Robo are shown in blue, whereas Robo2 elements are shown in yellow.

(B and D) Replacing all five Ig domains of Robo with those of Robo2 (Robo1^{R2I1-5}) confers lateral positioning activity equivalent to full-length Robo2.

(C and E) Replacing all five Ig domains of Robo2 with those of Robo (Robo2^{R1I1-5}) disrupts its lateral positioning activity. This receptor variant displays equivalent activity to Robo1:2 and full-length Robo; even at higher expression levels, *UAS-Robo2^{R1I1-5}* can barely match the weakest of the *UAS-Robo1^{R2I1-5}* lines.

(F) Swapping the first and second Ig domains between Robo and Robo2 results in two complementary receptors with equivalent lateral positioning activity, similar to Robo1^{R2I1-5}. Therefore, both Ig1+2 and Ig3–5 of Robo2 can independently confer Robo2-like lateral positioning activity in the context of an otherwise Robo-specific receptor.

(G) Replacing the first and third Ig domains of Robo with the corresponding domains of Robo2 (Robo1^{R2I1+3}) confers lateral positioning activity equivalent to full-length Robo2, whereas replacing Ig1–3 of Robo2 with the corresponding region of Robo (Robo2^{R1I1-3}) disrupts this activity.

(H) Ig1 (Robo1^{R2I1}) or Ig3 (Robo1^{R2I3}) of Robo2 are individually sufficient to confer Robo2-like lateral positioning activity to Robo. We are unable to formally rule out a contribution from Robo2 Ig2 in this context, because the chimeric receptors that would allow us to do so (Robo1^{R2I2} and Robo2^{R1I1+3}) were unstable when expressed in neurons or cultured cells (data not shown). See Figures S4 and S5 for additional quantification and expression data. See Supplemental Experimental Procedures for method of quantifying lateral position. n denotes number of hemisegments scored for the lines shown.

Robo2-expressing apterous axons to move outside of the collapsed axon scaffold.

We next asked whether Robo2 could reposition axons without its Slit-binding region. To ensure complete disruption of Slit binding, we deleted both the first and second Ig domains from Robo2 and found that Robo2$^{\Delta Ig1+2}$ was completely unable to reposition the apterous axons (Figure 4C). Deleting these two domains did not interfere with expression or localization of Robo2 (Figure 4D). Together, these results provide evidence that Robo2-directed lateral positioning is dependent on interactions with Slit; however, we note that in addition to disrupting Slit binding, deletion of Ig1 and Ig2 would also disrupt other potentially important functions of these domains. Genetic analysis of the role of *robo3* in the regulation of lateral chordotonal axon arborization within the CNS also supports Slit-dependent control of lateral position by Robo receptors [9].

Robo2's Ability to Promote Midline Crossing Depends on Ig2

Interestingly, pan-neuronal misexpression of Robo2 results in phenotypes that are inconsistent with a strictly repulsive function for Robo2 [5]. At the highest levels of overexpression, Robo2 prevents all midline crossing. However, moderate levels of Robo2 overexpression lead to ectopic midline

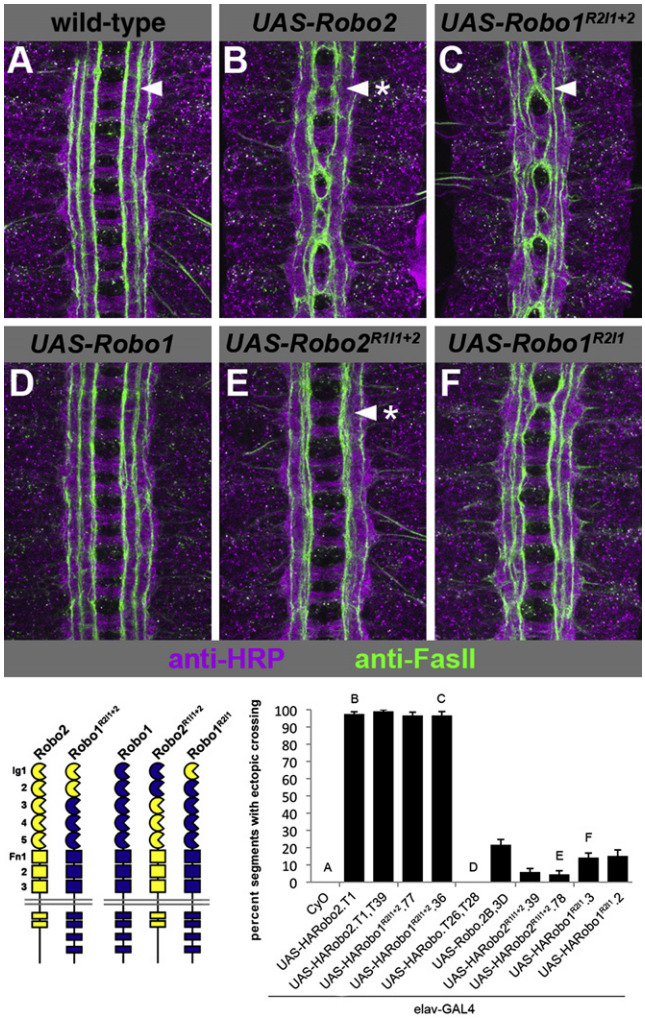

Figure 3. Differential Functions of Robo2's Ig1, Ig2, and Ig3 Domains in Regulating Longitudinal Pathway Formation and Midline Crossing

HA-tagged wild-type and chimeric receptors were crossed to *elavGAL4*, and the embryonic central nervous system axon scaffold and major longitudinal pathways were detected with anti-HRP (magenta) and anti-FasII (green) antibodies.

Promidline crossing activity of Robo2: misexpression of any receptor variant that includes Ig2 of Robo2 (B, Robo2; C, Robo1^{R2I1+2}) resulted in thickened commissures and ectopic midline crossing of FasII-positive axons (compare to wild-type, A). This phenotype resembles a reduction in *robo* function. Full-length Robo1 or chimeric receptors possessing the cytodomain, Fn domains, or Ig1, Ig3, Ig4, or Ig5 of Robo2 did not enhance midline crossing (D, Robo1; E, Robo2^{R1I1+2}; F, Robo1^{R2I1}). Graph shows frequency of ectopic midline crossing of FasII-positive axons for two transgenic lines each for the variants shown in (B)–(F). Error bars indicate standard error of the mean.

Disruption of FasII pathway formation: pan-neuronal misexpression of Robo2 disrupts the formation of the intermediate FasII pathway (B, arrowhead with asterisk; compare to wild-type, A); the axons that normally select this pathway apparently instead join the medial FasII tract, which becomes thicker in *elavGAL4;UAS-Robo2* embryos. Although Robo1^{R2I1+2} recapitulates the promidline crossing activity of Robo2, it does not mimic Robo2's effect on FasII pathway formation (C, arrowhead). In contrast, Robo2^{R1I1+2} is unable to promote midline crossing in this context but does reproduce Robo2's disruption of the intermediate FasII pathway (E, arrowhead with asterisk). A similar reduction of the intermediate pathway is produced by Robo1^{R2I3} but not Robo2^{R1I1-3} (data not shown), indicating that it is due solely to Robo2's Ig3 domain. Ig1 of Robo2 neither promotes midline crossing nor affects formation of the FasII longitudinal pathways (F).

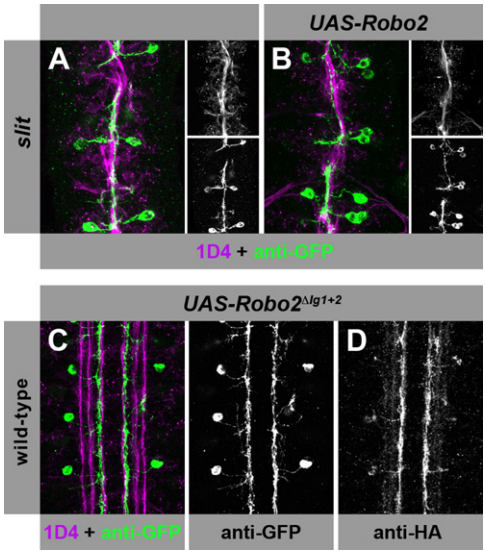

Figure 4. Evidence for Slit Dependence of Lateral Positioning by Robo2

(A) In *slit* mutants, all axons collapse at the midline, reflecting a complete absence of midline repulsion. The apterous axons project directly to the midline and do not leave.

(B) High levels of Robo2 misexpression (conferred by *EPRobo2*) are insufficient to direct apterous axons away from the midline in the absence of Slit.

(C) A Robo2 receptor variant lacking its first and second Ig domains (Robo2$^{\Delta Ig1+2}$) is unable to alter the lateral position of the apterous axons, even when its expression level is increased by adding a second copy of *UAS-Robo2*$^{\Delta Ig1+2}$ (data not shown).

(D) Robo2$^{\Delta Ig1+2}$ is expressed in apterous neurons and is preferentially localized to axons; therefore, the lack of activity we observed is not due to protein instability or mislocalization.

crossing, suggesting that in some contexts Robo2 can promote midline crossing. Perhaps Robo2, like the divergent Robo receptor Rig-1/Robo3 in vertebrates [10], can antagonize Slit-Robo repulsion.

We used our panel of chimeric receptors to map this activity of Robo2. All of the receptor variants that contain Ig2 of Robo2 promoted midline crossing when misexpressed with *elavGAL4*, whereas those that contain regions of Robo2 apart from Ig2 did not (Figure 3; Figures S2–S5). Thus, the promidline crossing activity of Robo2 is conferred by Ig2. Interestingly, rather than being excluded from the crossing portions of axons like all other Robo receptor variants, Robo2 proteins that promoted midline crossing were expressed strongly on crossing axons (Figures S2–S4). This localization to crossing axons was not shared by any of the Robo3 or Robo3-Robo1 receptors (Figures S2 and S3).

Although we cannot at present address the mechanism of Robo2's procrossing function, the fact that it is dependent on Ig2 alone suggests that it is probably not due to Robo2 binding Slit and sequestering Slit away from endogenous Robo. We also note that this crossing activity does not correlate with lateral positioning activity, because some variants with strong lateral positioning activity (e.g., Robo2^{R1I1+2}, Robo1^{R2I1+3}, Robo1^{R2I1}, and Robo1^{R2I3}) do not promote ectopic midline crossing. It will be interesting to determine whether Robo2 in *Drosophila* promotes midline crossing through inhibition of Robo or, alternatively, whether it mediates midline attraction in certain contexts. If, like Rig-1/Robo3, Robo2 acts as an antirepellent, it is likely to achieve this function through a distinct mechanism because Rig-1/Robo3's antirepellent function is

Table 1. Contribution of Individual Ig Domains to Robo2 Guidance Activities

Ig Domain	Lateral Position	Antirepulsion	Expression on Commissures	FasII Pathway Formation
Ig1	Y	N	N	N
Ig2	?	Y	Y	N
Ig3	Y	N	N	Y
Ig4	N	N	N	N
Ig5	N	N	N	N

Summary of functional contributions from each of the five Ig domains of Robo2. Both Ig1 and Ig3 can individually confer lateral positioning activity, whereas Ig4 and Ig5 cannot. Ig2's contribution to lateral positioning activity has not been individually verified. Ig2 is the only Robo2 Ig domain that confers antirepellant activity; Ig2-containing variants that promote ectopic midline crossing are also detectable on commissural axons. Ig3-containing variants disrupt intermediate FasII pathway formation when misexpressed pan-neuronally. Lateral positioning activity is not correlated with antirepellant activity.

specified by its cytoplasmic domain [11]. A summary of the contributions of Robo2's Ig domains to its different guidance functions is presented in Table 1.

Robo2's Lateral Positioning Activity Is Not Due to Increased Slit Affinity

Because Robo2's Ig domains control lateral positioning, one possibility is that Robo2 may have a higher affinity for Slit, encouraging Robo2-expressing axons to seek out positions farther down the Slit gradient. To test this possibility, we purified the Ig domain-containing portions of the Robo and Robo2 ectodomains and compared their affinities for the Robo-binding domain of Slit (Slit D2) with surface plasmon resonance (SPR). We found that Robo2 does not exhibit a higher Slit affinity than Robo; instead, the Ig1–5 region of Robo binds Slit D2 around 4-fold as strongly as the equivalent region of Robo2 (apparent affinity [K_D] of 235 ± 165 nM for Robo1 versus 1098 ± 193 nM for Robo2) (Figure 5). Thus, the functional distinction between Robo and Robo2 for longitudinal pathway choice is not increased Slit affinity of Robo2. Furthermore, these observations suggest that the promidline crossing activity of Robo2 does not result from greater Slit affinity.

Differential Receptor Multimerization Partially Accounts for the Distinct Activities of Robos

Apart from modest affinity differences, we observed a second distinction between the Slit binding profiles of Robo and Robo2. When tested against a constant amount of immobilized Slit, the maximum equilibrium binding response for Robo was approximately half of that for Robo2 (44% ± 7% maximal binding) (Figure 5B). Thus, at equilibrium, the same amount of Slit can bind twice as much Robo2 as Robo, suggesting a difference in receptor-ligand stoichiometry. Size-exclusion

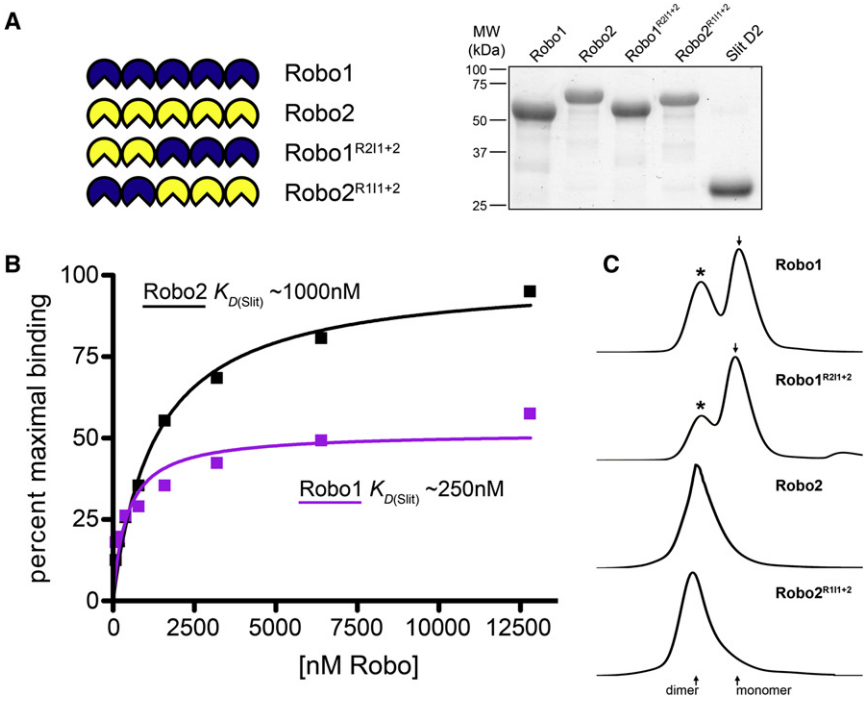

Figure 5. Robo1 and Robo2 Ig Domains Exhibit Distinct Multimerization and Slit Binding Properties

(A) Ig1–5 fragments of Robo1, Robo2, Robo1^{R2I1+2}, and Robo2^{R1I1+2} were purified and used for the biochemical analyses described below. Left: protein schematics showing domains present in wild-type and chimeric receptor fragments. Right: Coomassie-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel of purified proteins, loaded at a concentration of ~0.3 mg/ml.

(B) Surface plasmon resonance analysis indicates that Ig1–5 fragments of Robo1 and Robo2 exhibit distinct Slit affinities and maximal binding responses. Slit D2 was immobilized on a sensor chip, and increasing concentrations of soluble Robo fragments were flowed over the chip. Normalized response values at equilibrium were plotted against Robo concentration and fitted to a one-site binding curve, from which affinity (K_D) and maximum response (B_{max}) values were derived. Robo1 Ig1–5 binds Slit D2 with an apparent K_D of 235 ± 165 nM, which is around 4-fold stronger than the calculated Robo2 Ig1–5 K_D of 1098 ± 193 nM. In contrast, the Robo1 B_{max} was only around half that of Robo2 (44% ± 7%), indicating that a given amount of Slit D2 is capable of binding around twice as much Robo2 as Robo. Representative curves from a single experiment are shown; reported values are average ± standard deviation for three experiments.

(C) His-tagged wild-type and chimeric Robo receptor Ig1–5 fragments were subjected to size-exclusion chromatography; results are presented as normalized ultraviolet absorbance at 280 nm versus elution time. The Ig1–5 fragments of Robo1 and Robo1^{R2I1+2} migrate as a single monomeric peak (arrow). In contrast, Ig1–5 fragments of Robo2 and Robo2^{R1I1+2} are present nearly exclusively in dimeric form and migrate at a significantly larger apparent molecular weight than Robo1 and Robo1^{R2I1+2}. A large (~120 kDa) His-rich endogenous contaminant (*) was copurified from the initial nickel affinity column and is present in all samples. The His-rich contaminant is a large multimeric complex that commonly copurifies with His-tagged proteins derived from Sf9 cells (peak with asterisk in top two traces). On an SDS-PAGE gel, the individual denatured components of this complex are significantly smaller than our Ig1–5 Robo fragments and are thus easily distinguishable. The presence and distribution of receptor fragments in column fractions were analyzed by SDS-PAGE (data not shown), which confirmed that virtually no Robo1 or Robo1^{R2I1+2} was present within the contaminant peak, whereas Robo2 and Robo2^{R1I1+2} overlapped strongly with this peak.

chromatography (SEC) confirmed that the Ig1–5 fragment of Robo is almost exclusively monomeric in solution, whereas Robo2 Ig1–5 appears almost exclusively as a dimer (Figure 5C). These experiments were performed in the absence of Slit, indicating that the observed multimerization of Robo2 is at least partially ligand independent. However, the differences in maximum Slit binding response in our SPR experiments indicate that the multimerization states of Robo and Robo2 remain distinct even upon Slit binding.

To determine which region(s) of Robo2 are responsible for dimerization and whether the observed differences in receptor multimerization correlate with the two distinct lateral positioning activities we observed in vivo, we examined equivalent Ig1–5 fragments derived from the chimeric receptors Robo1^{R2I1+2} and Robo2^{R1I1+2} via SEC. These reciprocal chimeric receptors contained distinct portions of Robo2 and exhibited distinct large-scale effects on FasII tract formation (Figures 3C and 3E). We found that the Robo2^{R1I1+2} receptor fragment (containing Ig3–5 of Robo2) exhibited Robo2-like Slit-independent dimerization, whereas the Robo1^{R2I1+2} fragment (containing Ig1+2 of Robo2) did not (Figure 5C). Thus, ectodomain-dependent dimerization of Robo2 correlates with its ability to influence large-scale longitudinal pathway choice by FasII-positive axons and may account for Ig3's contribution to the lateral positioning activity of Robo2.

How do closely related axon guidance receptors, responding to a common ligand, generate diverse and, in some cases, opposing guidance outcomes? Here we have shown that the differential roles of the Robo receptors in directing longitudinal pathway choice are determined by structural differences between receptor ectodomains. In addition, we have provided evidence that a second function of Robo2 to promote midline crossing also depends on structural features of its ectodomain. We conclude that the diversification of Robo receptor axon guidance activities is facilitated by the functional modularity of individual receptor ectodomains. Although the importance of guidance receptor cytoplasmic domains in controlling guidance decisions has been known for a decade, our results reveal that Robo receptor Ig domains play an important part in the functional diversification of this ancient and evolutionarily conserved guidance receptor family.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cub.2010.02.021.

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