Repulsive Axon Guidance: Abelson and Enabled Play Opposing Roles Downstream of the Roundabout Receptor

Greg J. Bashaw,* Thomas Kidd,* Dave Murray,¹ Tony Pawson,² and Corey S. Goodman*³

In which Berkeley, California 94720 have undertaken a genetic and biochemical analysis of... in a mutant screen for genes that control the decision downstream of Robo to mediate repulsion. Here, we present genetic and biochemical evidence that the Abelson (Abl) tyrosine kinase and its substrate Enabled (Ena) play direct and opposing roles in Robo signal transduction. Genetic interactions support a model in which Abl functions to antagonize Robo signaling, while Ena is required in part for Robo’s repulsive output. Both Abl and Ena can directly bind to Robo’s cytoplasmic domain. A mutant form of Robo that interferes with Ena binding is partially impaired in Robo function, while a mutation in a conserved cytoplasmic tyrosine that can be phosphorylated by Abl generates a hyperactive Robo receptor.

Introduction

Neuronal growth cones follow specific pathways and navigate a series of choice points to find their correct targets. At each decision point, growth cones encounter a plethora of guidance cues in their extracellular environment. Guidance decisions are shaped by a balance of both attractive and repulsive signals and these signals can act at short or long range (Tessier-Lavigne and Goodman, 1996). Many ligands and receptors have been discovered and have been found to be members of phylogenetically conserved protein families. An emergent theme from several recent studies is that often, ligands are indeterminate, capable of generating either attractive or repulsive responses, and that it is the type of receptor or receptor complex expressed on the surface of the growth cone that determines the response (Bashaw and Goodman, 1999; Hong et al., 1999). Cytoplasmic domain swaps between attractive Netrin and repulsive Slit receptors indicate that the sign of the growth cone response is encoded in the cytoplasmic domains of these receptors.

If attraction versus repulsion is encoded in the cytoplasmic domain of the guidance receptor, then what are the signaling components within the growth cone that interact with a given receptor’s cytoplasmic domain to generate specific outputs? To address this question, we have undertaken a genetic and biochemical analysis of the Roundabout (Robo) repulsive axon guidance receptor in Drosophila (Kidd et al., 1998a, 1998b).

Drosophila Robo is the founding member of a conserved family of repulsive axon guidance receptors (Kidd et al., 1998a; Zallen et al., 1998) that respond to secreted Slit proteins (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999). Robo is a member of the immunoglobulin (Ig) superfamily and has an ectodomain with five Ig domains and three fibronectin (FN) type III repeats, a single transmembrane domain, and a long 457 amino acid cytoplasmic tail. The cytoplasmic domain does not contain any obvious catalytic signaling motif, but it does have proline-rich regions, potential phosphorylation sites, and other short stretches of evolutionarily conserved sequences (Kidd et al., 1998a), robo was identified in a mutant screen for genes that control the decision by axons to cross or not to cross the CNS midline (Seeger et al., 1993). In robo mutant embryos, too many axons cross and recross the midline.

How does Robo transmit its repulsive signal in response to Slit? A comparison of the Drosophila and Human Robo sequences revealed four short blocks of conserved cytoplasmic sequence, which were called CC0, CC1, CC2, and CC3 (CC for conserved cytoplasmic). CC2 (LPPPP) is a consensus binding site for a hyperactive Robo receptor.

Summary

Drosophila Roundabout (Robo) is the founding member of a conserved family of repulsive axon guidance receptors that respond to secreted Slit proteins. Little is known about the signaling mechanisms which function downstream of Robo to mediate repulsion. Here, we present genetic and biochemical evidence that the Abelson (Abel) tyrosine kinase and its substrate Enabled (Ena) play direct and opposing roles in Robo signal transduction. Genetic interactions support a model in which Abl functions to antagonize Robo signaling, while Ena is required in part for Robo’s repulsive output. Both Abl and Ena can directly bind to Robo’s cytoplasmic domain. A mutant form of Robo that interferes with Ena binding is partially impaired in Robo function, while a mutation in a conserved cytoplasmic tyrosine that can be phosphorylated by Abl generates a hyperactive Robo receptor.

Introduction

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Figure 1. Dominant Enhancement of ena Loss-of-Function

Each panel shows three segments of the embryonic CNS. Anterior is up. The stage 16 embryos in (A)-(D) are stained with monoclonal antibody (Mab) BP102 to reveal the CNS axon scaffold. The stage 13 embryos in (E)-(G) are stained with anti-Fas II Mab to reveal the longitudinal pCC axon pathway. (A and E) Wild type. (B and F) ena210/enaGC1. (C and G) ena210/enaGC1; robo4. (D and H) robo4.

(A) Arrowheads show the anterior and posterior commissures.
(B) Note the general disorganization of the CNS axon bundles. Arrows indicate typical deviations in commissural axon bundles.
(C) The bottom two segments have thicker than normal commissures and the longitudinal pathways show a marked lateral constriction towards the midline.
(D) Commissures are much thicker and appear fused. Longitudinal pathways are reduced and constricted laterally.
(E) The arrow indicates the pCC axon, which extends anteriorly and slightly away from the midline.
(F) pCC behaves normally.
(G) The pCC axon inappropriately crosses the midline in the top and bottom segments (arrows with asterisks). In the middle segment, pCC has a normal trajectory (arrow).
(H) The pCC axon inappropriately crosses the midline in every segment (arrows with asterisks).

that Ena family members can nucleate actin polymerization and bundle actin in vitro, have led to the suggestion that Ena/VASP proteins stimulate actin polymerization and cell motility. However, the presence of an Ena binding site in a repulsive guidance receptor points to a more complex role for Ena/VASP function. Does Ena lead to an increase or decrease in motility? Is Ena involved in attraction or repulsion? The results we present here strongly implicate Ena in repulsive axon guidance downstream of Robo. In a companion paper, Bear and colleagues (Bear et al., 2000 [this issue of Cell]) show that inhibition of Ena/VASP function actually increases the rate of cell migration of cultured fibroblasts, and that increased levels of Mena slow cells down. These two papers challenge the notion that Ena/VASP proteins play a stimulatory role in whole cell and growth cone motility, and instead focus attention on their role in repulsion and inhibition.

In the fly, mutations in the two genes—ena and robo—display strikingly different phenotypes; ena is weaker than robo in terms of midline guidance defects, but ena affects a broader range of guidance decisions than robo. Thus, if Ena functions downstream of Robo, then it can not function in a one receptor/one adaptor linear pathway. To determine if Ena plays a more subtle role in Robo signaling, we looked for genetic and biochemical interactions between Robo and Ena. ena and robo show several dosage-sensitive genetic interactions. In light of the known interactions between Ena and the Abl tyrosine kinase, the observation that robo interacts with ena suggested that Abl too could be involved in Robo signaling. While we were testing for robo/ena interactions, we recovered abl in a screen for genes that could modify the Fra-Robo chimeric receptor overexpression phenotype. This convergence led us to examine interactions between Robo and Abl as well.

A role for Abl in CNS axon guidance has been previously revealed by removing abl and a number of other gene functions (Gertler et al., 1989). For example, double mutants of abl and Fasciclin I show a dramatic reduction in the thickness of commissural axon bundles (Elkins et al., 1990). Like ena, mutations in abl also alter ISNb motor axon guidance (Wills et al., 1999a). Here, we show that whereas Ena mediates part of Robo’s repulsive signal, in contrast, Abl antagonizes Robo signaling.

Both Abl and Ena can directly bind to Robo’s cyto-
Abl and Ena Function in Robo Signaling

plasmic domain. A mutant form of Robo which interferes with Ena binding has impaired function, while a mutation in a conserved cytoplasmic tyrosine that can be phosphorylated by Abl generates a hyperactive Robo receptor. Taken together, our genetic and biochemical data are consistent with a model in which Abl and Ena play direct and opposing roles in Robo mediated repulsion.

Results

Reducing robogenedose in ena mutants reveals a role for ena in midline repulsion

In wild-type embryos, the CNS axon scaffold has a characteristic ladder-like appearance with longitudinal axon bundles running along either side of the midline and commissural axon bundles crossing the midline in the anterior and posterior commissures in each segment (Figure 1A). In ena mutants, the overall pattern of the CNS axon scaffold is fairly normal; the longitudinal axon tracks and anterior and posterior commissures are spaced normally and are usually of wild-type thickness. However, in contrast to wild type, the axons in ena mutants appear to be less tightly fasciculated, and commissural bundles sometimes appear abnormal and wander between commissures (Figure 1B) (Gertler et al., 1995).

Also, there are occasional errors in midline guidance, and axon pathways that do not normally cross the midline sometimes do (Table 1). Mutations in robo dramatically disrupt the CNS axon scaffold, causing far too many axons to cross and recross the midline; anterior and posterior commissures appear to be much thicker, or even fused together, and the longitudinal connectives are greatly reduced (Figure 1D) (Seeger et al., 1993).

To see if ena could play a role in Robo-mediated repulsion that is not apparent in animals mutant for ena alone, we reduced robo gene dose in ena mutants. We find that ena mutants that are heterozygous for mutations in robo exhibit striking defects in CNS axon guidance, indicative of a loss of midline repulsion; the anterior and posterior commissures are significantly thicker, longitudinal connectives are reduced and are sometimes closer to the midline. These animals have variable phenotypes, with some segments showing defects that approach those seen in animals that completely lack robo function (Figure 1C and Table 1A). We also looked at the axons that pioneer one of the longitudinal axon pathways. Normally, the pCC cell body, positioned at a distance of several cell diameters from the midline, extends an axon anteriorly to join with descending axons from the MP1 and dMP2 neurons. Together these axons pioneer the pCC pathway, which runs parallel to but does not cross the midline (Figure 1E). In robo mutants, pCC initially extends anteriorly, but instead of continuing its anterior trajectory, it turns and crosses the midline (Figure 1H). In ena mutants, pCC behaves normally. But if ena is mutant, and robo is reduced by 50%, then

Table 1A. Dominant Enhancement of ena

<table>
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<th>Genotype</th>
<th>Segments scored</th>
<th>Thicker commissures</th>
<th>Segments with lateral constriction</th>
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<tr>
<td>ena210/ena210</td>
<td>77</td>
<td>8%</td>
<td>1.3%</td>
</tr>
<tr>
<td>ena210/ena210;robo4/1</td>
<td>209</td>
<td>37%</td>
<td>25%</td>
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Stage 15–16 embryos stained with MAb BP102 were scored.

Figure 2. Genetic Interactions between ena, slit, and robo

(A–C) Late stage 16 embryos stained with anti-Fas II Mab to reveal longitudinal axon pathways. Anterior is up. (A) A wild-type embryo. The arrow indicates the medial longitudinal pathway. No Fas II-positive axon bundles cross the midline. (B) A slit1,robo5/11 transheterozygous embryo with mild midline crossing defects. Arrows with asterisks indicate axon bundles that are inappropriately crossing the midline. (C) A slit1,robo5,+/+,enaGC1 embryo with severe midline crossing defects. The medial axon pathway wanders back and forth across the midline (arrows with asterisks).

(D–F) Stage 16 embryos stained with Mab BP102. (D) A wild-type embryo. (E) A UAS-roboY-F/elavGAL4 embryo. Commissural axon pathways are absent. (F) The same genotype as in (E), but also heterozygous for the enaGC1 allele. Removing one copy of ena partially suppresses the commissureless phenotype.
Table 1B. Suppression of Gain-of-Function Phenotypes

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<th>Partial genotype</th>
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<td></td>
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<td>UASroboY-F</td>
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<td>132</td>
<td>76%</td>
</tr>
<tr>
<td>UASfra-robo;enaGC1</td>
<td>99</td>
<td>90%</td>
</tr>
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<td>EProbo2</td>
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<td>EProbo2,enaGC1</td>
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<td>comm</td>
<td>84</td>
<td>11%</td>
</tr>
<tr>
<td>enaGC1;comm</td>
<td>121</td>
<td>99%</td>
</tr>
<tr>
<td>slit2,robo4,enaGC1</td>
<td>88</td>
<td>76%</td>
</tr>
</tbody>
</table>

Stage 15–16 embryos stained with MAb BP102 were scored.

pCC frequently crosses the midline (Figures 1F and 1G). Normally, heterozygosity for robo does not cause defects. This kind of dose-sensitive genetic interaction, referred to as dominant enhancement, is often observed when two genes function together in the same process.

Complementary Dosage-Sensitive Genetic Interactions between slit, robo, and ena

Another common genetic test is to determine if 50% reduction of two genes reveals a phenotype not observed in the individual heterozygous mutants. This kind of transheterozygous interaction is very specific and is usually indicative of direct interaction in a common process. For example, robo and its ligand slit display such an interaction (Kidd et al., 1999).

We generated transheterozygous combinations of robo and ena, but we never observed significant ectopic midline crossing in these animals (Table 1C). Similar transheterozygous tests with slit and ena mutants revealed mild, but significant, midline crossing defects (Table 1C). Reasoning that a 50% reduction of either robo or slit alone did not create a sufficiently sensitized genetic background to see strong effects of reducing ena levels, we limited the gene dose of slit, robo, and ena.

Transheterozygous slit,robo/+/+ embryos typically display two to four axon bundles of the medial longitudinal pathway inappropriately crossing the midline per animal; such defects are never observed in wild-type embryos (Figures 2A and 2B; Table 1B). If now in this slit,robo background we also reduce ena by 50% a dramatic enhancement of the slit,robo phenotype is revealed; instead of 2–4 crossovers per embryo of the medial longitudinal pathway, we now see many more crossovers, often several per segment (Figure 2C and Table 1C). Thus, on the background of a 50% reduction in both ligand and receptor, we see a dramatic increase in inappropriate midline crossing by removing 50% of Ena. These observations further support a role for ena in robo-mediated repulsion.

We have also examined whether loss or reduction of ena function can suppress robo gain-of-function phenotypes. High level panneural expression of Robo generates a commissureless phenotype indicating that Robo repulsion can overcome all endogenous attraction to the midline (Figure 2E). This gain-of-function phenotype can be generated by overexpressing either wild-type Robo (Kidd et al., 1999), a mutated form of Robo, the Fra-Robo chimera (Bashaw and Goodman, 1999), or Robo2 (J. Simpson, personal communication). Furthermore, loss of commissureless(chema) function arguably represents the ultimate gain of robo function, since in the absence of Comm, Robo can not be down-regulated on commissural axons (Kidd et al., 1998b).

ena can partially suppress all of these types of gain-of-function robo phenotypes. With the exception of the tyrosine-phenylalanine (Y-F) mutant form of Robo (see transgenic expression of mutant forms of Robo below), significant suppression of the commissureless phenotype is not observed unless both copies of ena are removed (Table 1B). The suppression of the phenotype of the Y-F mutant receptor is most striking, where 50% reduction of ena function allows significant commissure formation (Figure 2F and Table 1B). These observations complement the dominant enhancement data, and together the genetic interaction results provide strong evidence that robo and ena work together in a common pathway to mediate midline repulsion.

Table 1C. Genetic Interactions between ena, robo and slit**

<table>
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<td>0</td>
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<tr>
<td>slit2, +/+robo4, enaGC1</td>
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<td>50</td>
<td>41</td>
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<td>32</td>
<td>27</td>
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<td>3.2</td>
<td>29%</td>
</tr>
<tr>
<td>slit2,robo4/+</td>
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<td>31</td>
<td>62</td>
<td>44</td>
<td>12</td>
<td>74%</td>
</tr>
<tr>
<td>slit2,robo4/+</td>
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<td>35</td>
<td>18</td>
<td>8</td>
<td>69%</td>
</tr>
<tr>
<td>slit2,robo4/+</td>
<td>187</td>
<td>43</td>
<td>35</td>
<td>47</td>
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Stage 16–17 embryos stained with anti-FasII MAb were scored. Column designations are as follows: +, thinner than normal fascicle ectopically crossing the midline; ++, normal fascicle ectopically crossing the midline; ++++, thicker than normal fascicle ectopically crossing the midline. **, neither slit, robo, nor ena show significant ectopic midline crossing as individual heterozygotes (data not shown).
Overexpression of Abl Mimics robo Loss-of-Function
Overexpression of the Fra-Robo chimeric receptor leads to dose dependent CNS axon guidance defects and reduced viability. We screened the EP collection (a collection of P-element inserts that allows targeted misexpression of flanking genes) (Rørth, 1996) for genes that when overexpressed in combination with Fra-Robo, would enhance the lethality of the chimera. In this screen, we recovered an EP insert in abl as one strong enhancer. This finding was intriguing in light of the genetic interactions between robo and ena, and prompted us to investigate a role for Abl in Robo signaling.

We used the EP insert in abl to overexpress Abl in all neurons with elavGAL4 and scabrousGAL4, and examined the CNS with antibodies to Fas II and BP102. Varying the copy number of EPabl and GAL4 driver allowed us to generate animals with a range of Abl expression levels. Overexpression of Abl results in dose-dependent ectopic midline crossing reminiscent of the phenotype observed in robo mutants. Low levels of Abl expression result in occasional errors in midline guidance; Fas II-positive bundles are sometimes observed to inappropriately cross the midline (Figure 3A and Table 1D). Higher levels of Abl expression caused more dramatic phenotypes that appear similar to loss of robo function (Figure 3B). Similar results were obtained with both GAL4 drivers, and substituting UASabl for EPabl also gave comparable results (Table 1D and data not shown). Overexpression of Abl also disrupts the guidance of axons that pioneer the longitudinal pathways (e.g., the pCC neuron), causing them to inappropriately cross the midline (Figure 3C). The midline crossing defects caused by Abl overexpression are largely kinase dependent; only very weak effects are observed when a mutant form of Abl that lacks kinase activity (Henkemeier et al., 1990) is similarly overexpressed (data not shown). This suggests that phosphorylation of substrates by Abl is important in generating the Abl overexpression phenotype.

Reducing Robo Function Enhances the Abl Overexpression Phenotype
The Abl overexpression phenotype suggests that Abl could act to antagonize Robo function. We reasoned that if increasing Abl expression produced its effects by directly antagonizing Robo, then genetically limiting robo and/or slit copy number might enhance the abl overexpression phenotype. We used low level expression of Abl (i.e., single copy), with the idea that it would be easy to detect enhancement of the mild phenotype caused by these levels of Abl. Indeed, although we

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<th>Genotype</th>
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<td>3%</td>
</tr>
<tr>
<td>UASabl^{hws}/elav</td>
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<td>0</td>
<td>0</td>
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<tr>
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Stage 16-17 embryos stained with anti-Fasll MAb were scored. Column designations are as follows: ++, thinner than normal fascicle ectopically crossing the midline; ++++, normal fascicle ectopically crossing the midline; ++++, thicker than normal fascicle ectopically crossing the midline.
Figure 4. Genetic Interactions between abl, slit, and robo
Late stage 16 embryos stained with anti-Fas II MAb to reveal longitudinal axon pathways. Anterior is up.
(A) A UASabl/elavGAL4 embryo. Note the wild-type appearance of the longitudinal pathways.
(B) A robo+/+; UASabl/elavGAL4 embryo. Removing a copy of robo enhances the midline defects caused by Abl overexpression (arrows with asterisks).
(C) A robo+/+; UASablkinase dead/elavGAL4 embryo. Overexpression of kinase dead Abl in a robo heterozygote does not result in significant defects.
(D) A slit1,robo5/11; abl1/1 embryo. Removing a copy of abl partially suppresses the slit,robo transheterozygous phenotype. Note the wild-type appearance of the longitudinal pathways and compare with (E).
(E) A slit1,robo5/11 embryo. Arrows with asterisks indicate inappropriate midline cross-overs.
(F) A slit1,robo5/11; EPabl,elavGAL4 embryo. The midline crossing defects of slit1, robo5/11 are enhanced by overexpression of Abl. (arrows with asterisks).

Sometimes observe ectopic midline crossing in this background (Figure 3A and Table 1D), most animals carrying one copy of EPabl and one copy of elavGAL4 are essentially wild type (Figure 4A and Table 1D). If, in this background we also remove one copy of robo, we see a marked enhancement of the midline crossing defects, with some animals showing many inappropriate cross-overs (Figure 4B and Table 1D). This effect is observed with two independent robo alleles (data not shown). Removing one copy of slit has a similar, but slightly weaker, effect. In addition, simultaneous pan-neural expression of Abl and a truncated Robo receptor, which acts as a weak dominant-negative, also enhances the ectopic midline crossing phenotype (Table 1D).

Interestingly, the phenotype resulting from increasing Abl and decreasing Robo is dependent on an intact Abl kinase domain; overexpressing a kinase-dead Abl in robo heterozygotes does not cause significant guidance errors (Figure 4C and Table 1D). This observation again suggests that phosphorylation of Abl substrates is important in generating these phenotypes. Removing one copy of ena, a known substrate of Abl and a logical candidate to be a potentially important target of Abl kinase activity in the context of midline repulsion, does not enhance the Abl overexpression phenotype (Table 1D), whereas removing one copy of robo does (Figure 4B and Table 1D).

Another prediction of the model that Abl inhibits Robo signaling is that decreasing Abl function could lead to an increase in Robo activity. To see if reducing abl gene dose could suppress the effects of partial loss of robo function, we tested whether removing one copy of abl could suppress the slit,robo/+ transheterozygous phenotype. We found that reducing abl (using either the abl1 or abl4 allele) results in significant suppression of the slit,robo/+ midline crossing defects (compare Figure 4D to 4E; Table 1D; and data not shown), arguing further for a role of abl in regulating Robo signaling. In contrast, increasing abl in the slit,robo/+ background has the reciprocal effect of enhancing the midline crossing phenotype (Figure 4F and Table 1D).

Both Abl and Ena Can Bind to the Cytoplasmic Domain of Robo
To test whether the genetic interactions observed between abl, ena, and robo could reflect direct interaction between these three proteins, we have performed in vitro and in vivo assays for protein/protein interactions. For these experiments, a panel of Abl, Ena, and Robo protein expression constructs were generated (Figure 5A).

GST pull down assays reveal robust in vitro interactions between Ena and Robo, as well as Abl and Robo (Figures 5B-5D). In the case of Ena, bidirectional interactions have been examined: a GST Robo cytoplasmic domain fusion (GST RoboC) can effectively pull down in vitro translated Ena, while GST alone can not, and GST Ena can bind in vitro translated Robo, while GST alone can not (Figures 5B and 5C). In both cases, deletion of the CC2 motif results in a similar significant reduction in Robo/Ena binding, while mutations in either CC3 or the Y-F mutant in CC1 do not (Figures 5B and 5C). In addition, deletion of the CC1 motif also results in decreased binding of Ena, suggesting that there might be multiple sites of association between Ena and Robo. These interactions are observed in the context of full-length Ena, as well as with the isolated Ena EVH1 domain (Figure 5D).
GST Abl SH3 and GST Abl SH2 can also efficiently pull down in vitro translated Robo (Figure 5B). Binding of Robo to the Abl SH3 domain is strongly reduced when the CC3 motif is deleted, while the other mutant forms do not significantly affect the observed binding (Figure 5B). In contrast to the interactions of Ena and the Abl SH3 domain with Robo, none of the Robo cytoplasmic domain mutants consistently affect the binding observed with the Abl SH2 domain (Figure 5B). In addition to efficiently binding to in vitro translated Robo, the GST Ena, GST Abl SH3, and GST Abl SH2 can precipitate full-length Robo from Robo-expressing S2 cells (Figure 5E).

We also examined in vivo interactions between Robo and Ena by commounoprecipitation from embryonic extracts. For this we generated a monoclonal antibody to the N terminus of Ena and used the previously described Robo monoclonal antibody. Our Ena antibody specifically recognizes Ena in whole-mount embryos as well as in extracts from embryos and S2 cells (Figures 5F and 5G; data not shown). Extracts were prepared from wild-type embryos as well as embryos expressing myc-tagged forms of Robo. We found that in wild-type embryonic extracts, the Robo monoclonal antibody can commounoprecipitate a small amount of Ena protein, while a preimmune serum can not (Figure 5F). In addition, in extracts from embryos expressing myc-tagged Robo, the anti-myc monoclonal antibody can also precipitate a small amount of Ena. Furthermore, in extracts from embryos expressing myc-tagged Robo, the anti-myc monoclonal antibody can also precipitate a small amount of Ena. However, in extracts from embryos expressing an equivalent amount of the myc-tagged RoboΔCC2 mutant, the myc antibody precipitates less Ena (Figure 5G). We have been unable to perform similar in vivo interaction experiments between Abl and Robo because we do not have an Abl antibody that efficiently recognizes Abl in embryonic extracts. However, the strong in vitro binding, together with the genetic interactions support the idea that Abl, like Ena, plays a direct role in Robo signal transduction.

Robo Is a Substrate for Abl Kinase Activity

To see if Abl could antagonize Robo by directly phosphorylating it, we tested whether Robo could be phosphorylated by Abl in mammalian Cos1 cell culture. When expressed in Cos1 cells, neither full-length human Robo1 (hRobo1), nor a Flag epitope-tagged hRobo1 cytoplasmic polypeptide, containing residues 930-1651 of the full-length protein, were detectably tyrosine phosphorylated. However cotransfection of a constitutively active c-Abl variant lacking the SH3 domain (Jackson and Baltimore, 1989), resulted in significant tyrosine phosphorylation. However cotransfection of a constitutively active c-Abl variant lacking the SH3 domain (Jackson and Baltimore, 1989), resulted in significant tyrosine phosphorylation of both full length hRobo1, as well as

**Figure 5. Ena and Abl Bind Robo’s Cytoplasmic Domain**

(A) A diagram of constructs used for in vitro binding assays. The left column shows Robo cytoplasmic domain constructs. The positions and sequences of the four CC motifs are indicated. Tyrosines (Y) in CC0 and CC1 that can be phosphorylated in vitro are in bold. The right column shows Abl and Ena constructs. GST/pCite indicates that two versions of the plasmids were made.

(B) Pull-down assays using GST Ena and Abl fusions and in vitro translated Robo cytoplasmic domains. The relative input of each version of Robo is shown in the top panel indicated above each lane. Breaks in panels are due to removal of irrelevant lanes. The fusion protein used is indicated above each gel. None of the pCite Robo products bind to GST alone. GST-Ena shows reduced binding to both ΔCC1 and ΔCC2—the reduction is more pronounced for ΔCC2. GST-AblSH3 shows reduced binding to ΔCC3.

(C) Pull down assays using GST-Robo and in vitro translated Ena. A small amount of Ena is bound by GST alone. GST-Robo binds much more strongly to Ena and this binding is reduced in the ΔCC2 mutant.

(D) Similar interactions are detected using the isolated Ena EVH1 domain.

(E) GST pull downs from S2 cells transfected with and without Robo. The + or – indicates Robo-expressing cells (+) or control cells (–). Western blots were probed with anti-Robo monoclonal antibody. GST-Ena, GST-Abl SH3, and GST-Abl SH2 all pull down full-length Robo from S2 cell extracts, while GST alone does not.

(F) Coimmunoprecipitation of Robo and Ena from wild-type embryos. Lanes are indicated above the gel and sizes are indicated next to the gel. An aliquot of extract is included as a positive control for the detection of Ena in the far left lane.

(G) Coimmunoprecipitation of Ena with myc-tagged Robo proteins. Lanes are as indicated. The left half of the IP gel consists of immunoprecipitates from embryos expressing UASrobomyc, the right half of the IP gel is from embryos expressing UASroboΔCC2myc. Note the strong reduction of Ena signal in the Myc IP from the ΔCC2 extract. The two types of extracts contained similar amounts of Ena as shown in the small gel on the left.

(F and G) Ena protein is detected at the expected size of ~80 kDa. The strong signal at ~50 kDa in the IP lanes corresponds to mouse heavy chain.
nano-electrospray-tandem mass spectrometry was used wild type (Figure 7E). In contrast to the near complete resemblance the c-Abl phosphorylation site in c-Crk forth across the midline (Figure 7D). When a wild-type contains the principal phosphotyrosine sites. The sequence ences were most striking in the case of the juxtamembrane region of hRobo1 containing, it does not act as a substrate for Abl, sug-

Figure 6. hRobo1 Is a Substrate for Abl in Cos1 Cells

(A and B) The hRobo1 cytoplasmic domain is tyrosine phosphorylated by active Abl in Cos1 cells. Flag-tagged cytoplasmic domain hRobo1 constructs were transfected alone, or in combination with Abi\(\Delta\)SH3, into Cos1 cells as indicated. hRobo1 proteins were immunoprecipitated from cell lysates with an anti-Flag monoclonal antibody and analyzed by Western blotting using an anti-phosphotyrosine monoclonal antibody (A) or an anti-Flag monoclonal antibody (B). Lane designations in (B) are the same as those in (A). The breaks in the gels in (A) and (B) are due to removal of irrelevant lanes. (C) Full-length hRobo1 is tyrosine phosphorylated by Abl. Full-length hRobo1 was transfected alone, or in combination with Abi\(\Delta\)SH3 into Cos1 cells. hRobo1 was immunoprecipitated from cell lysates with an anti-hRobo1 polyclonal antibody and analyzed by Western blotting using an anti-phosphotyrosine monoclonal antibody. (D and E) Decreased tyrosine phosphorylation of the hRobo1 cytoplasmic domain resulting from deletion of the CC1 motif or mutation of its conserved tyrosine. Wild-type, CC1, or Y1073F hRobo1 cytoplasmic domain constructs were transfected alone or in combination with Abi\(\Delta\)SH3 into Cos1 cells. hRobo1 proteins were immunoprecipitated from cell lysates with an anti-Flag monoclonal antibody and analyzed by Western blotting using an anti-phosphotyrosine monoclonal antibody (D) or an anti-Flag monoclonal antibody (E). Lane designations in (E) are the same as those in (D).

Transgenic Expression of Mutant Forms of Robo

To determine if the \(\Delta\)CC mutations in the cytoplasmic domain that effect the binding of Robo to Abl and Ena effect the in vivo function of Robo, we expressed them transgenically in Drosophila using the GAL4-UAS system. We did the same for the Y-F point mutant that leads to decreased in vitro tyrosine phosphorylation by Abl (Figure 7A). We put the \(\Delta\)CC deletion mutant transgenes into a robo mutant background and compared their ability to rescue robo function with that of wild-type robo transgenes. All three of the \(\Delta\)CC mutant Robo constructs showed some ability to rescue robo loss of function, reducing the amount of ectopic midline crossing when expressed in a robo mutant background (Figure 7; Table 2; and data not shown), indicating that none of the three CC motifs are absolutely required for complete Robo repulsive output.

However, when compared with the rescue observed using wild-type transgenes, there were significant differences seen in the relative degree of rescue. Both the \(\Delta\)CC2 and \(\Delta\)CC3 mutants showed consistent reduction in their ability to rescue the robo phenotype. The differences were most striking in the case of the \(\Delta\)CC2 mutant (Figures 7E and 7F; Table 2). In robo null mutants, the innermost Fas II positive pathway wanders back and forth across the midline (Figure 7D). When a wild-type Robo transgene is expressed in all CNS neurons in this background, the midline phenotype is restored to near wild type (Figure 7E). In contrast to the near complete rescue observed for the wild-type and \(\Delta\)CC1 transgenes,
robo mutants that express the ΔCC2 mutant transgene still exhibit moderate robo phenotypes in which many Fas II axon bundles still cross the midline (Figure 7F). Similar, but weaker effects are seen in the case of the ΔCC3 mutant (Table 2). The fact that the ΔCC1 mutant showed no reduction in its ability to rescue the robo mutant is consistent with the idea that CC1 is a site of negative regulation by Abl. The impaired ability of the ΔCC2 mutant to rescue robo function further supports a direct role for Ena in Robo repulsion.

In contrast to all wild-type robo transgenes examined thus far, several independent inserts of the roboY-F mutant transgene generate a completely commissureless phenotype when expressed in single copy (Figure 7C). Animals that express a relatively higher transgene dose of wild-type UASrobo (two copies of UASrobo and one copy of elavGAL4) have only a mild phenotype in which some commissures appear somewhat thinner (Figure 7B). Semi-quantitative immuno-histochemical comparison of the Robo protein levels of these two genotypes, showed no reduction in its ability to rescue the robo mutant is consistent with the idea that CC1 is a site of negative regulation by Abl. The impaired ability of the ΔCC2 mutant to rescue robo function further supports a direct role for Ena in Robo repulsion.

Table 2. Comparative Rescue of robo Mutants with Wild-Type and ΔCC Mutant Transgenes

<table>
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<th>Genotype1</th>
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<th>Class II mild robo</th>
<th>Class III moderate robo</th>
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<td>(21%)</td>
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<td>17</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>(59%)</td>
<td>(34%)</td>
<td>(7%)</td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>4</td>
<td>11</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>(13%)</td>
<td>(37%)</td>
<td>(50%)</td>
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<td></td>
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</tr>
<tr>
<td>robo5; elavGAL4</td>
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<td>7</td>
<td>12</td>
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<td>(30%)</td>
<td>(52%)</td>
<td>(18%)</td>
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</tbody>
</table>

1 Multiple inserts of each of the different transgenes were examined for their ability to rescue robo. Different lines of each transgene behaved similarly and the data were pooled. Three independent lines each for UASrobo, UASrobo,ΔCC1, and UASrobo,ΔCC2 were examined. Two lines of UASrobo,ΔCC3 were examined.

2 The data were grouped into three phenotypic classes: Class I (wild type), animals that had 0-2 ectopic Fas II axon bundles crossing the midline; Class II (mild robo), animals that had 3-4 ectopic Fas II axon bundles crossing the midline; Class III (moderate robo), animals with 5 or more ectopic Fas II axon bundles crossing the midline.
In combination with the in vitro phosphorylation data and the genetic interactions of abl and robo, these results support a model in which Abl can directly antagonize Robo signaling by the phosphorylation of Robo at tyrosine 1040 (1073 in hRobo1). It will be of interest to determine whether additional sites of Robo tyrosine phosphorylation are also functionally significant. It should be stressed that although the in vitro, transgenic, and genetic interaction data are consistent with the proposed role of Abl in regulating Robo function, they do not directly prove that this interaction occurs in the organism. This will require future analysis of in vivo Robo tyrosine phosphorylation and determination of the effect of mutations in abl and/or overexpression of Abl on Robo phosphorylation.

Discussion

In this paper, we have identified Abl and Ena as complementary components of the signaling machinery downstream of the Robo repulsive axon guidance receptor. Genetic interactions indicate that loss of ena function partially disrupts Slit- and Robo-mediated repulsion from the midline. Limiting or removing ena function enhances partial loss-of-function robo phenotypes and suppresses robo gain-of-function phenotypes. In contrast, reduction of abl has the opposite consequence, suppressing the effects of a partial loss of robo function, while panneural overexpression of Abl antagonizes Robo function, leading to a phenotype resembling that of robo mutants.

Both Abl and Ena bind directly to Robo's cytoplasmic domain in vitro and Robo can act as a substrate for Abl kinase activity in vitro and in cell culture. Robo and Ena also show in vivo physical interactions. Furthermore, cytoplasmic domain mutants that reduce Ena binding to Robo result in impaired ability to rescue robo loss of function, while a Y-F mutation in a conserved tyrosine that can be phosphorylated by Abl in vitro has the opposite consequence, generating a hyperactive Robo receptor. These genetic and biochemical data support a model in which Abl and Ena play direct and opposing roles in the transmission of Robo's repulsive signal.

The implication of Ena in repulsive axon guidance is somewhat surprising in light of the previous results from the pathogen Listeria monocytogenes indicating that Mena is required for Listeria's actin-polymerization dependent motility. The Listeria data, together with Ena/VASP proteins in vitro effects on actin, has frequently been interpreted to suggest that Ena/VASP proteins function to promote actin polymerization, thereby promoting motility. On the contrary, our results indicate that Ena is partially required for axon repulsion from the midline. These data suggest that Ena may have the opposite function, namely, to inhibit forward growth cone motility at sites where Robo encounters Slit.

In a companion paper (Bear et al., 2000), an independent study in mammalian cell culture has reached a similar conclusion. By expressing a multimerized EVH1 domain binding site attached to specific subcellular localization sequences, Ena/VASP family members can be efficiently targeted to different areas of cultured fibroblasts. This system has allowed a direct examination of the role of Ena/VASP proteins in cell motility. Surprisingly, when Ena/VASP proteins are directed away from the cell membrane, using a mitochondrial targeting sequence, the cells actually migrate more quickly. Conversely, targeting Ena/VASP proteins to the membrane, or overexpressing Mena, leads to a dose-dependent decrease in the rate of cell migration. A major conclusion of this study is that Ena/VASP proteins function in part to decrease the rate of whole cell motility. Whether Ena/VASP proteins achieve the observed in vivo effects on whole cell and growth cone motility by stimulating or inhibiting actin polymerization awaits future investigation.

Ena Function Does Not Account for All of Robo Repulsion

While the dosage-sensitive genetic interactions between ena and robo support a role for Ena in midline repulsion, Ena clearly can not explain all of Robo's repulsive output. Indeed, although mild midline crossing defects are observed in ena mutants, on the whole, Robo-mediated repulsion works fairly well in the absence of Ena. In this light, it is perhaps not surprising that the Robo ΔCC2 mutant receptor (in which the Ena binding site is deleted) still provides some repulsive activity and can partially rescue robo loss-of-function mutants. These results indicate that there must be other proteins that function downstream of Robo to mediate repulsion. One would predict that simultaneously removing ena and the as yet unknown additional factors would reveal stronger disruptions of midline repulsion.

Thus, Ena is only part of what must be a more complex repulsive output from Robo. Ena helps strengthen the output (perhaps by locally putting the break on the actin-based motility machinery), but is only part of the output. In this light, it is interesting to note that Robo2 also binds Slit and mediates repulsion (although apparently more weakly than Robo), but Robo2 does not have the Ena binding site and does not bind Ena (J. Simpson, personal communication).

An important question for future studies concerns whether Ena is always docked on Robo, or alternatively, whether Slit binding to Robo leads to the recruitment of Ena to Robo's cytoplasmic domain. From what we know about other receptor systems, this second alternative seems more likely, but is an open question and needs to be directly tested.

Abl Phosphorylation and the Regulation of Robo Signaling

Our genetic analysis shows that Abl antagonizes Robo-mediated repulsion. The two most likely possibilities are that Abl functions to antagonize this pathway by phosphorylating Robo or by phosphorylating Ena. Three results argue in favor of a direct interaction with Robo. First, we observe certain kinds of dose-dependent genetic interactions between abl and robo that we do not observe between abl and ena, suggesting that the Abl and Robo proteins might directly interact. Second, our biochemical experiments have shown that Abl can directly phosphorylate Robo's cytoplasmic domain at one or more tyrosine residues. Third, a Y-F mutation in a conserved tyrosine that can be phosphorylated by Abl
in vitro generates a hyperactive Robo receptor. Taken together, these genetic and biochemical data suggest that it is the dephosphorylated form of Robo which is most active.

How might Abl normally regulate the output of Robo signaling? On the one hand, Abl-mediated phosphorylation might normally modulate the output of Robo signaling. Alternatively, this phosphorylation might participate more directly in the ligand-gated signal. It is interesting to speculate that if it is the binding of Robo to its ligand Slit that triggers dephosphorylation, and that this in turn activates the repulsive response.

The CNS-specific receptor protein tyrosine phosphatases (RPTPs) RPTP10D and 69D are candidates to be additional factors that contribute to Robo repulsion. Simultaneous removal of these two RPTPs results in substantial ectopic midline crossing, and the double mutant shows dose-sensitive genetic interactions with slit (Sun et al., 2000). Whether these two phosphatases interact directly with Robo and whether their phosphatase activity is required for their observed roles in repulsion awaits future investigation.

In the model presented above, it is attractive to speculate that these two RPTPs function in opposition to the Abl kinase activity by directly dephosphorylating Robo upon Robo’s interaction with Slit. Interestingly, the other two Robo family members in Drosophila (Robo2 and Robo3; J. Simpson, personal communication) share the phosphorylation sites in Robo that are phosphorylated by Abl in vitro. In addition, genetic interactions are observed between the RPTPs and Robo2 (J. Simpson, personal communication). Together these observations suggest that perhaps a common mechanism is employed to regulate the signaling output of the three Robo receptors. It will be of interest to determine the in vivo significance of the conserved tyrosine phosphorylation sites in the three Robo receptors. The future elucidation of the events set in motion by ligand binding will require the development of cell culture systems that will allow analysis of the phosphorylation state and cytoplasmic domain associations of the Robo receptors before and after Slit stimulation.

Robo Signaling Components Are Likely to Function in a Similar Fashion in Many Other Pathways

In addition to their function during Robo signaling shown here, it is clear that both Abl and Ena function in multiple guidance signaling pathways, and thus that they are not committed to repulsion downstream of Robo. In the nematode C. elegans, ena acts as a suppressor of the axon migration defects associated with ectopic expression of the UNC5 repulsive Netrin receptor (Colavita and Culotti, 1998). This raises the possibility that ena functions downstream of diverse repulsive guidance receptors.

In Drosophila, during motor axon pathfinding, ena and abl play roles in ISNb choice point control (Wills et al., 1999). Overexpression of abl or loss of ena generates an ISNb “bypass” phenotype, where the ISNb fails to defasciculate and branch off at the appropriate location to enter its muscle target region. This phenotype is also observed in mutations in Dlar, the gene encoding a receptor protein tyrosine phosphatase (RPTP) (Knueger et al., 1996). Mutations in all three of these genes (ena, abl, and Dlar) give rise to only partially penetrant ISNb guidance phenotypes and appear to modulate guidance decisions at this choice point.

At the midline, mutations in the genes encoding the ligands (Slit and a key receptor (Robo) have strong and highly penetrant midline guidance phenotypes. In contrast, mutations in the genes encoding Ena, Abl, and RPTP10D and RPTP69D on their own have weaker and less penetrant phenotypes. This is consistent with Abl and the RPTPs modulating Robo receptor output, and Ena mediating only part of Robo output. If we apply this same logic to the motor axon ISNb choice point, then it is likely that some of the key components are still missing. At present, the only gene with a nearly 100% penetrant bypass phenotype at this choice point is side-step (Sink and Goodman, 1994). Side is an Ig superfamily transmembrane protein that is expressed on muscle surfaces and appears to function as an attractive ligand for motor axons (H. Sink, personal communication). The Side receptor is not known. Whether the key receptor is the Side receptor or not, it is likely that the major growth cone receptor for the ISNb choice point has not yet been identified.

In this context, it is tempting to speculate, by analogy with the proposed model for Robo signaling, that at the ISNb motor axon choice point, DLAR and Abl play complementary roles in modulating the output activity of the hypothetical guidance receptor, while Ena functions as part of the receptor output. In this way, the two guidance decisions— to cross or not to cross the midline, and to fasciculate or defasciculate from other motor axons — use different signals on the outside of the growth cone, but similar signaling and regulatory mechanisms on the inside. We suggest that once the signal crosses the membrane, in both cases the output is regulated in opposing directions by Abl vs. one or more RPTPs, and that the output is partially mediated by Ena. This model provides a unifying way of viewing signal transduction during these two different guidance decisions. It will be interesting in the future to see to what degree this model holds up in terms of both the role of phosphorylation in regulating receptor output, and the role of Ena in mediating part of repulsive signaling.

Experimental Procedures

Genetics

The following stocks were used: 1) ena(n2)CyO[Wg]gal, elav GAL4, aro10b; elav GAL4, 3hrIr(Cyo[Wg]gal, elav GAL4, 4Pab1, elav GAL4/TM3actin;gal, 5) ena(n2)EP202/Cyo[Wg]gal, 6ena(n2)CyO[Wg]gal; UASrobo1B, 3D, 7) ena(n2)CyO[Wg]gal; UASrobo, elav GAL4/TM6.8) ena(n2)CyO[Wg]gal; UASfra-robo, elav GAL4/TM2, 9) ena(n2)CyO[Wg]gal2X UASfra-robo. The following stocks were also used: abl(red), elav actin; gal, abl(red), elav actin, comm1/TM3-actin;gal, 8Probo+/CyO[Wg]gal, elav GAL4 and slitscalarsactin50. Genes were generated by recombination. robo, robo, ena(n2) and ena(n2) were kept over a wingless(Wg)[gal] balancer chromosome.
Molecular Biology
The pharmacia pGEX system was used for GST fusions of the Abl SH2, Abl SH3, Ena, Ena EVH1, and Robo cytoplasmic domain constructs. Regions of interest were amplified by PCR, or subcloned using native restriction sites. CC mutants and the Y-F point mutant were made using Stratagene's ExSite mutagenesis kit. CC1 deletes residues 1027–1046. CC2 deletes residues 1104–1119. CC3 deletes residues 1262–1269. Novagen's pcIt was used to make Robo plasmids for in vitro translation. All PCR constructs were sequenced. Human GST-fusion constructs were made by subcloning hRobo1 fragments (GST-hRobo1 1430–1651 and GST-hRobo1 962–1217) into pGEX-4T2. For expression in mammalian cells, the full-length hRobo1 cDNA was subcloned into pCDNA3.1 (Invitrogen). Flag-tagged mammalian expression constructs (hRobo1 cytoplasmic 930–1651, 1174–1651, and 1430–1651) were made by subcloning into pFLAG-CMV-2 (Kodak). hRobo1 cDNA mutants were made by PCR mutagenesis. All constructs were sequenced. GST fusion proteins were expressed and purified according to manufacturer's instructions. In vitro translation with S35-labeled methionine was performed using 5% desalted using ZipTip columns (Millipore), equilibrated in 5% methanol/5% formic acid. MS/MS analysis was performed on the QSTAR mass spectrometer (PE-Sciex) with a nanoelectrospray source (Protana A/S). Product ion spectra were generated by collision-induced dissociation (CID). Sequencing was performed using PeptideScan (EMBL). The following three fragments were found to be tyrosine phosphorylated: (1) QTNL METHVGYDVLNSK, (2) FWNPSSGQPTYPATTQIQSLNSNMMNGSGDSEK, and (3) QEVA PVYNHIEQNK.

Protein-Protein Interactions
Glutathione-Sepharose beads loaded with fusion proteins were equilibrated in binding buffer (20 mM HEPES-KOH [pH 7.9], 50 mM KCl, 2.5 mM MgCl2, 10% Glycerol, 1 mM DTT, 0.2% NP40, and 1 mM PMSF 1% goat serum) for 1 hr at 4°C. Equivalent GST loading was assessed by Coomassie staining of SDS-PAGE gels. For binding assays using in vitro translated proteins, 1/3 of a 50 µl reaction was added to equilibrated beads. Binding was overnight at 4°C on Nutator. Beads were washed several times in 10 mM Tris-HCI (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.2%. NP40 and separated on 12% SDS-PAGE gels. Gels were fixed for 30 min in 50% methanol, 10% glacial acetic acid, and 40% water and dried on a BioRad gel dryer and exposed to film for 2–24 hr, or for 2 hr on a BioRad phoso-imager screen. Input was normalized prior to binding, by quantitating an aliquot of each translation reaction by Phospho-imager analysis. For S2 cell binding assays, 10 µl of Robo-expressing S2 cells were pelleted and lysed in 1 ml of cold lysis buffer for 2–3 min (20 mM HEPES-KOH [pH 7.9], 50 mM KCl, 2.5 mM MgCl2, 10% Glycerol, 1 mM DTT, 1 mM PMSF, 1 mM NaVO4, and 0.5% Triton X-100), spun at 14000 rpm and transferred to a fresh tube. Ten to twenty milliliters of extract were used in each assay.

For Co-IP, extracts were prepared from 25–50 µl of wild-type embryos or embryos overexpressing one copy of UASrobo-myc or UASrobo.CC2-myc. Lysis and immunoprecipitation were performed in PBS/1% NP40 (0.8% Tween for Robo IPs), 1 mM PMSF, and 1 µg/ml each of apamin and leupeptin. The monoclonal antibodies were coupled to protein A Sepharose beads with bridging antibody for 1 hr at room temperature. Binding was performed for 60–90 min at 4°C. Samples were washed three times in lysis buffer, separated on 8% PAGE gels, transferred to membranes, and blotted with anti-Robo or anti-Myc antibodies at 1:50 or anti-Ena antibodies at 1:200. Signal was detected using the ECL detection system and autoradiography.

Rabbit antibodies were raised against GST-hRobo1 (amino acids 1430–1651) and affinity purified from crude sera with the original antigen. Cells were lysed in 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.2 mM sodium orthovanadate, and 1 mM PMSF. Full-length hRobo1 or the cytoplasmic hRobo1 proteins were immunoprecipitated from the Triton X-100 soluble fraction with 1 µg/ml anti-hRobo1 polyclonal antibody and protein A Sepharose beads or 1 µg/ml anti-Flag M2 monoclonal antibody (Sigma) and goat anti-mouse agarose beads, respectively, for 1.5 hr at 4°C. Immunoprecipitates were washed three times (20 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 0.2 mM sodium orthovanadate, and 1 mM PMSF) separated by SDS-PAGE, and blotted with Pan-phosphotyrosine monoclonal antibody (Upstate Biotechnology) at 0.5 µg/ml. Western blots were stripped in 0.1 M glycine (pH 2.5) and reprobed with anti-Flag M2 monoclonal antibody at 1.5 µg/ml.

Mammalian Cell Culture
Cos1 cells were cultured in Dulbecco’s modified Eagle medium containing 10% FBS. Cells were transfected using lipofectin reagent (Gibco BRL) overnight, after which time the media was replaced. Cells were harvested 48 hr after replacement of the media.

Phosphopeptide Mapping
GST-hRobo1 962–1217 was phosphorylated in vitro with purified v-Abl (Oncogene Research Products). Kinase reactions were performed using 3–25 units of v-Abl/µg of GST-hRobo1 fusion protein at 37°C for 30 min (in 20 mM MgCl2, 0.2 mM DTT, 60 mM HEPES [pH 7.5], 2 mM ATP, and 2 µg/ml BSA). Protein was trypsin digested at 37°C for 4 hr using a 50:1 ratio of protein to protease. Peptides were desalted using ZipTip columns (Millipore), equilibrated in 5% formic acid, washed with equilibration buffer and eluted in 60% methanol/5% formic acid. MS/MS analysis was performed on the QSTAR mass spectrometer (PE-Sciex) with a nanoelectrospray source (Protana A/S). Product ion spectra were generated by collisionally induced dissociation (CID). Sequencing was performed using PeptideScan (EMBL). The following three fragments were found to be tyrosine phosphorylated: (1) QTNL METHVGYDVLNSK, (2) FWNPSSGQPTYPATTQIQSLNSNMMNGSGDSEK, and (3) QEVA PVYNHIEQNK.

Immunohistochemistry and Antibody Production
Embryo staining and monoclonal antibody production procedures (Ena amino acids 105–370) were as previously described (Kidd et al., 1998a).

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