Neuron, Vol. 40, 113–127, September 25, 2003, Copyright ©2003 by Cell Press

Slit Stimulation Recruits Dock and Pak to the Roundabout Receptor and Increases Rac Activity to Regulate Axon Repulsion at the CNS Midline

Xueping Fan,[1] Juan Pablo Labrador,[1] Huey Hing,[2] and Greg J. Bashaw[1,*]
[1]Department of Neuroscience
University of Pennsylvania School of Medicine
421 Curie Boulevard
Philadelphia, Pennsylvania 19104
[2]Department of Cell and Structural Biology
University of Illinois at Urbana-Champaign
Urbana, Illinois 61801

Summary

Drosophila **Roundabout (Robo) is the founding member of a conserved family of repulsive axon guidance receptors that respond to secreted Slit proteins. Here we present evidence that the SH3-SH2 adaptor protein Dreadlocks (Dock), the p21-activated serine-threonine kinase (Pak), and the Rac1/Rac2/Mtl small GTPases can function during Robo repulsion. Loss-of-function and genetic interaction experiments suggest that limiting the function of Dock, Pak, or Rac partially disrupts Robo repulsion. In addition, Dock can directly bind to Robo's cytoplasmic domain, and the association of Dock and Robo is enhanced by stimulation with Slit. Furthermore, Slit stimulation can recruit a complex of Dock and Pak to the Robo receptor and trigger an increase in Rac1 activity. These results provide a direct physical link between the Robo receptor and an important cytoskeletal regulatory protein complex and suggest that Rac can function in both attractive and repulsive axon guidance.**

Introduction

During development, neuronal growth cones interpret a balance of attractive and repulsive cues to find their correct targets. Many evolutionarily conserved ligands and receptors that control axon guidance decisions have been discovered (Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001). For example, in the embryonic *Drosophila* CNS, midline glia cells secrete Netrin and Slit; Netrin attracts some axons across the midline, while Slit repels axons, preventing them from crossing more than once (Battye et al., 1999; Harris et al., 1996; Kidd et al., 1999; Mitchell et al., 1996). Slit repulsion is mediated by the Robo family of receptors (Brose et al., 1999; Kidd et al., 1998; Zallen et al., 1998). *robo* encodes an Immunoglobin (Ig) superfamily protein with five Ig domains, three Fibronectin repeats, a transmembrane domain, and a long cytoplasmic tail, which contains four conserved cytoplasmic (CC) sequences: CC0, CC1, CC2, and CC3. CC0 and CC1 are tyrosine-containing motifs that can be phosphorylated in vitro; CC2 is a proline-rich sequence (LPPPP) that matches the consensus binding site for the EVH1 domain of the *Drosophila* Enabled protein; and CC3 is a polyproline stretch.

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

Studies of Slit-mediated axon repulsion in *Drosophila* and *C. elegans*, together with studies of Slit-mediated neuronal cell migration in mammals, have implicated a number of molecules in Robo repulsive signaling. For example, the GTPase-activating proteins (GAPs) srGAP1 and srGAP2 interact with Robo's cytoplasmic domain and are required for Slit's repulsive effect on the migration of cultured precursor cells of the anterior subventricular zone (SVZa). Activation of Robo leads to the srGAP-dependent downregulation of the small GTPase Cdc42 (Wong et al., 2001). Genetic evidence in *Drosophila* suggests that downregulation of Cdc42 may also be important for regulating axon repulsion at the midline (Fritz and VanBerkum, 2002).

In *Drosophila*, the cytoplasmic tyrosine kinase Abelson (Abl) and its substrate, the actin binding protein Enabled (Ena), have been shown to play direct and opposing roles during Robo repulsion (Bashaw et al., 2000). Genetic and biochemical data are consistent with a model in which Ena functions to transduce part of Robo's repulsive signal by binding to Robo's CC2 motif, while Abl functions to antagonize Robo signaling—likely through a mechanism involving direct phosphorylation of the Robo receptor on the CC0 and CC1 motifs (Bashaw et al., 2000). In addition to its role in negatively regulating Robo function, recent evidence supports the idea that Abl can also promote repulsion downstream of multiple Robo receptors, suggesting that Abl function in the context of Robo repulsion may be more complex than previously appreciated (Wills et al., 2002). Recent studies in *C. elegans* have also demonstrated a direct role for Ena in Robo repulsion (Yu et al., 2002). Additional studies suggest a potential role for the Ras/Rho GEF Son of Sevenless (SOS) and Calmodulin in Robo repulsive signaling (Fritz and VanBerkum, 2000). Although Enabled appears to play a positive role in mediating Robo repulsion, Ena function is not sufficient to account for all of Robo's repulsive output. First, the phenotype of *ena* mutants is much weaker than that of *robo* mutants. Second, deletion of the CC2 motif in Robo's cytoplasmic domain, the site of Ena and Robo interaction, only partially impairs the function of the Robo receptor.

The SH3-SH2 adaptor protein Dock is a good candidate to play a role in Robo repulsion (Garrity et al., 1996). Dock is expressed in axons of the embryonic CNS, and mutations in Dock cause errors in embryonic axon guidance (Desai et al., 1999). Dock interacts with key regulators of the actin cytoskeleton, including members of the SCAR/WAVE family of actin regulatory proteins (Rohatgi et al., 2001), and the p21-activated protein kinase (Pak) (Hing et al., 1999), which in turn can interact with members of the Rho family of small GTPases, such as Rac1 and Cdc42 (Burbelo et al., 1995; Eby et al., 1998; Manser et al., 1994). Dock, together with its binding partner Pak, is known to play important roles during axon guidance in both the *Drosophila* visual system and in a special larval sensory structure, Bolwig's organ (Rao and Zipursky, 1998; Hing et al., 1999; Schmucker et al., 2000).

The Rho GTPases and their upstream positive and negative regulators—guanine nucleotide exchange fac-

tors (GEFs) and GTPase activating proteins (GAPs)—play important roles in the control of growth cone guidance in the developing nervous system (Dickson, 2001; Luo, 2000). Genetic disruption of various Rho family GTPases leads to defects in axon guidance. For example, mutations in *C. elegans* Rac genes or expression of dominant-negative Rac in the developing nervous system of *Drosophila* results in guidance errors at specific choice points (Kaufmann et al., 1998; Lundquist et al., 2001). More recently, loss of function mutations in the three *Drosophila* Rac genes (Rac1, Rac2, and Mtl) have been characterized and found to have defects in both axon growth and guidance as well as dendrite morphogenesis and branching, further supporting an in vivo role of the Rho GTPases in axon guidance (Hakeda-Suzuki et al., 2002; Ng et al., 2002). The opposing roles of the Rho family of GTPases during neurite outgrowth have led to the suggestion that repulsive axon guidance cues could exert their effects by causing local activation of Rho, while attractive guidance cues could work through the activation of Rac and/or Cdc42 (Dickson, 2001; Patel and Van Vactor, 2002). Studies of the Rho GTPases in signaling downstream from these receptors are generally consistent with the idea that Rho is involved in growth cone collapse and/or repulsion, although there are reported exceptions (reviewed in Luo, 2000).

Here we present genetic and biochemical evidence that Dock, Pak, and Rac1/Rac2/Mtl function during Robo-mediated midline axon repulsion. Mutations in *dock* result in axon guidance defects that are similar to, but weaker than, defects observed in *robo* mutants, and *dock* mutants display dosage-sensitive genetic interactions with *slit* and *robo*. Dose-sensitive genetic interactions between *slit, robo, dock, rac*, and *pak* provide the first reported evidence of an in vivo role for Rac in axon repulsion and argue against the simple model that Rac functions solely during attractive responses. Dock can directly bind to Robo's cytoplasmic domain, and this binding depends on the SH3 domains of Dock and the CC2 and CC3 motifs in Robo. Stimulation of the Robo receptor by Slit increases the amount of Dock bound to Robo and leads to the recruitment of the Pak serine-threonine kinase and a stimulation of Rac activity. Finally, mutations in the CC2 and CC3 motifs in Robo that prevent the in vitro association of Dock and Robo and that prevent the increase in Rac activity in response to Slit disrupt the in vivo function of the Robo receptor.

Results

Dock and Robo Share Common Mutant Phenotypes and Are Coexpressed in Developing Axons

Consistent with previously published reports (Desai et al., 1999), strong defects in embryonic axon guidance are only observed when both the maternal and zygotic components of *dock* function are removed. In these maternal minus *dock* mutants ($dock^{mat}$), phenotypes reminiscent of loss of *robo* function can often be seen (Figure 1). $dock^{mat}$ embryos examined with an antibody that labels all axons frequently show thickening of commissural axon bundles and a commensurate reduction in the thickness of longitudinal axon bundles (Figure 1C). Staining these embryos with an antibody that selectively labels noncrossing axons (anti-fasII) reveals a significant degree of ectopic midline crossing (Figure 1F; Table 1). These phenotypes are similar to, but considerably less severe than, those observed in *robo* mutants (Figure 1E). The similarity in mutant phenotypes that is observed provides genetic support for the idea that *dock* could contribute to Robo repulsion.

If *dock* and *robo* function together during midline guidance, they should be coexpressed in embryonic axons. This is indeed the case (Figures 1G–1I). Double labeling of embryos with antibodies raised against Dock and Robo reveals substantial coexpression of the two proteins (Figure 1I). Both Dock and Robo show enriched expression on CNS axons beginning as early as stage 12, corresponding to the time of initial axon outgrowth. At these early stages of axon growth, Dock is detected in the pCC axon, a cell known to express Robo, as revealed by double labeling with FasII (Figures 1J–1L). Interestingly, while Robo shows a regionally restricted expression pattern with high levels of expression on longitudinal portions of axons and low levels in commissural axons, Dock is expressed equivalently in both commissural and longitudinal axon segments (Figures 1G and 1H). This observation raises the possibility that Dock could have additional roles in the guidance of commissural axons not shared by Robo. These observations show that Dock and Robo are both present at the right time and place to function together during midline repulsion.

Genetic Interactions between *slit, robo, dock*, and *pak*

We next tested whether there are dosage-sensitive genetic interactions between *slit* and *robo* and *dock*. Such genetic interactions are often indicative of direct physical association between two proteins that function in a common process. When we examined embryos obtained from crossing flies that carry one copy each of *slit* and *robo* with flies that carry one copy of *dock*, a moderate enhancement of the mild *slit, robo*/+ transheterozygous phenotype is observed (Table 1). Further limiting *dock* function by performing a similar cross using female flies that lack *dock* maternal contribution reveals an even more striking enhancement of the midline crossing defects (Figures 2B and 2C; Table 1). Importantly, these embryos have one wild-type copy of *dock* from their fathers and no defects are observed in embryos lacking maternal *dock* that have two wild-type copies of *slit* and *robo* (Figure 2A; Table 1). In contrast, overexpression of *dock* in the *slit, robo* transheterozygous background did not significantly alter the midline crossing phenotype (Figure 2I; Table 1). In an independent test for dose-sensitive genetic interactions, we found that removing one copy of *dock* also enhanced the defects associated with panneural expression of a truncated dominant-negative Robo receptor (Table 1).

To provide support for the significance of the genetic interactions between *robo* and *dock*, as well as to give additional evidence for the similarity of the $dock^{mat}$ and *robo* mutant phenotypes, we examined the early projection patterns of individually defined FasII-positive neurons. Together, axons of the identified pCC, MP1, and

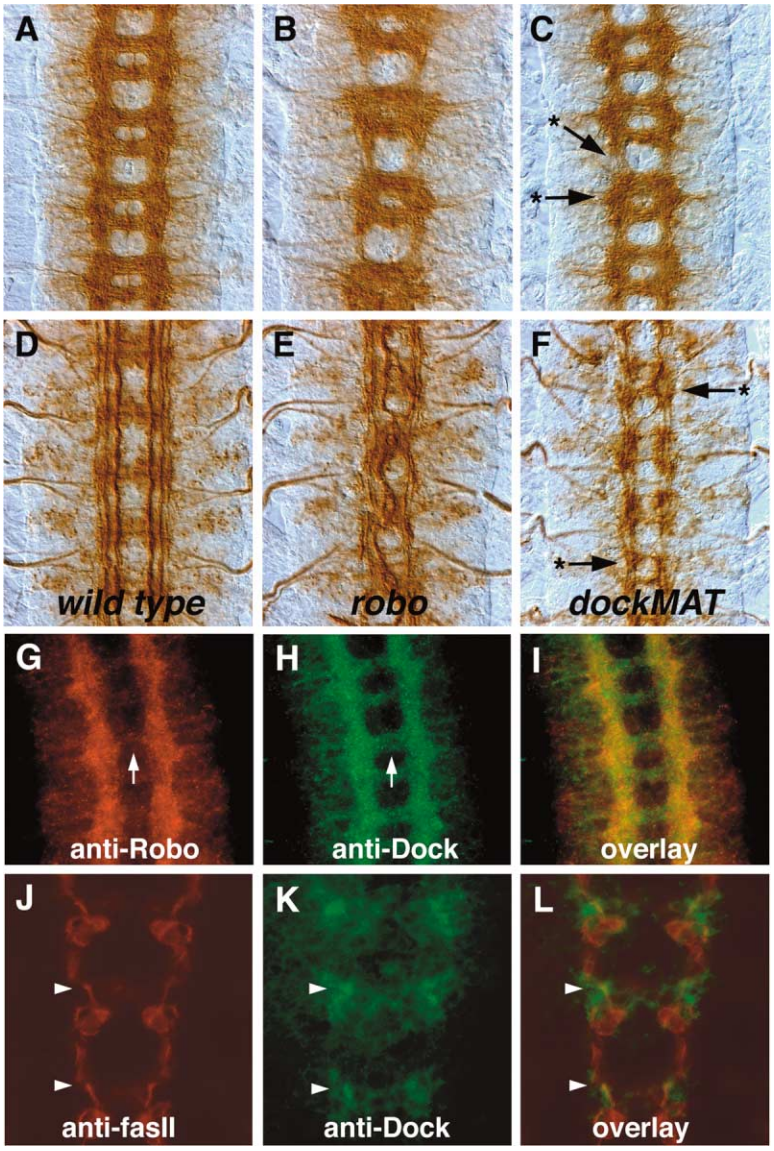

Figure 1. A Comparison of *robo* and *dock* Mutant Phenotypes and Expression Patterns

(A–F) Stage 16 filleted embryos stained for all CNS axons, anti-BP102 (A–C), and a subset of noncrossing axons, anti-FasII (D–F). Anterior is up.

(A and D) Wild-type embryos.

(B and E) *robo* mutant embryos. Note the thickening of commissural axon bundles and the reduction of the longitudinal bundles in the BP102 stained embryo (B) and the ectopic midline crossing of the innermost FasII bundle of axons in (E).

(C and F) $dock^{mat}$ mutant embryos (embryos that lack both maternal and zygotic *dock* function) show similar phenotypes to *robo*. In the BP102 stained animal (C), commissures appear to be generally thicker, while the longitudinals are reduced. Arrows with asterisks label segments that exhibit strong examples of these phenotypes. In the FasII stained animal (F), many axons inappropriately cross the midline—arrows with asterisks.

(G–I) Wild-type stage 16 embryos costained with anti-Robo and anti-Dock.

(G) Robo staining. Robo accumulates at high levels in longitudinal portions of CNS axons, while it is excluded form the commissures. The white arrow points to one of the axon commissures, where Robo is not detected.

(H) Dock staining. Dock is expressed in most CNS axons including the commissural portions of axons (white arrow).

(I) An overlay of the two staining patterns reveals substantial overlap in expression.

(J–L) Wild-type stage 13 embryos stained with anti-FasII (red) and anti-Dock (green).

(J) FasII expression is detected in the pCC axons (arrowheads).

(K) Anti-dock staining of the same embryo in (J). Arrowheads indicate the position of the pCC axons.

(L) An overlay of FasII and Dock shows that Dock is expressed in pCC.

dMP2 neurons pioneer the ipsilateral MP1 pathway. One of the hallmarks of the *robo* mutant phenotype is the completely penetrant abnormal midline crossing of these axons. Though infrequent, ectopic crossing of these axons is sometimes observed in embryos that completely lack *dock* function ($dock^{mat}$) (Figure 2D; Table 1). This finding is consistent with the idea that *dock* mutants have similar, but weaker, phenotypes to *robo*. Pioneer axon crossing defects are considerably less frequent in animals that are heterozygous for *slit* and *robo*, in comparison to the ectopic crossing observed in later stage animals of the same genotype (Table 1). Importantly, limiting *dock* function in embryos heterozygous for *slit* and *robo* strongly enhances these early guidance defects (Figures 2E and 2F; Table 1). Taken together, these results suggest that *dock* and *robo* function together during midline repulsion.

Based on the observation that removing one copy of the *enabled* gene strongly enhances the defects observed in *slit*, *robo* transheterozygotes, we performed a pilot enhancer screen of more than a hundred chromosomal deletions and a smaller number of candidate genes, including dominant-negative versions of Rho, Rac, and Cdc42 (see legend to Table 1 for details). From this screen, we identified a small number of chromosomal deletions that enhance the mild *slit, robo*/+ phenotype, including a deletion that removes the gene encoding the Pak serine-threonine kinase (Table 1). Since the deletion that removes *pak* removes other genes as well, we next tested whether *pak* mutant alleles also act as enhancers and observed a modest enhancement of the *slit, robo*/+ phenotype in animals where one copy of *pak* is also removed (Figures 2G and 2I; Table 1), suggesting that *pak*, like its binding partner *dock*, could play a role in *robo* repulsion. Interestingly, the pak^4 allele, which specifically mutates the dock binding domain in pak, was found to significantly enhance the *slit, robo* heterozygous phenotype (Table 1).

pak is expressed in embryonic CNS axons (Harden et al., 1996). In addition, *pak* mRNA is expressed ubiqui-

Table 1. Genetic Interactions between *dock, pak, robo*, and *slit*

Partial Genotype[a]	Segments Scored[b]	+[c]	++[d]	+++[e]	Defects/ Animal	% Defects[f]	% Embryos w/ Early Defects in pCC/MP1[g]
dock ^{MAT}/+	110	1	0	0	0.1	0	0 (0/17)
dock ^{MAT}*/dock*	110	36	34	7	7.7	70	18 (3/17)
slit^{1},*robo*^{5}/+	242	29	29	1	2.7	24	27 (4/15)
dock ^{MAT},+,+/+, *slit*^{1},*robo*^{5}	209	46	78	17	7.4	67	72 (13/18)
ena^{GC1}/*ena*^{GC5}	154	16	12	0	2	18	0 (0/20)
dock^{3}*ena*^{GC1}/*dock*^{3},*ena*^{GC5}	231	43	34	2	3.8	34	26 (8/31)
slit^{1},*robo*^{5}/+;*UASDock*	209	24	25	7	2.9	27	ND
slit^{1},*robo*^{5},+/+, +, *dock*^{3}	154	29	47	4	5.7	52	ND
slit^{1},*robo*^{5},+/+, +, *fra*^{3}	143	9	9	0	1.4	13	ND
UASRoboDN	110	14	8	0	2.2	20	ND
dock^{P1}/+ ;*UASRoboDN*	132	25	39	5	5.8	52	ND
slit^{1},*robo*^{5}/+;*DfPak*/+	132	27	37	6	5.8	53	ND
slit^{1},*robo*^{5}/+;*pak*^{4}/+	121	26	34	7	6.1	54	ND
slit^{1},*robo*^{5}/+;*pak*^{11}/+	198	37	62	6	5.8	53	ND
slit^{1},*robo*^{5}/+;*UASPak*	242	47	72	15	6.1	55	ND
slit^{1},*robo*^{5}/+;*UASPak*^{Myr}	132	20	96	56	14.3	130	ND

Stage 16–17 embryos stained with anti-FasII MAb were scored.

A deficiency for *pak* was uncovered in a screen of 103 nonoverlapping autosomal deletion chromosomes for enhancers of the *slit, robo* transheterozygous phenotype. Not all available intervals were examined. In total this screen revealed only 6/103 enhancing deficiencies. Of these 6, one was a deficiency for *robo1* and a second was a deficiency that removes *robo2 and robo3*. A deficiency that removes the *dscam* locus did not act as an enhancer.

[a] Unless otherwise indicated, animals carrying UAS transgenes also carry one copy of *elavGAL4*.

[b] Eight abdominal and three thoracic segments were scored in each animal for a total of eleven segments.

[c] Thinner than normal fascicle ectopically crossing the midline.

[d] Normal fascicle ectopically crossing the midline.

[e] Thicker than normal fascicle ectopically crossing the midline.

[f] Percent defects is defined as total number of defects divided by segments scored. Since embryos can show multiple defects per segment, it is possible to have defects exceeding 100%.

[g] % Stage 13 embryos with pioneer defects in two or more segments. The numbers in parentheses represent the number of embryos showing defects divided by the total number of embryos examined. ND, not determined.

tously in early embryos, suggesting that it is provided maternally (Harden et al., 1996). Zygotic loss-of-function mutations in *pak* do not result in obvious embryonic guidance phenotypes when examined with antibodies that label all axons, or antibodies that label the FasII subset of noncrossing axons (data not shown). Unfortunately, the strategy used for removing maternal *dock* function (a mosaic rescue of zygotic *dock* lethality by CNS expression of *UASdock*) did not permit us to examine the consequences of removing maternal *pak*. The absence of a clear *pak* loss-of-function phenotype in the CNS weakens somewhat the argument, based on the observed genetic interactions, that endogenous *pak* plays a vital role in *robo* repulsion. In this context it is worth noting that there is a second uncharacterized *pak*-like kinase in *Drosophila* that could also contribute to axon guidance.

slit, robo, and the Rho GTPases

We used dominant-negative (DN) UAS transgenes of Rho, Rac, and Cdc42 to determine if panneuronal expression of any of the DNs in the *slit, robo*/+ transheterozygous background would modify the ectopic midline-crossing phenotype. Panneuronal expression of the three different DNs in an otherwise wild-type background did not result in significant ectopic midline crossing (Table 2), although as previously reported, expression of *UASRacN17* did result in significant levels of motor axon path finding defects, including ISNb bypass (Kaufmann et al., 1998). In addition, panneural expression of *UASRhoN19* resulted in a somewhat surprising defect in embryonic head involution (data not shown), a phenotype observed in zygotic loss of function *rho* mutants (Magie et al., 1999). We used these secondary phenotypes associated with the Rho and Rac DNs as an independent confirmation of their expression.

Only the Rac1 DN showed strong enhancement of the *slit, robo*/+ defects when assayed in this way (data not shown). We next tested the same three DN constructs in animals where only *slit* is heterozygous. This is a more stringent genetic interaction test, since heterozygosity for *slit* rarely causes any defects in midline crossing (Figures 3A–3C; Table 2). Again, only *UASRacN17* resulted in striking levels of ectopic midline crossing (Figure 3B). *UASRhoN19* had a modest but significant enhancing effect, while *UASCdc42N17* (Figures 3A and 3C) had little if any effect (Table 2). *UASRacN17* was also able to enhance the mild DN defects associated with the expression of truncated Robo receptors (data not shown).

The following two additional approaches were taken to compare the relative effects of blocking *rac* and *rho*. (1) The effects of expressing the Rac and Rho DNs in smaller subsets of neurons (Fushi-tarazu [Ftz] and Apterous [Ap] neurons) in embryos heterozygous for *slit* were examined, and (2) the effects of loss of function mutations in the *rac* and *rho* genes in embryos heterozygous for *slit* or *robo* were examined. In contrast to the results using panneural expression, the Rho DN showed strong enhancement of the midline defects of *slit* hetero-

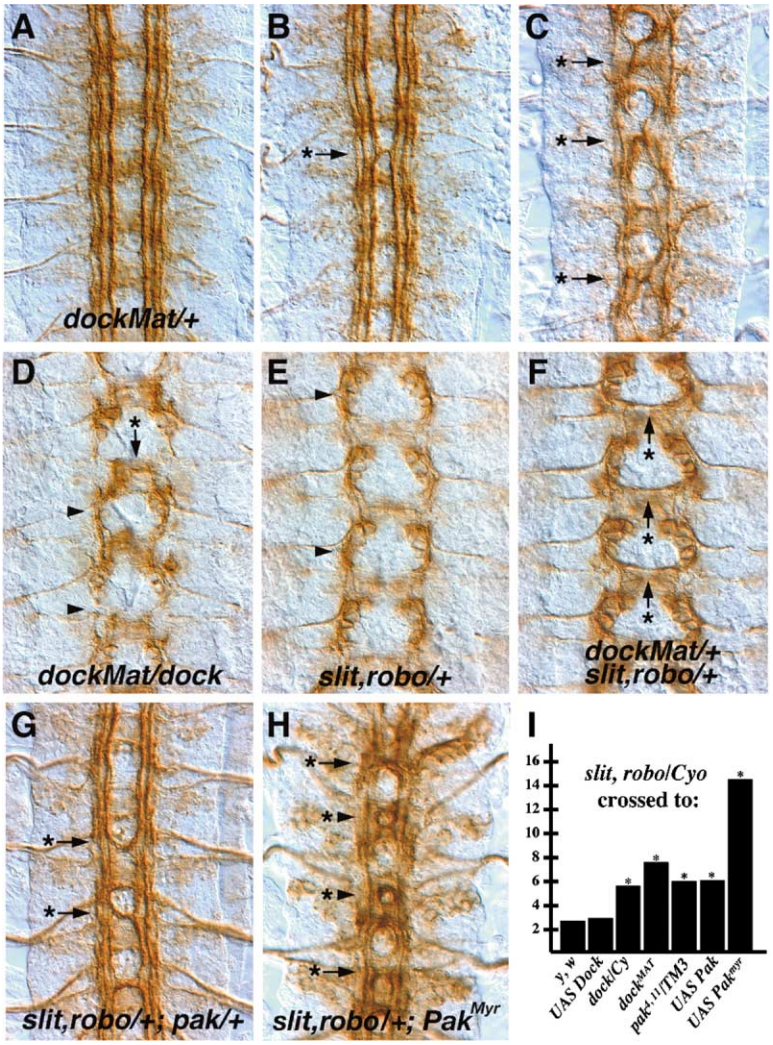

Figure 2. Genetic Interactions between *slit, robo*, *dock*, and *pak*

Stage 16 (A–C and G–I) or stage 13 (D–F) embryos stained with anti-FasII to reveal a subset of longitudinal axons. Anterior is up. Partial genotypes are indicated below each panel.

(A) A *dock*^{mat}/+ embryo (an embryo missing maternal *dock*, carrying one wild-type copy of zygotic *dock*) The FasII axons appear wild-type.

(B) A *slit, robo*/+ transheterozygous embryo with a mild midline crossing defect. An arrow with asterisk indicates a single bundle of axons that crosses the midline.

(C) A *dock*^{mat}/+// *slit, robo*/+ transheterozygous embryo exhibiting strong midline crossing defects reminiscent of *robo* homozygous mutants (arrows with asterisks). Compare with Figure 1E.

(D–F) Stage 13 embryos stained with Fas II to reveal the pCC, MP1, and dMP2 pioneer neurons.

(D) *dock*^{mat} mutant embryos occasionally show pioneer axon guidance defects (arrow with asterisk), but most segments appear normal (arrowheads).

(E) *slit, robo*/+ embryos show mostly normal pCC/MP pioneer projections (arrowheads).

(F) *dock*^{mat}/+// *slit, robo*/+ transheterozygous embryos frequently show strong defects in pioneer axon guidance, with pCC and dMP2 axons frequently crossing the midline (arrows with asterisks).

(G) *slit, robo*/+; *pak*/+ embryos show a mild enhancement of the midline crossing phenotype.

(H) *slit, robo*/+ embryos expressing one copy of *UASPak*^{Myr} exhibit striking defects. In addition to individual bundles of axons abnormally crossing the midline (arrows with asterisks), many segments have multiple axon bundles crossing the midline and forming circles reminiscent of *robo* mutants (arrowheads with asterisks).

(I) A quantitative analysis of selected genetic interactions between *slit, robo, dock*, and *pak*. Genotypes are indicated on the *x* axis. Average number of defects per embryo are shown on the *y* axis. Asterisk denotes phenotypes that are statistically different from the *slit, robo*/+control with a p value of less than 0.001 in a one-tailed t test.

zygotes in both the Ap neurons and the Ftz neurons (Figure 3E; Table 2; data not shown). The Ftz effects of the Rho DN on midline guidance are consistent with recently published findings (Fritz and VanBerkum, 2002). Although we do not observe striking defects in *robo*/+, *rho*/*rho* embryos that have half the normal levels of *robo* and are completely missing zygotic *rho* function (Figure 3G; Table 2), we believe that the small but significant effect of the panneural Rho DN expression in *slit* heterozygotes, and the more striking defects observed in the Ftz and Ap neurons in *slit* heterozygotes, support a role for Rho in Robo's repulsive output (see Discussion).

In light of the strong genetic interactions observed between *slit, robo*, and the Rac1 DN construct, it is somewhat surprising that the Rac1 DN does not result in a strong midline phenotype on its own. Recent characterization of the mutant phenotypes of all three Rac-related genes in *Drosophila* helps shed some light on this issue (Hakeda-Suzuki et al., 2002; Ng et al., 2002). Individual mutants of the *rac* genes do not result in obvious defects in midline guidance; however, double and triple mutant combinations do result in significant midline crossing defects that are qualitatively similar to *robo* mutants (Hakeda-Suzuki et al., 2002). This is particularly striking in the case of *rac1, mtl* double mutants, where many animals display characteristics of the *robo* mutant phenotype (Figure 3D and previously published data). Thus, loss-of-function mutations in the *rac* genes support our observations using the Rac1 DN construct.

Dominant-negative transgenes for the Rho GTPases are thought to act by sequestering endogenous GEF proteins. Since different Rho family members can share common GEFs, it is not clear which of the three Rac GTPases is most relevant to the observed genetic interaction with *slit*, or alternatively whether the observed interactions could reflect simultaneous downregulation of multiple Rac genes. (Based on the differences observed in the effects of the Rho, Rac, and Cdc42 dominant-negatives and the loss-of-function phenotypes of double *rac1, mtl* mutants, we believe it is unlikely that the Rac1 DN transgene could function to block the other

Table 2. Genetic Interactions between *slit, robo*, and the Rho GTPases

Partial Genotype	Segments Scored	+	++	+++	Defects/ Animal	% Defects	T Test p Value[a]
slit/+	154	6	0	0	0.4	4	reference
URhoN19	88	2	0	0	0.3	2	ND
URacN17	110	5	1	0	0.6	5	ND
UCdc42N17	88	0	0	0	0	0	ND
slit/+;URhoN19	187	17	13	4	2	18	8.27E-05
slit/+;URacN17	253	29	74	88	8.3	75	1.68E-25
*slit/+;*UCdc42N17	110	1	1	0	0.2	2	NS
slit/+;UPak^{Myr}	165	25	58	0	5.6	51	3.18E-10
slit/+;UPak	110	6	6	0	1.2	11	NS* 0.062
slit/+;UTau^{GFP};URacN17	154	48	38	9	6.8	62	NS* (0.022)
slit/+;UPak^{Myr};URacN17	198	19	25	46	5	45	(2.55E-07)
slit/+;URhoN19 (ftz)[b]	121	27	41	13	7.4	67	5.83E-05
slit/+;URacN17 (ftz)	55	11	9	0	4	36	0.004
robo, rho/+, rho/	132	6	1	0	0.6	5	ND
slit/+;rac1^{J11}	242	39	59	7	4.8	44	9.55E-09
slit/+;rac2$^{\Delta}$	121	5	11	0	1.4	13	ND
slit/+;mtl$^{\Delta}$	176	9	13	0	1.4	13	ND

Column designations are the same as for Table 1.

[a] A one-tailed t test was used to calculate the statistical significance of the % defects of selected genotypes relative to the *slit/+* control. P values are indicated. The two genotypes where a p value is given in parentheses are comparisons between *slit/+; UASRacN17* and genotypes where additional transgenes are also present. *U=UAS*. ND, not determined; NS, not significant. NS* indicates comparisons that fell below the 0.02 significance threshold but that were near this value. In these cases p values are indicated.

[b] Some of these embryos showed such strong midline crossing phenotypes that they were not quantifiable using this method.

Rho family members.) To address the relative contributions of the three Rac genes, we performed genetic interaction tests with each of the individual Rac gene mutants. We generated embryos heterozygous for *slit* and homozygous for *rac1, rac2*, or *mtl* and examined the axonal phenotypes. In this assay, only the *rac1* mutant had a significant effect on midline crossing (Figure 3H; Table 2). Interestingly, in our in vitro experiments, Slit stimulation had the most marked effect on Rac1 activity and only a small influence on Rac2 and Mtl (see below). The fact that the enhancing effect of the *rac1* mutant is considerably milder than that of the Rac1DN transgene leads us to conclude that either the *rac1* mutant does not limit *rac1* function as strongly as the dominant-negative does (perhaps due to maternal contribution), or the functions of multiple Rac genes need to be limited in order to observe strong enhancement.

Overexpression of Pak Modulates Robo Repulsion

To determine whether panneural overexpression of *pak* could suppress the *slit, robo/+* phenotype, we made use of two kinds of *pak* transgenes, either wild-type *UASPak* or a modified version of Pak that is constitutively targeted to the membrane with a myristilation tag, *UASPak^{Myr}*. Surprisingly, we found that expression of multiple independent inserts of *UASPak* and *UASPak^{Myr}* led to a striking enhancement of the ectopic midline crossing phenotype of *slit, robo* heterozygotes, rather than the predicted suppression (Figures 2H and 2I; Table 1). The enhancement observed with *UASPak* was quite similar to that seen with *pak* loss-of-function alleles, while the effects of *UASPak^{Myr}* were considerably stronger, resulting in phenotypes similar to the complete loss of *robo* function (Figure 2H). *UASPak^{Myr}* expression also enhanced midline-crossing defects in animals heterozygous for *slit* (Table 2). Taken together, the similar qualitative effects of *pak* loss and gain of function suggest that both too much and too little *pak* activity is disruptive for *robo* repulsion.

The Pak serine-threonine kinase has been shown to be an important effector of Rac in many systems (Burbelo et al., 1995; Eby et al., 1998; Manser et al., 1994). To further address the question of how alterations in *pak* expression/function can modulate Robo repulsion, we tested whether expression of *UASPak^{Myr}* could alter the ectopic midline crossing defects associated with limiting Rac activity in embryos heterozygous for *slit*. If the defects in midline repulsion observed in *slit/+; Rac1 DN* embryos are due in part to reduced signaling through *pak*, we would predict that by adding more *pak*, the *slit* and *Rac1 DN* interaction could be suppressed. If, on the other hand, increasing *pak* expression results in a perturbation of *robo* repulsion independent of the effects of *rac*, we would predict that expressing *UASPak^{myr}* and the *Rac1DN* together would result in even greater disruptions than those observed when *UASPak^{myr}* and the *Rac1DN* are expressed independently. Examination of embryos heterozygous for *slit* and expressing both the Rac1 DN and the *UASPak^{Myr}* transgenes reveals a partial suppression of the Rac1-dependent defects (Figures 4A–4E; Table 2). Since overexpression of a control UAS construct in combination with the Rac1 DN caused only a slight alleviation of the phenotype, we believe it is unlikely that the suppression observed with *UASPak^{Myr}* can be explained solely by titration effects on GAL4 (Figure 4E; Table 2). This result supports the idea that at least part of *pak* function is required downstream of *rac* and provides genetic evidence that *pak* function during *robo* repulsion is cell autonomous.

In addition to the rescuing effect of *UASPak^{Myr}* in the context of the *slit,* Rac1 DN genetic interaction, we also observe a reciprocal effect of *UASPak^{Myr}* (but not *UASPak*) in the context of *robo* gain of function. Low-level

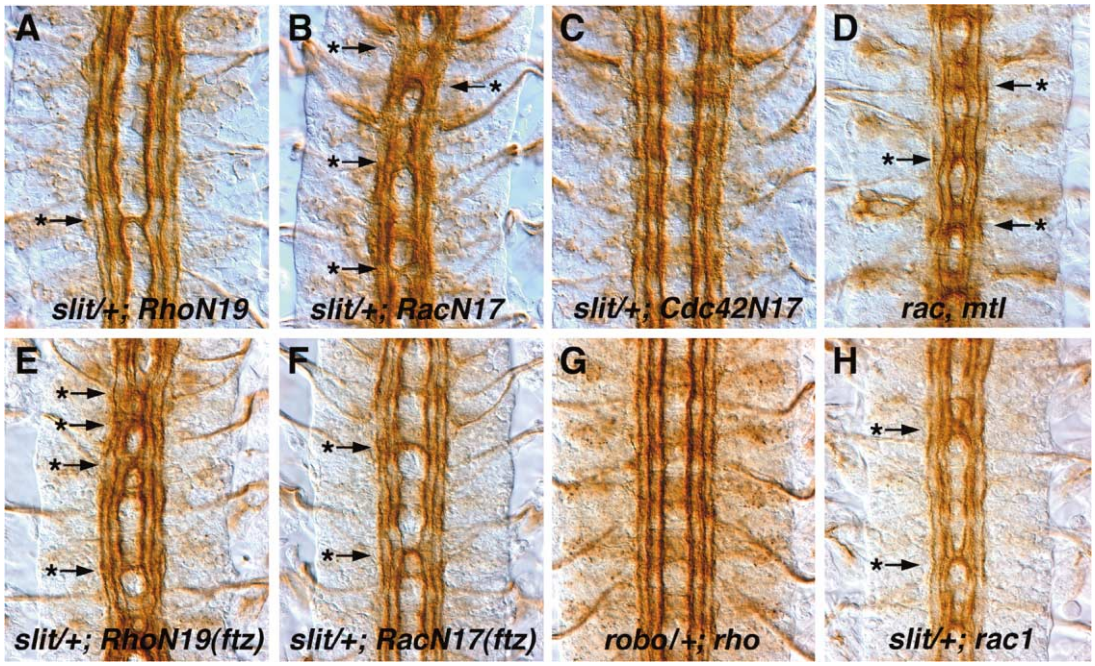

Figure 3. Genetic Interactions between *slit, robo*, and the Rho GTPases

Stage 16–17 embryos were stained with anti-FasII. Anterior is up.

(A) A *slit/+; UASRhoN19/ElavGal4* embryo with a single bundle of axons crossing the midline (arrow with asterisk).

(B) A *slit/+; UASRacN17/ElavGal4* embryo shows strong defects in midline repulsion reminiscent of *robo* mutants. Some of the defects are indicated by arrows with asterisks.

(C) Embryos of the genotype *slit/+; UASCdc42/ElavGal4* do not show any defects in midline repulsion.

(D) A *rac1mtl* double mutant exhibits defects indicative of reduced repulsion (arrows with asterisks).

(E) A *slit/+; UASRhoN19/ftz^{ng}Gal4* embryo shows striking defects in midline guidance with many bundles of axons inappropriately crossing the midline. The effects are much stronger than those observed in (A).

(F) A *slit/+; UASRacN17/ftz^{ng}Gal4* shows a mild enhancement in ectopic midline crossing, considerably weaker than the effects seen with *UASRhoN19*.

(G) No ectopic crossing defects are observed in *robo/+; rho/rho* mutant embryos.

(H) The mild defects observed in embryos of the genotype *slit/+; rac1/rac1* are indicated by arrows with asterisks.

panneural expression of the Frazzled-Robo (FraRobo) chimeric receptor (Bashaw and Goodman, 1999) does not result in an obvious increase in midline repulsion (Figure 4F). Similarly, panneural overexpression of *UAS-Pak^{Myr}* (single copy) does not result in significant defects in commissural axon thickness (Figure 4G). However, simultaneous overexpression of both *UASPak^{Myr}* and *UASFraRobo* leads to a synergistic effect, in which striking defects indicative of enhanced repulsion are observed—axon commissures are greatly reduced or even absent (Figure 4H). This interaction suggests that the presence of *UASPak^{Myr}* is enhancing the output of the Fra-Robo chimera and supports the idea that *pak* function can modulate Slit-Robo repulsion.

Biochemical Interactions between Dock and Robo

As a first step to test for physical interactions between Dock and Robo, we used the yeast two-hybrid system and found that the cytoplasmic domain of Robo shows robust interactions with the full-length Dock protein (Figure 5A). Further yeast two-hybrid analysis pointed to the importance of the region containing the proline-rich motifs CC2 and CC3 for the observed interaction (Figure 5A and data not shown). Mapping the regions of Dock involved in the two-hybrid interaction revealed that the SH3 domains of Dock, especially SH3-1 and SH3-2, are required to mediate Robo binding (Figure 5B). To test whether the binding observed in the yeast two-hybrid assay could be observed in intact cells, we coexpressed Robo and Dock in 293 cells and performed coimmunoprecipitation experiments. We observed binding of Robo and Dock that was dependent on the presence of the CC2 and CC3 motifs; removal of CC2 or CC3 alone resulted in a strong reduction in binding, while removing both motifs completely blocked the interaction (Figure 5C). To determine whether Robo and Dock interact in the organism, we attempted coimmunoprecipitation experiments using embryos expressing various myc-tagged versions of the Robo receptor and were able to coimmunoprecipitate wild-type Robo and Dock (Figure 5D). Furthermore, a mutant form of Robo where *both* CC2 and CC3 were deleted showed a dramatic reduction in Dock binding (Figure 5D). Taken together, the two-hybrid and coimmunoprecipitation data support a direct in vivo interaction between Robo and Dock.

Slit Stimulation Can Recruit Both Dock and Pak to the Robo Receptor

To test whether Slit stimulation of Robo regulates the association of Dock, cultured 293 cells transfected with

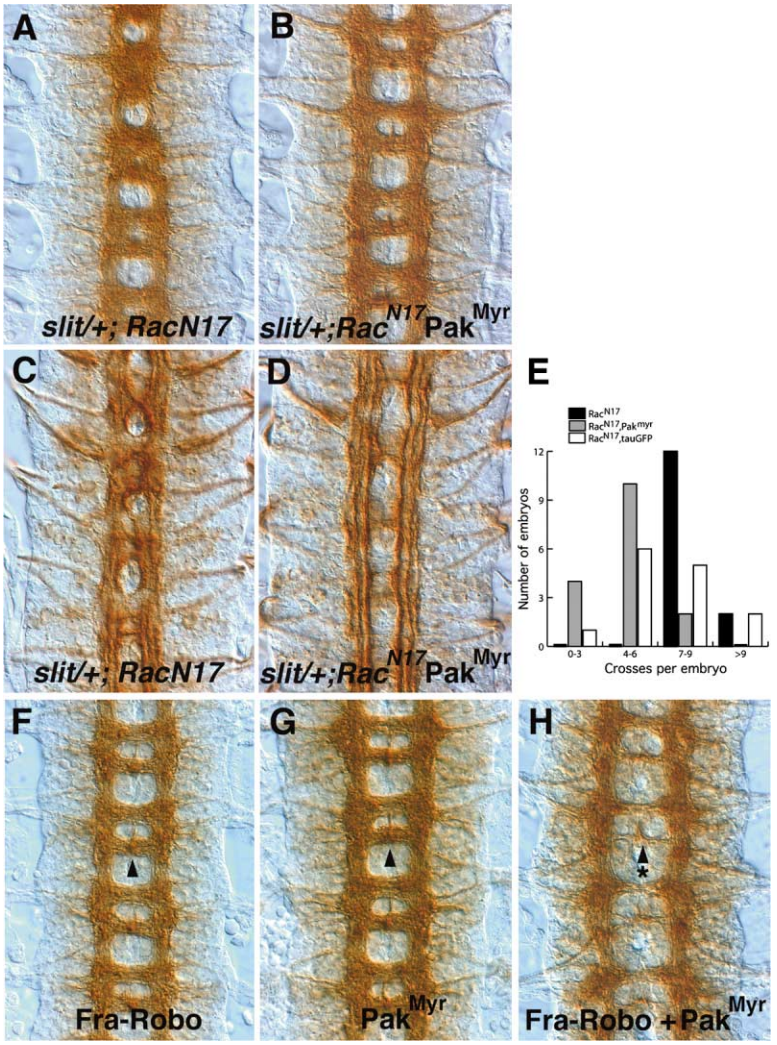

Figure 4. Overexpression of Pak Modulates Robo Repulsion

Stage 16 filleted embryos of the indicated genotype stained with antibodies that label all CNS axons, anti-BP102 (A, B, and F–H) and a subset of noncrossing axons, anti-FasII (C and D). Anterior is up in all panels.

(A–D) *slit/+*; *RacN17* embryos reveal strong defects in midline repulsion when examined with either anti-BP102 (A) or anti-FasII (C). These defects are partially suppressed by the expression of a membrane tethered Pak transgene, *PakMyr* (B and D).

(E) A graphic representation of the quantitative suppression of the *slit, RacN17* interaction by *PakMyr*. The *x* axis represents the severity of the crossing phenotype observed with the Fas II Mab, and the *y* axis represents the number of embryos observed for the indicated phenotypic class. The presence of *UASPakMyr* results in a leftward shift in the distribution of mutant phenotypes.

(F–G) Embryos expressing either low levels of the *UASFra-Robo* chimeric receptor (F) or the *UASPakMyr* transgene (G) in all neurons do not show significant reduction in commissural axon thickness (arrowheads). Embryos that coexpress both *UAS Fra-Robo* and *UAS PakMyr* (H) show striking reduction in commissural axon thickness (arrowhead with asterisks).

dock and *robo* cDNAs were treated with supernatants from cells transfected with *Drosophila* Slit or with supernatants from mock transfected cells. Under these conditions, Dock and Robo association is strongly stimulated by the addition of Slit, suggesting that Dock is recruited to Robo upon ligand binding (Figure 6B). Since previously published data has shown that Dock can bind directly to Pak (Hing et al., 1999), we tested whether a multiprotein complex consisting of Robo, Dock, and Pak could be detected, and if so, whether its assembly was regulated by Slit. We used a yeast two-hybrid approach to confirm known interactions between Dock and Pak and between Pak and Rac1 and to see whether we could detect direct interactions between Robo and Pak and/or Rac1 (Figure 6A). In agreement with published reports, robust interactions were observed between Dock and Pak and between Pak and Rac1 (Figure 6A). However, no direct interaction was observed between Robo and Pak or Robo and Rac (Figure 6A). This of course is exactly what the expectation would be if Dock were functioning to link Pak to Robo.

To determine whether we could detect a Dock-dependent interaction between Pak and Robo and whether such an interaction is regulated by Slit, cells were either transfected with Pak and Robo or with Dock, Pak, and Robo and then subsequently treated with or without Slit and processed for coimmunoprecipitation assays. Only in cells expressing all three proteins could an interaction between Pak and Robo be detected (Figure 6B). Similar to the interaction between Robo and Dock, the recruitment of Pak to Robo is Slit dependent. These biochemical data support the idea that Slit engagement of the Robo receptor leads to the recruitment of both Pak and Dock and potentially Rac1 as well into a multiprotein complex with Robo's cytoplasmic domain. Genetic data argue that these protein-protein interactions are important for Robo repulsion in vivo.

Slit Stimulation of Robo Upregulates Rac Activity

We next addressed the question of whether Slit stimulation would affect the activity of any of the *Drosophila* Rac genes, Rac1, Rac2, and Mtl, and whether any effect on Rac activity was dependent on the formation of the Robo, Dock, and Pak complex. Such a stimulation of Rac1 activity by Slit activation of Robo has previously been observed using vertebrate proteins (Wong et al., 2001), but the biological significance of the Rac1 activity increase in this context remains unclear (see Discus-

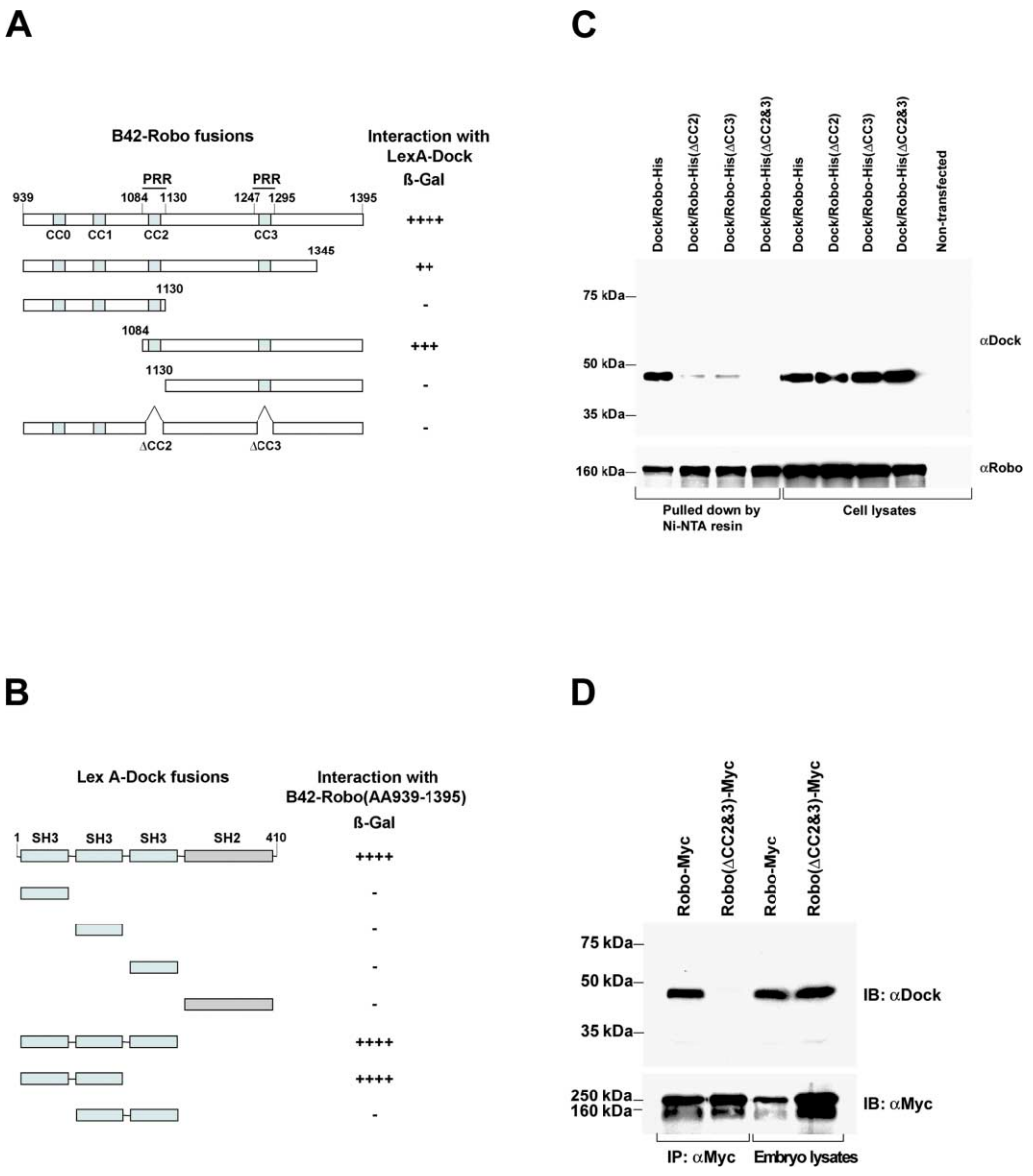

Figure 5. Physical Interactions between Robo and Dock

(A) The intracellular domain of Robo (aa 939–1395) and its different sized forms were fused to the B42 transcription activation domain. Full-length Dock was fused to the LexA DNA binding domain. Our analysis indicates that the C-terminal half of Robo's cytoplasmic domain, which includes the conserved proline-rich regions, is important for the interaction. Deletions of CC2 and CC3 in Robo disrupt the interaction.

(B) The same strategy was used to identify the potential interacting domain in Dock for Robo. LexA-fused truncated forms of Dock were tested with B42-fused Robo intracellular domain. Strong binding was mediated by a combination of the SH3-1 and SH3-2 domains. ++++, yeast turned dark blue (strong interaction); +++, blue; ++, light blue; -, white (no interaction) in 24 hr in the presence of 80 μg/ml X-Gal. Abbreviations: PRR, proline-rich region; CC, conserved cytoplasmic region; SH3, Src-homology 3 domain; SH2, Src-homology 2 domain. Numbers indicate positions of the amino acids in the full-length protein.

(C) Deletion of CC2 and CC3 in Robo blocks its interaction with Dock. 293T cells coexpressing both Dock and Robo-myc, RoboΔCC2-myc, RoboΔCC3-myc, or RoboΔCC2&ΔCC3-myc were treated with Slit for 10 min before performing precipitation. The left four lanes show the coprecipitated proteins while the right five lanes indicate Robo and Dock expressions in cells.

(D) In vivo Co-IP of Robo and Dock. Embryos overexpressing Robo-myc or RoboΔCC2ΔCC3-myc were lysed. Soluble extracts were immunoprecipitated with an anti-myc antibody. The precipitated Robo-myc and Dock were detected using anti-myc and anti-Dock antibodies respectively (left two lanes). Robo and Dock were also detected in the total lysates to control for protein levels in the two different extracts (right two lanes).

sion). Our genetic data in *Drosophila* provide a powerful argument for the potential importance of increasing Rac activity in response to Robo activation, since blocking or reducing Rac gene function partially disrupts Robo repulsion. To test whether we could detect a similar stimulation of Rac in the *Drosophila* system, we took advantage of a GST fusion protein of the Rac binding domain in Pak to specifically pull down active GTP bound Rac1, Rac2, or Mtl from 293T cells that had been transfected with Robo and Rac and treated with and

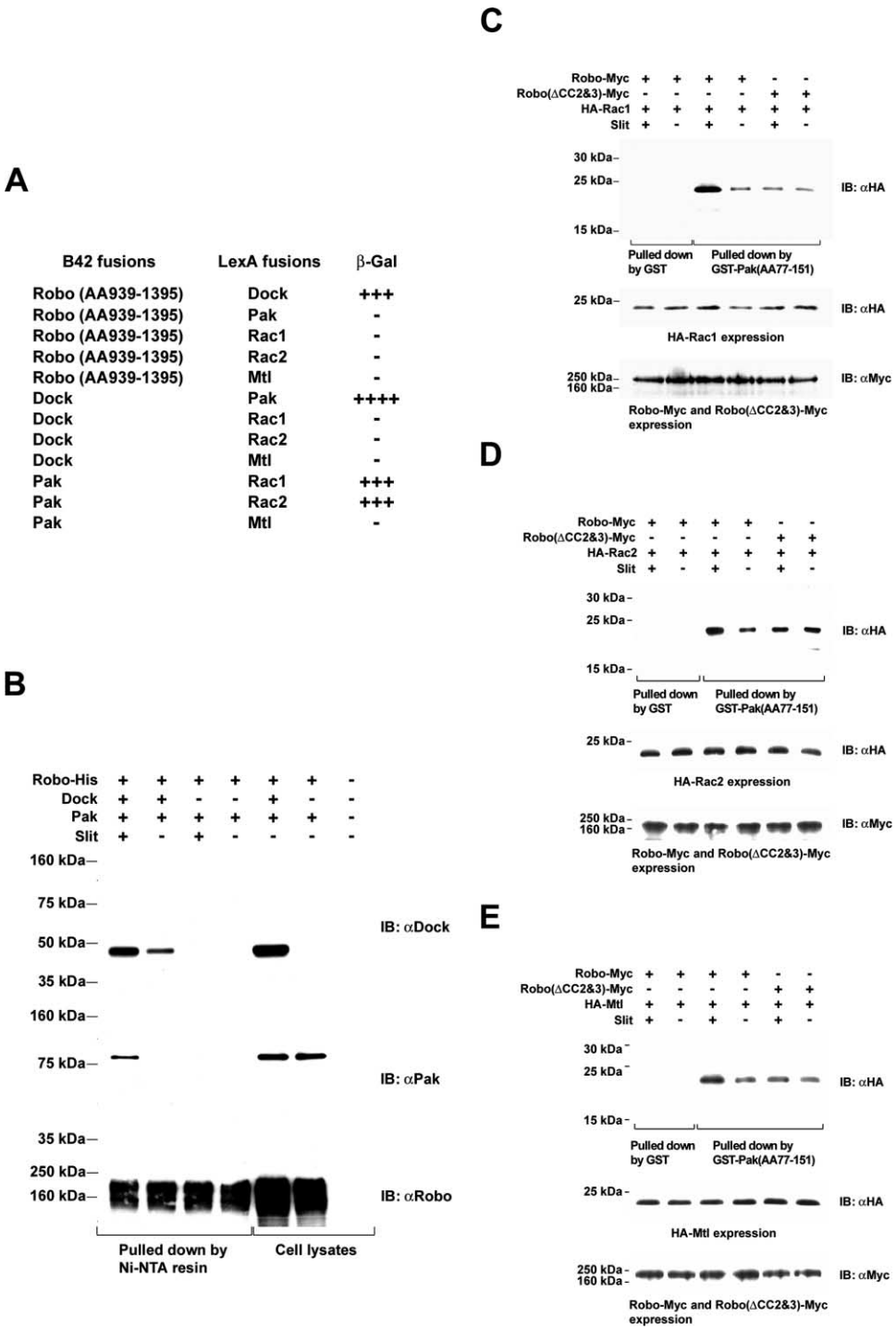

Figure 6. Slit Recruits Dock and Pak to the Robo Receptor and Regulates Rac Activity

(A) Yeast two-hybrid analysis of interactions among Robo, Dock, Pak, Rac1, Rac2, and Mtl. The intracellular domain of Robo (aa 939–1395), Dock, and Pak were fused to the B42 transcription activation domain. Dock, Pak, Rac1, Rac2, and Mtl were also fused to the LexA DNA binding domain. ++++, yeast turned dark blue (strong interaction) in 12 hr in the presence of 80 μg/ml X-Gal. +++, blue (positive interaction); -, white (no interaction).

(B) Slit regulation of the Robo, Dock, and Pak interaction. 293T cells were transiently transfected with C-terminal His-tagged Robo, Dock, and Pak (lanes 1 and 2) or with Robo-His and Pak (lanes 3 and 4) and were treated with Slit (lanes 1 and 3) or without Slit (lanes 2 and 4) for 10 min before performing coprecipitation with Ni-NTA beads. The precipitated Robo-His, Dock, and Pak were detected using anti-Robo, anti-Dock, and anti-Pak antibodies, respectively. Expression of the proteins from total lysates is shown on the right side of the gel (lanes 5 and 6).

(C) Slit regulation of Rac1 activity. 293T cells were transiently transfected with C-terminal Myc-tagged Robo and N-terminal HA-tagged Rac (lanes 1–4), or Myc-tagged Robo (ΔCC2ΔCC3) and HA-Rac (lanes 5 and 6). Cells were treated with Slit for 10 min (lanes 1, 3, and 5) or without Slit (lanes 2, 4, and 6). The active form of HA-Rac was pulled down using GST-Pak (aa 77–151), the active Rac-interacting domain of Pak. GST alone was used as a negative control. The precipitated HA-Rac was detected using anti-HA antibody (top panel). Expression of HA

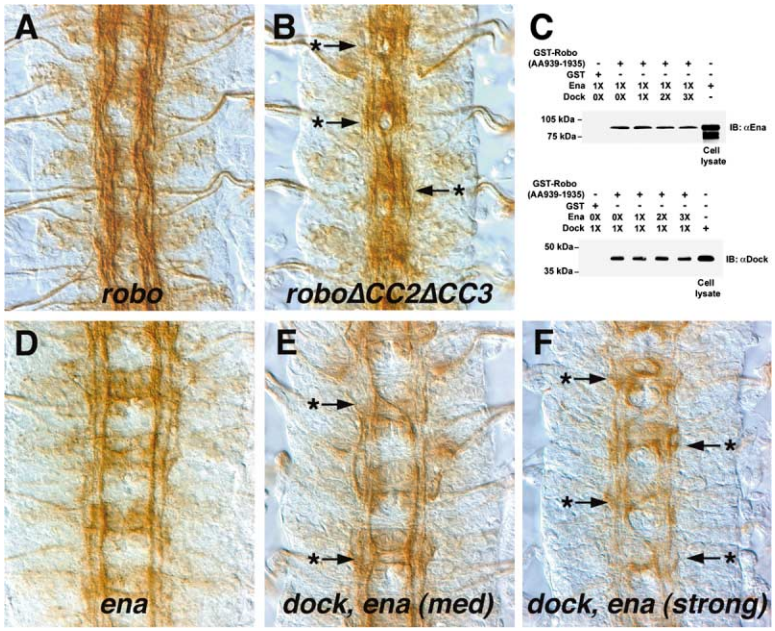

Figure 7. Expression of Mutant Forms of Robo and *dock, ena* Double Mutants

Stage 16 homozygous *robo* mutant embryos stained with FasII to label a subset of longitudinal axons. In addition to carrying *robo* mutations, each animal also expresses one copy of the indicated transgene. Anterior is up.

(A) *robo/robo; UASrobo/elavGal4*. A *robo* mutant embryo rescued by a wild-type *robo* transgene. Expression of the wild-type transgene restores the normal appearance of the FasII axons, which now stay on their own side of the midline (compare with Figure 1E).

(B) *robo/robo; UASroboΔCC2ΔCC3/elavGal4*. Expression of the ΔCC2ΔCC3 mutant transgene fails to rescue loss of *robo* function. Note the presence of characteristic circles of FasII axons (arrows with asterisks) and the regions of the embryo where the inner FasII bundle of axons has collapsed onto the midline (arrows with asterisks), both phenotypes seen in *robo* mutants. Also compare with Figure 1E.

(C) Dock and Ena can independently bind Robo. In the top blot, GST-Robo was used to pull down Ena in the presence of increasing amounts of Dock. Lanes are indicated above the gel. The bottom blot shows the reciprocal experiment. In neither case is significant competition for binding observed.

(D) *enaGC1/enaGC5* mutant embryos do not frequently show ectopic midline crossing.

(E and F) *dock,enaGC1/dockenaGC5* double mutant embryos show a range of phenotypes including mild midline crossing defects (E) and in some cases stronger disruptions (F). Axons abnormally crossing the midline are indicated by arrows with asterisks.

without Slit (Figure 6). Using this system, we are able to detect a modest increase in Rac1 activity and smaller increases in Rac2 and Mtl activity when Robo-expressing cells are stimulated with Slit (Figures 6C–6E). Importantly, the ΔCC2ΔCC3 mutant version of Robo that cannot bind to Dock, and hence cannot recruit Pak, is unable to mediate the Slit-dependent increase in Rac activity (Figure 6C). These data are consistent with the idea that Slit binding of Robo leads to an increase in Rac activity that is dependent on the recruitment of Dock and Pak to the Robo cytoplasmic domain; however, an alternative possibility is that other unidentified proteins interacting through CC2 and CC3 could function to recruit Rac (see Discussion).

A Mutant Form of Robo that Lacks CC2 and CC3 Fails to Rescue *robo* Mutants

To determine the in vivo effect of preventing Robo's association with Dock and Pak and its ability to increase Rac activity, we expressed a mutant form of Robo that is missing both the CC2 and CC3 motifs (RoboΔCC2ΔCC3) in *robo* mutants and tested for rescue of the *robo* phenotype. Similar experiments using Robo receptors mutated for the CC2 and CC3 motifs individually indicate that neither CC2 nor CC3 are absolutely required for complete Robo repulsive output (Bashaw et al., 2000). In contrast, the double mutant ΔCC2ΔCC3 receptor completely fails to rescue loss of *robo* function (Figure 7B and compare with Figure 1E). This is true for multiple inserts of the ΔCC2ΔCC3 transgene and is consistent both for untagged and myc-epitope tagged transgenic receptors. Immunohistochemical comparison of transgenic protein expression did not reveal dramatic differences in the levels of protein expression between the ΔCC2ΔCC3 and the wild-type Robo receptor (data not shown); we therefore do not believe that the inability of the ΔCC2ΔCC3 receptor to rescue results from reduced expression levels. This result suggests that it is the combined action of the CC2 and CC3 motifs that is critical for proper function, and is consistent with an important role for recruitment of Dock and Pak and the regulation of Rac activity during Robo repulsion.

dock and *ena* Likely Function in Parallel during Midline Repulsion

The failure of the ΔCC2ΔCC3 receptor to rescue the *robo* mutant cannot be exclusively attributed to the inability to bind Dock. Indeed, in addition to disrupting the ability to bind to Dock, the ΔCC2ΔCC3 receptor also disrupts the ability to bind Enabled and likely other proteins as well. We have performed genetic and biochemical experiments to determine whether *ena* and *dock* function together or independently during Robo repulsion. Since Ena and Dock binding sites in Robo appear to be partially overlapping, we tested whether Dock and Ena can simultaneously and independently bind to Robo or whether there is cooperation or competition for binding. Our results are consistent with independent binding of

Rac, Robo-Myc, and Robo (ΔCC2&3)-Myc was confirmed by Western blot using anti-HA antibody (middle panel) and anti-Myc antibody (bottom panel), respectively.

(D) Slit regulation of Rac2 activity.

(E) Slit regulation of Mtl activity.

the two proteins, since we do not observe significant competition for binding, nor do we observe striking stimulation of the binding of Ena or Dock to Robo when both proteins are present (Figure 7C and data not shown). We have also tested whether Dock and Ena influence each others' binding in the context of Slit stimulation. We detect a small but consistent increase in Robo and Ena association upon Slit stimulation; however, the presence of Dock does not appear to modulate the association of Ena, nor does Ena appear to modulate Dock binding (data not shown). We have also examined the effects of *dock, ena* double mutants and have observed only a modest additivity to the midline crossing defects (Figures 7D–7F; Table 1). However, these embryos do not phenocopy *robo* mutants, suggesting that *dock* and *ena* do not account for all of *robo* signaling. One caveat to this interpretation is that we have not been able to remove maternal *dock* in the *ena* mutant background due to the poor viability/fertility of $dock^{mat}$, *ena*/*dock* flies. Taken together, these observations support the idea that *dock* and *ena* function in Robo signaling are likely to be independent/parallel outputs.

Discussion

In this paper we provide evidence for the ligand-regulated assembly of a multiprotein complex that contributes to Robo receptor-mediated repulsive axon guidance. Genetic and biochemical evidence suggest that Dock, Rac, and Pak function together, most likely as a protein complex, to couple Robo receptor activation to the regulation of the actin cytoskeleton. Dose-sensitive and loss-of-function genetic analyses suggest that limiting Dock, Pak, or Rac function disrupts Robo repulsion. These data provide the first reported evidence of an in vivo role for Rac in a specific repulsive axon guidance response and support the idea that Rac can function in both attractive and repulsive responses.

The Mechanism of the Dock/Nck and Robo Interaction

Our biochemical data suggests that the interaction between Dock and Robo is an SH3-dependent interaction and that the first two SH3 domains of Dock are most important for mediating Robo binding. Based on the observations that we can detect a three-protein interaction between Robo, Dock, and Pak and that Pak has been shown to interact with the SH3-2 domain of Dock (Hing et al., 1999), we believe that the SH3-1 domain is the most important for Robo and Dock binding. Furthermore, we have found that Slit stimulation enhances Dock's ability to bind to Robo, suggesting a ligand-regulated SH3 domain interaction. This represents a different kind of adaptor interaction to many that have been observed previously, where Nck appears to interact with a number of tyrosine-kinase receptors through an SH2 domain/phosphotyrosine interaction (Kochhar and Iyer, 1996; Stein et al., 1998). In the latter case, how ligand binding to the receptor regulates the Nck SH2 domain interaction is quite well understood. Our observation that the Robo receptor shows a ligand-regulated SH3 domain interaction with Dock/Nck suggests that somehow ligand binding results in an increased availability of the SH3 binding sites in the receptor.

The regions of Robo that appear to be most important for the interaction are the proline-rich regions CC2 and CC3. Individual mutations in these motifs strongly reduce the amount of Dock that coimmunoprecipitates with Robo in cell culture, while removing both of these motifs completely abolishes binding. Furthermore, expression of Robo receptors that lack the CC2 and CC3 motifs in transgenic *Drosophila* disrupt the in vivo function of the receptor. It is important to stress that the CC2 and CC3 sequences are not only involved in Dock binding, but also bind Ena, Abl, and potentially other proteins as well. In addition, CC2 and CC3 are also required for the observed upregulation of Rac activity. The fact that many proteins bind Robo at these sites prevents clear conclusions about why the ΔCC2ΔCC3 mutant receptor is nonfunctional. In the future, more precisely defining the binding requirements of the many proteins that interact with Robo may allow us to create forms of Robo that specifically disrupt the binding of some partners and not others, which in turn should provide insight into the relative roles of different Robo signaling outputs.

A Role for Rac in Repulsion

The implication of Rac in Robo repulsion was unexpected in view of the well-established role of Rac as a positive regulator of axon outgrowth (Luo, 2000). On the surface, this finding appears quite contradictory to the function of Rac to promote actin polymerization at the leading edge of motile cells and axons. One possible explanation of this finding is that perhaps Rac can have different or even opposite effects on the actin cytoskeleton, depending on the molecular context in which it is activated and its overall level of activity. For example, depending on the coordinate local function of other small GTPases and actin regulatory proteins, the consequences of Rac function could be different. It is interesting to note that in addition to a role for Rac, our genetic analysis and previously published data also support an important role for Rho in midline repulsion (Fritz and VanBerkum, 2002). Furthermore, in addition to strongly stimulating Rac activity, Slit has been shown have a modest stimulatory effect on Rho activity (Wong et al., 2001). The implication of both Rac and Rho in mediating repulsive responses has also been suggested to explain the output of the Plexin receptor (Driessens et al., 2001). It will be interesting in the future to determine the interrelationship between Rac and Rho outputs in the context of Robo repulsion as well as in signaling downstream of other attractive and repulsive axon guidance receptors.

As an alternative to the context- and level-dependent explanation of the role of Rac in Robo repulsion, the observed axon steering defects in embryos where both Rac and Slit function are reduced, or in embryos deficient for multiple *rac* genes, could be explained as a secondary consequence of defects in the rate of axon extension. In this scenario, Rac's role in repulsive axon guidance would be intimately coupled with its role in axon outgrowth. That is to say, that appropriate steering decisions go hand and hand with the appropriate regulation of the rate of axon outgrowth (e.g., you are more

likely to miss your exit if you are driving too fast). In this regard, it is important to emphasize that even repulsive cues can have stimulatory effects on axon extension. For example, in addition to repelling *Xenopus* spinal neurons, Slit also has a stimulatory effect on the rate of axon extension (Stein and Tessier-Lavigne, 2001).

The Role of Pak in Robo Repulsion?

Perhaps the most difficult observation to explain is how reciprocal shifts in Pak levels can lead to similar consequences for Robo repulsion. Since the enhancing effects of Pak overexpression in partial loss-of-function *robo* backgrounds are more dramatic with the membrane-tethered form of Pak, it is tempting to speculate that in order to signal properly, turning Pak activity on and off needs to be tightly controlled. Little is known about how Pak signaling is terminated and it seems quite possible that the membrane-tethered version of *pak* is not as effectively regulated as the wild-type form of *pak*. Interestingly, in genetic backgrounds where *robo* signaling is specifically compromised in its output through reduction of *rac,* introducing the *UASPak^{Myr}* transgene can partially suppress the midline crossing defects. Given the clear ability of alterations in *pak* expression to modulate midline repulsion and the observation that Slit can promote the formation of a Robo, Dock, and Pak protein complex, it is somewhat surprising that complete removal of zygotic *pak* does not have major consequences for embryonic axon guidance. Indeed, in the absence of clear loss-of-function phenotypes in *pak* mutants, it is difficult to argue unequivocally for a critical role of endogenous *pak* in *robo* function. There are a number of potential explanations for these observations including, but not limited to, maternal *pak* contribution and the potential redundant function of a second *pak*-like gene. Future experiments should address these possibilities in order to link *pak* more firmly to *robo*.

Dock/Nck Functions Downstream of Many Axon Guidance Receptors

Drosophila Dock has been suggested to act downstream of the Dscam axon guidance receptor during pathfinding of Bolwig's nerve (Schmucker et al., 2000), and the vertebrate homolog of Dock, Nck, has also been linked to several guidance receptors in vitro, including Eph receptors and c-Met receptors (Kochhar and Iyer, 1996; Stein et al., 1998). More recently, Nck has been shown to directly interact with the cytoplasmic domain of the vertebrate attractive Netrin receptor DCC (Li et al., 2002). The Nck and DCC interaction is important for DCC's function to stimulate axon extension in vitro. Together these observations raise the question of whether a similar DCC/Nck interaction occurs in *Drosophila*, and if so whether the interaction is important for the in vivo function of *Drosophila* DCC (encoded in the fly by the *frazzled* gene [Kolodziej et al., 1996]), to attract commissural axons across the midline. Interestingly, in addition to its substantial overlap in expression with the Robo receptor, Dock protein is also expressed in commissural portions of axons, as is the Frazzled receptor. While the *dock* mutant phenotype is most consistent with a role in midline repulsion, we cannot rule out an additional function in attraction. In the future it will be interesting to test for genetic interactions between *frazzled, dock, Rac*, and *pak* to determine if this signaling module is also employed during midline axon attraction in *Drosophila*.

How Are Attraction and Repulsion Specified?

The implication of Dock/Nck and Rac in both DCC-mediated attraction and Robo-mediated repulsion raises the obvious question of how the specificity of attraction and repulsion is controlled and argues against a committed role of either of these signaling molecules to either one or the other type of responses. This is perhaps not too surprising, given the fact that Robo and DCC receptors themselves are intimately connected through their ability to form a heteromeric receptor complex with potentially unique signaling properties (Stein and Tessier-Lavigne, 2001). Although it remains possible that signaling molecules or adaptors will be identified that can account for the specificity, an alternative possibility is that it is the coordinate regulation, relative activity levels, and combinatorial action of a core group of common signaling molecules that makes the difference in attraction versus repulsion.

Dock, Rac, Pak, and "Missing" Components of Robo Repulsion

Our biochemical data support the idea that Slit stimulation of Robo can regulate the recruitment of Dock and Pak to the Robo receptor and also trigger an increase in Rac activity. Both of these events are dependent on the CC2 and CC3 sequences in Robo's cytoplasmic domain. Thus, our observations are consistent with either a Dock-dependent or a Dock-independent recruitment of Rac to Robo. Based on the known physical interactions between Dock and Pak and between Pak and Rac, we believe it likely that the recruitment of Rac is dependent on Dock. Alternatively, another protein interacting through CC2 and/or CC3 could function to recruit Rac in a Dock-independent fashion.

Regardless of whether the recruitment of Rac to Robo is dependent on Dock and Pak or is an independent event, our data cannot explain how Slit stimulation of Robo results in increased Rac activity. Two obvious types of molecules that are missing from the model and the protein complex are the upstream regulators of Rac, the GEF and GAP proteins. Intriguingly, in the course of a genome-wide analysis of all RhoGEFs and RhoGAPs in *Drosophila*, one Rac-specific GAP has been identified that when overexpressed results in phenotypes reminiscent of *robo* loss of function (H. Hu et al., submitted). There are a number of candidate GEFs that could explain how Rac activity is upregulated by Slit activation of Robo, most notably *Sos, rtGEF (pix)*, and *Trio* (Newsome et al., 2000). It will be interesting to determine which if any of these molecules could play such a role in Robo signaling.

Experimental Procedures

Genetics

dock^{mat} maternal mutants were generated as previously described (Desai et al., 1999). Rescue crosses using *robo* transgenes were performed as previously described (Bashaw et al., 2000). The Gal4-UAS system was used to express transgenes in all neurons (Elav-

Gal4), in the Ftz ipsilateral neurons (Ftz^{ng}Gal4), or in the apterous (Ap) ipsilateral neurons (ApGal4). The following stocks were generated for this study: (1) *slit^2/CyoWgßgal; ftz^{ng}GAL4*, (2) *slit^2/CyoWgßgal; pak^{11}/TM3ßgal*, (3) *robo,rho/CyOwgßgal*, (4) *slit^2/Cyoelavßgal/rac1^{J11}/TM2*, (5) *slit^2/Cyoelavßgal/rac2$^{\Delta}$/TM2*, (6) *slit^2/Cyoelavßgal/mtl$^{\Delta}$/TM2*, (7) *UASPak^{Myr}; UASRac^{N17}*, (8) *dock^3, ena^{GC1} /CyoWgßgal*, and (9) *dock^3, ena^{GC5} /CyoWgßgal*. *apGal4, UASTau-Myc-GFP/CyoTubGal80* was a gift from John Thomas. Rho mutants were obtained from Chris Magie and Susan Parkhurst. *rac1, rac2*, and *mtl* mutants were provided by Barry Dickson. Liqun Luo provided dominant-negative versions of Rho, Rac, and Cdc42. All crosses were conducted at 25°C.

Immunohistochemistry

HRP immunohistochemistry was performed as previously described. Fluorescent double staining of wild-type embryos for Robo and Dock was performed using the Robo mouse MAb (1:50) and Cy3 secondary antibody (Molecular Probes) (1:2000), a Dock rabbit antibody (1:1000), and AlexaFluor488 secondary antibody (1:1000). Images were obtained using a Zeiss Axiocam, Openlab software (Improvision), and a 63× oil immersion objective.

Yeast Two-Hybrid Assay

The DupLEX-A yeast two-hybrid system (OriGene Tech, Rockville, MD) was used to (1) detect Robo-Dock interaction, (2) detect Dock-Pak and Pak-Rac interactions, and (3) test for interactions between Robo and Pak and between Robo and Rac. Different forms of the Robo intracellular domain were fused to the activation domain B42 (pJG4-5), while Pak, Rac1, Rac2, Mtl, Dock, and its truncated forms were fused to the DNA binding domain LexA (pEG202). Robo constructs were confirmed to be full length by Western blotting.

Cell Transfection and Immunoprecipitation

293T cells were transfected at 80% confluency with 2 μg plasmid DNA using Effectene Transfection Reagent (Qiagen). To detect Slit regulation of Robo-Dock-Pak interaction, C-terminal His-tagged Robo, Dock, and Pak were coexpressed in 293T cells. 48 hr post-transfection, cells were stimulated with conditioned medium from Slit-expressing 293T cells or conditioned medium from mock transfected cells at 37°C with 5% CO_2 for 10 min, then were lysed (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, 0.5% TX100, 1× protease inhibitor [pH 8.0]). Cell lysates were incubated with Ni-NTA resin (Qiagen) at 4°C for 2 hr to precipitate Robo-His. The resin was washed three times (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, 0.5% TX100 [pH 8.0]) and heated at 95°C for 10 min. The precipitates were resolved on 12% SDS-PAGE and blotted with monoclonal anti-Robo antibody and polyclonal anti-Dock and anti-Pak antibodies. For in vivo Co-IP, lysates were prepared from 50 μl of embryos expressing one copy of *UASrobo-myc* or *UASrobo*Δ*CC2*Δ*CC3-myc*. Embryos were smashed and lysed (50 mM NaH_2PO_4, 300 mM NaCl, 0.5% TX100, 1× protease inhibitor [pH 7.4]). Extracts were centrifuged for 15 min at 14,000 rpm. Supernatants were incubated with monoclonal anti-myc antibody and protein A Sepharose 4B beads for 2 hr at 4°C. Beads were washed three times with lysis buffer and then heated at 95°C for 10 min. Precipitates were resolved on 12% SDS-PAGE and blotted with polyclonal anti-Dock antibody and monoclonal anti-myc antibody. Signals were detected by chemi-luminescence (ECL) and exposure to Kodak X-OMAT AR film.

Rac GTPase Activity Assay

To test Slit regulation of Rac activity, 293T cells were transfected with both Robo and HA-Rac, stimulated with Slit as described above, and then lysed (50 mM NaH_2PO_4, 300 mM NaCl, 0.5% TX100, 1× protease inhibitor [pH 7.4]). Lysates were incubated with 5 μg GST-Pak (aa 77–151) or GST alone and Glutathione Sepharose 4B beads at 4°C for 2 hr. Beads were washed three times with PBS, suspended in 30 μl of protein sample buffer, and heated at 95°C for 10 min. Precipitates were resolved on 15% SDS-PAGE and blotted with monoclonal anti-HA antibody.

GST Pull-Downs

GST-Robo (aa 939–1395) was expressed in *E. coli* and purified using the Bulk and RediPack GST purification modules (Amersham). Dock and Ena were expressed in 293T cells in 100 mm Petri dishes and lysed in 2 ml lysis buffer/dish. 150 μl Dock or Ena cell lysate, which contains excess Dock or Ena for 5 μg GST-Robo binding (predetermined), was treated as 1×. Glutathione Sepharose 4B bead bound 5 μg GST-Robo (aa 939–1395) was mixed with 1× Dock and 0×, 1×, 2×, or 3× Ena. Nontransfected cell lysate was used to adjust the total volume to 600 μl. The mixtures were incubated at 4°C for 2 hr. The beads were washed three times with PBS, suspended in 30 μl protein sample buffer, and heated at 95°C for 10 min. Precipitated proteins were resolved on 12% SDS-PAGE and detected with anti-Dock antibody. The parallel experiment was done using 1× Ena and 0×, 1×, 2×, or 3× Dock. The precipitated Ena was blotted with monoclonal anti-Ena antibody.

Acknowledgments

We thank Long Yang and Fernanda Musa for outstanding technical support. We are grateful to Corey S. Goodman, in whose lab this project was initiated. We thank Barry Dickson, Liqun Luo, Larry Zipursky, Craig Magie, and Susan Parkhurst for various fly strains. We are indebted to Liqun Luo for the gift of cDNAs encoding the small GTPases. Larry Zipursky generously supplied us with polyclonal antibodies to Dock. We thank Hailan Hu for critical comment on the manuscript. This work was supported by a Human Frontier Science Program postdoctoral fellowship to J.P.L. and a Burroughs Wellcome Career Award and Whitehall Foundation Research Grant to G.J.B.

Received: January 23, 2003
Revised: July 31, 2003
Accepted: August 21, 2003
Published: September 24, 2003

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