Cytoplasmic domain requirements for Frazzled-mediated attractive axon turning at the *Drosophila* midline

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The conserved DCC ligand-receptor pair Netrin and Frazzled (Fra) has a well-established role in axon guidance. However, the specific sequence motifs required for orchestrating downstream signaling events are not well understood. Evidence from vertebrates suggests that P3 is important for transducing Netrin-mediated turning and outgrowth, whereas in *C. elegans* it was shown that the P1 and P2 conserved sequence motifs are required for a gain-of-function outgrowth response. Here, we demonstrate that *Drosophila fra* mutant embryos exhibit guidance defects in a specific subset of commissural axons and these defects can be rescued cell-autonomously by expressing wild-type Fra exclusively in these neurons. Furthermore, structure-function studies indicate that the conserved P3 motif (but not P1 or P2) is required for growth cone attraction at the *Drosophila* midline. Surprisingly, in contrast to vertebrate DCC, P3 does not mediate receptor self-association, and self-association is not sufficient to promote Fra-dependent attraction. We also show that in contrast to previous findings, the cytoplasmic domain of Fra is not required for axonal localization and that neuronal expression of a truncated Fra receptor lacking the entire cytoplasmic domain (FraΔC) results in dose-dependent defects in commissural axon guidance. These findings represent the first systematic dissection of the cytoplasmic domains required for Fra-mediated axon attraction in the context of full-length receptors in an intact organism and provide important insights into attractive axon guidance at the midline.

KEY WORDS: Axon guidance, Midline, Attraction, Netrin, DCC, Frazzled, Signaling

INTRODUCTION

During development neuronal growth cones navigate a series of intermediate choice points to find their correct targets. At each decision point, axons encounter many guidance cues in their extracellular environment (Dickson, 2002; Yu et al., 2002). Growth cones translate these cues using signaling mechanisms downstream of conserved guidance receptors and ultimately steer an axon to its target. Several conserved families of guidance cues and receptors have been discovered including the Netrin-family of ligands and their DCC family of receptors including Frazzled (Fra) and Unc-40 (Kennedy, 2000).

The DCC family of Netrin receptors, including UNC-40 in *C. elegans*, Fra in *Drosophila*, and DCC in vertebrates contain extracellular domains consisting of six immunoglobulin (Ig) repeats and four fibronectin type III (FNIII) repeats and cytoplasmic domains consisting of three conserved sequence motifs, P1, P2 and P3 (Kolodziej, 1997). Previous studies have shown that DCC family members bind to Netrin and mediate axon outgrowth and growth cone attraction (Chan et al., 1996; de la Torre et al., 1997; Keino-Masu et al., 1996; Kolodziej et al., 1996; Stein et al., 2001). For example, in *C. elegans*, during circumferential axon guidance, the UNC-40 receptor is required in ventrally migrating cells that respond to Netrin (Chan et al., 1996). Genetic analysis in *Drosophila* also illustrates a role for Netrin-Frazzled signaling in axon attraction. *Netrin* and *fra* mutants exhibit a decrease in the number of commissural axons crossing the ventral midline suggesting that attraction is compromised (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996). In addition, using the *Xenopus* spinal axon turning assay, it was shown that axons are attracted to an

exogenous source of Netrin, and this response depends on DCC (de la Torre et al., 1997). Together, these data establish that the DCC/Frazzled/UNC-40 family of receptors responds to Netrin to mediate axon outgrowth and attraction.

Although the biological relevance of the Netrin-DCC pathway is becoming clear, the receptor motif requirements and signaling mechanisms downstream of the receptor are not well understood. Recent studies from vertebrate systems and *C. elegans* have investigated which of the cytoplasmic motifs of DCC/UNC-40 play a role in signaling. For example, in vertebrates it was reported that the cytoplasmic P3 sequence motif is necessary for receptor multimerization and Netrin-induced attractive turning of stage 22 *Xenopus* neurons (Stein et al., 2001). Versions of DCC lacking the P3 motif are not able to mediate axon attraction or stimulate outgrowth. However, replacing the P3 motif with the SAM self-association domain (from the EphB receptor) is sufficient to restore function, suggesting the major role of P3 is to mediate self-association (Stein et al., 2001). More recently, several independent reports demonstrated that one of the major functions of P3 is to recruit focal adhesion kinase (Fak). This interaction is important for tyrosine phosphorylation of DCC and for the ability of DCC to elicit axon outgrowth and turning (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004). Specifically, the LD-like motif within P3 of DCC was critical for FAK binding; however, another P3-independent binding site was also proposed (Ren et al., 2004).

Alternatively, a study from *C. elegans* showed that the P1 and P2 motifs, but not *P3*, are required to promote an Unc-40 gain-of-function outgrowth response (Gitai et al., 2003), suggesting P1 and P2 function in endogenous UNC-40 signaling. It should be noted that the potential function of P3 in full-length Unc-40 receptor signaling in the context of a normal in vivo attractive decision has not been examined. Further genetic analysis from this report argues that *unc-34*/*ena* and the Rac GTPase, *ced-10*, likely contribute to signaling via the P1 and P2 cytoplasmic domains, respectively (Gitai et al., 2003). In *Drosophila*, *ena* also genetically interacts with *fra*

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further supporting a role for Ena during attractive midline guidance (Forsthoefel et al., 2005); however, in this case, the specific sequence motifs required were not investigated.

In this study, we report a detailed structure-function analysis of the *Drosophila* DCC family receptor Fra to identify the domains required for attractive guidance at the midline. Using a specific subset of commissural neurons, we demonstrate that *fra* function is required cell autonomously for attraction toward and across the midline. We also show that the conserved cytoplasmic P3 motif is vital for this attractive function, whereas P1, P2 and the SH3 domain interacting PXXP motifs are dispensable. Importantly, a mutant construct that retains the LD-like motif and lacks only the last 12 amino acids of the P3 motif fails to promote midline attraction suggesting that only the second half of P3 is required during guidance. We also discovered that a Fra receptor lacking the entire intracellular domain (FraΔC) is normally localized to CNS axons and is able to disrupt commissural axon guidance in a dose-dependent fashion. Expression of this truncated receptor in a *fra* heterozygous background causes a more severe phenotype, whereas expression in *fra* homozygous mutants leads to a complete commissureless phenotype suggesting the exciting possibility that, in addition to acting as a dominant-negative for Frazzled signaling, FraΔC also interferes with additional guidance pathways.

MATERIALS AND METHODS
Genetics
The following *Drosophila* stocks were used: (1) *yw*, (2) *UAS-TauMycGFP/CyTubGal80; eagleGal4*, (3) *Df-NP5/FM7βactin*, (4) *NetA,B/FM7βactin*, (5) *fra³/CyWglacZ*, (6) *fra⁴/CyWglacZ*, (7) *fra⁴, UAS-TauMycGFP/CyTubGal80; eagleGal4*, (8) *fra³, UAS-FraMyc^{1.1}/CyWglacZ*, (9) *fra³/CyWglacZ; UAS-FraMycII*, (10) *UAS-FraΔP1-Myc^{#2}; fra³/CyWglacZ*, (11) *fra³, UAS-FraΔP1-Myc^{#1.1}/CyWglacZ*, (12) *UAS-FraΔP2-Myc^{#1.1}; fra³/CyWglacZ*, (13) *fra³/CyWglacZ; UAS-FraΔP2-Myc^{#2.1}*, (14) *fra³, UAS-FraΔP3-Myc^{#2}/CyWglacZ*, (15) *fra³, UAS-FraΔP3-Myc^{#1.1B}/CyWglacZ*, (16) *fra³/CyWg; UAS-Myr-Fra-Myc^{#1.1}*, (17) *fra³/CyWg; UAS-Myr-Fra-Myc^{#2.1}*, (18) *fra³/CyWglacZ; UAS-FraΔP3-SAM-Myc^{#4}*, (19) *fra³/CyWglacZ; UAS-FraΔP3-SAM-Myc^{#5.2}*, (20) *fra³, UAS-FraΔC-HA^{#4}/CyWglacZ*, (21) *fra³/CyWglacZ; UAS-FraΔC-HA^{#2}*, (22) *fra³/CyWglacZ; UAS-FraΔC-HA^{#8}*, (23) *fra³/CyWglacZ; UAS-FraΔC-HA^{#9}*, (24) *fra⁴/CyWglacZ; elavGal4*, (25) *fra³/CyWglacZ; UAS-FraΔC^{Robo67}Myc*, (26) *elavGal4^{3A}*, (27) *UAS-FraΔC-HA^{#4}/CyWglacZ; elavGal4/TM2*, (28) *robo^{Ga285}/CyWglacZ*, (29) *robo^{Ga285}/CyWg; elavGal4*, (30) *fra³, robo/CyWglacZ*, (31) *fra⁴, robo/CyWglacZ*, (32) *fra³, robo, UAS-FraΔC-HA^{#4}/CyWglacZ*, (33) *fra⁴, robo/CyWglacZ; elavGal4*, (34) *fra³, slit²/CyWglacZ*, (35) *fra⁴, slit²/CyWglacZ*, (36) *fra³, slit²/CyWglacZ*, (37) *fra⁴, slit², UAS-FraΔC-HA^{#4}/CyWglacZ*, (38) *fra³, slit²/CyWglacZ; elavGal4*.

Molecular biology
All constructs were generated using standard molecular biology techniques. P1 (aa 1123-1140), P2 (aa 1270-1302), and P3 (aa 1349-1372) motifs are based on those described by Kolodziej et al. (Kolodziej et al., 1996) and precise deletions were made using a 'sewing PCR' strategy. Details provided upon request. For detail of the constructs used, see Fig. 3 and Fig. S1 in the supplementary material.

Immunofluorescence
Embryos were collected, fixed and stained as previously described (Kidd et al., 1998a). The following primary antibodies were used: (1) Ms-anti-1D4/FasII [(Developmental Studies Hybridoma Bank (DSHB); 1:100], (2) Ms-mAb BP102 (DSHB; 1:100), (3) Rb-anti-Myc (Sigma-Aldrich, 1:500), (4) Ms-anti-Sex Lethal (DSHB; M18, 1:1000), (5) Ms-anti-β-gal (DSHB; 40-1a, 1:250), (6) Rb-anti-GFP (Molecular Probes; 1:500), (7) Rb-anti-HA (Covance; 1:2000). The following secondary antibodies were used: (1) Alexa Fluor 488 gt-anti-Rb (Molecular Probes; 1:500), (2) Cy3 gt-anti-Ms (Jackson Laboratories; 1:1000). Stacks of images were obtained using a

Leica DMIRE2 confocal and a 63× oil immersion objective. After despeckling the stacks, a maximum projection was generated with NIH Image/ImageJ software.

Biochemistry
For co-ip, S2R+ cells were transfected using the Effectene reagent (Qiagen) along with 0.5 μg pMT-Gal4, then induced on day 2 with (0.5 mM) CuSO₄. 40 ng of plasmid was transfected per reaction for Myr constructs and 200 ng for full-length constructs. Cells were lysed in 1 ml lysis buffer [50 mM Tris-HCl pH 7.4, 500 mM NaCl, 1% NP40, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1× complete protease inhibitor cocktail (Roche) and 1 mM PMSF] on day 3, followed by overnight incubation with 2 μl of either rabbit anti-Myc antibody (Sigma) or rabbit anti-HA antibody (Covance). 30 μL of protein G-Sepharose (Zymed) beads were used followed by three washes in lysis buffer. For immunoblots, mouse anti-Myc (9E10 1:1000) and mouse anti-HA (Covance 1:1000) antibodies were used.

RESULTS
frazzled mutant embryos exhibit defects in a subset of commissural axons similar to *Netrin* mutants
Previously, axon guidance phenotypes of *frazzled* (*fra*) mutants were analyzed at a gross level using mAb BP102 to label all axons (Fig. 1, magenta). However, this strategy is qualitative and subjective since commissural bundles are very thick and contain many axons. Additionally, commissure thickness is quite stage dependent and slight differences in embryo age can make unambiguous analysis difficult. In order to establish a more quantitative technique to analyze the guidance defects seen in *fra* mutants, we examined a
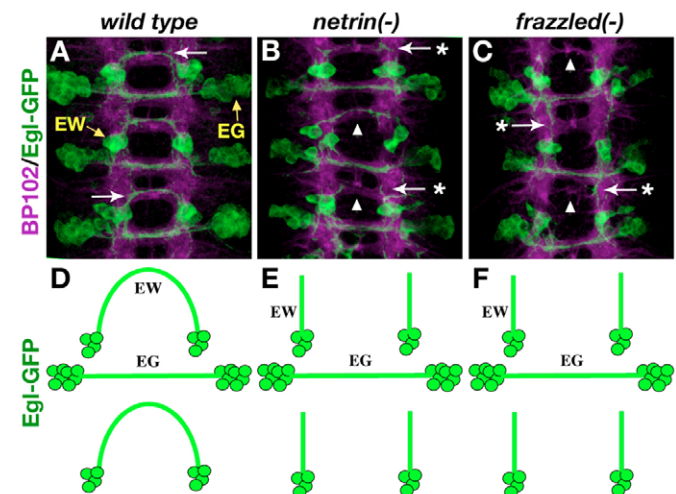

Fig. 1. *fra* and *Netrin* mutants have defects in a subset of commissural neurons. (**A-C**) Stage 16 embryos stained with mAb BP102 to display all axons and anti-GFP to visualize a subset of commissural neurons, the Egl neurons. Indicated genotypes also contain *UAS-TauMycGFP* and *eagleGal4*. Anterior is up. (A) In wild-type embryos, EW and EG neurons cross the midline in every segment (white arrows). (B) EW axons fail to cross the midline in many segments of *Netrin* mutants (starred arrow). Note the thinning of CNS commissures (arrowhead) when stained with BP102 (magenta). (C) Similar to *Netrin* mutants, the EW axons frequently fail to cross the midline in *fra* mutant embryos (starred arrow). Note the thinning of commissures (arrowhead) when stained with BP102 (magenta). (**D-F**) Cartoons depicting the trajectories of the EW and EG neurons in each of the indicated genotypes.
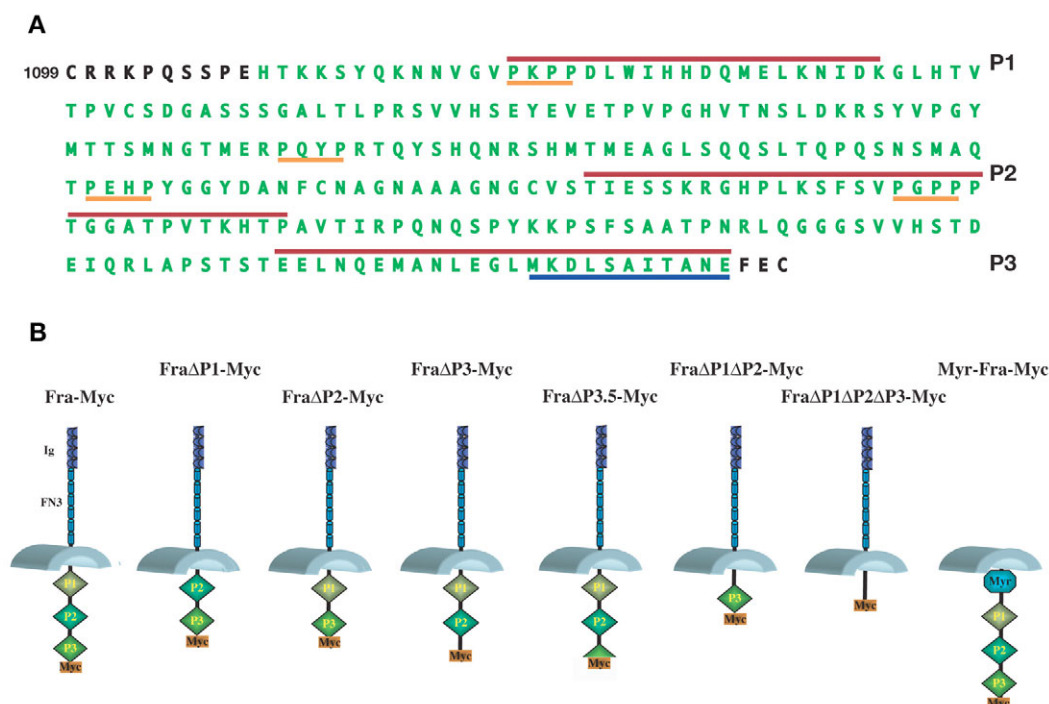
A

1099 C R R K P Q S S P E H T K K S Y Q K N N V G V P K P P D L W I H H D Q M E L K N I D K G L H T V P1

T P V C S D G A S S S G A L T L P R S V V H S E Y E V E T P V P G H V T N S L D K R S Y V P G Y

M T T S M N G T M E R P Q Y P R T Q Y S H Q N R S H M T M E A G L S Q Q S L T Q P Q S N S M A Q

T P E H P Y G G Y D A N F C N A G N A A A G N G C V S T I E S S K R G H P L K S F S V P G P P P P2

T G G A T P V T K H T P A V T I R P Q N Q S P Y K K P S F S A A T P N R L Q G G G S V V H S T D

E I Q R L A P S T S T E E L N Q E M A N L E G L M K D L S A I T A N E F E C P3

B

Fig. 2. Schematic of the Fra cytoplasmic domain indicating conserved motifs. (**A**) Sequence of the Fra cytoplasmic domain. Red lines over the sequence indicate the conserved P1, P2 and P3 motifs as defined by Kolodziej et al. (Kolodziej et al., 1996). These sequences were deleted in the constructs shown in B. Orange lines under the sequence indicate the four PXXP motifs found in the Fra cytoplasmic domain. The blue line delimits the region deleted in the construct ΔP3 1/2; this construct leaves the critical Fak-binding residues, defined in vertebrate DCC, intact. (**B**) Schematic diagram of some of the constructs used in this study. Each construct carries a six-Myc epitope at its carboxyl terminus for comparison of expression levels and localization. Ig, immunoglobulin; FN3, fibronectin type III.

specific and easily quantifiable subset of commissural neurons, the eagle (Egl) neurons, labeled with Tau-MycGFP (Fig. 1) (Dittrich et al., 1997; Higashijima et al., 1996).

In wild-type embryos the Egl neurons consist of two clusters of commissural neurons (Fig. 1A, yellow labels): one cluster (the EG neurons) contains 10-12 cells (located laterally) that extend axons across the midline in the anterior commissure. The second more medial cluster (the EW neurons) consists of four cells, three of which project axons across the midline in the posterior commissure of the adjacent segment (Dittrich et al., 1997; Higashijima et al., 1996). Wild-type axons from both the EG and EW clusters cross the midline in all segments (Fig. 1A and see Fig. S1 in the supplementary material). Conversely, in approximately three-quarters of *fra* mutant segments, the EW axons display defects in midline crossing [not crossing (51%) or significantly thinner (16%) than wild type]; however the trajectories of the EG axons are unaffected (Fig. 1C and see Fig. S1 in the supplementary material). Importantly, these phenotypes are similar although somewhat stronger than those reported for *Netrin* mutants (Fig. 1B) (Brankatschk and Dickson, 2006). Our own quantification of the defects in the *Netrin-A Netrin-B* (*NetAB*) double mutants reveals that the EW axons fail to cross the posterior commissure in 34.2% of segments (n=27 embryos, 216 segments), whereas the EG axons fail to cross in only 1% of segments (n=27 embryos, 214 segments).

The Fra receptor cell autonomously guides commissural axons across the midline
Since it has been reported that Fra has non cell-autonomous functions (Gong et al., 1999; Hiramoto et al., 2000), we next asked if the EW cluster requires Fra exclusively in neurons for proper guidance.

Expressing a wild-type full-length Fra receptor (UAS-Fra-Myc or UAS-Fra-HA) in the Egl neurons under the control of *eagleGal4* (Brand and Perrimon, 1993) cell-autonomously rescues EW guidance defects (Fig. 2B, Fig. 3B and see Fig. S1 in the supplementary material). By contrast, a myristolated form of Fra lacking the entire extracellular domain (UAS-MyrFra-Myc) is unable to rescue the mutant phenotypes, presumably because this truncated protein is unable to bind Netrin and mediate a directional response. Unlike observations in *C. elegans* where expression of a myristolated UNC-40 leads to ectopic neurite outgrowth (Gitai et al., 2003), our Myr-Fra construct did not cause similar gain-of-function phenotypes when expressed in all neurons or within a defined subset of neurons (data not shown). Together these results are supportive of previous findings demonstrating that Fra plays an important role in attracting certain classes of neurons towards and across the midline in response to Netrin (Kolodziej et al., 1996). Since the *fra* defects can be rescued cell autonomously within an easily quantifiable subset of neurons, this system allows a thorough investigation of the domains necessary for mediating Netrin attraction.

The P3 motif of Fra is required for midline attraction
To determine the domain requirements for Fra-mediated signaling, we generated mutant transgenes containing various deletions within the receptor (Fig. 2A,B and see Fig. S1 in the supplementary material; see Materials and methods) and tested whether these mutated receptors could rescue the *fra* defects in EW neurons. We found that whereas expression of a mutant receptor lacking either P1 or P2 (or both; UAS-FraΔP1-Myc, UAS-FraΔP2-Myc or UAS-FraΔP1ΔP2-Myc) efficiently rescues
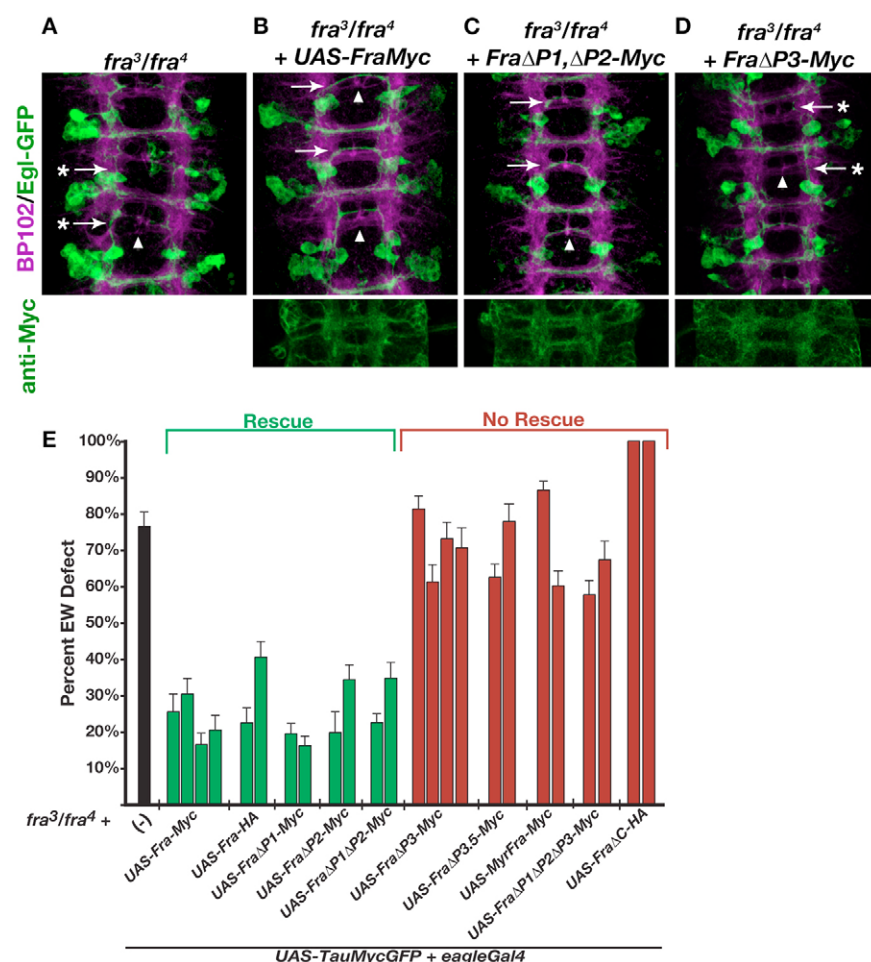
Fig. 3. Fra is required cell autonomously to guide axons and the P3 motif is critical for mediating Fra attractive function. (A-D) Stage 16 embryos stained with mAb BP102 to visualize all axons, and anti-GFP to highlight the Egl neurons. Indicated genotypes also contain *UAS-TauMycGFP* and *eagleGal4*. Anterior is up. (A) EW axons fail to cross the midline in many segments of *fra* mutants (starred arrows). Also notice the overall thinning of CNS commissural bundles (arrowhead). (B) *fra* mutant defects can be rescued (arrows) by specifically expressing UAS-Fra-Myc in the Egl neurons. (C) Similar to UAS-Fra-Myc, expression of UAS-FraΔP1ΔP2-Myc in the Egl neurons results in rescue of the *fra* guidance defects. (D) A Fra receptor lacking the P3 motif cannot rescue the guidance defects of *fra* mutants. (B-D, bottom panels) Anti-Myc staining (green) of embryos expressing UAS-Fra-Myc (B), UAS-FraΔP1ΔP2-Myc (C) or UAS-FraΔP3-Myc (D) under the control of *elavGal4* shows that the transgenes are expressed at comparable levels and are similarly localized to CNS axons. One segment is shown. (**E**) The rescuing ability of each of the constructs used in this study. Green bars indicate rescue that is comparable to that of wild type; red bars indicate a failure to rescue. Data is presented as the percentage of EW axons that fail to cross the midline. Mutant defects were scored as the complete absence or thinning of EW axons across the midline. Each bar represents an independent transgenic line (see Fig. S1 in the supplementary material; Materials and methods). A total of 60-80 embryos were scored for each genotype (or approximately 15-20 embryos for each line). Error bars indicate s.e.m. For statistical analysis, see Fig. S1 in the supplementary material.

the guidance errors of EW axons, a version of Fra missing P3 (UAS-FraΔP3-Myc) does not (Fig. 2A,B and Fig. 3C-E). Furthermore, a mutant construct missing only the last 12 amino acids of P3 (UAS-FraΔP3.5) also does not rescue the attractive guidance defects, suggesting a critical requirement for these 12 residues during Fra-mediated attraction at the *Drosophila* midline (Fig. 2A,B and Fig. 3E). Importantly UAS-FraΔP3.5 still contains the LD-like motif, the presumed FAK binding motif identified in vertebrate DCC (Ren et al., 2004). In addition, individually mutating each of the PXXP motifs (Fig. 2) did not result in decreased Fra function; all of these mutant receptors rescued the EW neurons as well as a wild-type transgene (data not shown). We note that one of the FraΔP3 and FraΔP3.5 lines as well as both FraΔP1,P2,P3 lines were significantly different from *fra* mutants alone, suggesting that these receptors may still provide some attractive function, albeit at much lower levels than the other mutant Fra receptors. We tested at least two independent transgenic inserts for each construct (Fig. 3B and see Fig. S1 in the supplementary material) and confirmed that they are expressed at similar levels to wild-type transgenes (Fig. 3 and data not shown).

Self-association of Fra is not dependent on P3
Studies using cultured *Xenopus* spinal neurons have suggested that Netrin-dependent attractive responses mediated by DCC require P3-dependent self-association (Stein et al., 2001). To test this in an in vivo system, we assessed whether replacement of the P3 motif of Fra

with the SAM self-association domain of the EphB receptor could rescue function of this mutated receptor when expressed in Egl neurons in *fra* mutants. In contrast to observations in vertebrate systems, substitution of the P3 motif with a SAM multimerization domain could not rescue EW crossing defects (data not shown), suggesting that multimerization (at least not P3-dependent multimerization) is not sufficient for normal Frazzled guidance responses.

To determine whether the inability of FraΔP3-SAM to rescue crossing defects in EW neurons is not simply a failure of receptor multimerization, we sought to confirm that P3 directs receptor self-association in *Drosophila* through expression of Fra constructs in cultured *Drosophila* S2R+ cells. It has been previously demonstrated that myristolated DCC cytoplasmic domains exhibit Netrin-independent constitutive multimerization (Stein et al., 2001). Interestingly, a myristolated c-terminal version of Fra (Myr-Fra-Myc) bound to both a myristolated cytoplasmic construct (Myr-Fra-HA) as well as a full-length construct lacking a P3 domain (Fra-ΔP3-HA), indicating that multimerization of Fra can occur independently of the P3 domain (Fig. 4A, lanes 1 and 3). Additionally, association was observed between Myc and HA-tagged constructs in which both P3 domains were deleted, eliminating the possibility of P3-dependent interaction with a distinct motif (Fig. 4B). Deletion of P1 and P2 motifs also had no effect on self-association (data not shown). It will be interesting to determine which cytoplasmic motif(s) mediate this interaction in Fra and whether multimerization is indeed required at the *Drosophila*
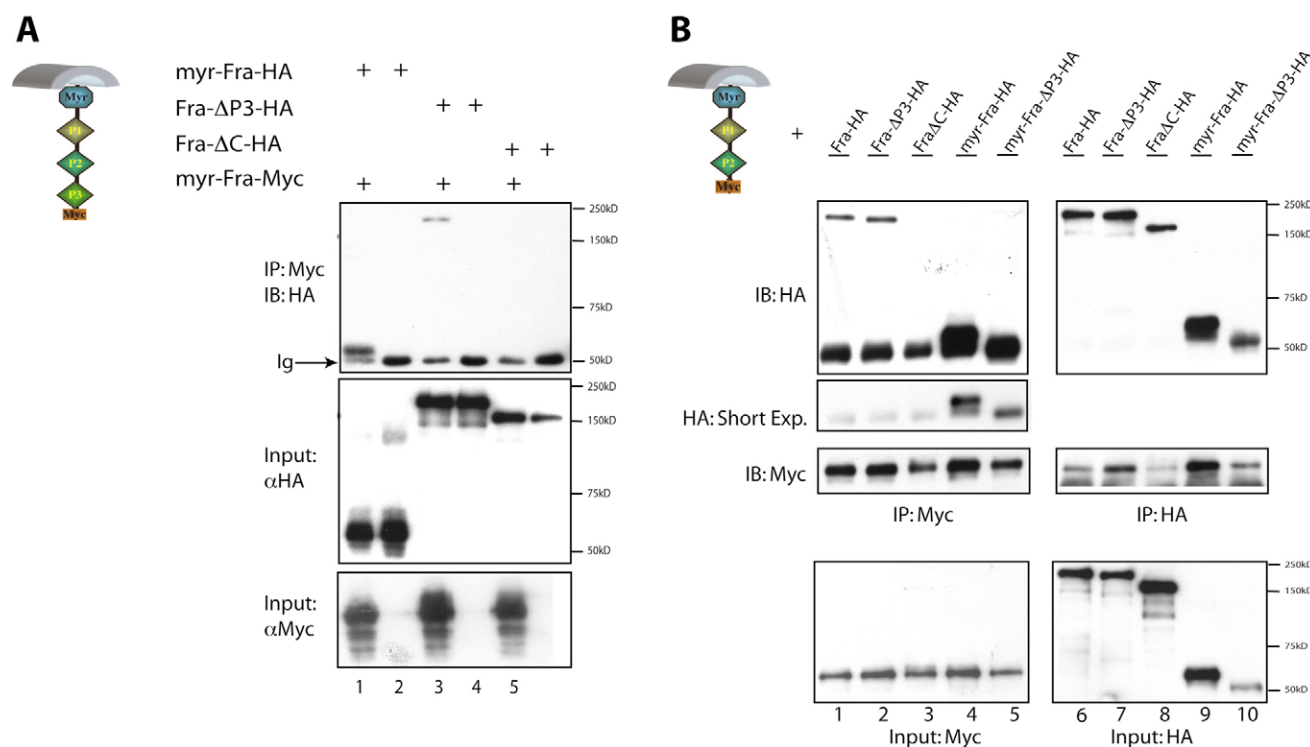
Fig. 4. The P3 motif is not required for Frazzled multimerization. (A,B) Co-immunoprecipitation of Myc and HA-tagged Frazzled constructs expressed in *Drosophila* S2R+ cells. Lysates were immunoprecipitated with either anti-Myc antibody (A, and upper left in B), or anti-HA antibody (upper right B). (A) Myr-Fra-Myc co-immunoprecipitates with HA-tagged Fra constructs, including one lacking the P3 motif (lane 3). FraΔC-HA serves as a control (lane 5). (B) A P3 motif is not required in either construct for interaction, as Myr-FraΔP3-Myc binds to ΔP3 constructs as efficiently as to those containing wild-type cytoplasmic domains (compare lanes 2, 5, 7 and 10 to lanes 1, 4, 6 and 9).

midline; however, these results suggest that whatever role Fra multimerization plays in mediating Fra function, it is not sufficient to direct axon attraction in the absence of the P3 motif.

The cytoplasmic domain of Fra is not required for normal receptor distribution

Previously, it was reported that the cytoplasmic domain of Fra was necessary for proper axonal localization (Hiramoto et al., 2000). Our Myc-tagged mutant transgenes presented the opportunity to establish whether any of the conserved sequence motifs play a role in receptor localization. Expression of our singly deleted ΔP1, ΔP2 or ΔP3 mutant transgenes in all neurons using *elavGal4* did not lead to a dramatic alteration in Fra receptor distribution when compared to a wild-type transgene (Fig. 3, and data not shown). Additionally, mutating all three motifs in combination (UAS-FraΔP1ΔP2ΔP3-Myc) does not disrupt Fra localization (data not shown). In fact, overexpressing a Fra receptor missing almost the entire cytoplasmic domain (UAS-FraΔC-HA; amino acids 1109-1372 were deleted; see Fig. 2) still does not cause a substantial difference in the localization pattern when compared to a wild-type transgene (Fig. 5D,E). For the purpose of this study, we remade UAS-FraΔC-HA (Fig. 6F) because previous experiments (Hiramoto et al., 2000) were done using a FraΔC receptor that contained the transmembrane domain and 67 juxtamembrane cytoplasmic amino acids of the Robo receptor (FraΔC^{Robo67}-Myc). We suspect that this exogenous sequence might interfere with normal Fra distribution. Given that our newly generated construct localizes similarly to wild-type and differently from the original FraΔC^{Robo67}-Myc protein (Fig. 5,

compare C′ or D′ to E′), we consider this a reasonable hypothesis. At least two independent transgenic lines were tested in our localization studies. Our data suggest that the cytoplasmic domain of Fra is not required for robust axonal localization. However, it is important to point out that these overexpression experiments may not reflect the true localization of endogenous proteins with similar deletions.

FraΔC interferes with normal axon guidance and depends on Netrin

In the course of investigating the localization pattern of UAS-FraΔC, we noticed that overexpression of this receptor in a wild-type background causes commissures to become thin, suggesting some commissural axons fail to cross the midline (Fig. 6A,B). The severity of this phenotype increases when one copy of *fra* is removed (Fig. 6D) suggesting that FraΔC could be acting as a dominant negative for Fra. When FraΔC is expressed at high levels in a wild-type background, most axons do not cross the midline (Fig. 6C) demonstrating that the effect is dose dependent. Strikingly, homozygous *fra* mutants overexpressing only one copy of UAS-FraΔC exhibit a near complete loss of axon commissures (Fig. 6E), a phenotype that closely resembles that of *commissureless* (*comm*) loss-of-function mutant embryos (Seeger et al., 1993). We observe this strong commissureless ectopic expression phenotype in four independent transgenic lines; however, one line, which is expressed at lower levels (Fig. 5 and data not shown), gives only a mild overexpression phenotype. The overexpression phenotype cannot be the result of the exogenous HA tag as a full-length Fra-HA receptor does not show this phenotype and in fact, can rescue the *fra*
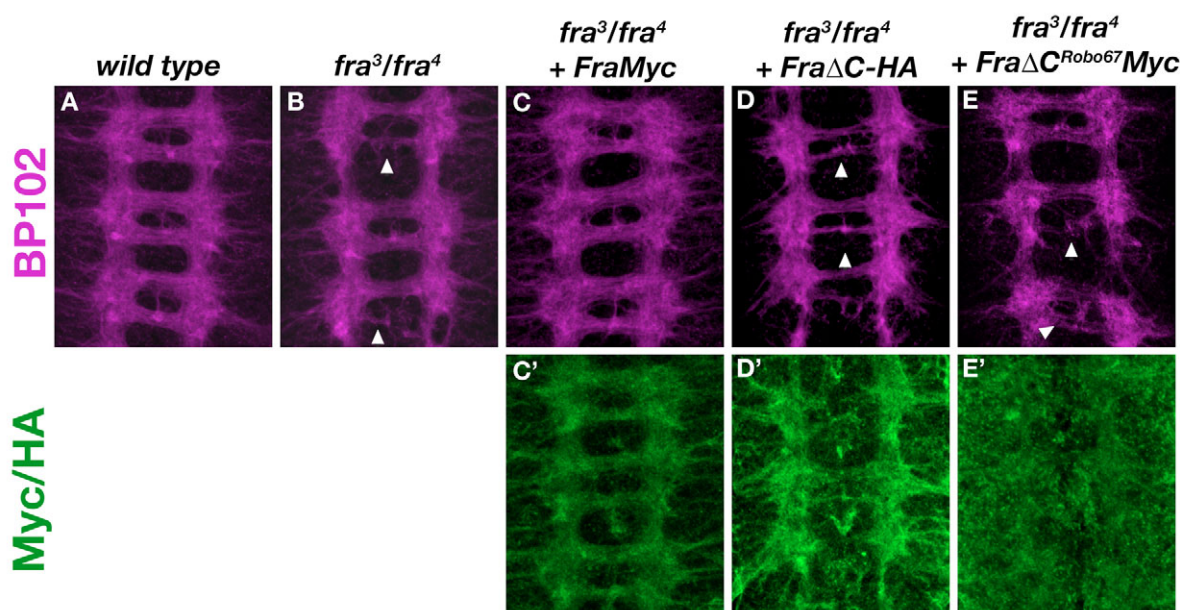
Fig. 5. The cytoplasmic domain of Fra is not required for proper localization. (**A-E'**) Stage 16 embryos stained with BP102 to visualize all axons. (C'-E') Same embryos as C-E. Staining with anti-Myc or anti-HA reveals Fra receptor expression. Anterior is up. (A) Wild-type embryos stained with BP102 exhibit a ladder-like CNS scaffold with distinct thick anterior and posterior commissures. (B) Commissural bundles are thin or absent in *fra* mutant embryos (arrowheads). (C) Expressing a full length Fra receptor in all neurons using *elavGal4* rescues *fra* mutant defects. (C') Wild-type Fra localizes to the axons scaffold when expressed in all neurons. (D) When a weak insert of FraΔC is expressed panneurally in a *fra* mutant background, many axons fail to cross the midline (arrowheads). (D') Despite the inability of FraΔC to rescue *fra* guidance defects, this truncated receptor is still localized to the axon scaffold as well as the wild-type full-length receptor. (E) Expression of FraΔC[Robo67]Myc does not rescue the defects seen in *fra* mutant embryos (arrowheads); however, in contrast to FraΔC, FraΔC[Robo67]Myc does not efficiently localize to axons (E') perhaps because the exogenous Robo sequence interferes with normal Fra receptor distribution.

mutant defects in the EW neurons (Fig. 3B). This suggests that in addition to acting as a dominant negative for wild-type Fra function, FraΔC also affects additional guidance signals including the exciting possibility that it is inhibiting novel attractive pathways.

We next investigated the consequence of expressing FraΔC in a subset of commissural neurons; once again we turned to the Egl neurons. Expression of FraΔC in Egl neurons of wild-type embryos causes a decrease in EW attraction towards the midline (Fig. 6G), though in many segments the EW neurons cross normally. When FraΔC is expressed in *fra* homozygous embryos, 100% of the EW axons fail to cross the midline (Fig. 6H). In some segments, we also observe defects in EG axon guidance (Fig. 6H), a phenotype never observed in *fra* mutants. These data support the hypothesis that in addition to acting as a dominant negative for Fra function, FraΔC is also interfering with an additional attractive pathway (one required for EG guidance) at the *Drosophila* midline. An alternative although not mutually exclusive possibility is that FraΔC might somehow be increasing axon repulsion thereby preventing axons from approaching the midline (see below).

To test whether Netrin is required for the commissureless phenotype caused by FraΔC expression, we expressed UAS-FraΔC in a *Netrin* mutant background. Two different strategies were used to eliminate Netrin function. First, we used a X-chromosomal deletion (NP5) known to completely remove both *Netrin* genes (Mitchell et al., 1996), and second we used a specific *NetAB* double mutant stock generated by homologous recombination (Brankatschk and Dickson, 2006). Expression of FraΔC did not cause a commissureless phenotype in either mutant background (Fig. 7A,B and data not shown) suggesting that this truncated receptor depends on Netrin to produce the phenotype.

The FraΔC phenotype is independent of Unc-5 signaling and partially dependent on Slit-Robo signaling

Since Slit and the Robo family of receptors are essential for midline repulsion (Brose et al., 1999; Kidd et al., 1999; Kidd et al., 1998a; Long et al., 2004), we reasoned if FraΔC is causing an increase in repulsion, then Slit-Robo might be required. To test this possibility, we expressed this mutant construct, in a *fra* homozygous background (the background in which we see a strong commissureless phenotype) in combination with mutations in either *slit* or *robo*. In the *Drosophila* CNS, axons collapse at the midline in *fra, slit* double mutant embryos (Garbe and Bashaw, 2007); this phenotype is quite strong (Fig. 7G). Panneural expression of FraΔC in *fra, slit* embryos still prevents many axons from crossing the midline (Fig. 7H) suggesting that FraΔC can prevent axon collapse in the absence of all midline repulsion. Consistent with this result, expression of FraΔC in a *fra, robo* double mutant background dramatically repels axons, though the defect is less severe than that seen a *fra* single mutant background (Fig. 7E,F; compare with Fig. 6E). These data suggest that Slit-Robo signaling is partially required to generate the strongest FraΔC-induced misexpression phenotype. Similarly, expressing FraΔC in all neurons leads to a partial suppression of the robo single mutant phenotype (Fig. 7C,D). Taken together, the above data suggests that repulsion via the Slit-Robo pathway is not strictly required for FraΔC to elicit a phenotype. In other words, in the absence of either the repulsive ligand Slit or its receptor Robo, axons still avoid the midline when FraΔC is overexpressed suggesting that some other mechanism generates the phenotype.
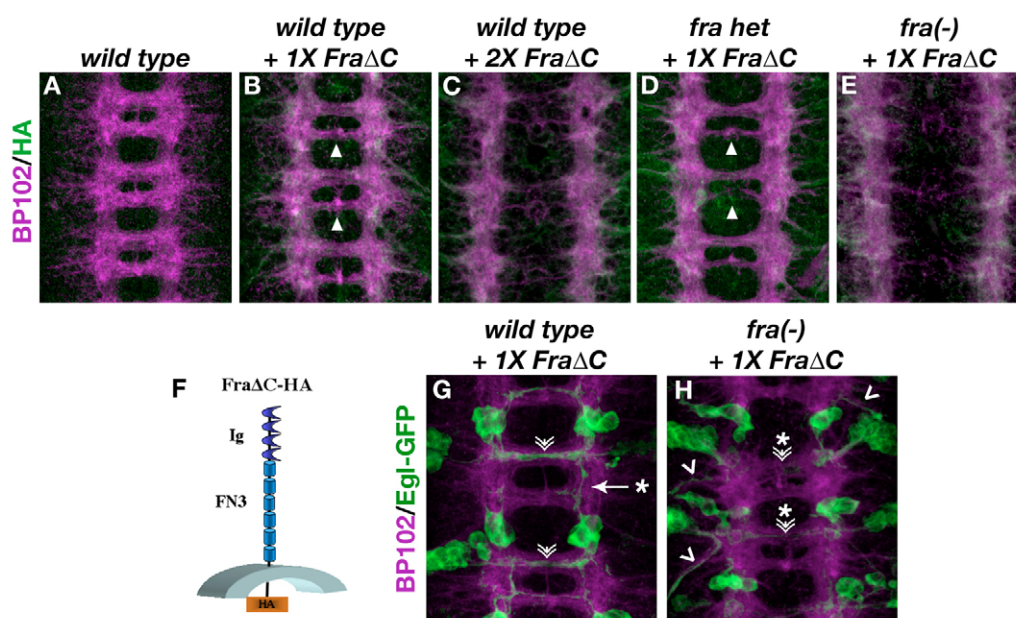
Fig. 6. Axons expressing FraΔC are not attracted toward the midline. (**A-E**) Stage 16 embryos stained with mAb BP102 to visualize all axons (magenta) and anti-HA to reveal FraΔC-HA expression (green). Anterior is up. (A) Wild-type embryos stained with BP102 exhibit a ladder-like CNS scaffold. (B) Commissural bundles are thin when one copy of FraΔC is panneurally expressed in a wild-type background (arrowheads). (C) Axon commissures are almost completely absent when two copies of FraΔC are expressed. (D) Fewer axons cross the midline when one copy of FraΔC is expressed in a *fra* heterozygous background (arrowheads; compare this phenotype to the one in B). (E) When one copy of FraΔC is expressed in a *fra* homozygous mutant background, axons are no longer attracted toward the midline. (**F**) Diagram of the FraΔC-HA construct used in these experiments. (**G,H**) Stage 16 embryos stained with mAb BP102 and anti-GFP to highlight the Egl neurons. (G) When one copy of FraΔC is expressed specifically in the Egl neurons in a wild-type background, EW axons fail to cross the midline in many segments (arrow with star). (H) None of the EW axons cross the midline when FraΔC is expressed in a *fra* mutant background. Additionally, we also see defects in EG axon guidance (starred feathered arrow), a phenotype never observed in *fra* mutants (feathered arrows in G). Specifically, both EW and EG axons are often observed exiting the midline (carets).

Unc-5 is another guidance receptor known to promote axonal repulsion in many organisms (Hamelin et al., 1993; Hedgecock et al., 1990; Hong et al., 1999; Keleman and Dickson, 2001), and in *Drosophila*, ectopic expression of Unc-5 causes a Netrin-dependent phenotype similar to that seen in *comm* mutants (Keleman and Dickson, 2001). Therefore, we tested whether repulsion via Unc-5 is required for the FraΔC overexpression phenotype. This does not appear to be the case; panneural overexpression of FraΔC in a *fra, unc-5* double mutant background still causes a commissureless phenotype indicating that the Unc-5 receptor is not required to prevent axons from approaching the midline (Fig. 7I,J; compare with Fig. 6E).

DISCUSSION
Growth cone steering is a complex process that requires the integration of multiple guidance pathway outputs (Garbe and Bashaw, 2004). Although many molecules have been implicated in mediating guidance decisions, how these factors function together to regulate axon guidance in vivo is poorly understood. Additionally, our knowledge of how non-catalytic receptors (for example DCC/Fra) elicit a response and what domains are required for signaling is not complete. In this study, using a specific subset of commissural neurons in vivo, we confirm the observation of in vitro experiments that the P3 sequence motif of Fra/DCC is required at the *Drosophila* midline for an attractive response. Additionally, our data show that neither P1 nor P2 are required for attractive guidance; neurons are properly guided by receptors missing both of these domains. Our work also demonstrates that the cytoplasmic domain

is not required for normal Fra distribution in axons as was previously reported. Furthermore, while investigating these truncated receptors, we noticed that expression of a receptor lacking the entire cytoplasmic domain causes defects in midline guidance and produces a completely commissureless phenotype in *fra* homozygous embryos. This phenotype is dependent on Netrin, is partially dependent on Slit-Robo signaling, and is independent of the Unc5 receptor suggesting that this misexpression phenotype is not a consequence of repulsion via Unc5 and cannot be completely explained by an increase in Slit-Robo signaling. Therefore, the emergence of a commissureless phenotype is quite exciting as it implies that our truncated Fra receptor is interfering with additional pathways, perhaps those playing a role in axon attraction at the *Drosophila* midline.

Domain requirements for Fra-mediated attraction
At first glance, the domain requirements for DCC/Fra/Unc-40 signaling might appear to differ among species. For example, in vertebrates it has been demonstrated that the P3 conserved sequence motif is essential for the outgrowth and turning of cultured *Xenopus* spinal neurons. Whereas in *C. elegans*, P3 is not required to generate a gain-of-function phenotype associated with expression of MyrUnc-40; however, P1 and P2 are essential for this response (Gitai et al., 2003). Can the difference in motif requirements between vertebrates and *C. elegans* simply be attributed to a divergence in conserved functions for these domains throughout evolution? Given the high degree of sequence similarity of these motifs, this seems unlikely. Here it should be noted that the potential
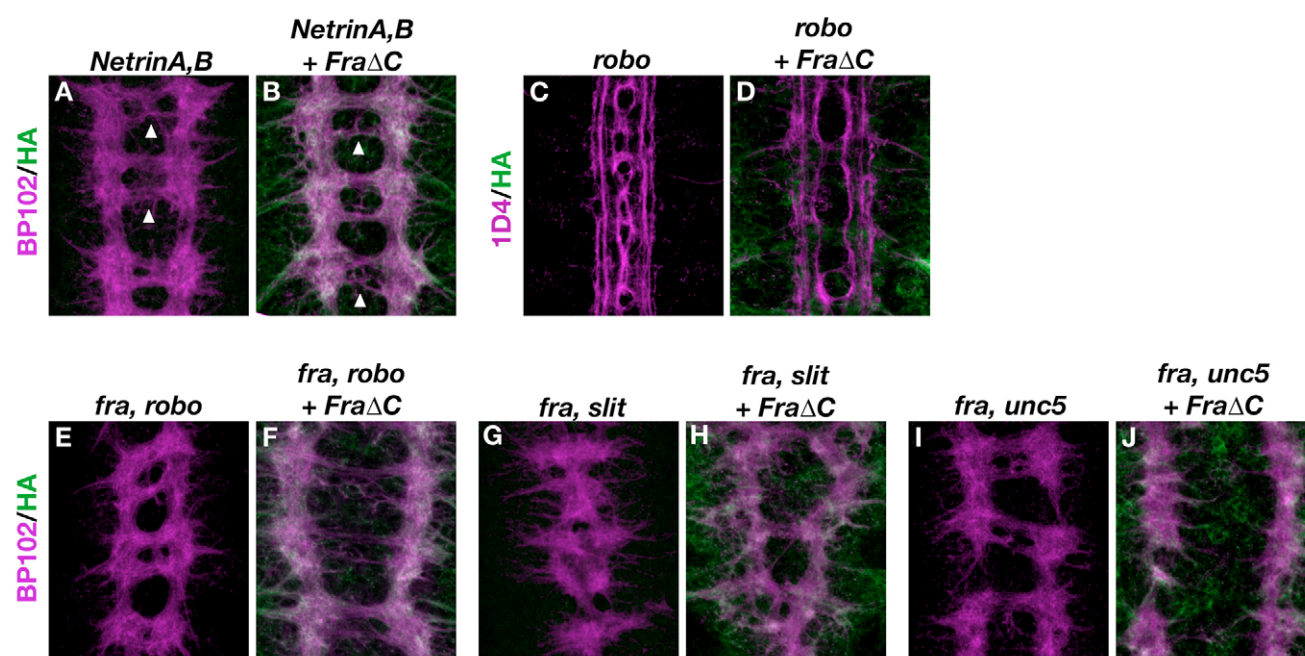
Fig. 7. The FraΔC misexpression phenotype depends on Netrin but not Slit-Robo or Unc-5 signaling. (**A,B,E-J**) Stage 16 embryos stained with mAb BP102 to display all axons and anti-HA to reveal FraΔC-HA expression. Anterior is up. (A) *Netrin* mutants contain many segments with absent or thin commissures suggesting a decrease in midline attraction (arrowheads). (B) Expression of FraΔC cannot enhance the *Netrin* phenotype suggesting that the receptor depends on Netrin to generate an overexpression phenotype (compare this phenotype to the one in Fig. 5E). (**C,D**) Late stage 16 embryos stained with anti-FasII (1D4) to display three ipsilateral bundles of axons and anti-HA to reveal FraΔC-HA expression. Anterior is up. (C) Medial FasII-positive bundles frequently cross and re-cross the midline in *robo* mutants. (D) Fewer FasII-positive axons cross the midline when FraΔC is overexpressed. (E) *fra, robo* double mutants have many extra axons crossing the midline, though the defects are slightly less severe than *robo* single mutants. (F) Expression of FraΔC in all neurons in *fra, robo* double mutants causes a dramatic defect in commissure formation, although some commissures still do form relatively normally. (G) In *fra, slit* double mutants almost all axons collapse at the midline. (H) Expression of FraΔC is able to push axons laterally in a *fra, slit* mutant background. (I,J) The FraΔC misexpression phenotype is not dependent on the presence of the Unc-5 repulsive Netrin receptor.

function of the conserved P1-P3 motifs in full-length Unc-40 receptor signaling in the context of an in vivo attractive decision have not been examined. Therefore, perhaps the apparent divergence in function could simply be attributed to the phenotypic readout of each assay in the individual systems.

In the case of axonal attraction, our data are in agreement with previous reports from vertebrates suggesting that P3 is required for a Netrin-mediated turning response whereas they are inconsistent with P1 and P2 playing obligatory roles. Our deletion receptors also offer the exciting opportunity to study the signaling and/or domain requirements for additional Netrin-Fra-mediated decisions such as the promotion of axon outgrowth and the steering of motor neurons to their appropriate ventral muscle targets.

Involvement of the P3 sequence motif during attractive guidance

In vertebrate systems, P3 has been implicated in mediating two distinct functions. Initially, it was reported that the conserved cytoplasmic P3 sequence motif is necessary and sufficient for ligand-gated receptor multimerization and Netrin-induced attractive turning in cultured *Xenopus* spinal neurons (Stein et al., 2001). Versions of DCC lacking the P3 motif cannot self-associate and neurons expressing this form of DCC are no longer able to respond to Netrin. Replacing the P3 motif with the SAM multimerization domain from Eph tyrosine kinase receptors (Bruckner and Klein, 1998; Thanos et al., 1999) is sufficient to restore an appropriate Netrin response, suggesting that the major function of the P3 domain

is to mediate self-association (Stein et al., 2001). Surprisingly, we have found that P3 does not appear to mediate self-association of the *Drosophila* Fra receptor, as mutants lacking P3 show equivalent biochemical interactions. It seems clear from our data that P3 function in the context of midline attraction is through a mechanism that is independent of mediating receptor multimerization. Although it remains an open question whether the receptor-receptor interactions that we observe in vitro are necessary for attractive guidance, it is clear that in the absence of an intact P3 domain they are not sufficient.

Another set of studies proposed that P3 recruits Fak and this recruitment leads to tyrosine phosphorylation of DCC by Src family non-receptor tyrosine kinases (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004). Both Fak recruitment and tyrosine phosphorylation are important in mediating Netrin-induced outgrowth and attractive turning in cultured neurons in vitro (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). Specifically, the LD-like motif within P3 was shown to play a critical role in FAK association, although a P3-independent binding site was also suggested (Ren et al., 2004). Interestingly, we have found that mutant Fra receptors in which the LD motif is intact, but the rest of P3 is disrupted are still unable to mediate Fra attraction (Fig. 3), suggesting that Fak binding may not be important for Fra function in *Drosophila*. This is also consistent with the observation that *fak* mutants do not have a disrupted CNS phenotype (D.S.G. and G.J.B., unpublished). Nevertheless, future studies will test for genetic interactions between mutations in *fra* and mutations in *fak* and/or *src* in the context of the

Drosophila midline. Here it is worth noting that the tyrosine residue in DCC identified as the principal target of Fyn/Src kinases is not conserved in *Drosophila* Fra or *C. elegans* UNC-40, suggesting that the precise mechanisms by which Fra/DCC/UNC-40 signaling is regulated by tyrosine kinases may differ between organisms. However, the facts that, (1) tyrosine phosphorylation of UNC-40 has been observed in *C. elegans* (though the responsible kinase has not been identified) and (2) UNC-40 signaling appears to be negatively regulated by a receptor tyrosine phosphatase is consistent with an evolutionarily conserved role for tyrosine phosphorylation of the DCC/Fra/UNC-40 family of receptors (Chang et al., 2004; Tong et al., 2001). Furthermore, the Abl kinase has also been implicated in Netrin-Fra signaling in *Drosophila* (Forsthoefel et al., 2005) suggesting that additional phosphorylation events may be important for DCC/Fra/UNC-40 output.

Requirements for Fra localization

Previous data determined that the cytoplasmic domain of Fra is necessary and sufficient for normal distribution of the receptor (Hiramoto et al., 2000). Therefore, we were surprised to find that our newly generated FraΔC-HA localized similarly to the wild-type protein. The original experiments from Hiramoto et al. (Hiramoto et al., 2000) were performed using a truncated Fra receptor (Bashaw and Goodman, 1999) that contained the transmembrane domain and 67 juxtamembrane cytoplasmic amino acids of the Robo receptor (FraΔC^{Robo67}-Myc). We hypothesized that this exogenous protein sequence could be interfering with proper localization of the Fra receptor. Indeed this seems to be the case; whereas the FraΔC-HA construct mimics wild-type receptor localization when expressed in all neurons by *elavGal4*, the original FraΔC^{Robo67}-Myc does not (Fig. 5F′). Therefore, these data suggest that normal Fra localization does not require the cytoplasmic domain. Additional experiments will help determine the specific domains of Fra that are sufficient to control receptor distribution. Finally, all of the above observations are based on overexpression studies and therefore may not completely reflect the localization of endogenous proteins with similar deletions.

How does FraΔC disrupt guidance?

Intriguingly, expression of FraΔC in a *fra* mutant background results in a complete commissureless phenotype, suggesting the possibility that it is capable of inhibiting Fra-independent axon attraction. Although this is one of the simplest interpretations, other hypotheses exist. For example, double mutants between *fra* null alleles and either *abl* or *trio* also exhibit a near commissureless phenotype (Forsthoefel et al., 2005). These data are consistent with Abl and Trio participating in other pathways that are important for guidance toward and across the *Drosophila* midline. Accordingly, one possibility could be that FraΔC is interfering with an independent Abl and/or Trio signaling pathway.

Previous results demonstrated that panneural overexpression of Netrin leads to a phenotype where few axons cross the midline (Kolodziej et al., 1996; Mitchell et al., 1996). It was suggested that wild-type Netrin distribution provides a directional cue that attracts axons across the midline and when Netrin is misexpressed in all neurons, axons become confused and are no longer able to decipher the appropriate path. Along these lines, perhaps the extracellular domain of FraΔC is binding Netrin and 'presenting' it everywhere thereby confusing the axons. Indeed, the Fra receptor has been shown to redistribute Netrin laterally away from its midline source (Hiramoto et al., 2000). However, this theory would have to assume that this specific truncation is somehow misregulated upon ligand binding (for example, perhaps it is not efficiently internalized) since

our other wild-type and deletion receptors – which presumably bind to and relocalize Netrin similarly to FraΔC – do not produce this effect when expressed panneurally. Since we only see a commissureless phenotype when FraΔC is overexpressed in a *fra* background, we would also have to argue that another receptor elicits the response to this un-internalized and redistributed Netrin.

Finally, FraΔC expression may cause increased midline repulsion, thereby preventing axons from crossing the midline. Accordingly, the FraΔC misexpression phenotype shows a striking similarity to embryos deficient for the gene *comm*. Comm is a single-pass transmembrane protein that acts to downregulate Robo expression (Keleman et al., 2002; Keleman et al., 2005) and *comm, robo* double mutants resemble *robo* single mutants indicating that *comm* acts upstream of the Robo receptor (Kidd et al., 1998b). Therefore, if FraΔC misregulates Comm, then perhaps Robo levels are increased and axons are repelled away from the midline. This argument would imply that overexpressing FraΔC in a *robo* mutant background should produce *robo*-like mutants (similar to the *comm, robo* double mutants). Contrary to this hypothesis, we observe that FraΔC expression can partially suppress a *robo* loss-of-function phenotype suggesting that FraΔC is not simply misregulating Comm. However, since FraΔC cannot prevent all axons from approaching the midline in *fra, slit* double mutants, and given that the FraΔC overexpression phenotype in a *fra, robo* double mutant background is weaker than that seen in *fra* single mutants, we cannot rule out the possibility that signaling through Robo is partially required and/or that the upregulation of another Robo family member, for example Robo2, prevents axons from approaching the midline when FraΔC is expressed in all neurons. Whatever the mechanism by which FraΔC exerts its influence on commissural axon guidance, it may provide an important route to a further dissection of the missing factors that function in addition to Netrin and Fra to guide axons across the midline.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/24/4325/DC1

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