The phylogenetically conserved Netrin family of chemoattractants signal outgrowth and attractive turning of commissural axons through the Deleted in Colorectal Carcinoma (DCC) family of receptors. Src family kinases are thought to be major signaling effectors of Netrin/DCC. In vertebrates, Src and the closely related Fyn kinases phosphorylate DCC and form a receptor-bound signaling complex leading to activation of downstream effectors. Here we show that, in the Drosophila embryonic CNS, Src kinases are dispensable for midline attraction of commissural axons. Consistent with this observation, tyrosine phosphorylation of the Netrin receptor DCC or its Drosophila ortholog, Frazzled, is not necessary for attraction to Netrin. Moreover, we uncover an unexpected function of Src kinases: inhibition of midline axon crossing through a novel mechanism. We propose that distinct signaling outputs must exist for midline axon crossing independent of Src kinases in commissural neurons.
poset the existence of a novel Netrin–DCC signaling output that is Src independent.

Materials and Methods
Molecular biology. For Fra-Myc and DCC-Myc, all generated transgenic constructs were cloned into a pUAST vector containing 10× UAS and an attB site for PhoC31-mediated targeted insertion (p10UAST-attB). All were cloned along with a C-terminal 6× Myc epitope. Fra-Myc was cloned as an EcoR1/Not1 fragment from pUAST-Fra-Myc (Garbe and Bashaw, 2007). Rat DCC and DCCY1418F were cloned from pRKn5-DCC (Li et al., 2002) and pRKn5-DCCY1418F (Meriane et al., 2004) in two steps into pUAST-attB using an EcoR1/Xba1 fragment followed by an EcoR1/EcoR1 fragment. Fra-YF was generated by stepwise PCR mutagenesis of individual or multiple sites in close proximity. Mutated tyrosine residues were Y1113, Y1170, Y1189, Y1193, Y1207, Y1212, Y1247, Y1250, and Y1313. All constructs were fully sequenced. Transgenic flies were generated by Best Gene.

Genetics. The following alleles were used in this study: for fra, fra1, fra3, Df(2R)vg135 (Kolodziej et al., 1996), and fra4 (Yang et al., 2009); for Netrin, NetAB (Brankatsch and Dickson, 2006); for Scr42A, Scr42ASt1 (Tatenoo et al., 2000), Scr42ASt10 (Lu and Li, 1999); for Src64BB, Src64BBKO (O’Reilly et al., 2006); for drr1 (Callahan et al., 1995); for Unc-5, Unc-52 (Labrador et al., 2005); for myospheroid, mys1 (Wright, 1990); for roundabout, rob2 (Kidd et al., 1998); for egl-6 (egl-6) (Dittrich et al., 1997); and for apertuous, apet1 (Benveniste et al., 1998). The following transgenes were used: (1) P[UAS-Fra-Myc]68EFb, (2) P[UAS-Fra-YF-Myc]68Fa, (3) P[UAS-DCC-Myc]68EFb, (4) P[UAS-DCCY1418F-Myc]68EFb, (5) P[UAS-Fra-AC-HA]46 (Garbe et al., 2007), (6) P[UAS-TauMycGFP]II, (7) P[UAS-TauMycGFP]III, (8) constitutively active Src64B, P[UAS-Src64BE1]27, and (9) P[Gal4-clav-L3]. All crosses were performed at 25°C. Embryos were genotyped using a combination of marked balancer chromosomes, the presence of linked transgenes, or, in the case of NetABΔ, the absence of fluorescent mRNA in situ hybridization signal. Where possible, all comparative phenotypes were analyzed in the same embryo, the percentage of noncrossing segments was calculated. A segment was considered noncrossing when both clusters of EW axons (six axons per segment) failed to make an orthogonal turn toward the midline. SEM as depicted in figures was based on the number of embryos per genotype. For apertous ectopic crossing phenotypes, whole-mount embryos were analyzed at Stage 17. Eight abdominal segments were scored per embryo. When a segment contained a continuous crossing of at least the thickness of incoming axons from ap cell bodies, it was considered an ectopic cross. For muscle 6/7 innervation defects, Stage 17 embryos were filleted. Ten abdominal hemisegments were analyzed per embryo. An innervation was considered absent when no projection of FasII-positive axons could be detected originating from the intersegmental nerve b in the muscle 6/7 cleft. Only segments where muscles and nerve had not been disrupted in the dissection process were analyzed. Muscles were identified using DIC optics. For quantification of phenotypes using mAb BP102, posterior commissures were scored as defective if they were absent or substantially thinner than in wild-type (WT) embryos. For statistical analysis of guidance phenotypes, comparisons were made using generalized estimate equations for clustered binary data, using R software. Correlation structure was chosen based on calculation of quasi-log-likelihood under the independence model information criterion and correlation information criterion as described previously (Pan, 2001; Hin and Wang 2009). For multiple comparisons, a post hoc Bonferroni correction was applied. The p values are based on corresponding Wald statistics.

Results
Drosophila Src mutants are not sufficient in midline axon attraction, but resemble integrin loss-of-function mutants
Based on the model of receptor-associated kinase signaling in vertebrates (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004), we expected that Src mutants in Drosophila would have defects in midline axon attraction, similar to Netrin and fra mutants. Netrin and Fra are required primarily for the formation of axonal commissures of the embryonic CNS. We were surprised, however, to see that CNS axons appeared to cross relatively normally in embryos lacking both of the two Drosophila Src genes, Scr42A and Src64B (Wouda et al., 2008). We therefore decided to examine Src mutants more closely to determine whether these embryos have subtle axon crossing defects. Using an antibody to label all axons in single and double Src mutants, we found that most commissural axons appear to cross appropriately, although there are defects in the separation of the anterior and posterior commissures in double mutants, as reported previously (Fig. 1G) (Wouda et al., 2008). To evaluate commissural axon guidance more quantitatively, we labeled the eagle-positive subset of commissural neurons (EW neurons) using eg-Gal4 to drive expression of an axon marker, Tau-Myc-GFP. However, we found no defects in EW midline axon crossing, even in Scr42A; Src64B double mutants (Fig. 1J–N; Table 1).

In contrast to the relatively normal CNS in single Src mutants, in Scr42A; Src64B double mutants there are severe defects in FasII-positive ipsilateral axons, which often cross the midline inappropriately (Fig. 1G). These axons depend on repulsive Slit–Robo signaling for pathfinding (Seeger et al., 1993; Kidd et al., 1998), but often cross in embryos in which adhesion has been reduced as well, as seen in integrin loss-of-function mutants (Loureiro and Peifer, 1998; Speicher et al., 1998; Stevens and Jacobs, 2002). Accompanying these CNS malformations are profound patterning defects including partial head involution, defective dorsal closure, and a failure of germ-band retraction, as reported previously (Lu and Li, 1999; Takahashi et al., 2005). Because we observe these patterning defects, and because midline and lateral glia are frequently mispositioned in these mutants (Wouda et al., 2008) (data not shown), it is difficult to conclusively interpret the CNS phenotype in these embryos.

Src antagonizes midline axon crossing through an integrin-independent pathway
The pleiotropic defects in Src double mutants confound the interpretation of the midline crossing phenotype of EW neurons. It
is possible, though unlikely, that SFKs play an essential role in midline axon crossing that is masked in this genetic background due to a requirement for Src function in an independent process. In principle, this function should be revealed in sensitized genetic backgrounds. If Src function is essential in Netrin-dependent attraction, this should be evident when Netrin signaling is partially traction, this should be evident when Netrin signaling is partially

| Genotype | Noncrossing (%) | SEM (%) | n (segments) | n (embryos) | p       |
|----------|-----------------|---------|--------------|-------------|---------|
| src mutants: |                 |         |              |             |         |
| fra[1] [UAS-TauMycGFP] fra[2] [eg-Gal4]/+ | 20.15 | 4.06 | 168 | 21 |         |
| Src42A[1]/Src42A[2] [UAS-TauMycGFP] [eg-Gal4]/+ | 0.63 | 0.63 | 160 | 20 |         |
| Src42A[1]/[UAS-TauMycGFP] [eg-Gal4]/Src64B[4]/ | 0 | 0 | 120 | 15 |         |
| Src42A[1]/ [UAS-TauMycGFP] Src42A[2] [10108] [eg-Gal4]/Src64B[4] | 0.89 | 0.89 | 110 | 14 |         |
| fra[1] [UAS-TauMycGFP] [Ssrc42A] [10108]/Src64B[4] [eg-Gal4] | 0 | 0 | 88 | 11 |         |
| fra rescue in EW neurons: |                 |         |              |             |         |
| fra[1] [UAS-TauMycGFP] fra[2] [eg-Gal4]/+ | 25.78 | 3.93 | 183 | 23 |         |
| fra[1] [UAS-TauMycGFP] fra[2] [eg-Gal4] | 11.88 | 3.20 | 160 | 20 | 0.027  |
| fra[1] [UAS-TauMycGFP] fra[2] [eg-Gal4] [UAS-DCCTW-Myc] | 3.75 | 1.60 | 160 | 20 | <0.0001 |
| fra[1] [UAS-TauMycGFP] fra[2] [eg-Gal4] [UAS-DCCTY1418B-Myc] | 6.34 | 2.95 | 159 | 20 | 0.009  |
| fra[1] [UAS-TauMycGFP] fra[2] [eg-Gal4] [UAS-Fra9YF-Myc] | 5.56 | 2.20 | 72 | 9 | <0.0001 |
| fra[1] [UAS-TauMycGFP] [DI2Ry1135] [eg-Gal4]/+ | 27.08 | 3.72 | 96 | 12 |         |
| fra[1] [UAS-TauMycGFP] [DI2Ry1135] [eg-Gal4]/ [UAS-Fra9YF-Myc] | 5.09 | 1.80 | 216 | 27 | <0.0001 |
| fra[1] [UAS-TauMycGFP] [DI2Ry1135] [eg-Gal4] [UAS-DCCTW-Myc] | 2.50 | 1.15 | 160 | 20 | <0.0001 |
| fra[1] [UAS-TauMycGFP] [DI2Ry1135] [eg-Gal4] [UAS-DCCTY1418B-Myc] | 5.15 | 2.16 | 136 | 17 | 0.00014 |
| fra[1] [UAS-TauMycGFP] [DI2Ry1135] [eg-Gal4] [UAS-Fra9YF-Myc] | 4.17 | 1.57 | 168 | 21 | <0.0001 |

Stage 15 and 16 embryos were whole mounted and scored for the EW noncrossing phenotype (see Materials and Methods). For rescue experiments, p values for each subgroup are relative to the control fra mutant phenotype (listed first).
Src42A and Src64B antagonize midline axon crossing. A–D, EW midline crossing defects were scored at Stages 15 and 16 using eg-Gal4 to express TauMycGFP, after immunostaining for anti-GFP. A, Wild-type embryo. B, An embryo expressing DN-Fra in, e.g., neurons. Most EW axons misproject (arrows). C, An fra+/fra hypomorphic mutant. EW axons fail to cross in ~20% of segments (arrow). D, quantification of EW crossing defects in DN-Fra (left) and frahyp (right) backgrounds. Reduction in Src gene dose rescues midline crossing defects, while increasing Src activity in EW neurons increases phenotypic severity. Error bars indicate SEM. p values are calculated from Wald statistics, relative to the control background, DN-Fra (left), and frahyp (right). *p < 0.05; **p < 0.001. See Materials and Methods for details on statistical analysis. E–G, DN-Fra expression is not reduced in Src mutants. Embryos expressing TauMycGFP (F, G, anti-GFP, green) and DN-Fra (E, J, anti-HA, magenta) in EW neurons exhibit severe crossing defects (E–G, arrows) in wild-type embryos (E–G) that are almost fully rescued in Src42A/+; Src64A–/– mutants (H–J). Specific genotypes are as follows: A, “wt”: [eg-Gal4];[UAS-TauMycGFP]/+; B, “DN-Fra”: [UAS-Frac-HA]4,[UAS-TauMycGFP]/+; C, “frahyp”: [UAS-Frac-HA]4,[UAS-TauMycGFP]/frahyp; D, left, “+” [UAS-Frac-HA]4,[UAS-TauMycGFP]/+; D, right, “−” [UAS-DN-Fra]4, [UAS-TauMycGFP]/+; E, F, H, I, J, “Src42k10108”: [UAS-frac-HA]4,[UAS-TauMycGFP]/Src42k10108; anti-GFP, green; anti-HA (DN-Fra), magenta. *p < 0.001. See Materials and Methods for details on statistical analysis. **p < 0.001. See Materials and Methods for details on statistical analysis.
While we detect Unc-5 mRNA expression in neuroblasts that give rise to EW neurons, this expression is eliminated in the EW neurons before axogenesis and is only maintained in their sibling, the GW motor neuron (data not shown). Moreover, Unc-5 mutations do not modify the fra loss-of-function phenotype in EW neurons (Fig. 3E). Thus, Src likely inhibits midline axon crossing through a pathway independent of Unc-5 and Netrin.

To determine whether Src acts in parallel to Fra in commissural guidance, we tested whether Src mutations suppress EW crossing defects in fra null mutants using the predicted null fra alleles. We find that in contrast to fra hypomorphs, heterozygosity for Src64KO does not suppress crossing defects in fra mutants (data not shown), suggesting that Src might play a role in the noncanonical, Netrin-independent fra pathway (Yang et al., 2009).

The existence of an additional attractive or repulsive pathway promoting midline axon crossing in Drosophila has been postulated due to the partially penetrant defects in Netrin and fra mutants. SFKs can function in multiple signaling pathways involved in axon guidance in Drosophila, which might account for these genetic interactions in commissural neurons. For example, Src64B acts in the Wnt5-Derailed (Drl)/Ryk pathway to promote anterior commissure choice (Wouda et al., 2008). In addition, in multiple systems, SFKs play a central role in integrin signaling, an important pathway in Drosophila axon guidance (Hoang and Chiba, 1998; Stevens and Jacobs, 2002; Legate et al., 2009), which could in principle account for our observed genetic interactions. To test these possibilities, we introduced mutations in components of these pathways into sensitized genetic backgrounds and quantified the EW crossing phenotypes (Fig. 3E). Drl heterozygous or homozygous mutations do not suppress the fra loss-of-function phenotype in EW neurons. Similar results were obtained using mutations in the single Integrin PS gene in Drosophila, mysophoroid. Midline crossing defects caused by DN-Fra expression are not suppressed in robo mutants, suggesting Src’s effects on midline crossing are not exclusively through regulation of the Slit–Robo pathway. These results indicate that Src likely inhibits midline axon crossing through a novel Integrin- and Derailed/Ryk-independent signaling pathway.

**DCC receptor phosphorylation is dispensable for Netrin-dependent axon attraction in Drosophila**

One mechanism by which Src has been proposed to mediate Netrin-signaling is through direct receptor phosphorylation, presumably leading to the assembly of a downstream signaling complex that causes Rac activation (Li et al., 2004; Meriane et al., 2004). This precise mechanism of Src-dependent Netrin signaling is unlikely to occur in Drosophila because the essential tyrosine residue implicated in these studies is not conserved in Fra; however, a similar process could occur centering on one or multiple alternative tyrosine residues. To directly address whether a similar mechanism occurs in Drosophila, we sought to rescue fra loss of function phenotypes using rat DCC or Fra receptors in which tyrosine residues were mutated to phenylalanines. We generated transgenic flies expressing DCC or Fra with C-terminal Myc tags under Gal4/UAS control. To eliminate position effects, all DCC and Fra constructs used in these studies were inserted at the same genomic location, and are expressed and localized comparably...
when driven by the pan-neural elav-Gal4 (see Fig. 5H,I). To first determine whether rat DCC can signal in response to Drosophila Netrin, we made use of a gain-of-function assay in an ipsilaterally projecting subset of neurons using apterous-Gal4 (ap-Gal4). When either Fra or DCC is expressed in these neurons, their axons aberrantly cross the midline (Fig. 4A–D). Importantly, the DCC-dependent crossing defects in this background are suppressed in NetABA mutants, suggesting that this receptor can signal in response to Drosophila Netrin (Fig. 4D).

To determine whether DCC can functionally compensate for Fra in commissural neurons, we expressed DCC constructs in EW neurons in fra mutants. DCC rescues fra midline crossing defects in EW neurons to a similar degree as Drosophila Fra (Fig. 5A–D,G; Table 1). Based on experiments in Xenopus neurons, we expected that a DCC receptor with a mutation in the Fyn target tyrosine site, DCCY1418F, would behave like a dominant-negative receptor. Surprisingly, however, DCCY1418F fully rescues EW crossing defects (Fig. 5E,G; Table 1). DCCY1418F also generates a quantitatively similar phenotype to wild-type DCC when expressed in ap neurons (Fig. 4D). From these data, we conclude that the essential signaling motifs for Netrin-dependent commissural axon guidance are conserved between DCC and Fra, and that tyrosine phosphorylation of DCC at Y1418 is not required for its function in these neurons.

Tyrosine phosphorylation of Fra is not required for CNS or motor axon guidance

Based on these results, it appears that the role of Src family kinases in Drosophila axon guidance is distinct from that proposed in vertebrates. These results do, however, leave open the possibility that another nonreceptor tyrosine kinase may have a similar function in Drosophila. To determine whether tyrosine phosphorylation of Fra is involved in Netrin signaling, we tested whether a Fra receptor bearing mutations in all nine of the cytoplasmic tyrosines (Fra-9YF) can functionally replace endogenous fra in embryonic axons. We thus generated flies that express Fra-9YF under Gal4/UAS control. Using elav-Gal4 to drive expression in all neurons, Fra-9YF fully rescues fra commissural axon defects as visualized using the BP102 antibody to label CNS axons (Fig. 6A–D). Fra-9YF also rescues EW midline crossing defects in fra mutants to a similar extent as wild-type Fra (Fig. 5F; Table 1). These results suggest that tyrosine phosphorylation of Fra is not necessary for commissural axon guidance. fra mutants also have defects in motor axon guidance; in particular, the innervation of the Netrin-expressing ventral muscles 6/7 is frequently absent (Fig. 6G,H,K) (Mitchell et al., 1996), as visualized using the motor axon marker anti-FasII. Both wild-type Fra and Fra-9YF rescue these motor axon guidance defects when driven by elav-Gal4 (Fig. 6J,L), indicating that tyrosine phosphorylation of Fra is dispensable for both commissural and motor axon guidance. Pan-neural expression of DCC does not, however, rescue motor guidance defects or longitudinal connective defects (Fig. 6E,L), and only mildly rescues the commissural guidance phenotype in fra mutants as assayed using BP102 (Fig. 6E), precluding the analysis of DCCY1418F in these contexts. Fra regulates the formation of longitudinal connectives through a nonautonomous function involving localization and presentation of Netrin (Hiramoto et al., 2000). These nonautonomous functions may not be conserved in DCC, which may explain the failure to rescue other fra-dependent embryonic phenotypes.

Discussion

We have found that in Drosophila, tyrosine phosphorylation of the attractive Netrin receptor Frazzled is not required for its embryonic axon guidance functions, and that Src tyrosine kinases...
antagonize Netrin-dependent axon attraction. These results contrast with the prevailing model of Src-dependent signal transduction through the DCC family of receptors (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004; Round and Stein, 2007). There are three explanations that could potentially account for this discrepancy, which we will discuss here.

First, species-specific differences in signal transduction may have evolved between Drosophila and vertebrates. Supporting this possibility are the combined observations that DCC family members have multiple signaling outputs encoded by distinct cytoplasmic domains. For example, in Caenorhabditis elegans, the cytoplasmic P1 motif regulates branching and outgrowth through unc-34/enabled, and the P2 motif does so through a Rac-dependent pathway (Gitai et al., 2003). The P1 motif also regulates local mRNA translation in vertebrates (Tcherkezian et al., 2010), while the P3 motif interacts with phosphatidylinositol transfer protein alpha (Xie et al., 2005), Myosin X (Zhu et al., 2007), and FAK (Li et al., 2004; Ren et al., 2004; Lai Wing Sun et al., 2011). Only a subset of these signal transduction mechanisms may be required in a particular species. We do not favor this interpretation, although we cannot rule it out based on our observations. Because DCC can fully rescue the fra mutant phenotype in EW commissural neurons, we suggest that if there are Drosophila-specific signaling outputs downstream of Netrin in commissural neurons, these are retained in the vertebrate receptor. Also, with few exceptions, the diverse signaling outputs mentioned above are all associated with highly conserved cytoplasmic domains, the P1, P2, and P3 motifs, though functional conservation between species has not been directly tested using these domains.

A second explanation for these contrasting results is that DCC’s function in different cell types may reflect distinct cell-biological outputs, such that a particular signaling mechanism may only be necessary in a specific cell type or process. Support for this possibility comes from the observation that in response to Netrin, neurons expressing DCC family members can undergo multiple changes in cell morphology including polarization, axon outgrowth, axon turning, axon branching, and synaptic growth (Round and Stein, 2007; Lai Wing Sun et al., 2011). The particular changes in cell morphology that occur in response to Netrin depend on the cell type being evaluated, as well as the intracellular complement of signaling effectors and second messengers expressed at a given point in time. In some cases, intracellular effectors that have been implicated in mediating one of these diverse cell-biological outputs are not necessary for a different cellular response. For example, the tripartite motif protein encoded by the C. elegans gene madd-2 is required for axon branching and attractive guidance, but not for axon outgrowth induced by a constitutively active myristoylated Unc-40 receptor (Hao et al., 2010). While we cannot assay the intracellular environment in the cell types we tested, we provide evidence here that in at least two different neural cell types, embryonic commissural...
Figure 6. Fra9YF is equivalent to wild-type Fra in motor and CNS axon guidance. A–E, Stage 16 embryos immunostained with mAb BP102 to visualize CNS axons. Genotypes are boxed below panels. A, Control embryo. B, fra2 mutant. Posterior commissures are thin or absent (arrow), and occasional breaks in longitudinal connectives occur (asterisk). C–E, Pan-neural rescue of fra2 mutants using elav-Gal4. C, Fra-WT rescues both commissural and longitudinal defects. D, Fra-9YF rescue similar to Fra-WT. E, DCC-WT shows marginal rescue of commissural thickness and fails to rescue longitudinal defects (asterisk). F, Diagram shows the location of nine cytoplasmic tyrosines (Y, blue) in wild-type Fra and the corresponding phenylalanine (F, red) residues in Fra-9YF. G–J, Stage 17 embryonic ventral motor field showing motor axons immunostained with anti-FasII. Arrows indicate muscle 6/7 innervation. G, Control embryo. Most muscle 6/7 clefts show a FasII-positive axon projection. H, fra3 mutant. Two segments show proper targeting (arrows), but in one segment (asterisk), the 6/7 projection is absent. In this case, the RP3 axon has apparently stalled (right of the asterisk). I, J, Pan-neural rescue of fra2 using elav-Gal4. I, Fra-WT rescue. Most 6/7 clefts are properly targeted. J, Fra-9YF rescue. This phenotype is indistinguishable from Fra-WT. K, Diagram depicting the location of Netrin-expressing muscle 6/7 (green), whose cleft is innervated by a FasII-positive axon (magenta, arrow). L, Quantification of muscle 6/7 defects. Fra9YF rescues to a similar extent as Fra-WT, though DCC-WT does not. Error bars indicate SEM. *p < 0.05.

interneurons and motor neurons, tyrosine phosphorylation of Fra is dispensable for Netrin-dependent guidance functions. Based on these observations, we conclude that if differences in intracellular milieu account for these distinct signaling requirements, then these must be shared between the two neural cell types we have assayed here.

An alternative to these possibilities, which are not mutually exclusive, is based on the observation that the substrate of adhesion dictates the intracellular signaling requirements and/or the directional growth of a migrating axon. Thus, navigating growth cones in vivo, which are likely to encounter distinct substrates than cultured cells, may respond differentially to perturbations in a signaling cascade. This is perhaps best exemplified by the observation that in retinal ganglion cells expressing DCC, culturing on Laminin converts the normal attractive turning responses to repulsion (Höpker et al., 1999). The experiments performed by Meriane et al. (2004) and Li et al. (2004) using tyrosine mutant DCC receptors involved cultured cells, which were likely exposed to a different complement of adhesive substrates than the Drosophila neurons we have assayed here. However, experiments performed by Liu et al. (2004) showed that in spinal cord explant cultures, presumably exposed to the normal in vivo extracellular environment, inhibition of Fyn blocks turning responses to Netrin. Thus, culture conditions are unlikely to fully explain the differing results here. Rescue experiments in vertebrates should allow help distinguish between these possibilities. For example, if DCC Y1418F can rescue guidance defects in commissural neurons in dce mutants, then this result would suggest that culture conditions are likely to explain these discrepancies. The alternative outcome would suggest that either species or cell-type-specific differences in signaling are more likely to explain these results.

We have also shown that in addition to being dispensable for Netrin-dependent attraction in commissural neurons, Src family kinases actually antagonize midline axon crossing. Our observed dose-dependent genetic interactions are consistent with Src functioning to inhibit Fra, although our results suggest there must be Netrin-independent functions as well. So how, then, does Src antagonize midline crossing? We have tested multiple guidance pathways that use Src as a signaling effector that could, in principle, account for the genetic interactions we have observed here. However, this effect does not appear to be regulated by signaling downstream of integrins, the Drl/Ryk receptor, or Unc-5. Moreover, it is unlikely that the mechanism of Src-dependent inhibition of midline crossing occurs through direct phosphorylation of Fra, because we do not observe increased activity of the Fra9YF receptor when expressed in EW or apterous neurons.

Together, our observations suggest that Src likely functions in a novel parallel pathway to inhibit midline axon crossing. The partially penetrant phenotype of fra and Netrin mutants suggests that there must be a additional pathway promoting midline crossing in the Drosophila CNS. This Src-regulated pathway could potentially be either attractive or repulsive. Fra has been shown to regulate midline crossing through a canonical, Netrin-independent pathway as well as a noncanonical Netrin-independent pathway (Yang et al., 2009). This Netrin-independent pathway occurs through transcriptional regulation of the Robo inhibitor, com-
misssureless. Our results in fra-null mutants are consistent with Src functioning in part to antagonize this pathway. However, the role of SFKs in commissural guidance is unlikely to exclusively involve repulsive Slit–Robo signaling because robo homozygous mutants do not suppress defects in the same genetic background that we have seen strong suppression using Src alleles. In vertebrates, the morphogen Sonic Hedgehog attracts commissural neurons to the floor plate through a SFK-dependent pathway (Yam et al., 2009). However, there is no evidence that Hedgehog directs commissural axons in Drosophila, and, given our results, Src kinases are unlikely to play a similar role as they antagonize midline crossing here. Two additional guidance cues regulate commissural axon guidance in the vertebrate CNS: ephrins and semaphorins (Evans and Bashaw, 2010). While there is evidence that SFKs play a role in ephrin and semaphorin signal transduction (Arvanitis and Davy, 2008; Zhou et al., 2008), data linking these cues to commissural guidance in signal transduction (Arvanitis and Davy, 2008; Zhou et al., 2008), there is evidence that SFKs play a role in ephrin and semaphorin signal transduction (Arvanitis and Davy, 2008; Zhou et al., 2008), data linking these cues to commissural guidance in Drosophila are lacking. Thus, the future identification of this novel pathway, which is likely regulated by Src activity, will yield a more complete understanding of mechanisms of midline axon crossing.

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