Development/Plasticity/Repair

Src Inhibits Midline Axon Crossing Independent of Frazzled/Deleted in Colorectal Carcinoma (DCC) Receptor Tyrosine Phosphorylation

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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.

Introduction

Bilaterally symmetric animals must coordinate left and right sensorimotor information. Contralateral connectivity is in part achieved during embryogenesis when commissural neurons project axons across the midline, a source of instructive cues. In bilaterians, midline-derived Netrin and its neuronal receptor Deleted in Colorectal Carcinoma (DCC) promote commissural axon crossing (Evans and Bashaw, 2010). Embryos lacking Netrins or DCC have profound commissural axon defects in all animals studied, though much of the mechanism of Netrin–DCC signal transduction has been revealed through *in vitro* approaches (Round and Stein, 2007). DCC family members have no known catalytic motifs, and axon attraction to Netrin through DCC is thought to involve a combination of locally induced changes in second messengers as well as activation of intracellular kinase-dependent signaling cascades (Lai Wing Sun et al., 2011).

One output of Netrin signaling is the regulation of the Rho family GTPases, Rac and Cdc42 (Li et al., 2002; Shekarabi and Kennedy, 2002; Gitai et al., 2003; Shekarabi et al., 2005). Though the precise mechanism of Rac regulation is not known, it has been proposed that tyrosine phosphorylation of DCC by Src family kinases (SFKs) results in the formation of a signaling complex that activates Rac (Meriane et al., 2004). Consistent with this model, Netrin stimulation recruits SFKs to the DCC receptor cytoplasmic domain through focal adhesion kinase (FAK) (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). Pharmacological inhibition or genetic disruption of SFK activity blocks Netrin-dependent responses in cultured neurons (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004). Moreover, a DCC receptor bearing a mutation of the Fyn/Src target tyrosine (Y1420F) acts as a dominant negative when expressed in cultured *Xenopus* spinal neurons (Li et al., 2004). These data suggest that the phenotype of *Src* loss-of-function mutants should mimic the loss of Netrin or DCC. Knock-out (KO) embryos deficient for the two SFKs implicated in these studies (Fyn and Src) develop relatively normally with few overt phenotypic defects. However, commissural axon pathfinding in these mice has not been closely analyzed (Soriano et al., 1991; Stein et al., 1992). Also, given the large Src gene family in vertebrates, other SFKs might compensate for the loss of Src and Fyn in these animals, as they do in other processes (Stein et al., 1994).

In *Drosophila*, only two genes encode SFKs: *Src42A* and *Src64B.* Therefore, we reasoned that the *Drosophila* embryonic CNS could be a simpler system to understand the *in vivo* contribution of SFKs to Netrin signaling. Embryonic commissural neurons require both Netrin (encoded by *NetA* and *NetB* genes) and the fly ortholog of DCC, Frazzled (Fra), for midline axon crossing (Kolodziej et al., 1996; Mitchell et al., 1996). We find here that, in contrast to the proposed function of SFKs as effectors of Netrin signaling, Src kinases antagonize midline axon crossing in *Drosophila* through a novel pathway. Additionally, we show that tyrosine phosphorylation of DCC receptors is dispensable for their roles in commissural and motor axon guidance. We therefore

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posit 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 PhiC31-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 pRK5-DCC (Li et al., 2002) and pRK5-DCCY1418F (Meriane et al., 2004) in two steps into p10UAST-attB using an EcoR1/Xba1 fragment followed by an EcoR1/EcoR1 fragment. Fra-9YF was generated by stepwise PCR mutagenesis of individual or multiple sites in close proximity. Mutated tyrosine residues are 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 *frazzled*, fra^3, fra^4, Df(2R)vg135 (Kolodziej et al., 1996), and fra^6 (Yang et al., 2009); for *Netrin*, *NetABΔ* (Brankatschk and Dickson, 2006); for *Src42A*, $Src42A^{E1}$ (Tateno et al., 2000), $Src42A^{k10108}$ (Lu and Li, 1999); for *Src64BB*, $Src64B^{KO}$ (O'Reilly et al., 2006); for *derailed*, drl^{R343} (Callahan et al., 1995); for *Unc-5*, $Unc\text{-}5^2$ (Labrador et al., 2005); for *myospheroid*, mys^1 (Wright, 1960); for *roundabout*, $robo^1$ (Kidd et al., 1998); for *eagle*, eg^{MZ360}(*eg*-Gal4) (Dittrich et al., 1997); and for *apterous*, ap^{Gal4} (Benveniste et al., 1998). The following transgenes were used: (1) *P{UAS-Fra-Myc}86Fb*, (2) *P{UAS-Fra-9YF-Myc}86Fb*, (3) *P{UAS-DCC-Myc}86Fb*, (4) *P{UAS-DCCY1418F-Myc}86Fb*, (5) *P{UAS-FraΔC-HA}#4* (Garbe et al., 2007), (6) *P{UAS-TauMycGFP} II*, (7) *P{UAS-TauMycGFP} III*, (8) constitutively active *Src64B*, $P\{UAS\text{-}Src64^{Y547F}\}$ *III*, *Src64* (O'Reilly et al., 2006), and (9) *P{GAL4-elav.L}3*. 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* mutants, the absence of fluorescent mRNA *in situ* hybridization signal. Where possible, all comparative phenotypes were analyzed in the same genetic background to limit the effects of potential modifier mutations. Exceptions to this are listed here. For Figure 3*E* (left) as well as Figure 2*D* (right), "fra^{hypo}" depicts the genotype fra^3,[UAS-TauMycGFP]/fra^6;*eg*-Gal4/+, whereas in Figure 3*E* (middle left), "fra^{hypo}" depicts the genotype fra^3/fra^6;*eg*-Gal4,[UAS-TauMycGFP]/+. For *Src64* genetic suppression experiments, the $Src64^{KO}$ allele was used in trans to *eg*-Gal4 in Figure 2*D* (right), whereas in Figure 3*E* a recombinant $Src64^{KO}$, *eg*-Gal4 chromosome was used.

Immunostaining/imaging. Dechorionated, formaldehyde-fixed, methanol-devitellinized embryos were fluorescently stained using standard methods. The following antibodies were used in this study: mouse mAb BP102 (1:100), mouse anti-Fasciclin-II/mAb 1D4 (1:100), rabbit anti-GFP (Invitrogen, catalog #A11122; 1:500), rabbit anti-c-Myc (Sigma C3956; 1:500), Alexa 647-conjugated goat-anti-HRP (Jackson ImmunoResearch, catalog #123-605-021; 1:250), Cyanine 3-conjugated goat anti-mouse (Jackson ImmunoResearch, catalog #115-165-003; 1:1000), and Alexa 488-conjugated goat anti-rabbit (Invitrogen, catalog #A11008; 1:500). Embryos were mounted in 70% glycerol/PBS. Fluorescent mRNA *in situ* hybridization was performed as described previously (Garbe and Bashaw, 2007). Phenotypes were analyzed, and images were acquired using a spinning disk confocal system (PerkinElmer) built on a Nikon Ti-U inverted microscope using a Nikon OFN25 60× objective with a Hamamatsu C10600-10B CCD camera and Yokogawa CSU-10 scanner head with Volocity imaging software. Images were processed using ImageJ.

Phenotypic quantification. For EW commissural neuron crossing phenotypes, whole-mount embryos were analyzed at Stages 15 and 16. Eight abdominal segments were analyzed per embryo where possible, and for each 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 *apterous* 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 projection 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 deficient 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, *Src42A* 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. 1*G*) (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 *Src42A; Src64B* double mutants (Fig. 1*J–N*; Table 1).

In contrast to the relatively normal CNS in single *Src* mutants, in *Src42A;Src64B* double mutants there are severe defects in FasII-positive ipsilateral axons, which often cross the midline inappropriately (Fig. 1*G*). 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

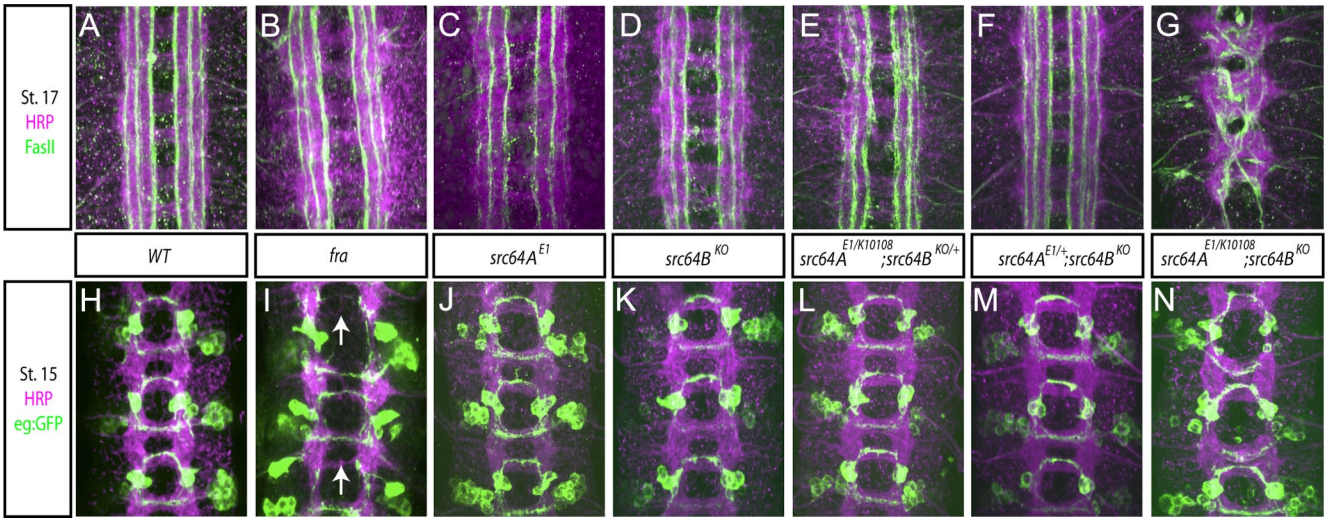

Figure 1. Commissural axon pathfinding is normal in *Src* mutant embryos. ***A–N***, Representative Stage 17 (***A–G***) and Stage 15 (***H–N***) embryos of indicated genotypes stained using anti-HRP (magenta) to label all axons, in addition to anti-FasII (***A–G***, green) and anti-GFP (***H–N***, green) to label ipsilateral and *eg*-positive commissural neurons, respectively. Anterior is up. ***A***, ***H***, Wild-type embryos. Three ipsilateral FasII-positive axon pathways have formed properly (***A***), *eg*-positive commissural axons have all properly crossed the midline at this stage (***H***). ***B***, ***I***, fra^3/fra^4 mutants. FasII-positive axons remain ipsilateral but occasional breaks in longitudinal pathways occur (***B***). *eg*-positive commissural axons frequently mistarget ipsilaterally (***I***, arrows). ***C–F***, ***J–M***, *Src* mutant embryos. FasII-positive axons display occasional wandering/defasciculation but remain ipsilateral (***C–F***). EW neurons project axons normally (***J–M***). ***G***, ***N***, *Src42A;Src64B* double mutants. Severe defects in FasII-positive axons including stalling and midline collapse (***G***). EW axons cross normally in *Src* double mutants despite substantial patterning defects (***N***). For quantification of the EW crossing phenotype, see Table 1.

Table 1. Quantification of EW crossing defects in *src* and *fra* mutants, including transgenic rescue

Genotype	Noncrossing (%)	SEM (%)	*n* (segments)	*n* (embryos)	*p*
src mutants					
fra^3,[*UAS-TauMycGFP*]/fra^3;[*eg-Gal4*]/+	20.15	4.06	168	21	
$Src42a^{E1}/Src42a^{E1}$,[*UAS-TauMycGFP*];[*eg-Gal4*]/+	0.63	0.63	160	20	
$Src42a^{E1}$,[*UAS-TauMycGFP*]/+;$Src64b^{KO}$,[*eg-Gal4*]/$Src64b^{KO}$	0	0	120	15	
$Src42a^{E1}$,[*UAS-TauMycGFP*]/$Src42a^{k10108}$;$Src64b^{KO}$,[*eg-Gal4*]/$Src64b^{KO}$	0.89	0.89	110	14	
fra^3,[*UAS-TauMycGFP*]/$Src42a^{k10108}$;$Src64b^{KO}$/[*eg-Gal4*]	0	0	88	11	
fra rescue in EW neurons					
fra^3,[*UAS-TauMycGFP*]/fra^4;[*eg-Gal4*]/+	25.78	3.93	183	23	
fra^3,[*UAS-TauMycGFP*]/fra^4;[*eg-Gal4*]/[*UAS-FraWT-Myc*]	11.88	3.20	160	20	0.027
fra^3,[*UAS-TauMycGFP*]/fra^4;[*eg-Gal4*]/[*UAS-DCCWT-Myc*]	3.75	1.60	160	20	<0.0001
fra^3,[*UAS-TauMycGFP*]/fra^4;[*eg-Gal4*]/[*UAS-DCCY1418F-Myc*]	6.34	2.95	159	20	0.009
fra^3,[*UAS-TauMycGFP*]/fra^4;[*eg-Gal4*]/[*UAS-Fra9YF-Myc*]	5.56	2.20	72	9	<0.0001
fra^3,[*UAS-TauMycGFP*]/*Df(2R)vg135*;[*eg-Gal4*]/+	27.08	3.72	96	12	
fra^3,[*UAS-TauMycGFP*]/*Df(2R)vg135*;[*eg-Gal4*]/[*UAS-FraWT-Myc*]	5.09	1.80	216	27	<0.0001
fra^3,[*UAS-TauMycGFP*]/*Df(2R)vg135*;[*eg-Gal4*]/[*UAS-DCCWT-Myc*]	2.50	1.15	160	20	<0.0001
fra^3,[*UAS-TauMycGFP*]/*Df(2R)vg135*;[*eg-Gal4*]/[*UAS-DCCY1418F-Myc*]	5.15	2.16	136	17	0.00014
fra^3,[*UAS-TauMycGFP*]/*Df(2R)vg135*;[*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).

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 reduced. We observed no effect on the guidance of EW neurons in embryos that are compound heterozygous mutant for *fra* and either *Src42A* or *Src64B* (data not shown). To further reduce Netrin signaling, we analyzed embryos expressing a truncated Frazzled receptor, FraΔC (DN-Fra), in EW neurons (Fig. 2*B*). We showed previously that this receptor acts as a dominant negative for Fra (Garbe et al., 2007). Surprisingly, instead of exacerbating the *fra* loss-of-function phenotype, *Src* mutations actually suppress the midline crossing defects caused by DN-Fra expression (Fig. 2*D*). We observed suppression of midline crossing defects in both *Src42A* and *Src64B* mutants, and these effects are dependent on the amount of endogenous *Src* gene dose. This suppression is not due to a reduction in DN-Fra transgene expression levels, as immunostaining for an epitope tag (HA) on this transgene appears identical in embryos that are wild-type and mutant for SFKs (Fig. 2*E–J*). The suppression of midline crossing defects in *Src* mutants is both potent and specific; we observed almost a full rescue of midline crossing in embryos in which three of four gene copies of *Src* are mutant, and this effect can be seen independent of any obvious patterning defects. *Src* mutations also suppress midline axon crossing defects in *fra* hypomorphic allelic combinations (Figs. 2*D*, 3*A*,*B*,*E*), suggesting that SFKs can antagonize endogenous Fra function in commissural neurons. Additionally, when we analyzed commissural guidance using mAb BP102 to label all axons, we observe a substantial reduction in defects in these embryos, similar to our observations in EW neurons (45 ±

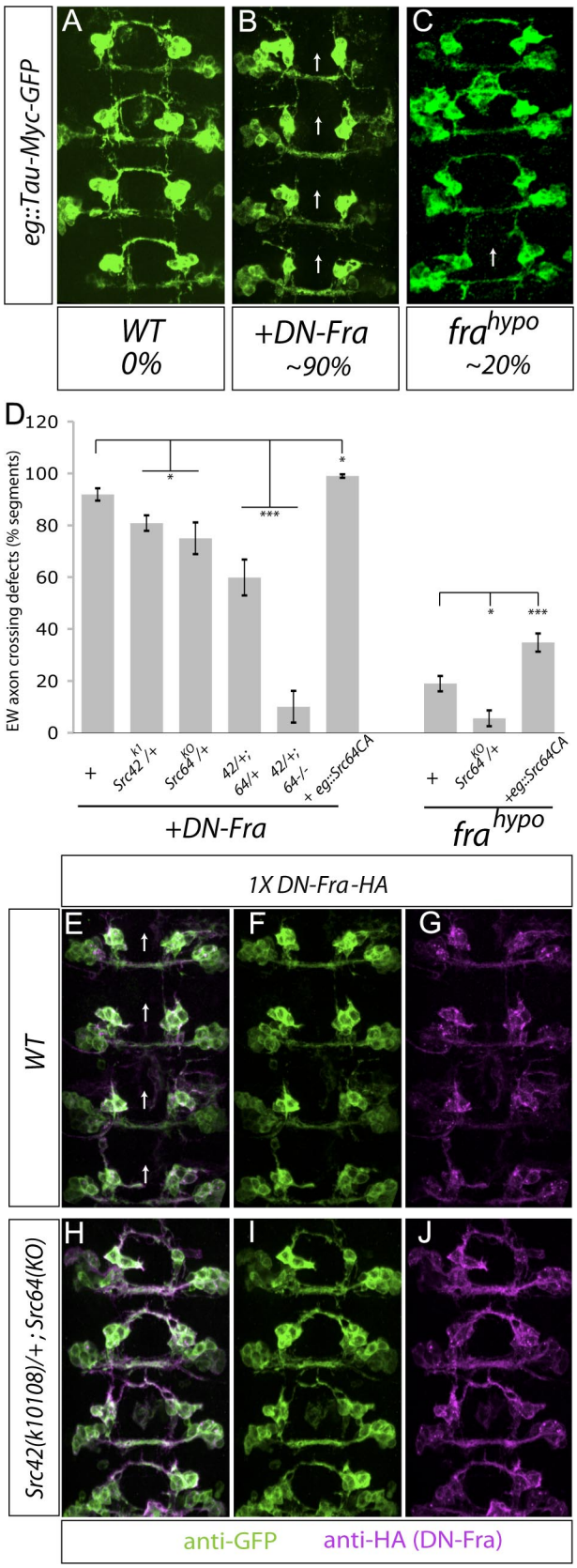

Figure 2. *Src42A* and *Src64B* antagonize midline axon crossing. ***A–D***, EW midline crossing defects were scored at Stages 15 and 16 using *eg*-Gal4 to express TauMycGFP, after immunostaining for anti-GFP. ***A***, Wild-type embryo. ***B***, An embryo expressing DN-Fra in, e.g., neurons. Most EW axons misproject (arrows). ***C***, An fra^3/fra^6 hypomorphic mutant. EW axons fail to cross in ~20% of segments (arrow). ***D***, quantification of EW crossing defects in DN-Fra (left) and fra^{hypo} (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 fra^{hypo} (right). $*p < 0.05$; $***p < 0.001$. See Materials and Methods for details on statistical analysis. ***E–J***, DN-Fra expression is not reduced in *Src* mutants. Embryos expressing TauMycGFP (***F***, ***I***, anti-GFP, green) and DN-Fra (***G***, ***J***, anti-HA, magenta) in EW neurons exhibit severe crossing defects (***E***, arrows) in wild-type embryos (***E–G***) that are almost fully rescued in *Src42A*/+;*Src64*−/− mutants (***H–J***). Specific genotypes are as follows: ***A***, "wt": [*eg*-Gal4],[UAS-TauMycGFP]/+; ***B***, "DN-Fra": [UAS- FraC-HA] 4,[UAS-TauMycGFP]/+; [*eg*-Gal4/+; ***C***, "fra^{hypo}": fra^3,[UAS-TauMycGFP]/ fra^6;[*eg*-Gal4]/+; ***D***, left, "+" [UAS- FraC-HA] 4, [UAS-TauMycGFP]/+; *eg*-Gal4/+, "Src42/+": [UAS-DN-Fra] 4, [UAS-TauMycGFP]/$Src42^{k10108}$; *eg*-Gal4/+, "Src64/+": [UAS-DN-Fra] 4, [UAS-TauMycGFP]/+; $Src64^{KO}$/+, "42/+;64/+": [UAS-DN-Fra] 4, [UAS-TauMycGFP]/$Src42^{k10108}$; *eg*-Gal4/$Src64^{KO}$, "42/+;64−/−": [UAS-DN-Fra] 4, [UAS-TauMycGFP]/$Src42^{k:10108}$; $Src64^{KO}$, *eg*-Gal4/$Src64^{KO}$, right, "+": fra^3,[UAS-TauMycGFP]/ fra^6; [*eg*-Gal4]/+, "Src64/+": fra^3,[UAS-TauMycGFP]/ fra^6; [*eg*-Gal4]/$Src64^{KO}$, "*eg*::Src64CA", fra^3,[UAS-TauMycGFP]/fra^6;[*eg*-Gal4]/[UAS-Src64-CA]; ***E–G***, [UAS-FraΔC-HA]#4,[UAS-TauMycGFP]/+;[*eg*-Gal4]/+; ***H–J***, [UAS- FraΔC-HA]#4,[UAS-TauMycGFP]/$Src42A^{k10108}$; $Src64B^{KO}$,[*eg*-Gal4]/$Src64B^{KO}$. See Materials and Methods for comments on genotypes.

4.2% defects, $n = 20$ in fra^3/fra^6 vs 19.8 ± 3.2% defects, $n = 21$ in fra^3/fra^6; $Src64^{KO}$/+; $p < 0.0001$). This suggests that Src inhibits midline crossing in many other commissural neurons in addition to EW neurons. To determine whether Src acts autonomously in commissural neurons to inhibit midline crossing, we expressed a constitutively active *Src64B* (*Src64CA*) in EW neurons. While expression of *Src64CA* has no effect in wild-type embryos (data not shown), expression in backgrounds with reduced Netrin–Fra signaling exacerbates midline crossing defects (Fig. 2*D*), suggesting that Src exerts its effect on midline crossing cell autonomously.

The observed genetic suppression of multiple *fra* loss of function phenotypes is consistent with Src functioning to antagonize Netrin signaling, but also could reflect a role for Src in a parallel pathway regulating midline axon crossing. If Src acts exclusively in the Netrin pathway, we would not expect to see similar suppression of midline crossing defects when *Src* mutations are introduced into *Netrin*-null mutants. However, the *NetA*, *NetB* double mutant phenotype is also suppressed in *Src64B* heterozygotes (Fig. 3*C–E*), suggesting that Src acts via a Netrin-independent pathway in these neurons in addition to any role it may play in inhibiting Netrin–Frazzled signaling. Because Src functions as an effector of Netrin-Unc-5 repulsive axon guidance, we tested whether Unc-5 signaling is active in these neurons (Itoh et al., 2005).

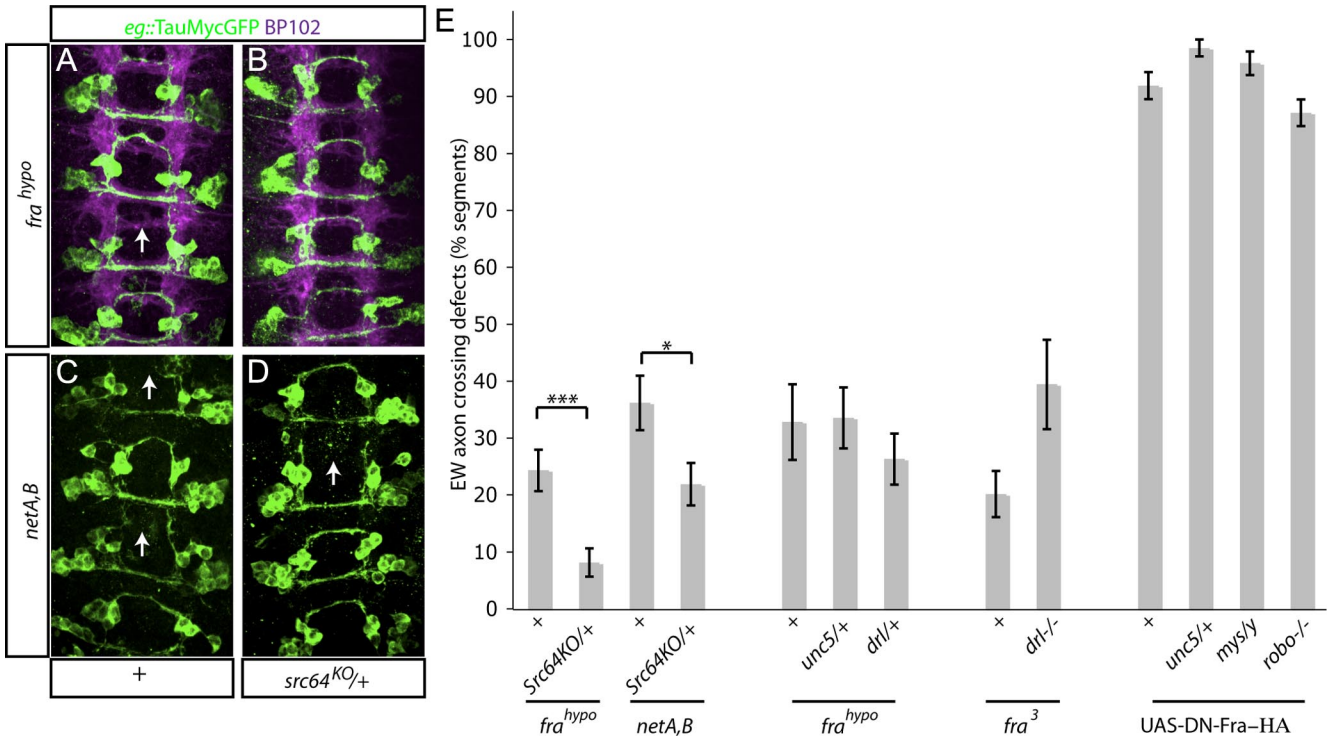

Figure 3. Inhibition of midline crossing by Src kinases occurs through a novel, Netrin-independent pathway that is not regulated through Derailed, Unc-5, or Integrin signaling. ***A–D***, Representative Stage 15 embryos immunostained with anti-GFP to visualize EW axons (***A–D***, green) and BP102 to visualize CNS axons (***A***, ***B***, magenta). ***A***, An fra^{hypo} embryo displays a partially penetrant EW crossing phenotype (arrow), which is suppressed in $Src64^{KO}$ heterozygous mutants (***B***, ***E***). ***C–E***, A *NetAB*-null mutant also displays partially penetrant EW axon defects (arrows), and these are similarly suppressed in $Src64^{KO}$ heterozygotes (***D***, ***E***). ***E***, Quantification of EW crossing defects in *netrin* and *frazzled* mutants bearing different candidate modifier mutations. Unlike *Src64B*, neither *unc-5* nor *drl* mutations modify the fra^{hypo} phenotype (middle left). Midline crossing defects are enhanced, not suppressed, in fra^3,drl^{it343} mutants compared to fra^3 (middle right). The DN-Fra phenotype is not suppressed in *unc-5* heterozygotes, *mys* hemizygotes, or *robo* homozygous mutants (right). Error bars indicate SEM. $*p < 0.05$; $***p < 0.001$. See Materials and Methods for comments on genotypes.

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. 3*E*). 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^3 allele. We find that in contrast to *fra* hypomorphs, heterozygosity for $Src64^{KO}$ does not suppress crossing defects in fra^3 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. 3*E*). *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*, *myospheroid*. 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. 5*H*,*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. 4*A–D*). Importantly, the DCC-dependent crossing defects in this background are suppressed in *NetAB*Δ mutants, suggesting that this receptor can signal in response to *Drosophila* Netrin (Fig. 4*D*). 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. 5*A–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. 5*E*,*G*; Table 1). DCCY1418F also generates a quantitatively similar phenotype to wild-type DCC when expressed in *ap* neurons (Fig. 4*D*). 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.

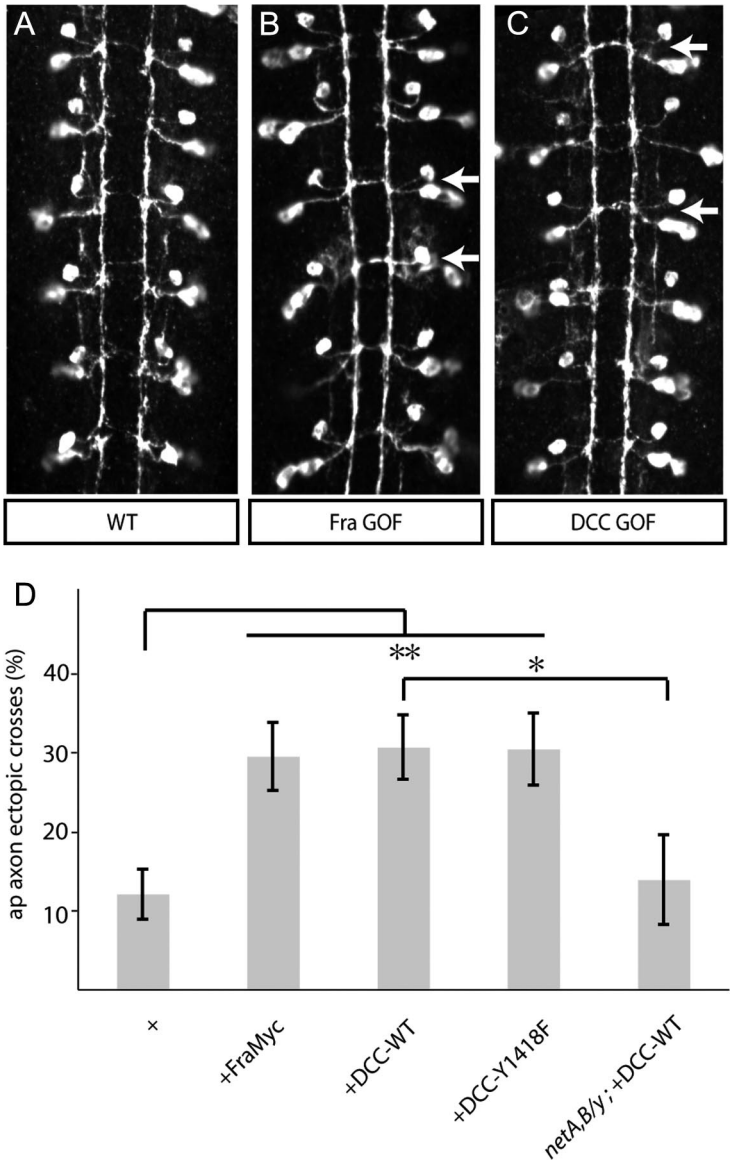

Figure 4. Vertebrate DCC can signal Netrin-dependent axon attraction in *Drosophila*. ***A–C***, Stage 17 embryos, expressing TauMycGFP under control of *ap*-Gal4, are immunostained with anti-GFP to label the ipsilateral *apterous* axons. Six abdominal segments are shown. ***A***, Wild-type embryo. The *ap* axons remain ipsilateral. ***B***, Fra gain-of-function embryo. Ectopic crossing of *ap* axons occurs sporadically (arrows). ***C***, DCC gain-of-function embryo. The *ap* axons display a similar ectopic crossing phenotype (arrows; compare ***C***, ***B***). ***D***, Quantification of *ap* ectopic crossing defects. DCC gain-of-function depends on Netrin (compare +DCC-WT and *netA,B/y;*+DCC-WT). Also, DCCY1418F gain-of-function is equivalent to DCC-WT in this assay. Error bars indicate SEM. $*p < 0.05$; $**p < 0.01$.

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. 6*A–D*). Fra-9YF also rescues EW midline crossing defects in *fra* mutants to a similar extent as wild-type Fra (Fig. 5*F*; 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. 6*G*,*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. 6*J*,*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. 6*E*,*L*), and only mildly rescues the commissural guidance phenotype in *fra* mutants as assayed using BP102 (Fig. 6*E*), 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

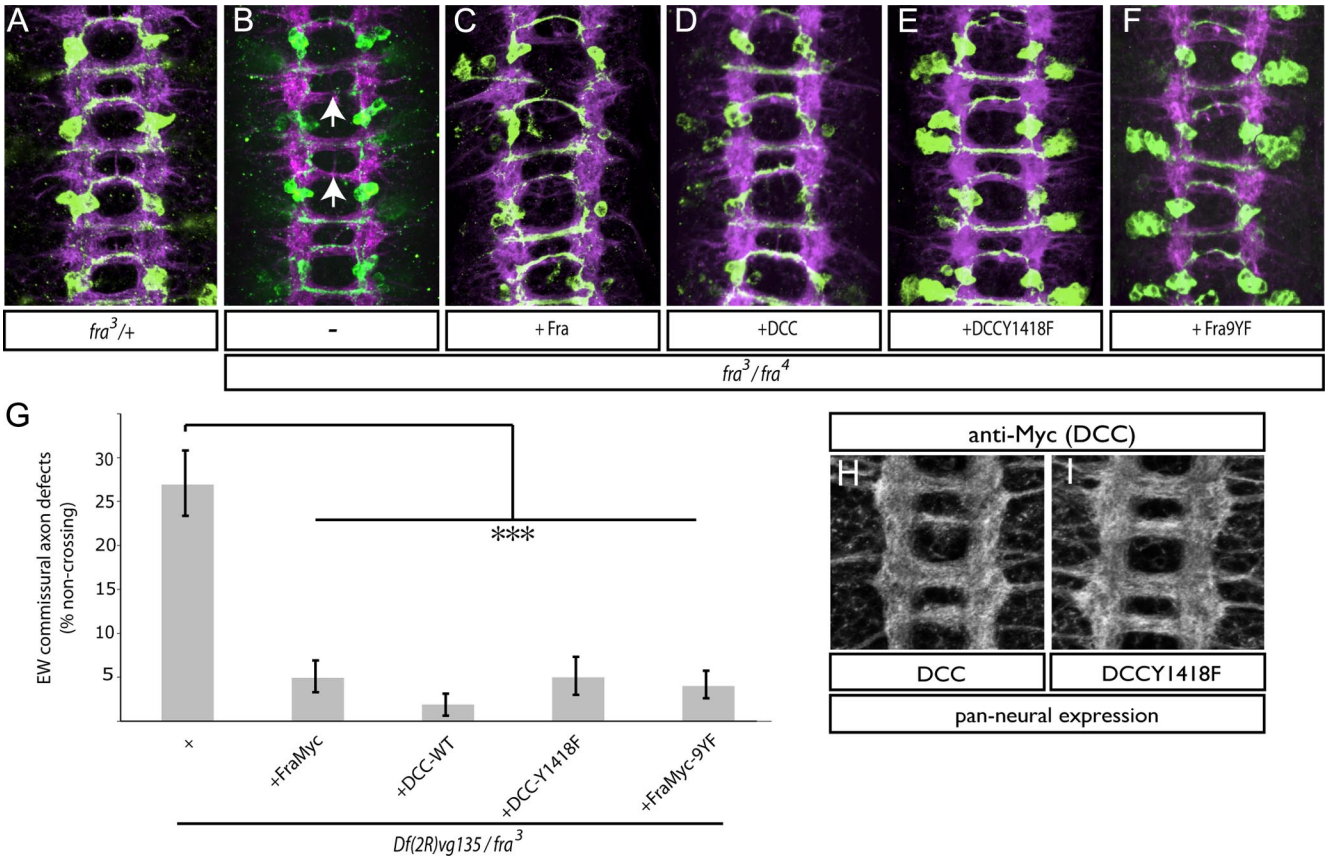

Figure 5. Tyrosine phosphorylation of DCC receptors is dispensable for midline axon guidance. ***A–F***, Stage 15 control (***A***) or *fra* mutant embryos (***B–F***) expressing TauMycGFP under control of *eg*-Gal4, along with various rescue transgenes indicated in boxes below. Embryos are immunostained with anti-GFP (green) to visualize EW axons and mAB BP102 (magenta) to visualize CNS axons. ***A***, Control embryo. All EW axons cross appropriately. ***B***, fra^3/fra^4 mutant. Many EW axons fail to cross (arrows). ***C***, Fra-WT rescue. Most EW axons cross appropriately. ***D***, DCC rescue. Rescue of EW crossing is similar to that of Fra-WT. ***E***, DCCY1418F rescue. The phenotype is indistinguishable from DCC-WT. ***F***, Fra-9YF rescue. The phenotype is indistinguishable from Fra-WT. ***G***, Quantification of EW crossing defects in fra^3/Df(2R)vg135 mutants. Error bars indicate SEM. $^{***}p < 0.001$. For quantification of fra^3/fra^4 rescue, see Table 1. ***H***, ***I***, Anti-Myc immunostaining to visualize DCC-Myc transgene expression levels, under control of pan-neural *elav*-Gal4. Transgenes are expressed at comparable levels.

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

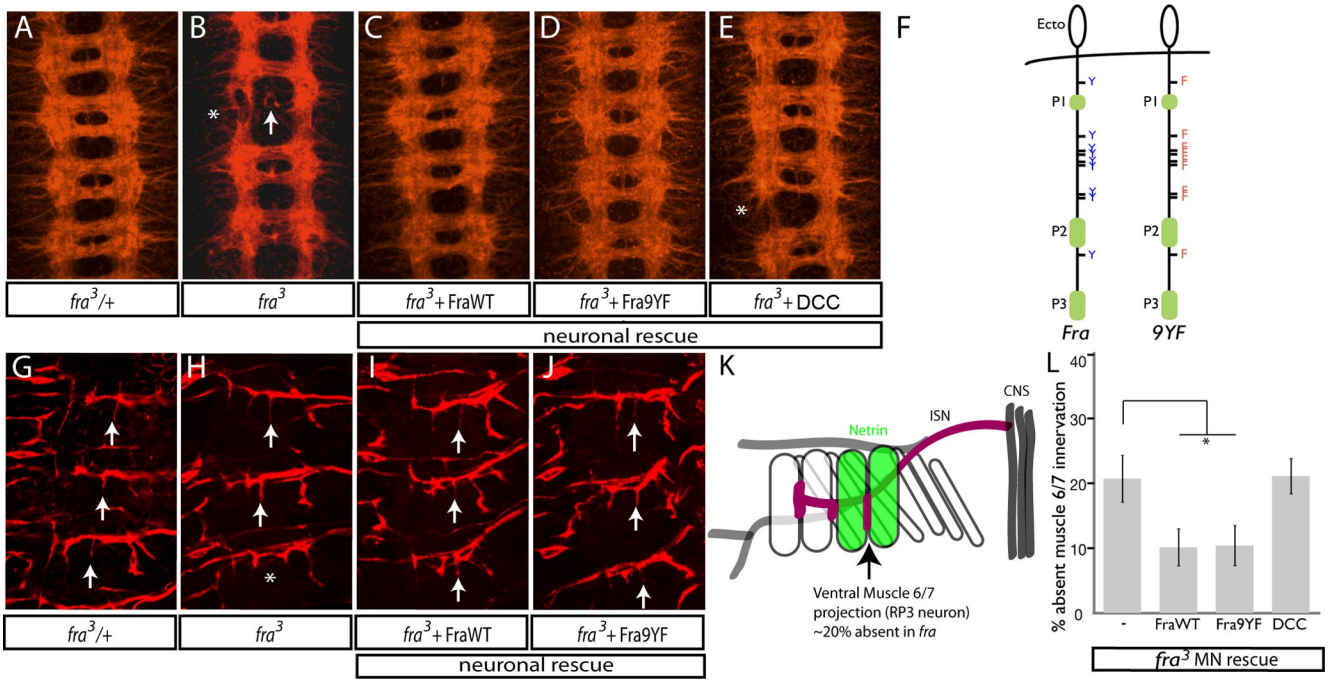

Figure 6. Fra9YF is equivalent to wild-type Fra in motor and CNS axon guidance. ***A–E***, Stage 16 embryos immunostained with mAb BP102 to visualize CNS axons. Genotypes are boxed below panels. ***A***, Control embryo. ***B***, *fra*^{3} mutant. Posterior commissures are thin or absent (arrow), and occasional breaks in longitudinal connectives occur (asterisk). ***C–E***, Pan-neural rescue of *fra*^{3} 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***, *fra*^{3} 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 *fra*^{3} 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 differently 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 *dcc* 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-dependent 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-*

missureless. 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 *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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