

# ***frazzled* Encodes a Drosophila Member of the DCC Immunoglobulin Subfamily and Is Required for CNS and Motor Axon Guidance**

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## **Summary**

**We have identified a *Drosophila* member of the *deleted in colorectal cancer* (DCC) gene family. The *frazzled* gene encodes transmembrane proteins that contain four immunoglobulin C2 type domains, six fibronectin type III repeats, and a cytoplasmic domain of 278 amino acids. Like vertebrate members of the DCC family, *Frazzled* is expressed on axons in the embryonic central nervous system and on motor axons in the periphery. *Frazzled* is also expressed on epidermis and gut epithelium. Null mutants in *frazzled* are defective in axon guidance in the central nervous system and in motor axon guidance and targeting in the periphery. The phenotypes strongly resemble those of a deletion of the two *Drosophila Netrin* genes. We have rescued the *frazzled* CNS and motor axon defects by expressing *Frazzled* specifically in neurons; expression in target tissues does not rescue the phenotype. These data, together with vertebrate studies showing binding of DCC to netrin, suggest that *Frazzled* may function *in vivo* as a receptor or component of a receptor mediating Netrin-dependent axon guidance.**

## **Introduction**

Secreted and membrane-bound guidance cues can function as either attractants or repellents to influence axon pathfinding (reviewed by Tessier-Lavigne, 1994; Goodman, 1996), and these molecules appear to be highly conserved among invertebrates and vertebrates. For example, netrins, secreted proteins related to laminin, are expressed at the midline in a variety of organisms, where they function via as yet unidentified receptors as both chemoattractants and chemorepellents (Hedgecock et al., 1990; Ishii et al., 1992; Kennedy et

al., 1994; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995; Harris et al., 1996; Mitchell et al., 1996; Wadsworth et al., 1996). Growth cone responses to these and other candidate guidance molecules vary with neuronal cell type, suggesting that the distribution and diversity of both the guidance ligands and their receptors play a key role in determining the patterns of neuronal connections.

The Deleted in Colorectal Cancer (DCC) protein and the related neogenin protein are immunoglobulin (Ig) superfamily members that are expressed on developing axons. These two molecules share ~52% amino acid identity and belong to a distinct Ig subfamily. Their extracellular domains contain four immunoglobulin C2 repeats and six fibronectin III repeats (Hedrick et al., 1994; Vielmetter et al., 1994) and their ~300 amino acid cytoplasmic domains show no significant homology to proteins outside the subfamily (Hedrick et al., 1994; Vielmetter et al., 1994).

The *deleted in colorectal cancer* gene was originally isolated as a possible tumor suppressor (Fearon et al., 1990), but the role of DCC in tumor progression and normal development is unclear. Both DCC and neogenin are also expressed in most epithelial tissues during development, particularly in the gut. The structure of DCC-like molecules and the timing of their expression during development of the nervous system and gut has led to proposals that they function as negative regulators of cell division or as receptors for morphogenetic information (Chuong et al., 1994; Hedrick et al., 1994; Pierceall et al., 1994; Vielmetter et al., 1994). Either role could explain why the loss of DCC appears to promote tumor progression, since tumors are abnormal in both cell growth and morphology.

We have identified a *Drosophila* gene, *frazzled* (*fra*), that encodes a DCC-related protein. *Frazzled* is also expressed on axons in the developing nervous system, as well as on a variety of epithelia. Null mutations in *fra* disrupt axon guidance in the central nervous system and motor axon pathfinding and target recognition in the periphery. The phenotypes strongly resemble those observed in embryos lacking the two *Drosophila Netrin* genes (Harris et al., 1996; Mitchell et al., 1996). We also provide evidence that *fra* functions cell autonomously, raising the possibility that it encodes a receptor or part of a receptor mediating Netrin-dependent guidance. Together with the observation that DCC and neogenin are vertebrate netrin receptors (Keino-Masu et al., 1996 [this issue of *Cell*]) and studies on a DCC-related protein, UNC-40, in *C. elegans* (Chan et al., 1996 [this issue of *Cell*]), these data suggest that mechanisms of netrin-dependent axon guidance are evolutionarily conserved.

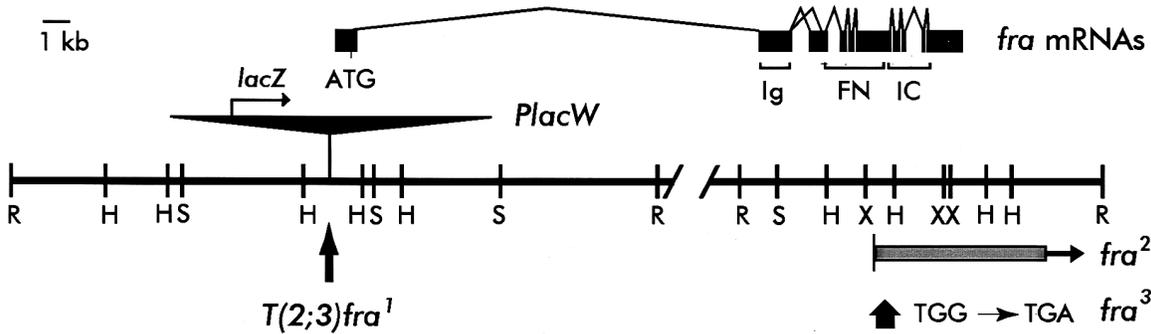
## **Results**

### **The *frazzled* Gene Encodes DCC-Related Proteins**

We identified a *P-lacZ* insertion at 49B in an enhancer trap screen for mutations that affect nervous system development and function (Bier et al., 1989). The locus

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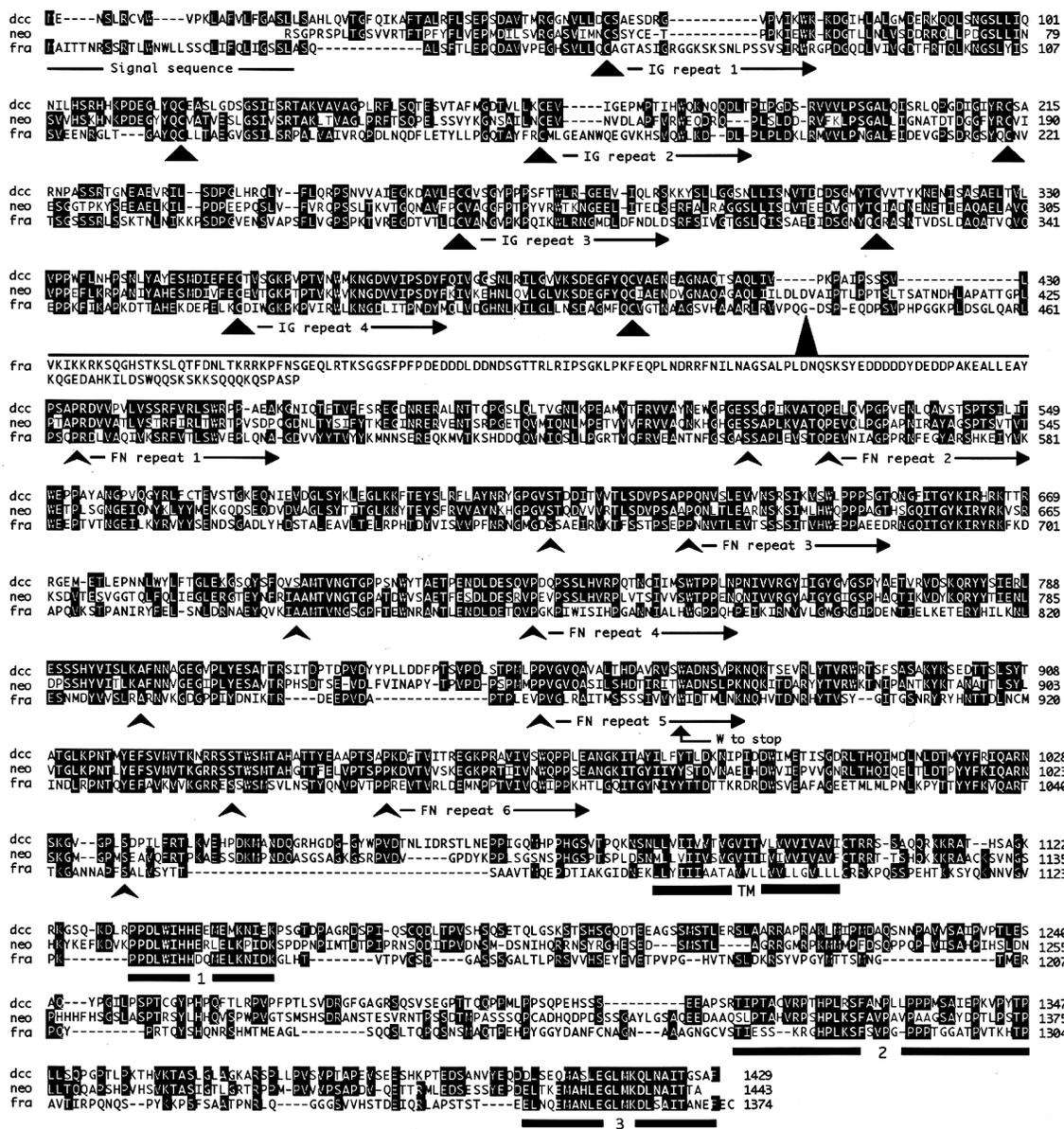


Figure 1. Genomic Organization of the *frazzled* (*fra*) Locus and Sequence Identities among DCC Family Members  
 (A) The bottom line represents a partial restriction map of the genomic region immediately flanking the P-element and encoding the *fra* transcripts. The probes used to seek transcripts in the vicinity of the insertion site were derived by labeling restriction fragments that span the region. Northern analysis and in situ hybridization with the 2.3 kb HindIII DNA fragment containing the P-element insertion site detected

was named *frazzled* (*fra*) because adult flies homozygous for the P-element shake upon revival from ether-induced anesthesia. Precise excision of the P element reverted the phenotype, thus establishing that the shaking defect is due to the insertion. Other excision lines were homozygous embryonic lethals that failed to complement each other and the existing deficiency for 49B, *Df(2R)vg135*. Since embryos bearing the 49B enhancer trap expressed *lacZ* in the CNS (data not shown), we investigated whether a nearby gene might play an essential role in nervous system development or function.

In situ hybridization experiments on embryos with probes covering ~10 kb of DNA flanking the *P-lacZ* insertion identified a single transcription unit in the region. *frazzled* cDNA clones encode two isoforms that exhibit ~43% overall sequence identity to DCC and neogenin (Figure 1) and the expression pattern of *frazzled* transcripts matches that of the enhancer trap (data not shown).

Frazzled, DCC, and neogenin belong to the same Ig subfamily. The extracellular domains of the two predicted Frazzled isoforms contain four immunoglobulin (Ig) C2 type repeats followed by six fibronectin repeats, as do those of DCC and neogenin (Hedrick et al., 1994; Vielmetter et al., 1994). The two isoforms differ by an insertion of 151 amino acids between the fourth immunoglobulin repeat and the first fibronectin repeat (Figure 1). They share a membrane-spanning domain and a cytoplasmic domain that is 278 amino acids in length. Within the cytoplasmic domain, there are several regions of amino acid identity with the vertebrate proteins (Figure 1); no other close matches were found in public sequence databases.

#### Frazzled Is Expressed on Extending Axons during Development

Antisera that specifically recognize the Frazzled C-terminal domain reveal that, like DCC and neogenin, Frazzled is expressed on developing axons and epithelia in the embryo. Frazzled is expressed at high levels on commissural and longitudinal axons in the developing CNS (Figure 2) and is detected at stage 13 on the earliest commissural axons (data not shown). Frazzled is present at lower levels on peripheral motor axons that extend outward in the intersegmental and segmental nerves (Figure 2 and data not shown) and also on the surfaces of midgut epithelial cells beginning at stage 12 and on epidermis (data not shown). Frazzled does not appear to be expressed on tissues that are thought to express ligands required for motor and CNS axon pathfinding

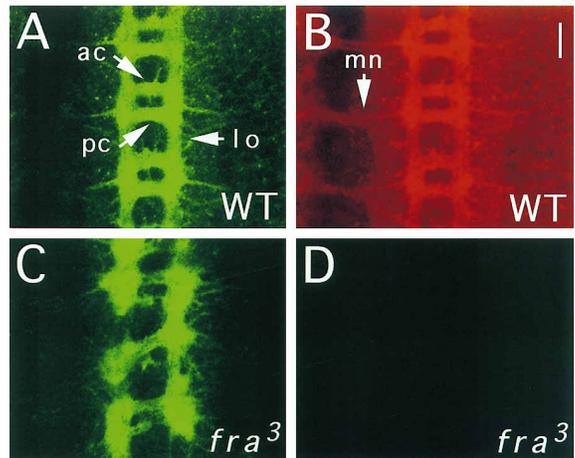


Figure 2. Frazzled Is Expressed on CNS Axons

(A) Commissural and longitudinal axons in the CNS of a stage 15 wild-type embryo visualized with MAb BP102. The axon pathways form a regular ladder-like pattern, with commissural axons forming the rungs, and longitudinal axons forming the sides. ac, anterior commissure; pc, posterior commissure; and lo, longitudinal axons. (B) The same embryo as in A), but stained with anti-Frazzled C-terminal domain. Frazzled is detected on commissural and longitudinal axons and on motor axons (mn) projecting from the CNS. (C) Commissural and longitudinal axons in the CNS of a stage 15 *fra<sup>3</sup>* embryo visualized with MAb BP102. The commissural axons are absent or reduced in number, and gaps appear in the longitudinal axon tracts. (D) The same embryo as in (C), but stained with anti-Frazzled. Frazzled is not detected in *fra<sup>3</sup>* embryos, or in *fra<sup>1</sup>*, *fra<sup>2</sup>*, or *fra<sup>4</sup>* mutant embryos (data not shown). Confocal images are from a 1  $\mu$ m thick optical section. Anterior is at the top here and in Figure 3.

such as muscle, glia, or midline cells (Figure 2 and data not shown). Frazzled immunoreactivity is absent in homozygous *fra<sup>1</sup>*, *fra<sup>2</sup>*, *fra<sup>3</sup>*, *fra<sup>4</sup>* mutant embryos (Figure 2 and data not shown).

#### Null Mutations in *fra* and *NetrinA/B* Similarly Affect CNS Axon Development and Motor Axon Pathfinding

To investigate the role of DCC-like proteins in development, we identified null mutants in *fra* among embryonic lethal lines generated either by imprecise excision of the P-element inserted adjacent to the *fra* transcription

an ~6 kb message that was abundantly expressed in the CNS and gut epithelium. Other probes did not identify additional transcripts. The positions of the *fra<sup>1</sup>* translocation, the *fra<sup>2</sup>* deletion breakpoints and the *fra<sup>3</sup>* point mutation are indicated. The *fra<sup>2</sup>* deletion extends 3' beyond the *fra* gene. The thick blocks in the top line indicate the relative size and positions of the ten exons in the identified *fra* mRNAs; the gaps indicate the relative sizes of the nine introns. The actual size of the first intron is unknown. The exons corresponding to the four immunoglobulin-like (Ig) repeats, the six fibronectin type III (FN) repeats, and the intracellular (IC) domain are indicated by brackets.

(B) *fra* encodes two DCC-related proteins 1355 and 1506 amino acids in length. BLAST (Altschul et al., 1990) was used to identify DCC, neogenin, and Frazzled as related, and the sequences were aligned (Higgins, 1994). Identical residues are shaded. The four Ig domains are shown and triangles indicate the position of the two conserved cysteines present in each domain (Williams and Barclay, 1988). Arrowheads indicate the amino acids at the ends of each fibronectin type III repeat (Patthy, 1990). The longer *fra* cDNA encodes a 151 amino acid sequence not found in other DCC-like proteins. The putative signal sequence, transmembrane region (TM), and three areas of particularly strong identity among the predicted intracellular domains of human DCC, chick neogenin, and Frazzled are underlined. The sequence alteration in the *fra<sup>3</sup>* allele changes the TGG encoding W 1028 to TGA, a stop codon.

start site or by ethyl methanesulfonate (EMS) mutagenesis (Experimental Procedures and Figure 1). Since the *fra<sup>3</sup>* and *fra<sup>4</sup>* EMS alleles were obtained in two different genetic backgrounds, we focused our investigations on the development of Frazzled expressing CNS and motor axons in transheterozygous *fra<sup>3</sup>/fra<sup>4</sup>* mutant embryos.

The CNS in each segment of wild-type embryos stained with MAb BP102 (Seeger et al., 1993) contains bilaterally symmetric longitudinal tracts and two commissural tracts (anterior and posterior), all of which express Frazzled. In *fra* mutant embryos, partially penetrant defects are observed in the earliest stages of the development of the commissures. In *fra<sup>3</sup>/fra<sup>4</sup>* mutant embryos, 12% of anterior commissures and 43% of posterior commissures ( $n = 182$ ) in abdominal segments A1–A7 are thin or absent. Commissures that appear to be relatively normal in thickness are often less well-organized than normal (Figures 2 and 3). Comparable results are observed in other *fra* alleles (data not shown). Occasional breaks are also observed in the longitudinal tracts (Figures 2 and 3).

Approximately 40 motor axons in each abdominal hemisegment of the *Drosophila* embryo extend into the periphery where they innervate 30 body wall muscles in a highly stereotyped pattern. A subset of motor axons exit the ventral CNS in the intersegmental nerve (ISN) and extend dorsally to innervate the *NetrinA/B* expressing dorsal muscles 1 and 2 (Mitchell et al., 1996) (Figure 4). In *fra* mutant embryos, these ISN axons, which normally express Frazzled (Figure 2 and data not shown), extend dorsally, but often branch or extend inappropriately when they reach the dorsal muscle region. In 20% of hemisegments (143 scored), these ISN axons wander into adjacent segments or toward the dorsal midline, appear to make contacts with inappropriate muscles, or branch more extensively over their normal muscle targets (Figure 4). The projections of motor axons in the segmental nerve b (SNb) to ventral muscles 6 and 7, which express *NetB*, are also occasionally aberrant in *fra* embryos (Figure 4). It is possible, however, that the SNb innervation defects, unlike the ISN defects, are secondary to previous routing errors made by these axons in the CNS. ISN axons exit the CNS normally in *fra* embryos, but SNb axons must first cross the midline.

The CNS defects and ISN motor axon defects in *fra* null mutant embryos strongly resemble those observed in embryos homozygous for a deletion on the X chromosome that removes the two tandem *Drosophila Netrin* genes (Harris et al., 1996; Mitchell et al., 1996). In *NetrinA/B* mutant embryos, the posterior commissure is also more severely affected than the anterior commissure (Figure 3). Occasional breaks in the longitudinal tracts are also observed in the *Netrin* double mutant. Moreover, in *Netrin* mutant embryos, the ISN axons display a similar frequency of dorsal muscle targeting errors and innervation of muscles 6 and 7 by the SNb motor axons is similarly affected (Mitchell et al., 1996). In *fra* and *Netrin* mutants the SNa axons project normally to their lateral muscle targets, which do not normally express *Netrin* (Figure 4). However, these axons do express Frazzled and their trajectory can be altered by ectopic *Netrin* expression on all muscles (Mitchell et al., 1996).

The *fra* mutant phenotypes do not appear to be due

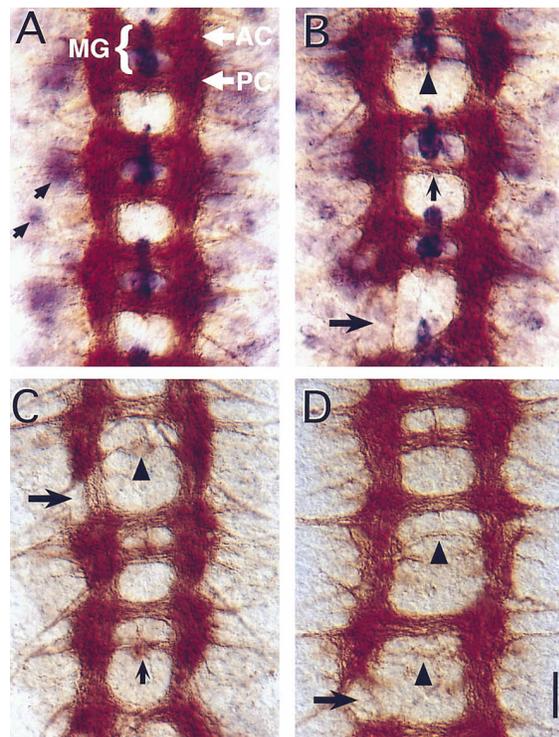


Figure 3. *frazzled* Is Required for Commissural Axons to Project toward *Netrin*-Expressing Midline Cells

(A) Expression of *Netrin* mRNAs (purple) in the CNS of a wild-type embryo. *Netrins A* and *B* are expressed at high levels by the midline glia (MG) and at lower levels by lateral clusters of CNS neurons (black arrows). AC, anterior commissure; PC, posterior commissure. (B) Expression of *Netrins* is normal in a *fra<sup>3</sup>/fra<sup>4</sup>* mutant embryo, but the posterior commissures are thinner (small arrow) or absent (arrowhead) (except for the median fascicle projecting out from the midline neurons). A break has occurred in the longitudinal tracts in one segment (large arrow). The anterior commissures also appear thinner than normal. (C) Another *fra<sup>3</sup>/fra<sup>4</sup>* embryo stained with MAb BP102. Some posterior commissures are considerably thinner (small arrow) or absent (arrowhead), the anterior commissures are slightly thinner than normal, and the longitudinal tracts are interrupted (large arrow). (D) Similar commissural and longitudinal axon tract defects are observed in embryos hemizygous for the deficiency (*Df(1)NP5*) that removes both *Netrin* genes (Mitchell et al., 1996). Arrowheads, missing posterior commissures; arrow, longitudinal break. Bar, 10  $\mu$ m.

to alterations in the survival, fate, or patterning of midline cells, CNS neurons, muscle, or motor axons. In addition to BP102, numerous markers for CNS development (*Engrailed*, *Eve*, *Ftz*, *Connectin*, *22C10*, *Fasciclin II*) are expressed normally in *frazzled* mutants (data not shown) and midline cells appear to express normal levels of mRNA of *NetrinA* and *NetrinB* (Figure 3).

#### Expression of Frazzled in Neurons, but Not in Target Tissues, Rescues the *fra* CNS and Motor Axon Guidance Phenotypes

We investigated whether expression of a *fra* cDNA in all neurons in *fra* mutant embryos could restore the abilities of commissural axons to cross the midline and ISN motor axons to find their dorsal muscle targets. As a control, we first tested whether overexpression of the shorter

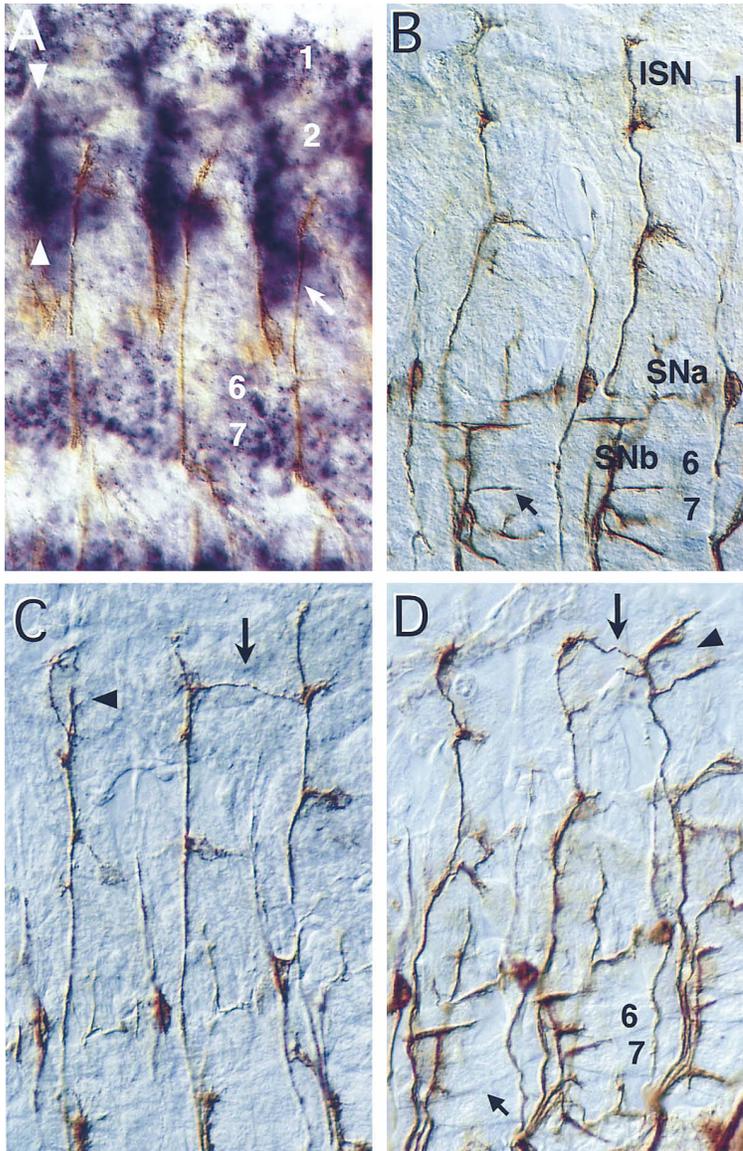


Figure 4. *frazzled* Is Required for Motor Axon Pathfinding to Netrin-Expressing Target Muscles

(A) A stage 15 embryo double-stained with MAb 1D4 (brown) (Van Vactor et al., 1993) and antisense probes to *Netrins A* and *B* (purple). The ISN (white arrow) is growing out dorsally, just posterior and internal to the epidermal stripe of *NetrinA* expression (white arrowheads) toward the dorsal muscle group. Dorsal muscles 1 and 2 express *Netrins* just prior to their innervation by ISN motor axons, and ventral muscles 6 and 7 express *NetrinB* (Mitchell et al., 1996).

(B) The pattern of muscle innervation in a wild-type embryo (late stage 16). The ISN has contacted the dorsal muscles, the SNa (forked structure, out of focus) projects to the lateral muscles, and the SNb innervates the ventral muscles. Innervation in the synaptic cleft between muscles 6 and 7 is indicated by an arrow.

(C) The pattern of muscle innervation in a *fra<sup>2</sup>/fra<sup>4</sup>* mutant embryo (early stage 16). ISN motor axons make inappropriate contacts with dorsal muscles (arrowhead) or extend a collateral branch into an adjacent segment (concave arrow).

(D) The pattern of muscle innervation in a *fra<sup>2</sup>/fra<sup>4</sup>* mutant embryo (late stage 16). ISN motor axons branch excessively and make inappropriate contacts with dorsal muscles (arrowhead) or project into adjacent segments (concave arrow). Ventral muscles 6 and 7 that lack SNb innervation in one segment are shown (small arrow).

Bar, 15  $\mu$ m.

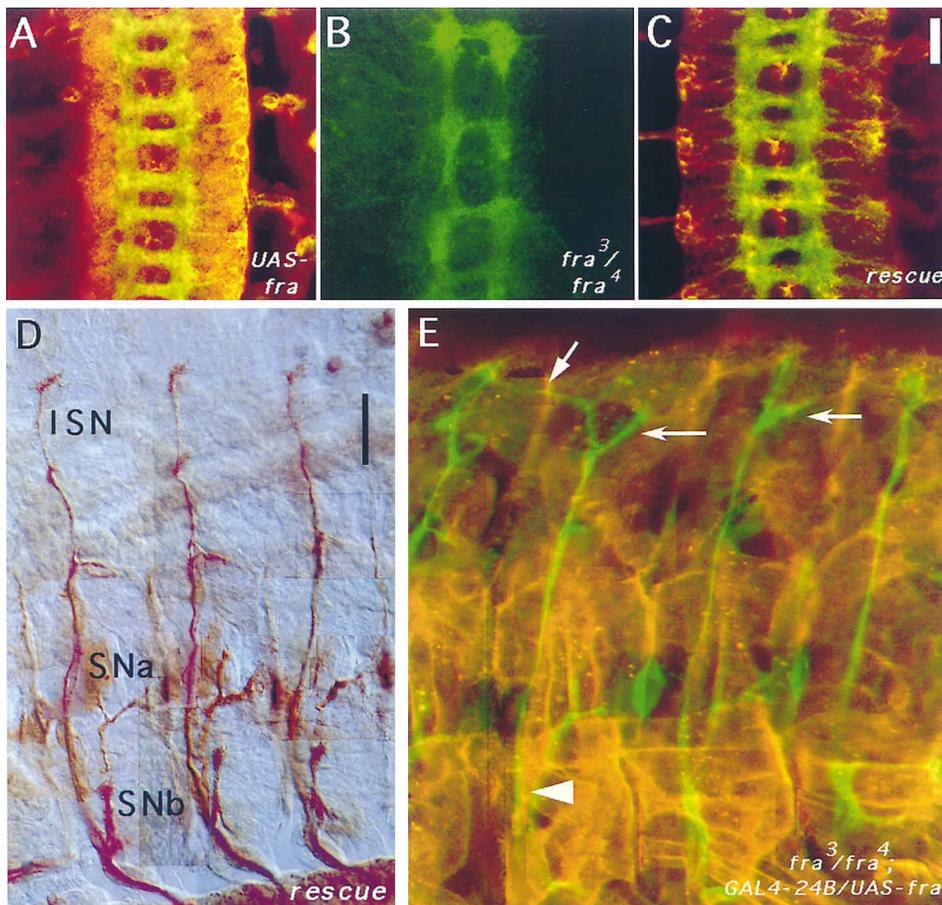
Frazzled isoform in all neurons could perturb axon development in otherwise wild-type embryos. We crossed flies in which the shorter cDNA was driven by the *GAL4 UAS* to the *1407 GAL4* line in which all neurons express the *GAL4* transcription factor (Brand and Perrimon, 1993; Luo et al., 1994). *1407-GAL4/+; UAS-fra/+* embryos express Frazzled on all axons; however, no defects in CNS or PNS projections are observed with MABs BP102, 22C10, or 1D4, and viable adult flies are recovered (Figure 5 and data not shown).

We created flies containing the *UAS-fra* transgene and a second chromosome bearing the *1407 GAL4* enhancer trap and *fra<sup>3</sup>* and crossed them to *fra<sup>4</sup>* flies. We identified transheterozygous *fra* mutant embryos as those lacking marked balancers; half of these embryos expressed Frazzled strongly in all neurons, but not in epithelia or gut. Commissures appear to form normally in these rescued *fra* embryos ( $n = 70$  anterior and 70 posterior commissures in abdominal segments A1–A7), but not in mutant embryos lacking the *UAS-fra* transgene (Figure

5). In rescued *fra* embryos, the ISN motor axons also innervated their targets at near wild-type levels (95/98 hemisegments scored) (Figure 5). Thus, this Frazzled isoform can function in neurons to promote proper axon extension across the midline and motor axon targeting to netrin expressing muscles.

We then used the pan-muscle *GAL4* line 24B (Luo et al., 1994) to test whether Frazzled was acting cell autonomously in motor axon targeting. We found that expression of Frazzled in all muscles in *fra* mutant embryos neither rescues nor enhances *fra* motor axon defects (Figure 5), and does not affect axon targeting in wild-type embryos (data not shown). From these experiments and the observed normal expression of Frazzled in CNS and motor axons, but not in nonneuronal tissues that affect pathfinding (midline cells and glia), we believe that Frazzled is likely to act as a receptor or component of a guidance receptor.

Not all *fra* CNS defects were rescued. Rescued embryos exhibited 1–3 gaps per embryo in the longitudinal



**Figure 5. Expression of the Shorter Frazzled Isoform in All Neurons, but Not in Target Tissues, Rescues Commissure and Motor Axon Guidance Defects**

All embryos were stained with rabbit anti- $\beta$ -galactosidase (1:1000), rabbit anti-Frazzled C-terminal domain (1:1500), and either mouse MAb BP102 (1:20) or 1D4 (1:5).

(A) Commissures (green, MAb BP102) form normally in a *GAL4-1407 fra<sup>3</sup>/CyO-elav-lacZ* (wild-type) embryo. The shorter Frazzled isoform and  $\beta$ -galactosidase (both in red) are expressed in all neurons.

(B) Commissures are absent or thinned in *GAL4-1407 fra<sup>3</sup>/+ fra<sup>4</sup>* embryos. Frazzled and  $\beta$ -galactosidase staining are absent.

(C) Commissure formation is restored in *GAL4-1407 fra<sup>3</sup>/+ fra<sup>4</sup>; UAS-fra/+* embryos. The shorter Frazzled isoform (red) is expressed in all neurons;  $\beta$ -galactosidase staining is absent. Bar for (A)–(C), 10  $\mu$ m.

(D) Restored dorsal muscle innervation in a *GAL4-1407 fra<sup>3</sup>/+ fra<sup>4</sup>; UAS-fra/+* embryo (early stage 16; SNb has not fully developed). The CNS is dark due to the expression of Fra in all neurons. Bar for (D) and (E), 15  $\mu$ m.

(E) Motor axons (green) branch excessively (concave arrows) or cross segments (arrow) in a *fra<sup>3</sup>/fra<sup>4</sup>; GAL4-24B/UAS-fra* embryo that expresses Frazzled (red) in all muscles. SNb axons are absent in one hemisegment (arrowhead).

axon tracts. These remaining defects may reflect a requirement for other Frazzled isoforms in the formation of these tracts or other differences between transgene and wild-type *frazzled* gene expression.

## Discussion

We have identified a *Drosophila* DCC-related gene called *frazzled*, and demonstrated that this Ig superfamily member is expressed on and required for the pathfinding of Netrin-responsive axons. We have also provided evidence that it mediates guidance cell autonomously by rescuing the defects in motor axon targeting by expression in neurons, but not by expression in muscle. Although we have not shown directly that Frazzled binds Netrin proteins, these data and Frazzled similarity

to DCC, a vertebrate netrin receptor (Keino-Masu et al., 1996), strongly suggest that Frazzled is a receptor or a ligand-binding component of a *Drosophila* Netrin receptor. A parallel study in *C. elegans* (Chan et al., 1996) shows that *UNC-40* encodes a DCC-related protein and has a mutant phenotype that overlaps with the netrin *unc-6* phenotype (Hedgecock et al., 1990). These results indicate that the mechanisms underlying netrin-dependent axon guidance are likely to be conserved among vertebrates, flies, and nematodes.

## *frazzled* and Other DCC Family Members May Encode Netrin Receptors

Netrins are secreted, laminin-related proteins that are expressed by midline cells and other tissues in worms, fruit flies, and vertebrates. Netrins are required in vivo

for commissural axon guidance (Hedgecock et al., 1990; Harris et al., 1996; Mitchell et al., 1996) and are chemoattractants for commissural axons in vitro (Kennedy et al., 1994; Serafini et al., 1994). Netrins can also repel certain axons in vitro (Colamarino and Tessier-Lavigne, 1995) and most likely in vivo as well (Hamelin et al., 1993; Wadsworth et al., 1996).

Analysis of mutations removing the two *Drosophila* *Netrin* genes (*NetrinA* and *NetrinB*) identifies commissure formation and ISN dorsal muscle targeting as *Netrin*-dependent guidance processes (Harris et al., 1996; Mitchell et al., 1996). Midline and muscle expression of *NetrinA/B* is appropriately placed and timed and is required to steer approaching *Frazzled*-expressing axons.

*frazzled* null mutants disrupt guidance events that also depend on *NetrinA/B*, but do not have other guidance phenotypes seen in other CNS or motor axon mutants (Seeger et al., 1993; Van Vactor et al., 1993; Kolodziej et al., 1995). Among the extensive collections of mutants affecting guidance in the CNS (Seeger et al., 1993; Kolodziej et al., 1995), the *fra* phenotypes most closely resemble those of *NetrinA/B* mutants. In embryos lacking *NetrinA/B* or *frazzled*, CNS axon commissures are partially missing or thinner, with the posterior commissure disrupted more severely than the anterior commissure. In addition, occasional breaks are observed in the longitudinal tracts. The *Netrin* double mutant and *fra* null mutants also show the same spectrum of motor axon projection abnormalities, particularly in the dorsal path of the ISN and in the SNb. Ectopic expression of the *Drosophila* *Netrin* genes, but not *frazzled*, leads to misrouting of CNS and motor axons that express *Frazzled* proteins, and the presence of *Frazzled* on motor axons (SNa) that normally do not innervate *Netrin* expressing targets can explain the responsiveness of these axons to ectopic *Netrin* expression.

From the binding of vertebrate netrin to DCC (Keino-Masu et al., 1996), the study of UNC-40 (Chan et al., 1996), and these data, we do not know whether DCC-like proteins constitute a complete receptor-mediating netrin-dependent chemoattraction or a key ligand-binding component. Expression of *Frazzled* in axons that do not normally express it does not appear to cause guidance defects. Further analysis of *frazzled* and *Netrins* in *Drosophila* may help resolve this and other issues and identify additional components of this guidance system.

#### Experimental Procedures

##### *frazzled* Cloning

DNA flanking the 49B insertion was isolated by plasmid rescue (Pirota, 1986) and used to isolate additional flanking DNA. Digoxigenin-labeled (Boehringer-Mannheim) DNA probes from these genomic clones were then used to identify transcripts in the region by in situ hybridization on embryos and to clone cDNAs from embryonic cDNA libraries. Multiple clones were isolated and sequenced on both strands to obtain the entire *fra* sequence. Corresponding genomic DNA fragments were sequenced to confirm the cDNA sequence and to identify intron/exon boundaries.

##### Isolation and Identification of *fra* Mutants

The P-element at 49B was mobilized by standard methods. 56/293 excision lines were lethal; the lethal lines that failed to complement

the 49B deficiency *Df(2R)vg135* comprised a single complementation group: *frazzled*. Embryos 12–16 hr old from 20 *frazzled* mutant lines balanced over *CyO*, *P[w+, T8-lacZ]* (Kolodziej et al., 1995) were screened by in situ hybridization with *fra* and *lacZ* cDNAs. One line (*fra*<sup>1</sup>) lacking detectable *fra* transcripts in mutant embryos was identified, and subsequently shown by chromosome squashes to be a 2;3 translocation. EMS alleles were provided by Troy Zars and David Hyde or were identified in a screen for mutations affecting axon development in the CNS (Seeger et al., 1993). Mutations were analyzed by Southern blotting. DNA fragments of *fra*<sup>3</sup> were sequenced after 24 rounds of PCR on single homozygous mutant embryos and the mutation was confirmed by sequencing three independent clones.

##### Production of Antisera, Histochemistry, and Microscopy

To raise antisera against the C-terminal domain of *Frazzled*, PCR-amplified DNA encoding the 278 amino acid *Frazzled* C-terminal domain (amino acid 1328 of the longer transcript to TAA) was cloned into p6 *Frazzled* fusion protein were isolated from 20 l of culture after purification on polyhistidine affinity resin (Qiagen). Antibodies were raised against SDS-PAGE purified fusion protein at Caltag Laboratories and further purified on protein-A agarose (Boehringer Mannheim). Histochemistry and microscopy have been previously described (Kolodziej et al., 1995).

##### *frazzled* Rescue

An EcoRI-XbaI partial digestion DNA fragment containing nucleotides 1–5038 from the shorter isoform of the *frazzled* cDNA was cloned into pGAL4-UAST (Brand and Perrimon, 1993). One homozygous insertion line (chromosome III) containing the resulting *pUAS-fra* was obtained. The *GAL4* expressing panneuronal enhancer trap line 1407 was recombined with the *fra*<sup>3</sup> mutation and balanced over the *CyO*<sup>276</sup> chromosome. *CyO*<sup>276</sup>-containing embryos express *lacZ* in the embryonic salivary glands and hindgut. *yw*; 1407 *fra*<sup>3</sup>/*CyO*<sup>276</sup> females were then crossed to *yw*; *Bc EIp/CyO*<sup>276</sup>; *pUAS-fra/TM6b P[Y+]* males, and males of genotype 1407 *fra*<sup>3</sup>/*CyO*<sup>276</sup>; *pUAS-fra/+* were crossed to *fra*<sup>4</sup>/*CyO-elav-lacZ* females (or *fra*<sup>4</sup>/*CyO*<sup>276</sup> females for the motor axon rescue experiment). *CyO-elav-lacZ*-containing embryos express β-galactosidase in all neurons. 1407 *fra*<sup>3</sup>/*fra*<sup>4</sup> adult flies containing the transgene were not recovered. Experiments using the *GAL4* pan-muscle line 24B were similarly designed. Dorsal muscle connectivity was scored under 250× or greater magnification.

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#### GenBank Accession Numbers

The GenBank accession numbers for the sequences of the two *frazzled* isoforms are U71001 and U71002, respectively.