Midline governs axon pathfinding by coordinating expression of two major guidance systems

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Formation of the neural network requires concerted action of multiple axon guidance systems. How neurons orchestrate expression of multiple guidance genes is poorly understood. Here, we show that *Drosophila* T-box protein Midline controls expression of genes encoding components of two major guidance systems: Frazzled, ROBO, and Slit. In *midline* mutant, expression of all these molecules are reduced, resulting in severe axon guidance defects, whereas misexpression of Midline induces their expression. Midline is present on the promoter regions of these genes, indicating that Midline controls transcription directly. We propose that Midline controls axon pathfinding through coordinating the two guidance systems.

Supplemental material is available at http://www.genesdev.org.

Axons navigate long distances to their targets by responding to a succession of molecular guidance cues expressed along the pathway (Tessier-Lavigne and Goodman 1996; Dickson 2002). Each neuron expresses multiple axon guidance receptors and also acts as a secretion source of guidance molecules. Two major evolutionarily conserved ligand-receptor systems are involved in controlling the axonal behavior: ligand Netrin and its receptor Frazzled/DCC, and ligand Slit and its receptor ROBO (for review, see Dickson 2002). Receptors of these guidance factors are localized not only on the growth cone, but also on the axon shaft, suggesting that they exert autonomous as well as nonautonomous functions (e.g., Hiramoto et al. 2000; Hivert et al. 2002). Because these two systems are thought to act in an antagonistic way, proper axonal

behavior depends on the balance between these two systems; how such coordination is accomplished is largely unknown.

Coordination of the two guidance systems is likely achieved, at least in part, via regulating the expression levels of these ligands and receptors. Forced transcription of guidance genes often causes pathfinding errors (Harris et al. 1996; Mitchell et al. 1996; Kidd et al. 1998, 1999), indicating that transcriptional regulation plays an important role in determining the expression levels. Identifying a transcriptional regulator of axon guidance genes is thus an essential step in understanding how the coordination of axon guidance systems is established and maintained. Here, we show that *Drosophila* T-box transcription factor Midline is a key regulator controlling the expression of multiple components of the two major guidance systems: Frazzled, ROBO, and Slit.

Results and Discussion

Identification of midline gene

midline (*mid*) was initially found as one of the genes affecting the larval cuticle pattern, having deletions in pattern elements along the ventral midline (Nüsslein-Volhard et al. 1984). We identified *mid* as a gene encoding a novel T box protein of tbx20 subclass (Fig. 1A; Ahn et al. 2000; Yamagishi et al. 2004; Reim et al. 2005; Takeuchi et al. 2005; Song et al. 2006). T box proteins are transcription factors that play key roles during development (Stennard and Harvey 2005). Of eight T-box genes present in the *Drosophila* genome, CG6634 maps close to the location of *mid* at the cytological location 25E2. Three independently isolated *mid* alleles all contained point mutations in the ORF of CG6634, indicating that this gene corresponds to the *mid* locus. *mid[1]* and *mid[2]* harbor a nonsense mutation before and within the T box domain, respectively, and *mid[D62]* carries an 11-base-pair (bp) deletion in the T box region (Fig. 1A). Identification of *mid* as CG6634 was also made independently by Buescher et al. (2004), Reim et al. (2005), and Gaziova and Bhat (2009).

MID is expressed in post-mitotic neurons and is required for the axon pathfinding

mid mRNA is initially expressed in a pattern of 14 stripes in stage 6 embryos (data not shown). Following gastrulation and germband extension, the developing nervous system exhibits a segmentally repeated striped pattern. In stage 16 embryos, high levels of *mid* mRNA and MID protein are seen in a subset of CNS neurons (Fig. 1B,C; Buescher et al. 2006). Expression of *mid* was also observed in sensory neurons of the peripheral nervous system (Fig. 1D–F). Previous studies on *mid* function focused on its role in segmentation and cell fate specification during neurogenesis (Nüsslein-Volhard et al. 1984; Buescher et al. 2004, 2006; Gaziova and Bhat 2009; Leal et al. 2009). Since MID is highly expressed in post-mitotic neurons during axonogenesis, we investigated the axon wiring phenotype of *mid* (Jacobs 1993) that might reflect the role at this stage. In normal embryos, axon tracts in the CNS are organized in a ladder-like scaffold, with
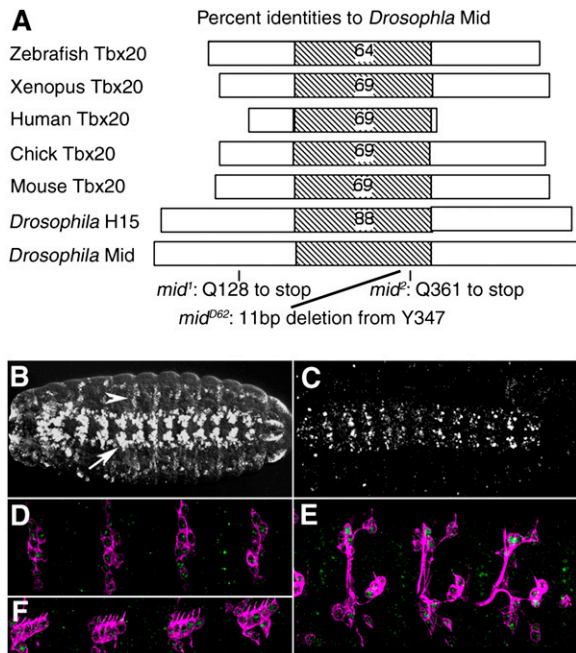
Figure 1. Molecular analysis of the *mid* gene. (*A*) The domain of *Drosophila* MID, aligned with other T-box proteins, showing the percentage of identical amino acid residues within the T-box domain. The domain consists of the amino acid residues 194–381 of MID. *mid¹* contains a nonsense mutation at amino acid 128. *mid²* contains a nonsense mutation at amino acid 361 in the T-box domain of MID. *mid^{D62}* contains an 11-bp deletion in the ORF, which results in deletion of Y347 and V348, and a frame shift. (*B*) In situ hybridization analysis of *mid* expression in a stage 16 whole-mount normal embryo. *mid* mRNA expression is confined to a subset of CNS neurons (arrow), epidermis, and sensory neurons in the periphery (arrowhead). (*C*) A normal stage 16 embryo stained with anti-MID. MID is detected in essentially the same pattern as in *B*. *mid¹*, *mid²*, *mid^{D62}*, or *Df(2L)cl-h1* mutants embryos exhibited no anti-MID immunoreactivity (data not shown). (*D–F*) Staining of MID (green) and 22C10 (magenta). MID is expressed in the 22C10-positive sensory neurons, including dp and dda (*D*), vp and vch (*E*), lch1 and lch5 (*F*).

longitudinal axon bundle running on both sides of the midline and two commissures, anterior and posterior, connecting the bilateral neuromeres. In *mid* mutant, longitudinal axon tracts were often interrupted (65%, *n* = 96), and the posterior commissure was much thinner than normal (71% of the commissures, *n* = 96), suggesting defects in pathway selection and/or axon elongation (Fig. 2B). These defects were largely rescued by pan-neuronal expression of *mid* (Fig. 2C). In the periphery, afferent axon of the sensory neurons often crossed the segment border (38%, *n* = 108) (Fig. 2G).

To identify the cellular defects of *mid* mutant embryos during axonogenesis we examined the axonal projections of several identified neurons, motoneurons RP1 and RP3, and a longitudinal pioneer neuron dMP2 (Hidalgo and Brand 1997), which do not express MID. Using specific cellular markers such as anti-Eve antibody, Lim3A-tau-myc transgene (Thor et al. 1999), or MAb 22C10, no alterations in gene expression pattern or cell position could be detected in *mid* mutants, suggesting that these cells do not have cell fate alterations caused by early MID function in segmentation or neurogenesis. In normal embryos RP1 and RP3 motor axons exit the CNS toward

the periphery. In *mid* mutant embryos, however, axons of these neurons stalled or followed longitudinal axons (Fig. 2E). Axon of the dMP2 neuron also showed a severe stall phenotype, failing to connect to the next neuromere (Supplemental Fig. S1). The absence of endogenous MID expression in these neurons suggests that these cellular phenotypes in *mid* mutant are caused by defects in axon guidance environments that depend on *mid* function. One possibility is that MID controls expression of secreted guidance factors, or their receptors that possess not only cell-autonomous functions but also nonautonomous activities.

MID controls the expression of frazzled

The axon scaffold phenotype of *mid* mutant embryo, such as the thinning of the commissure and interrupted longitudinal axons, contains features of defects in the two major axon guidance systems: Netrin/Frazzled and Slit/ROBO. To identify potential transcriptional target genes of MID in axon guidance, we examined whether or not *mid* exhibits genetic interactions with components of these two axon guidance systems. While animals heterozygous for *mid*-null allele were normal, *trans*-heterozygotes of *mid* and *frazzled* and *trans*-hetrozygotes of *mid*
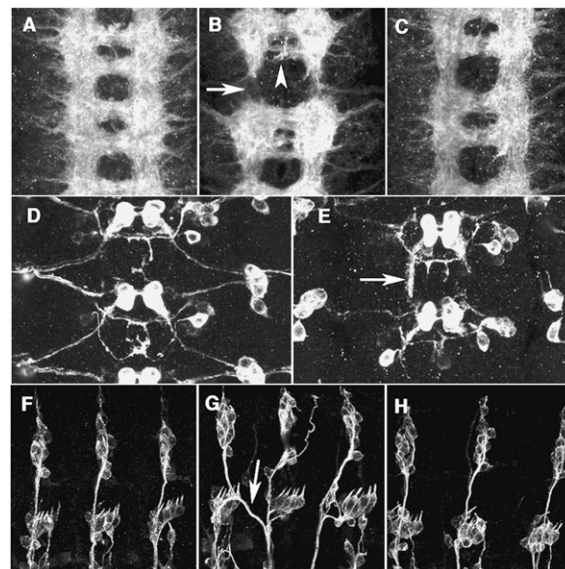

Figure 2. MID is required for CNS and PNS pathfinding. (*A–C*) A stage 16 embryo stained with the monoclonal antibody (mAb) BP102 to reveal the axonal scaffold of the CNS. Anterior is up. (*A*) Normal CNS. (*B*) In *mid¹* mutant, the longitudinal tracts were interrupted (arrow) and posterior commissures were thinner (arrowhead). (*C*) Normal axonal scaffold is restored by pan-neural expression of MID (*elav-Gal4/+; mid¹/mid¹; UAS-mid/+* embryo). (*D,E*) Visualization of lim3A⁺ motor neurons in a stage16 embryo using the *lim3A-tau-myc* transgene (anti-Myc epitope staining). (*D*) Normal. (*E*) Motor axons stall and follow a longitudinal path in *mid¹* mutant (arrow). Similar motoneuron projection defects were observed in *mid²* and *mid^{D62}* embryos (data not shown). (*F–H*) A stage16 embryo stained with mAb 22C10 to reveal the sensory axons in the PNS. Anterior is left and dorsal up. (*F*) Normal PNS. (*G*) In *mid¹* mutant, sensory axons branch excessively or cross the segment borders (arrow). (*H*) Restored dorsal sensory axons in an *elav-Gal4/+; mid¹/mid¹; UAS-mid/+* embryo. Similar phenotypes of CNS and PNS were also observed in *mid²* or *mid^{D62}* mutant embryos (data not shown).

and *slit* showed strong defects in the longitudinal tracts (Supplemental Fig. S2). This raised the possibility that MID is involved in the transcriptional regulation of components of the Netrin/Frazzled and Slit/ROBO systems.

We first examined the Netrin system, Netrin encoded by *NetrinA* and *NetrinB* genes, and their receptor Frazzled, which has a dual function as a cell-autonomous guidance receptor and a nonautonomous ligand presen-

tation receptor (Kolodziej et al. 1996; Hiramoto et al. 2000). In normal CNS, *Frazzled* mRNA is expressed in many neurons. In *mid* mutant embryo, cluster of *mid*-expressing neurons exhibited specific reduction of *Frazzled* mRNA (Fig. 3A,B; e.g., 47% reduction in the marked area). Reduction of Frazzled protein could also be detected in commissural and longitudinal axons in the CNS as well as in the mechanosensory neurons (ch and es) in the periphery (Fig. 3C,D). This reduction of Frazzled in either CNS or periphery was restored by pan-neuronal expression of *mid* (Fig. 3C,D). Moreover, misexpression of MID in salivary glands induced the expression of *frazzled* (Fig. 4A). These results indicate that MID can regulate the expression of *frazzled*. On the other hand, expression levels of *NetrinA* and *NetrinB* were not significantly altered, either in *mid* mutant or upon MID misexpression (Fig. 4A; data not shown). Thus, MID controls Netrin/Frazzled signaling through regulating *frazzled* expression.

MID controls the expression of robo and slit

Loss of MID function also affected the expression of components of the Slit/ROBO system. In situ hybridization and immunostaining revealed that expression of *robo* mRNA and protein was significantly reduced in *mid* mutant (Fig. 3E–G; e.g., 59% reduction in the marked area). This reduction of ROBO was restored by pan-neuronal expression of *mid* (Fig. 3G). Loss of *mid* activity also affected an aspect of *slit* expression. In normal embryos, *slit* mRNA is highly expressed in midline glia and weakly in neurons located lateral to the midline (Fig. 3H). Slit expression in these lateral cells also contributes to the guidance of the longitudinal axons (Hiramoto and Hiromi 2006). In *mid* mutant, *slit* mRNA expression in lateral cells was severely reduced (Fig. 3H; e.g., 90% reduction in the area marked by an arrowhead). As with *frazzled*, expression of *slit* and *robo* mRNA and Slit protein was induced upon MID overexpression in the
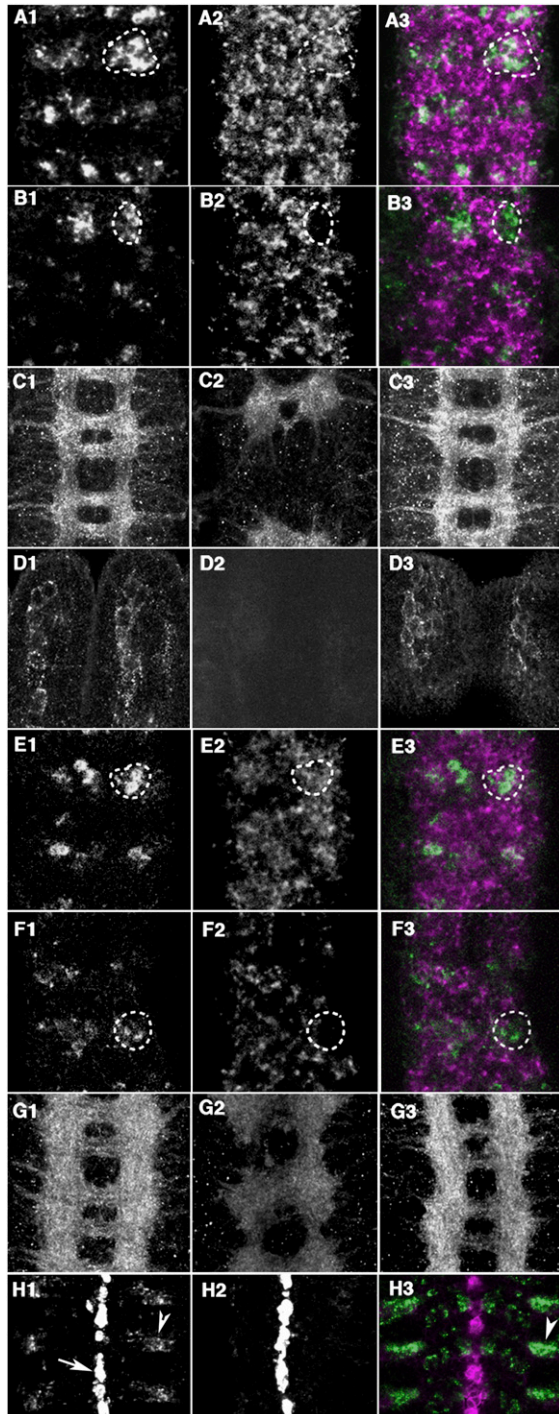

Figure 3. *mid* is required for the expression of multiple axon guidance molecules. (*A1–B3*) Stage 16 *mid*[1] mutant embryos and their heterozygous siblings were examined for *frazzled* mRNA expression. (*A1–A3*) A *mid*/+ heterozygous embryo shows clear expression of *frazzled* mRNA (*A2*) in *mid*-positive cells (*A1*). (*B1–B3*) *mid*[1] mutants show reduced or no expression of *frazzled* (*B2*) in *mid* mRNA-labeled cells (*B1*). (*C1–D1*) Stage 16 embryos stained with anti-Frazzled antibody. (*C1*) Normal CNS. (*C2*) *mid*[1] CNS is weakly stained than normal embryos. (*C3*) Restored Frazzled expression in an *elav-Gal4*/+; *mid1/mid1*; *UAS-mid*/+ embryo. (*D1*) Wild-type mechanosensory neurons. (*D2*) No signal was detected in the *mid*[1] mechnosensory neurons. (*D3*) Restored Frazzled expression in the mechnosensory neurons of an *elav-Gal4*/+; *mid*[1]/*mid*[1]; *UAS-mid*/+ embryo. (*E1–F3*) Stage 16 *mid*[1] mutant embryos and their heterozygous siblings were examined for *robo* mRNA expression. (*E1–E3*) A *mid*/+ heterozygous embryo shows clear expression of *robo* mRNA (*E2*) in *mid*-positive cells (*E1*). (*F1–F3*) *mid*[1] mutants shows reduced or no expression of *robo* mRNA (*F2*) in *mid* mRNA-labeled cells (*F1*). (*G1–G3*) Stage 16 embryos stained with anti-ROBO antibody. (*G1*) Normal CNS. (*G2*) *mid*[1] CNS is weakly stained than normal. (*G3*) Restored ROBO expression in an *elav-Gal4*/+; *mid1*/ *mid1*. (*H1–H3*) In situ hybridization analysis of *slit* expression in a stage 16 embryo. (*H1*) In normal embryo, the highest level of *slit* mRNA occurs in the midline glia (arrow), whereas low levels of *slit* mRNA are present in cells located lateral to the midline (arrowhead). (*H2*) In *mid*[1] mutant, *silt* mRNA expression in lateral regions is weaker than normal. (*H3*) Colocalization of *mid* (green) and *slit* (magenta) mRNA in lateral cells (arrowhead).

salivary gland (Fig. 4A; Supplemental Fig. S3). Thus, *mid* induces the expression of Frazzled, ROBO, and Slit, components of the two major axon guidance systems.

A direct control of frazzled, robo, and slit by MID

To address how MID activates expression of the three axon guidance genes, we first determined the binding
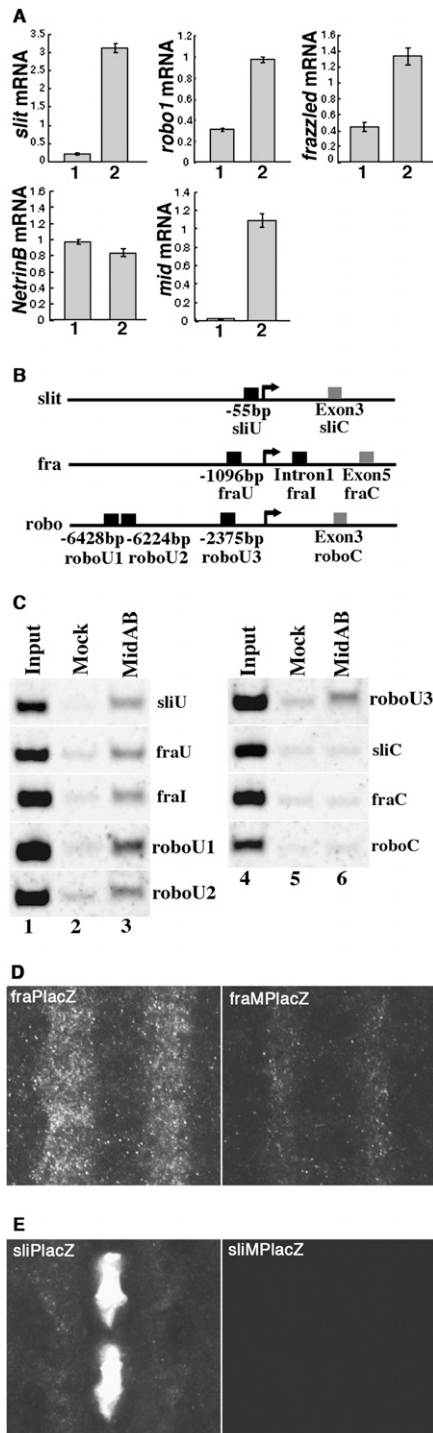
sequence of MID using an in vitro binding site selection method (Liu et al. 2003). MID-binding sequence was selected from a pool of random oligonucleotides using MID protein affinity-purified from an embryonic extract. The consensus sequence deduced from the selected oligonucleotides was (G/A/T)NA(A/T)N(T/G)(A/G)GGTCAAG (Supplemental Fig. S4). This sequence was found in the upstream regions or an intron of *slit*, *frazzled*, and *robo* (Fig. 4B), and all of these sites were conserved among several *Drosophila* species (Supplemental Fig. S5). To determine whether MID binds to these regions in vivo, we performed chromatin immunoprecipitation (ChIP) using anti-MID antibody. In all three genes, MID was present around the MID-binding sites, but not on regions without the binding site (Fig. 4C; Supplemental Fig. S6). In contrast, a potential MID-binding site 32-kb upstream of the *commisureless* gene, whose expression is not affected in *mid* mutants, was not occupied by MID (data not shown).

We next assessed the importance of the MID-binding sites in *frazzled* and *slit* by transgenic reporter assays. To test the role of the MID site in *frazzled*, reporter genes were constructed that contain the transcription start site of *frazzled* and an upstream region including a wild-type MID-binding site (fraPlacZ) or a mutated site (fraMPlacZ). Compared with the wild-type reporter gene, the reporter with a mutated binding site showed reduced expression levels (33% reduction) (Fig. 4D). Thus MID-binding site is indeed required for the proper expression of *frazzled*. Mutating the MID-binding site in *slit* also caused a severe effect on slit expression. The lacZ expression in sliPlacZ is driven by the *slit* regulatory element and the endogenous promoter. While sliPlacZ with the wild-type binding site recapitulated the *slit* expression in the midline glia and lateral cells, base substitutions in the MID-binding site in sliMPlacZ abolished the lacZ expression (Fig. 4E). It is possible that the MID-binding site resides in an essential promoter element of *slit*, and hence, the base substitutions abolished *slit* transcription in all cells. However, the same results were obtained using sli4.5HHlacZ and sliM4.5HhlacZ in which the *slit* regulatory element is fused to a heterologous *hsp70* promoter (Supplemental Fig. S7). As *mid* was expressed in the lateral cells but not in midline glia, these results suggest that MID-binding sites in *slit* control *slit*

Figure 4. MID coordinates expression of key axon guidance genes. (*A*) Levels of mRNA in the salivary gland of normal third instar larvae (lane *1*) or upon MID misexpression (*sgP[Gal4]/+; UAS-mid/+*) (lane *2*). Shown are levels of transcripts normalized to the corresponding levels of tubulin mRNA in each sample, as determined by quantitative real time PCR. (*B*) Schematic representation of the MID-binding sites on the genomic sequence of *slit*, *frazzled*, and *robo*. The black boxes indicate the MID-binding site and the gray boxes indicate regions without the MID-binding site adopted as negative controls. Only the sites with perfect match with the consensus sequence were considered. The arrows represent transcription start sites and the numbers in base pairs are distance from the start sites. (*C*) ChIP with the antibody against MID revealed occupancy of MID at sliU, fraU, fraI, roboU1, roboU2, and roboU3, but not at the control regions sliC, fraC, and roboC (shown in lanes *3,6*). The input was 0.1% (lanes *1,4*). No primary antibody was used for mock (lanes *2,5*). (*D,E*) Staining of stage 16 embryos with an anti-β-galactosidase antibody. (*D*) The reporter fraPlacZ was expressed in CNS and the mutant reporter fraMPlacZ exhibited significantly reduced expression in the CNS. (*E*) The reporter sliPlacZ but not the reporter sliMPlacZ was expressed in the midline glia and lateral cells.

transcription via binding to multiple factors: MID in lateral cells and unknown factor(s) in midline glia. Taken together, these results demonstrate a direct role for MID in the regulation of *frazzled* and *slit*, and suggest that MID governs the expression of multiple axon guidance genes through directly binding of the MID sites in their regulatory regions.

MID directs the expression of components of two major axon guidance systems

We showed that MID directly controls transcription of key components of the two major axon guidance systems: the Netrin/Frazzled system and the Slit/ROBO system. Because these two systems are considered to have opposing outputs, it is interesting that the expression of both systems are induced by the same transcription factor, MID. Dynamic expression of Frazzled and ROBO is required for growth cones to simultaneously respond to both attractants and repellents, integrate these signals, and then respond to the relative balance of forces (Bashaw and Goodman 1999; Garbe and Bashaw 2007). These molecules also provide nonautonomous functions required for cell motility, such as mediating cell adhesion and promoting axon elongation (Hiramoto et al. 2000; Rhee et al. 2002). The coordination of axon guidance systems by MID may thus ensure cooperative actions of multiple guidance molecules in growth cone dynamics, axonal adhesion, and elongation. The role of MID in the transcriptional regulation of axon guidance might be a conserved function, because its orthologs of human, mouse, and zebrafish Tbx20 are also expressed in motor neurons (Ahn et al. 2000; Takeuchi et al. 2005).

Materials and methods

Sequencing of mid mutant alleles

Homozygous *mid* mutants were identified in embryo collections by the use of the *CyOact-GFP* balancer chromosome. For each mutant allele, we sequenced both genomic exons and the cDNA after PCR amplification and subcloning. For each PCR, three clones were sequenced on both strands. Mutations appeared in all three clones; no other areas of the ORF showed reproducible sequence alterations.

Generation of transformants

fraPlacZ was made by inserting a 4.4-kb genomic fragment containing the upstream region and transcription start site of *frazzled* with a MID-binding site (CTTGACCCACATTT) into CaSpeR-AUG-β-gal vecter. fraMPlacZ was made from fraPlacZ and contained a mutated MID-binding site (ACCTGTTAGA CGGG). *slit* promoter reporter constructs were designed as described (Wharton and Crews 1993). sliPlacZ was constructed by placing 1.2NM to the upstream of 4.8 BV containing a MID-binding site (TCAGTTTGGTCAAG). sliMPlacZ was made from sliPlacZ and contained a mutated MID-binding site (CTG TCCCAACTCCA). sliM4.5HHlacZ containing the mutated MID-binding site was made from sli4.5HHlacZ. *UAS-mid* was made by inserting the *mid* cDNA into the pUAST plasmid (Brand and Perrimon 1993). Several independent transgenic lines were used, with identical results. The pan-neuronal expression of *mid* was achieved by driving *UAS-mid* transgene using *elav-Gal4* (Lin and Goodman 1994). Misexpression in the salivary gland was done using *sgP[Gal4]/+* driver strain (Liu et al. 2003).

Immunofluorescence staining

Embryos were fixed and processed as described (Liu et al. 2003). Rabbit anti-MID antibody was made using bacterially expressed C-terminal 83 amino acids of MID as an antigen, and was used at 1:1000 dilution. We obtained the following monoclonal antibodies from Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD: 22C10 (1:20; developed by S. Benzer), P102 (1:20; developed by C. Goodman); anti-Myc 9E10 (1:10; developed by J. M. Bishop); anti-Slit mAb C555.4 (1:50; developed by S. Artavanis-Tsakonas; Kidd et al. 1999); anti-ROBO mAb M13C9 (1:20; developed by C. Goodman; Kidd et al. 1998). Rabbit anti-Frazzled (1:500) was a gift from P. Kolodziej (Kolodziej et al. 1996); Rabbit anti-β-galactosidase (1:2000) was purchased from Cappel. Mutant embryos were identified using balancer chromosome carrying the β-galactosidase gene, or staining with anti-MID antibody.

DNA-binding selection and ChIP

In vitro DNA-binding selection was performed as described (Liu et al. 2003). ChIP was performed as described (Smith et al. 2001), using 10- to 20-h embryos. PCR primers were designed to amplify ~100-bp fragments (Supplemental Material).

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