The Homeobox Transcription Factor Even-skipped Regulates Netrin-Receptor Expression to Control Dorsal Motor-Axon Projections in Drosophila

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Summary
Homeobox transcription-factor codes control motor-neuron subtype identity and dorsal versus ventral axon guidance in both vertebrate and invertebrate nervous systems [1-4]; however, the specific axon guidance-receptors that are regulated by these transcription factors to control pathfinding are poorly defined. In Drosophila, the Even-skipped (Eve) transcription factor specifies dorsal motor-axon projection [5] through the regulation of unidentified guidance molecules. The Netrins and their attractive and repulsive receptors DCC and Unc-5, respectively, define important conserved cue and receptor families that control growth-cone guidance [6]. In Drosophila, the Netrins and frazzled (the fly homolog of DCC) contribute to motor-axon guidance [7-9]. Here, using genetics and single-cell mRNA-expression analysis, we show that expression and requirement of different Netrin receptor combinations correlate with distinct dorsal and ventral motor-axon projections in Drosophila. Misexpression of eve dorsalizes ventral axons in part through the upregulation of Unc-5, whereas loss of eve function in two dorsally projecting motor neurons results in aberrant axon projections and a failure to express Unc-5. Our results support a functional link between the expression of distinct Netrin receptor combinations and the transcriptional control of dorsal motor-axon guidance.

Results and Discussion
Unc-5 Mutants Display Multiple Defects in Motor-Axon Guidance
Genetic analysis indicates that the Drosophila Netrins (NetA and NetB) and the attractive Netrin receptor Fra guide subsets of embryonic motor axons [7-9]. Specifically, NetAB double mutants affect multiple motor projections, including the dorsally projecting intersegmental nerve (ISN), the laterally projecting segmental nerve (SN), and the ventrally projecting ISNb [7, 9], whereas fra mutants disrupt the dorsal ISN and the ventral ISNb [8]. In contrast to attraction mediated by DCC/Fra/Unc-40 receptors, Unc-5 receptors mediate repulsion [10-12]. Unc-5 can act either independently or together with DCC to mediate Netrin repulsion [13-15]. Previous studies of Unc-5 in Drosophila have examined the effects of mis- and overexpression of Unc-5 [10]. Analysis of endogenous Unc-5 function has been limited to RNA interference (RNAi) approaches, where Unc-5 function was reduced in embryos overexpressing NetB. In these experiments, Unc-5 RNAi partially suppresses the gain-of-function NetB phenotype, suggesting that Unc-5 functions as a repulsive Netrin receptor [10].

To further address the endogenous role of Unc-5, we have generated mutations in Unc-5 and examined their effects on motor-axon guidance in Drosophila embryos (see Supplemental Experimental Procedures and Tables S1 and S2 in the Supplemental Data available with this article online). Before describing the defects in Unc-5 mutants, it is useful to review the phenotypes observed in NetAB double mutants and fra mutants [7-9, 16]. NetAB and fra mutants both affect the trajectory of the ISN, which normally projects dorsally past the epidermal stripe of NetA expression to innervate dorsal muscles (Figure 1A). Specifically, the ISN is observed to inappropriately (1) stall, (2) branch excessively, (3) cross segment boundaries, and (4) project beyond the dorsal target muscles (Figures 1B and 1D). Both NetAB and fra mutants also frequently disrupt the normal ventral ISNb innervation of the NetB expressing muscles 6 and 7 (Figures 1B and 1D). In addition, lateral SNa axon projections are disrupted in NetAB double mutants. In wild-type, SNa projects to the lateral muscle field, where it bifurcates to innervate muscles 5 and 8 and transverse muscles 21, 22, 23, and 24 (Figure 1A). In NetAB double mutants, SNa sometimes stalls or is missing one of the two major branches (Figure 1B). We do not see these phenotypes in fra mutants (Figure 1D), suggesting that Netrin's influence on SNa guidance is fra-independent. This finding is consistent with previous observations that the NetB gain-of-function phenotype in SNa is fra-independent [16].

Loss of Unc-5 function results in defects that overlap with the phenotypes observed in fra and NetAB mutants (Figure 1C). Specifically, in Unc-5 mutants, like in NetAB and fra mutants, the dorsally projecting ISN inappropriately crosses the segment boundary (Figure 1C). The Unc-5 ISN defects are observed at similar frequency to NetAB and at a higher frequency than fra, which consistently shows weaker ISN crossover phenotypes (Figure 1E). We interpret these phenotypes as a failure to be repelled by the epidermal stripe of NetA expression (Figure 1B). In contrast to fra, where the SNa is not affected, Unc-5 mutants display SNa defects that are qualitatively similar to those observed in NetAB, including premature stalling and absence of one or both branches (Figure 1C). This suggests that Netrin influence on SNa guidance represents a repul-
Figure 1. Motor-Axon-Guidance Defects in NetAB, fra, and Unc-5 Mutants

Stage-17 embryos stained with anti-Fasll Mab to reveal the motor projections. Images represent the maximum projection of confocal stacks to reveal all projections in a single focal plane. Anterior is left and dorsal is up in all panels. Three adjacent abdominal hemisegments are shown. Partial genotypes are indicated below each panel. Top panels are schematic representations of the wild-type and mutant-motor-axon projections. Middle panels show the more dorsal projections, and bottom panels show the more ventral projections.

(A) Wild-type. The top panel indicates the normal projections of the ISN, SNa, and ISNb as well as the domain of Netrin expression. For simplicity, NetA and NetB expression are combined and shown as shaded ovals. Netrin is expressed in a presumptive gradient from the CNS midline (not shown), in ventral muscles 6 and 7, in a more lateral epidermal stripe, and in dorsal muscles 1 and 2. Note the normal trajectory of the ISN as it projects dorsally and respects the segment boundary (middle panel).

(B-D, middle panels) The ISN can be seen inappropriately crossing the segment boundary and fasciculating with the ISN in neighboring segments (arrows with asterisks). NetAB double mutants exhibit defects in 23.5% ± 3.3% of all segments examined (B), and Unc-5 mutants exhibit defects in 24.5% ± 3.5% of all segments examined (C). The ISN defects are observed less frequently in fra mutants, where defects were exhibited in 6.3% ± 1.8% of all segments examined (D).

(A, bottom panel) Wild-type. The SNa and ISNb nerve branches are indicated. Note the two branches of the SNa as they make appropriate contacts with their target muscles (arrowheads). The normal innervation of muscles 6 and 7 by the ISNb is also indicated (arrowheads).

(B-D, bottom panels) In NetAB double mutants (B), and Unc-5 mutants (C), one or both of the SNa branches are sometimes missing (arrowheads with asterisks). NetAB double mutants and Unc-5 mutants exhibit these SNa defects in 15.3% ± 4.3% and 19.4% to 28.2% ± 3.9% of all segments examined, respectively. In contrast, in fra mutants (D), SNa innervations are normal (arrowheads). In NetAB double mutants (B) and fra mutants (D), the ISNb innervation of muscles 6 and 7 is often absent (arrowheads with asterisks). NetAB double mutants and fra mutants exhibit this ISNb phenotype in 39.7% and 28.2% of all segments examined, respectively. In Unc-5 mutants (C), ISNb innervations are normal (arrowheads).

Differential Expression of Unc-5 and fra in Subsets of Motor Neurons

We next investigated the possibility that neurons with differential genetic requirements for Unc-5 and fra may show differential expression of the receptors. To examine expression patterns of Unc-5 and fra in specific dorsal, lateral and ventral motor neurons, we labeled embryos with fluorescent-RNA in situ probes and simultaneously detected protein markers that define dorsally projecting ISN motor neurons (RN2Gal4 driving UASTauMycGFP [RN2TMGJ] [17]), laterally projecting SN motor neurons (Fasll) [18], and ventrally projecting...
**Figure 2. Unc-5 and fra mRNA-Expression Patterns in Defined Motor Neurons**

(A) A schematic diagram summarizing the positions of cells and the expression of fra and Unc-5 for the dorsally projecting motor neurons labeled by RN2Gal4 (aCC and RP2 are colored blue) and some of the ventrally projecting neurons labeled by HB9Gal4 (RPs are colored red). (B-E) Stage-13 RN2Gal4::TauMycGFP embryos (B and C) or stage-17 dHB9Gal4::TauMycGFP embryos (D and E) were double labeled with RNA in situ probes for either Unc-5 (B and D) or fra (C and E) and antibodies to GFP to examine defined motor neurons. The top panels show RNA signals in green, and the bottom panels show the overlay of the RNA (green) and protein signals (red). Anterior is up in all panels. White hash marks in the in situ panels indicate the positions of the XZ and YZ sections displayed below and to the right of the main XV panels, respectively. In (B and C), two separate XZ sections are shown, one for aCC and one for RP2. (B) Unc-5 is expressed in both the aCC and RP2 motor neurons (arrowheads). (C) fra is also expressed in aCC and in RP2 (arrowheads). (D and E) A dorsal (internal) layer of stage-17 embryos stained with anti-GFP to reveal some of the midline RP neurons, and either Unc-5 in situ probe (D) or fra in situ probe (E) shows that the midline RP neurons express fra (arrowheads in E) but not Unc-5 (empty arrowheads in E). Note the clear localization of both unc-5 and fra in aCC and RP2 in the XZ and YZ sections (B and C) and the localization of fra but not Unc-5 in the midline RP neurons in the XZ and YZ sections (D and E).

ISNb motor neurons (HB9Gal4 driving UASTauMycGFP [Hb9TMG]) [19]. In addition to labeling axons, these markers clearly label neuronal cell bodies, allowing us to determine whether the fra and Unc-5 mRNAs are specifically expressed in these neurons (Figure 2). Here, it is critical to use fluorescence mRNA in situ, rather than antibody staining, to detect receptor expression because antibody staining does not allow us to resolve which individual neurons express the receptors.

Double labeling of stage-13 embryos with RN2TMG and Unc-5 or fra mRNA probes shows that both receptors are expressed by the RP2 and aCC neurons, pioneers of the ISN (Figures 2A–2C). Both receptors are also clearly expressed in additional cells not labeled by our protein markers, with fra showing a considerably broader expression pattern than Unc-5. Expression of fra and Unc-5 in the ISN is consistent with phenotypes of the fra and Unc-5 mutants and suggests that the ISN-guidance defects may reflect a loss of Netrin repulsion rather than a loss of attraction, which had been previously inferred from the similarity of fra and NetAB mutants [9]. Interestingly, and consistent with the inability to detect Unc-5 protein on the ISN in late-stage embryos [10], Unc-5 mRNA expression in aCC and RP2...
this observation, previous studies have shown that agreement between the mRNA expression data and the receptor expression in interneurons by the Lola transduction factor provides another example of functional transcription factor.
expression pattern (except for the expression in the
these fluorescence units 1.89 ± 0.13 SEM in stage-12 embryos and 2.57 ± 0.13 SEM in stage-13 embryos) and RP2 (green fluorescence units 0.20 SEM in stage-13 embryos) and RP2 (green fluorescence units 0.24 SEM in stage-12 embryos and 0.33 ± 0.20 SEM in stage-13 embryos) and RP2 (green fluorescence units 3.13 ± 0.15 SEM in stage-12 embryos and 7.46 ± 0.20 SEM in stage-13 embryos). Indeed, the cells are readily identified even in the absence of the LacZ probe (bottom panel, arrowheads point to RP2 and aCC). (B) eve mosaic mutants show reduced or absent expression of Unc-5 in both aCC (green fluorescence units 1.69 ± 0.13 SEM in stage-12 embryos and 2.57 ± 0.13 SEM in stage-13 embryos) and RP2 (green fluorescence units 0.86 ± 0.11 SEM in stage-12 embryos and 2.57 ± 0.13 SEM in stage-13 embryos). It is difficult to identify these neurons in the single label (bottom panel, empty arrowheads point to missing Unc-5 mRNA in RP2 and aCC). However, other cells that are not mutant for eve show robust Unc-5 expression (also [C], bottom panel, glia quantification). (C) Quantification of fluorescence in stage-12 embryos (top panel) and stage-13 embryos (bottom panel). Genotypes for each cell analyzed are indicated on the x axis, and green or red fluorescence intensity is indicated on the y axis. Note that whereas the red fluorescence is drastically reduced or at background levels in the mutants as compared to the heterozygous siblings. Green fluorescence (i.e., Unc-5 mRNA) in nonmutant cells (glia, bottom panel) is comparable between mutant and heterozygous animals.

The bottom panels show RNA signals in green and LacZ antibody in red. The top panels show fluorescence RNA in situ with probes to Unc-5 in green and LacZ antibody to identify the RP2 and aCC neurons in red. White hash marks indicate the positions of the XZ and YZ sections displayed below and to the right of the main XY panels, respectively. Two separate XZ sections are shown, one for aCC and one for RP2.

Figure 4. Endogenous eve Regulates Unc-5
Stage-12 mosaic eve mutant embryos (B) and their heterozygous siblings (A) were examined for Unc-5 mRNA expression. The top panels show fluorescence RNA in situ with probes to Unc-5 in green and LacZ antibody to identify the RP2 and aCC neurons in red. The bottom panels show RNA signals in green. Anterior is up in all panels. White hash marks indicate the positions of the XZ and YZ sections displayed below and to the right of the main XY panels, respectively. Two separate XZ sections are shown, one for aCC and one for RP2.

(A) A mosaic eve/+ heterozygous embryo shows clear expression of Unc-5 mRNA in both aCC (green fluorescence units 6.04 ± 0.24 SEM in stage-12 embryos and 8.33 ± 0.20 SEM in stage-13 embryos) and RP2 (green fluorescence units 3.13 ± 0.15 SEM in stage-12 embryos and 7.46 ± 0.20 SEM in stage-13 embryos). Indeed, the cells are readily identified even in the absence of the LacZ probe (bottom panel, arrowheads point to RP2 and aCC).

(B) eve mosaic mutants show reduced or absent expression of Unc-5 in both aCC (green fluorescence units 1.69 ± 0.13 SEM in stage-12 embryos and 2.57 ± 0.13 SEM in stage-13 embryos) and RP2 (green fluorescence units 0.86 ± 0.11 SEM in stage-12 embryos and 2.57 ± 0.13 SEM in stage-13 embryos). It is difficult to identify these neurons in the single label (bottom panel, empty arrowheads point to missing Unc-5 mRNA in RP2 and aCC). However, other cells that are not mutant for eve show robust Unc-5 expression (also [C], bottom panel, glia quantification).

There is a clear correlation between the expression and requirement of different combinations of Netrin receptors and the transcription-factor code controlling dorsal versus ventral motor-axon projection. Motor neurons that project in the dorsal ISN express the eve homeodomain transcription factor and both fra and Unc-5, whereas motor neurons that project in the ventral ISNb do not express eve (they instead express Islet, Nkx6, and dHB9 [19, 23–25]) and express fra but not Unc-5. Given this positive correlation between eve and Unc-5 expression, we next tested whether there is any link between eve function and Unc-5 expression.

Previous studies have established that eve specifies the dorsal growth of ISN motor axons [5, 17]. For example, loss of eve function in just two dorsally projecting ISN motor neurons, RP2 and aCC, results in a failure of these neurons to project dorsally [17]. Interestingly, in these eve mosaic mutants, generated by rescuing eve null mutants with expression of eve under the control of a promoter element that recapitulates the entire eve expression pattern (except for the expression in the RP2, aCC, and pCC neurons [17]), ISN axons are frequently observed to inappropriately cross segment boundaries (Figures 3A–3C), a phenotype reminiscent of Unc-5 mutants. In contrast to eve loss of function, misexpression of eve in ventrally projecting ISNb motor neurons redirects their axons dorsally, where they join and fasciculate with the dorsal branch of the ISN (Figures 3C and 3E) [5]. Importantly, neither loss nor gain of eve function dramatically alters cell fate; Fasll expression and eve enhancer function are maintained in RP2 and aCC in eve mosaic mutants [17], and normal numbers of Faslll and Connectin positive ventral motor neurons are generated in eve gain-of-function embryos [5].

To test whether ectopic expression of eve influences Unc-5 expression, we examined embryos misexpressing eve for Unc-5 mRNA levels and observed a striking increase in the recruitment of Unc-5-expressing cells to the dorsal ISN (Figures 3G and 3H). We reasoned that if the observed upregulation of Unc-5 contributes to the "dorsalization" of ISNb axons, ectopic expression of Unc-5 should also change the normal behavior of ISNb axons and cause them to project more dorsally. This is indeed the case: Unc-5 misexpression results in dramatic defects in the normal ventral guidance of ISNb, thereby redirecting these axons dorsally (Figures 3C and 3F). Importantly, the Unc-5 gain-of-function phenotype is dependent on endogenous Netrin expression (Figure 3I). To further confirm the specificity of the Unc-5 gain-of-function phenotype and to test the idea that the dorsalizing effect of Unc-5 in the ISNb is due to Unc-5 signaling, we misexpressed chimeric Unc-5-Fra and Fra-Unc-5 receptors [16] and found that only Fra-Unc-5 (consisting of fra's ectodomain fused to the cytoplasmic domain of Unc-5) showed effects on the ISNb (data not shown).
Figure 5. Expression of Unc-5 Rescues the CNS-Exit Defects in eve Mosaic Mutants.

Stage-16 RN2Gal4::CD8GFP embryos with the following genotypes: eve heterozygous (A), mosaic eve mutant (B), and mosaic eve mutant siblings expressing Unc-5 (RN2Gal4, UAS-HAUnc-5) (C) labeled with antibodies to GFP to examine the aCC and RP2 projections.

(A) eve heterozygous embryos have wild-type aCC and RP2 axon projections toward the muscle field (arrowheads).

(B) In eve mosaic mutants, most aCC and RP2 motor neurons fail to exit the CNS (89%, n = 80 hemisegments), instead projecting longitudinally within the CNS (arrows). Occasionally, individual thin axons do exit the CNS (11% of the hemisegments, n = 80, empty arrowheads). Other defects include mispositioning of the RP2 and aCC cell bodies.

(C) Expression of Unc-5 in RP2 and aCC in eve mosaic mutants results in increased motor-neuron exit (60% of the hemisegments, n = 100, arrowheads). Cell-body positioning remains defective. Anterior is up in all panels, and partial genotypes are indicated above each panel.

These results support the idea that the Unc-5 gain of function in ISNb is specifically caused by ectopic Unc-5 signaling in these neurons. In contrast to misexpression of eve, where both ventral ISNb axons and lateral SNa axons project dorsally (Figure 3E), misexpression of Unc-5 does not affect the lateral SNa (Figure 3F). This is perhaps not too surprising, because Unc-5 is normally expressed in SNa. Given that fra is normally not expressed in the lateral SNa, we wondered whether eve’s ability to drive the SNa dorsally could be caused by the upregulation of the fra receptor in these neurons. To test this idea, we ectopically expressed fra in order to change the Netrin-receptor combination in SNa to the combination present normally in the more dorsal ISN (i.e., fra + Unc-5); however, this manipulation did not significantly affect SNa or any other motor projections, suggesting that the Netrin-receptor combination alone is not sufficient to convert lateral projections into dorsal projections (data not shown).

Although these observations suggest that ectopic eve can upregulate Unc-5 expression, they do not establish whether eve normally functions to regulate Unc-5. To address this question, we again took advantage of the eve mosaic mutants, where eve is only mutant in two dorsally projecting ISN motor neurons per hemisegment: aCC and RP2 [17]. In wild-type animals or eve/+ heterozygotes, these neurons show robust expression of Unc-5 mRNA (Figures 2B and 4A), whereas in eve mosaic mutants, there is a clear reduction in Unc-5 mRNA expression in both aCC and RP2 (Figure 4B).

Importantly, Unc-5 expression is detected in other neurons and glia that are wild-type for eve at comparable levels to their heterozygous siblings (Figures 4A-4C). In contrast, expression of fra in eve mosaic mutants is not significantly affected (data not shown). To further test the relationship between eve and Unc-5, we examined whether the dorsal-extension defects in eve mosaic mutants could be “rescued” by expressing Unc-5 just in RP2 and aCC. In contrast to eve mosaic mutants, where aCC and RP2 seldom exit the CNS (Figure 5B), targeted expression of Unc-5 in these cells in eve mosaic mutants results in significant rescue of dorsal guidance (Figure 5C), a result that strongly supports the model that Unc-5 functions downstream of eve to contribute to dorsal motor-axon guidance.

These results support a role for Unc-5 in contributing to the translation of the dorsal versus ventral transcription-factor code into specific axon-guidance decisions, and provide one of the only examples of a functional link between translational regulation of motor-axon projection and expression of specific axon-guidance receptors. Furthermore, to our knowledge, these findings are among the first to link transcriptional identity to guidance-receptor expression in single identified neurons. Changing the transcription-factor code or changing the combination of Netrin receptors expressed by ventrally projecting neurons both lead to more dorsal axon projections, albeit to different extents. Not surprisingly, manipulating the transcription-factor code leads to a more profound transformation of motor projections than does the alteration of a single guidance receptor. Clearly, many guidance receptors and adhesion molecules contribute to the pathfinding of individual motor projections; indeed, previous studies have implicated complementary and combinatorial influences of Semaphorin and Netrin ligands and of IgCam cell-adhesion molecules for the guidance and target selection of subsets of embryonic motor neurons [16]. Thus, the Netrin receptors are likely to represent only a fraction of the differentially regulated targets of the transcription-factor code. It will be interesting in the future to identify additional molecules that constitute the readout of transcriptional identity in motor neurons and to assess the similarities and differences between invertebrate and vertebrate systems.

Supplemental Data

Supplemental Data include detailed Experimental Procedures, two figures, and one table and are available with this article online at: http://www.current-biology.com/cgi/content/full/15/15/1413/DC1/.

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