Specification of *Drosophila* motoneuron identity by the combinatorial action of POU and LIM-HD factors

Sarah J. Certel and Stefan Thor*,†

Department of Neurobiology, Harvard Medical School, 220 Longwood Avenue, Boston, MA 02115, USA
*Present address: Division of Biology-IFM, Linkoping University, Campus Valla, 581 83 Linkoping, Sweden
†Author for correspondence (e-mail: steth@ifm.liu.se)

Accepted 13 August 2004

Development 131, 5429-5439
Published by The Company of Biologists 2004
doi:10.1242/dev.01418

Summary

In both vertebrates and invertebrates, members of the LIM-homeodomain (LIM-HD) family of transcription factors act in combinatorial codes to specify motoneuron subclass identities. In the developing *Drosophila* embryo, the LIM-HD factors Islet (Tailup) and Lim3, specify the set of motoneuron subclasses that innervate ventral muscle targets. However, as several subclasses express both Islet and Lim3, this combinatorial code alone cannot explain how these motoneuron groups are further differentiated. To identify additional factors that may act to refine this LIM-HD code, we have analyzed the expression of POU genes in the *Drosophila* embryonic nerve cord. We find that the class III POU protein, Drifter (Ventral veinless), is co-expressed with Islet and Lim3 specifically in the ISNb motoneuron subclass. Loss-of-function and misexpression studies demonstrate that the LIM-HD combinatorial code requires Drifter to confer target specificity between the ISNb and TN motoneuron subclasses. To begin to elucidate molecules downstream of the LIM-HD code, we examined the involvement of the Beaten path (Beat) family of immunoglobulin-containing cell-adhesion molecules. We find that *beat Ic* genetically interacts with *islet* and *Lim3* in the TN motoneuron subclass and can also rescue the TN fasciculation defects observed in *islet* and *Lim3* mutants. These results suggest that in the TN motoneuron context, Islet and Lim3 may specify axon target selection through the actions of IgSF call-adhesion molecules.

Key words: *Drosophila*, *drifter*, *islet*, *Lim3*, Combinatorial code, Motoneurons

Introduction

Assembling the vast number of connections found in the developing nervous system depends upon both the precise spatiotemporal generation of distinct sets of neurons and the projection of their axons to the correct targets. Studies of CNS development in many systems have established that transcription factors, often acting in distinct combinatorial codes, play key roles during these events by functioning at multiple levels: anterior/posterior and dorsal/ventral patterning; determination of precursor cell identity; specification of neuronal fates; directing defined axonal pathways; and selection of specific synaptic partners (reviewed by Jessell, 2000; Shirasaki and Pfaff, 2002; Skeath and Thor, 2003). Two specific classes of regulators, LIM-homeodomain (LIM-HD) and POU domain transcription factors, have been shown to act primarily at late steps in these processes.

In both vertebrates and invertebrates, the activity of LIM-HD proteins is coordinated through multiple mechanisms to specify distinct neuron subpopulations. Studies in the vertebrate spinal cord show that Isl1 (Tup – FlyBase) and Lhx3 act in concert by forming a hexameric complex with the LIM co-factor NLI, to regulate motoneuron verses interneuron specification (Thaler et al., 2002). By contrast, other studies reveal that Lim1 and Isl1 exert mutual cross-repressive interactions to control neuronal cell body position and axon pathway selection (Kania and Jessell, 2003). POU proteins are also required for the production and positioning of neurons, as well as playing important roles in axon guidance. Brn1 and Brn2, both class III POU proteins, control the initiation of radial migration through the Cdk5 kinase pathway in neocortex neurons (McEvilly et al., 2002; Sugitani et al., 2002). The *Drosophila* class III member, Drifter/Ventral veinless, controls the distinct dendritic targeting of second order olfactory neurons (Komiyama et al., 2003), while the mouse class IV POU factor Brn3.2 (Pou4f2 – Mouse Genome Informatics) regulates the pathfinding of retinal ganglion cell axons (Erkman et al., 2000). In *C. elegans*, the POU protein UNC-86 is important for the terminal differentiation of several neuronal subtypes, in addition to controlling axon pathfinding in serotonergic neurons (Duggan et al., 1998; Sze et al., 2002). Furthermore, LIM-HD and POU members have been shown to function together to direct cell differentiation in both vertebrates and invertebrates. For example, UNC-86 and the LIM-HD factor MEC-3 interact genetically and physically to regulate tough sensory neuron differentiation in *C. elegans* (Rockelein et al., 2000; Xue et al., 1993).

In the *Drosophila* embryonic ventral nerve cord (VNC), three well-described motoneuron subtypes are distinguished by their axonal projections sent out either through the transverse nerve (TN) or the intersegmental nerve b (ISNb) or d (ISNd) fascicle. Previous studies have revealed that the LIM-HD proteins, Islet (Isl/Tailup) and Lim3, act in a combinatorial manner to dictate ISNb versus ISNd motoneuron subclass identity (Thor et al., 1999). However, both genes are also

expressed in, and are important for, TN motoneuron differentiation, and thus, how ISNb/d versus TN identity is determined was unknown. Here, we demonstrate that this LIM-HD combinatorial code requires the POU domain protein, Drifter/Ventral veinless (Dfr), to confer target specificity between ISNb and TN motoneuron subclasses. Dfr is co-expressed with Isl and Lim3 only in the ISNb motoneurons; when Dfr activity is decreased, a reduction in muscle innervation similar to *isl* and *Lim3* mutants is observed. In addition, when we add Dfr to the Isl/Lim3 TN combinatorial code, the TN motor axons are redirected to the ISNb muscle target field. The retargeting of TN motor axons upon Dfr misexpression does not occur without Isl function, suggesting cooperative actions between these transcription factors. Our studies indicate that a specific POU protein can modify a combinatorial LIM-HD code and act to regulate essential aspects of target selection by distinct neuronal subgroups.

To identify possible targets of these regulators, we focused on additional molecules that are differentially expressed between the TN and ISNb motoneuron subclasses. A recent report described the TN motoneuron expression of the cell-adhesion molecule (CAM) Beat Ic. *beat Ic* belongs to a multigene family in *Drosophila* that enclodes immunoglobulin superfamily (IgSF) proteins related to the Beat Ia axon guidance protein (Pipes et al., 2001). The new Beat family members, including *beat Ic*, have restricted neuronal expression patterns and appear to function in a pro-adhesive manner. Furthermore, loss of Beat Ic affects the adherence of the transverse motor nerve and the LBD sensory neuron projection (Pipes et al., 2001). We confirmed that the transverse nerve in *beat Ic* mutant embryos is often bifurcated and axons explore the ventral muscle surface. These TN defects are identical to the fasciculation defects we observe in *isl* and *Lim3* mutants. We also found strong genetic interactions between *isl*, *Lim3*, and *beat Ic*. In addition, increasing Beat Ic expression in *isl* and *Lim3* mutants partly rescues the TN axon fasciculation defects. These results indicate that the combinatorial code of two LIM-HD proteins, Isl and Lim3, can regulate not only the trajectory of a group of defined motor axons but also direct a single specific motoneuron synaptic connection, and that this axon targeting may be directed through the actions of IgSF CAMs.

Materials and methods

Fly stocks

Flies were raised on standard cornmeal-yeast-agar medium. Stocks and balancer chromosomes not specifically mentioned in the text are described by Lindsley and Zimm (Lindsley and Zimm, 1992). All genetic crosses were performed at 25°C unless otherwise specified. Stages of embryonic development are according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985).

The following strains were used in this study: isl^{37Aa} $Lim3^{Bd6}$ (S.T. and J. B. Thomas, unpublished); $Lim3^{BD1}$, *isl* alleles tup^{1} and tup^{2}; *Df(2L)OD15* removes the *isl* locus and is herein noted as isl^{Df}; *Df(2L)TW130* removes the *Lim3* locus (further designated as $Lim3^{Df}$); and *Df(2L)E71* removes both the *isl* and *Lim3* loci (Thor et al., 1999; Wright et al., 1976). The following *dfr* alleles were used: the strong hypomorph dfr^{E82} (Anderson et al., 1995), the null allele dfr^{B129} (truncation mutation before the POU domain) (W. A. Johnson, unpublished) and *Df(3L)XBB70* which removes the *dfr* locus and is further designated as dfr^{Df} (Anderson et al., 1995). To remove *beat Ic* function the deficiencies *Df(2L)TE35D-GW19* (herein noted as *beat* Ic^{Df}) and *Df(2L)RM5* (further designated as *beat* Ic^{Df1}) (Pipes et al., 2001) were used. The $hb9^{KK30}$ allele was used (Broihier and Skeath, 2002). The *islH-tau-myc*, *Lim3A-tau-myc* and *Lim3A-lacZ* transgenic lines were employed. Mutations were maintained over CyO or TM6B Tb balancers with *lacZ* or *GFP* markers.

Immunohistochemistry

Staged embryos were labeled using a modification of protocols previously described (Certel and Johnson, 1996). The following primary antibodies were used: rabbit anti-GFP (1:500) (Molecular Probes), rabbit anti-Glutactin (1:300) (Olson et al., 1990), mAb 1D4 anti-Fas2, (1:50), mAb 3A4 anti-Islet1/2, (1:20) (Tsuchida et al., 1994), mAb 9E10 anti-Myc (1:25) and mAb C555.6D anti-Slit, (1:50) (Rothberg et al., 1990). Slit staining was performed using 0.1% Tween 20 as the detergent (Crowner et al., 2002). β-Gal protein was detected either using a rabbit polyclonal anti-β-gal, (1:500) (Cappel) or a mouse monoclonal mAb 40-1a anti-β-gal (1:5). Dfr expression was detected using preabsorbed anti-Dfr rat sera at a 1:2000 dilution (Anderson et al., 1995). Monoclonal antibodies were obtained from the NIH supported Developmental Studies Hybridoma Bank maintained by the University of Iowa, Dept of Biological Sciences.

Secondary antibodies include biotinylated goat anti-mouse, goat anti-rabbit (1:1000) (Vector), Alexa Fluor 488-conjugated goat anti-rabbit, goat anti-mouse, goat anti-rat (Molecular Probes), Rhodamine-Red X-conjugated donkey anti-mouse, donkey anti-rat (Jackson ImmunoResearch Laboratories), Cy5-conjugated donkey anti-rat, anti-rabbit (Jackson ImmunoResearch Laboratories). All fluorescein-conjugated secondary antibodies were highly cross-adsorbed for use in multi-labeling experiments. In some figures, double-labeled images were false colored, converting red to magenta for the benefit of colorblind readers.

We generated additional Dfr rat serum by producing a glutathione S-transferase-Dfr (GST-Dfr) fusion protein containing amino acids 319-436 of the Dfr protein fused to the C-terminal end of the GST polypeptide. This region does not include the POU domain. The GST-Dfr fusion protein was expressed and purified using glutathione-agarose according to protocols provided by the manufacturer. Covance Research Products (Denver, PA) generated polyclonal rat antibodies.

Immunohistochemistry and in situ hybridization

In situ hybridization was carried out as previously described (O'Neill and Bier, 1994). The cDNA from EST GH22661 obtained from Research Genetics was used to generate digoxigenin-labeled RNA probes (Boehringer Mannheim). The sense (control) probe did not produce any specific signal. Double RNA and antibody labeling was performed by blocking the embryos with PBS/0.1%Tween20/10%BSA following hybridization with the RNA probe. Following blocking, the embryos were incubated with the rabbit anti-GFP antibody (1:500 dilution, Molecular Probes), followed by a biotinylated anti-rabbit secondary antibody (Vector Labs). The sheep anti-DIG antibody (1:2000 dilution, Roche) and the Vectastain Streptavidin system were added together. The antibody was detected through standard diaminobenzidine development followed by PBS/Tween20 washes and then the in situ signal was visualized with alkaline phosphatase development.

DNA manipulation and *P* element transformations

Germ-line transformants were generated as previously described (Spradling, 1986). Multiple insertion lines were isolated and established for each construct.

The *Lim3B-Gal4* construct was constructed by inserting the 3.9 kb *Eco*RI/*Nru*I fragment from *Lim3* fragment A (Thor et al., 1999) into a modified pCaSpeR 2/17 vector containing the Gal4-coding region [S.T., unpublished (details available upon request)]. *Lim3B-Gal4*

expression recapitulates the *Lim3A-lacZ* expression in the CNS (not shown).

UAS-dsdfr transgenes were generated by producing a 1582 bp inverted-repeat fragment through PCR amplification of the 5′ end of the *dfr* cDNA. The following primers were used to generate a *dfr* fragment with dyad symmetry. The first product had an *Eco*RI site at the 517 bp end, dfr5′RI-GTCGAATTCAAGACGGTTGCCTCACGGTTC and a *Sfi*I site at the 1308 end, dfr3′SfiI-GTCGGCCATCTTGGCCTGCTG-CAACTGATCGCTCGTG. The second product had an *Xho*I site at the 517 end dfr5′XhoI-GTCGAGAAGACGGTTGCCTCACGGTTC and a *Sfi*I site at the 1308 end, dfr3′SfiI(2)-GTCGGCCTAGATGGCCTGCT-GCAACTGATCGCTCGTG. Underlined sequences denote the central nonpalindromic core of each site. This 791 bp fragment does not include the POU domain. After digestion with *Sfi*I, the two products were ligated together. Dimers were digested with *Eco*RI and *Xho*I and cloned into the pUAST P-element transformation vector provided by Andrea Brand (Brand and Perrimon, 1993).

Results

The LIM-homeodomain proteins Islet and Lim3 co-localize with the Drifter POU protein in a subset of developing motoneurons

In each abdominal hemisegment of the *Drosophila* embryo, the axons of ~40 motoneurons exit the ventral nerve cord and specifically synapse with 30 identified muscle fibers. The axons of these motoneurons form six discrete fascicles and exit the VNC together, extending into the periphery to innervate specific muscle groups (or fields). The ventral muscles are innervated by the motoneurons of three subclasses: the transverse nerve (TN), intersegmental nerve b (ISNb) and intersegmental nerve d (ISNd) (Fig. 1A) (Landgraf et al., 1997). In addition, the segmental nerve c (SNc) innervates a set of externally located ventral muscles. The two TN motoneurons contact ventral muscle 25 or the ventral process of the lateral bipolar dendritic neuron (LBD) (Fig. 1A) (Gorczyca et al., 1994; Thor and Thomas, 1997). The ISNb motoneurons innervate the ventral muscles 6, 7, 12, 13, 14, 28 and 30, and the ISNd motoneurons muscles 15, 16 and 17 (Fig. 1A) (Landgraf et al., 1997; Schmid et al., 1999). The *Drosophila* LIM-HD genes, *islet* and *Lim3*, are co-expressed in a subset of CNS neurons, including several classes of motoneurons (Thor et al., 1999). Although Isl and Lim3 are required in these distinct motoneurons to specific neuronal identity (Thor et al., 1999), this two member 'LIM-code' cannot alone be responsible for the unique differentiation and function of each subclass. More specifically, as the TN and ISNb motoneuron subclasses both express a combination of Isl and Lim3, it is likely that other factors act to discriminate between these two subclasses.

To identify factors that act to further differentiate these motoneuron subgroups, we examined the expression pattern of previously characterized transcription factors. We found that Isl and Lim3 are co-expressed with the class III POU factor, Drifter (Dfr), in a limited number of embryonic VNC neurons (Fig. 1B). Immunohistochemical experiments using double transgenic animals reporting on Isl and Lim3 expression and antibodies directed against Dfr reveal restricted triple expression specifically in the ISNb motoneuron subclass (Fig. 1B). Dfr, Isl and Lim3 expression is clearly observed in the RP1, RP3, RP4 and RP5 motoneurons (arrowheads) that project out the ISNb fascicle to innervate ventral muscles 6, 7,

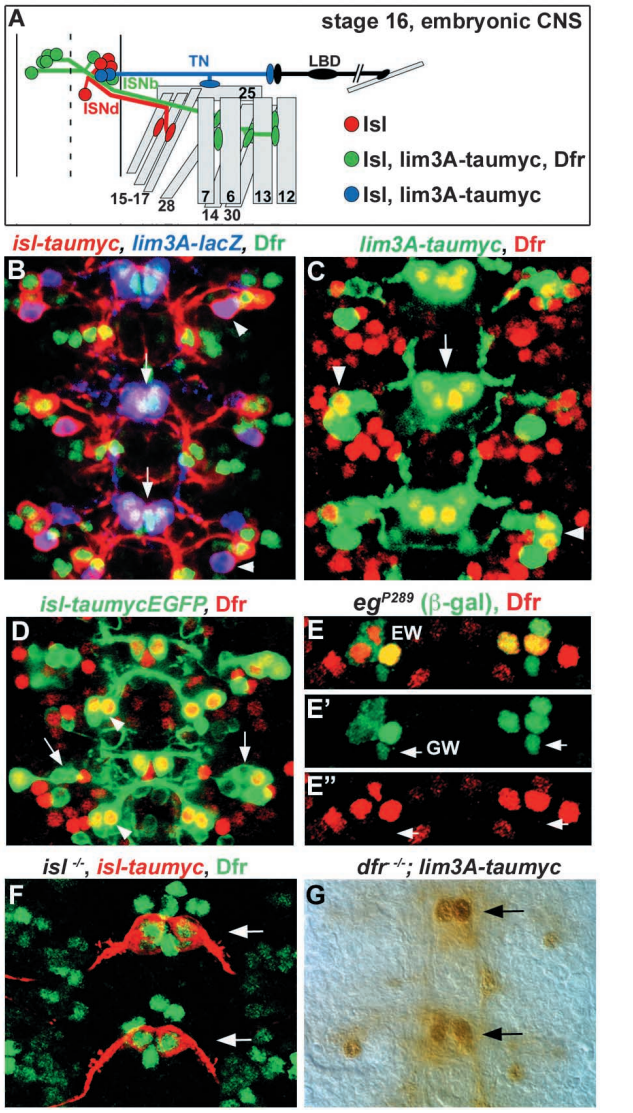

Fig. 1. Colocalization of Isl, Lim3 and Dfr in the embryonic ventral nerve cord. (A) Schematic representation of Isl, Lim3 and Dfr expression in the TN, ISNb and ISNd motoneuron subclasses. (B) Staining for Tau-Myc (red), β-galactosidase (blue) and Dfr (green) in a stage 16 embryo carrying the *isl-tau-myc* and *Lim3A-lacZ* transgenes. Isl, Lim3 and Dfr are co-expressed in the ISNb neurons, including the RP neurons (arrows). Only Isl and Lim3 are expressed in the TN neurons (arrowheads). (C) A stage 16 *Lim3A-tau-myc* transgenic embryo labeled for Tau-Myc (green) and Dfr (red) expression. Co-expression is seen in the RP neurons (arrow) and the lateral cluster of motoneurons that include MN14/30 and MN 28 (arrowheads). (D) An early stage 15 *isl-tau-mycEGFP* transgenic embryo labeled for EGFP (green) and Dfr (red) expression. Several lateral EGFP-expressing cells, presumably the ISNd neurons, do not express Dfr (arrows). Dfr and EGFP co-expression is observed in two serotonergic EW neurons at this stage (arrowheads). (E) An abdominal neuromere from a late stage 15 eg^{P289} *lacZ* enhancer trap line. β-Gal (green) expression is visible in the three EW neurons and the GW (ISNd) motorneuron. Dfr (red) is not expressed in the GW (ISNd) motoneuron (arrows). (F) Dfr expression (green) is unaffected in an isl^{37Aa}/isl^{Df} *isl-tau-myc* transgenic embryo. The RP (ISNb) motoneurons (arrows) are identified by Myc (red) expression from the transgene. (G) Tau-myc expression (arrows) is not altered in dfr^{B129}/dfr^{Df}*Lim3A-tau-myc* transgenic embryos.

12 and 13 (Fig. 1A,B). Dfr expression is not detected in the pair of Isl- and Lim3-expressing TN neurons (arrows, Fig. 1B). To confirm that the lateral co-expressing neurons belong to the ISNb subclass, we double-labeled embryos carrying the *Lim3A-tau-myc* transgene. This reporter construct drives expression in Isl-expressing motoneurons that project via the ISNb but not the ISNd fascicle (Thor et al., 1999). We find that the lateral Dfr-expressing neurons do belong to the ISNb subclass as they also express the Tau-myc fusion protein (arrowheads, Fig. 1C). We also further analyzed the co-expression of Dfr and Isl (Fig. 1D). In addition to the ISNb neurons, Dfr and Islet co-expression is also observed in two serotonergic EW neurons (Fig. 1D, arrowheads).

To verify the lack of Dfr expression in the ISNd subgroup, we focused on the well-characterized GW/7-3M motoneuron that innervates muscle 15 via the ISNd pathway (Dittrich et al., 1997; Higashijima et al., 1996). To identify this ISNd motoneuron, we used the enhancer-trap line, eg^{P289}, which drives *lacZ* expression in the 7-3M progeny (Dittrich et al., 1997). Although we see Dfr expression in the EW interneurons (arrows, Fig. 1E,E′), the ISNd GW/7-3M motoneuron does not express Dfr (arrow, Fig. 1E,E″). The results of these labeling experiments demonstrate that Dfr expression can be further used to subdivide the ISNb from the TN and ISNd neuronal classes.

Genetic interaction studies indicate that Drifter is required for the specification of ISNb motoneurons

The ISNb fascicle contains motor axons from at least eight motoneurons innervating ventral muscles 6, 7, 12, 13, 14, 28 and 30. Wild-type innervation of muscles 6, 7, 12 and 13 is shown in Fig. 2A. Loss of *isl* or *Lim3* function in ISNb motoneurons affects axon targeting resulting in a reduction of target muscle innervation (Thor et al., 1999; Thor and Thomas, 1997). The most common phenotype in both *isl* and *Lim3* mutants is a failure to innervate the cleft between muscles 12 and 13. In *Lim3* mutants, muscles 12/13 were not innervated by motor axons in 46% of hemisegments compared with 3% in wild-type hemisegments. In *isl* mutants, the lack of muscle contacts was also coupled with the ISNb motor axons, leaving the ventral muscle field and joining the TN (26% of hemisegments in *isl* mutants versus 0% in wild type) (Thor et al., 1999; Thor and Thomas, 1997).

Dfr is functionally required in a variety of tissues, including the midline glia, trachea, wing veins, sensory neurons and antennal lobe projection neurons (Anderson et al., 1995; Certel et al., 2000; Inbal et al., 2003; Komiyama et al., 2003). The early lethality of null *dfr* (*vvl* – FlyBase) alleles, and the VNC perturbations caused by midline glia defects made analyzing the requirement for Dfr function in ISNb neurons difficult. To circumvent this problem, and to examine whether Isl, Lim3 and Dfr are required to specify similar aspects of motor axon targeting, we first analyzed the ISNb fascicle in trans-heterozygous combinations. Removing a single copy of *isl*, *Lim3* and *dfr* results in motor axon targeting defects characterized by significant reductions in muscle innervation (Fig. 2B). The failure to innervate muscles 12 and 13 observed in both isl^{37Aa}/+;dfr^{B129}/+ and $Lim3^{Bd1}$/+;dfr^{B129}/+ trans-heterozygotes is easily quantifiable and observed in *isl* and *Lim3* mutant embryos (Fig. 2B-D, Table 1). Reducing the levels of Isl and Dfr also results in ISNb motor axons leaving

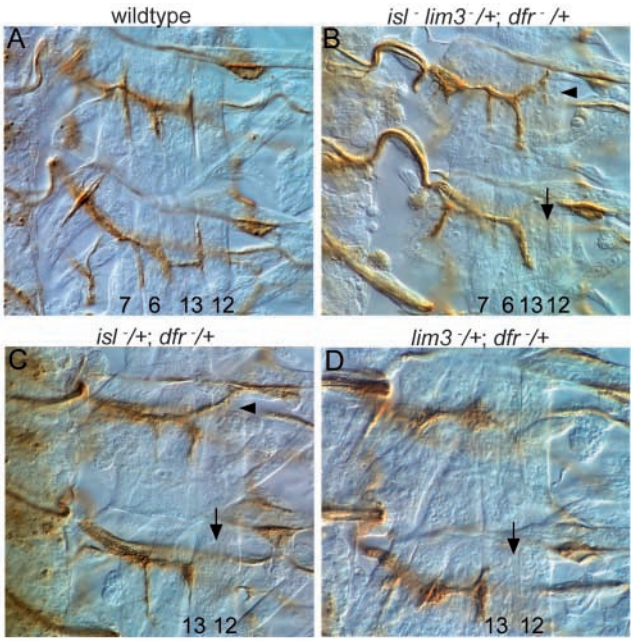

Fig. 2. Transheterozygous combinations between *isl*, *Lim3* and *dfr* show motoneuron innervation defects. In each panel, the dissected embryos are at stage 16 and stained with anti-Fas2 antibodies. The ventral nerve cord is towards the left, anterior is upwards. (A) In the wild-type embryo, the ISNb motoneurons innervate muscles 6, 7, 12 and 13 in a stereotypical fashion, extending processes into the muscle clefts. (B) In isl^{37Aa} $Lim3^{BD6}$/+; dfr^{B129}/+ embryos, the ISNb motoneurons fail to project to and innervate their specific target muscles, including muscles 12 and 13 (arrow). (C) Muscle innervation is also affected in isl^{37Aa}/+; dfr^{B129}/+ embryos (arrow) and in some hemisegments, the ISNb motor axons target the TN fascicle (arrowhead). (D) $Lim3^{BD6}$/+; dfr^{B129}/+ embryos also show severe defects in motor axon targeting and muscle innervation (arrow).

the ventral muscle field and targeting the TN fascicle (Fig. 2C, Table 1). Neither Dfr nor Isl is expressed in motoneurons that innervate the dorsal muscles or the externally located ventral muscles. In line with their restricted expression, we found no evidence of non-cell autonomous affects upon the targeting of the ISN and SNc motor axons in *dfr, isl* trans-heterozygotes (not shown).

In an attempt to address the specificity of the genetic interactions between POU and LIM-HD genes, we tested for genetic interactions between *dfr* and another key regulator of ISNb identity, the *Drosophila exex/hb9* gene (Broihier and Skeath, 2002; Odden et al., 2002). However, we found no evidence of ISNb axon pathfinding defects in embryos trans-heterozygotes for *dfr* and *hb9* (dfr^{B129}/$hb9^{KK30}$; not shown). Therefore, results from our genetic interaction studies indicate that Dfr may be required specifically in combination with Isl and Lim3 to specify the ISNb motoneuron subclass.

Reducing Drifter function results in ISNb motor axons redirecting to TN neuron targets

As described above, *isl* and *Lim3* mutants display axon targeting defects manifested by a reduction in muscle innervation (Fig. 4D). Results from the trans-heterozygous genetic interaction studies suggest that Dfr is necessary in

Table 1. Quantification of *isl*, *Lim3* and *dfr* genetic interactions

Genotype	Failure to innervate muscles 12 and 13 (%)	ISNb axons crossing to TN (%)	Number of hemisegments examined
Df(2L)E71/+	1.8	3.5	57
Df(2L)E71/+; dfr^{B129}/+	22.0	16.5	79
isl^{37Aa}/+	2.0	3.0	82
isl^{37Aa}/+; dfr^{B129}/+	21.0	16.0	129
$Lim3^{BD6}$/+	1.5	2.8	87
$Lim3^{BD6}$/+; dfr^{B129}/+	29.0	24.0	99
dfr^{B129}/+	1.5	4.0	136

Quantification of specific ISNb phenotypes observed in control and transheterozygous embryos. Control embryos were crossed to *w1118* and labeled for the absence of the balancer chromosome. For each genotype, abdominal hemisegments were scored at late stage 16.

combination with Isl and Lim3 to specify ISNb axon targeting. If this hypothesis is correct, than a loss of Dfr function in ISNb motoneurons should also result in a reduction in muscle innervation. To address the possible role of Dfr in ISNb specification, we used RNA interference to reduce or eliminate the expression of Dfr protein specifically in neurons, without affecting its expression and function in midline glia. We generated transgenic flies expressing double-stranded *dfr* RNA (*UAS-dsdfr)* under the control of neuronal-specific *Gal4* drivers. To further assist in the removal of Dfr function, two independent *UAS-dsdfr* insertions were recombined onto a chromosome containing a null *dfr* allele, dfr^{B129}. Although the protein produced by this allele is detected by the Dfr antiserum, it is non-functional because of a premature stop codon located before the DNA-binding POU domain (W. A. Johnson, personal communication). Several *Gal4* drivers were used to analyze the effectiveness of the *UAS-dsdfr* transgenes. Using both the *C155(elav)-Gal4* and the *Lim3B-Gal4* drivers (see Materials and methods), Dfr protein was reduced or eliminated in the majority of ISNb motoneurons, including the RP motoneurons (Fig. 3A,B). As a control, the Dfr-expressing midline glia showed no loss of Dfr protein and, accordingly, no loss of the midline glia-specific marker Slit (Fig. 3A,B). This showed that *UAS-dsdfr* could be used together with neuronal-specific *Gal4* drivers to address how loss of Dfr function affects ISNb neuron specification. To verify that the *Lim3B-Gal4* driver expression correlates with the endogenous Lim3 expression, we labeled *Lim3B-Gal4;UAS-taumycEGFP* double-transgenic embryos with the Lim3 antibody (Broihier and Skeath, 2002). We can visualize the TN process leaving the VNC (arrow, Fig. 3C) and Lim3 is expressed in these neurons (arrowhead, Fig. 3C).

Using both early (*ftz_{ng}-Gal4.20*) and post-mitotic (*Lim3B-Gal4*) drivers to reduce Dfr activity, we observed two axon outgrowth phenotypes. First, ISNb motor axons can now leave the ventral muscle field and contact or target the TN fascicle (Fig. 4B,D). This re-targeting suggests that losing Dfr leaves the ISNb neurons in an Isl/Lim3-only specified state generating a TN fate. Second, reducing Dfr function causes an overall decrease in muscle innervation by the ISNb motor axons similar to defects observed in *isl* mutants (Fig. 4A-C). Both of these axon targeting and innervation phenotypes indicate that Dfr plays an important role in ISNb neuronal specification.

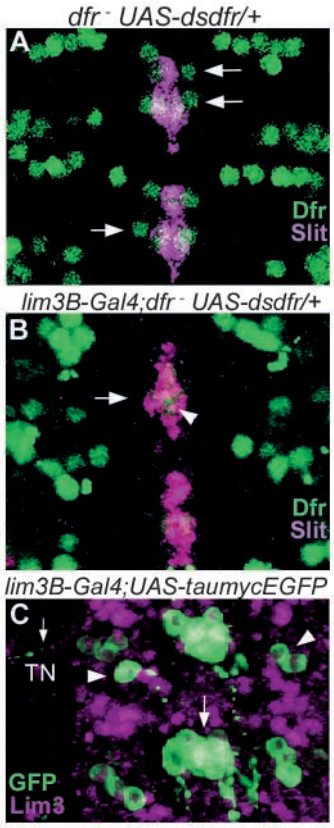

Fig. 3. Dfr expression can be reduced in the ISNb motoneurons. (A) Wild-type Dfr expression (green) in the ventral nerve cord of a stage 16 *dfr^{B129} UAS-dsdfr/+*transgenic embryo. Dfr is expressed in the ISNb neurons, including the RP neurons (lower arrow). Slit expression (magenta) is used to mark the Dfr-expressing midline glia (white/colocalization). (B) Dfr expression (green) is severely reduced or eliminated in *Lim3B-Gal4;dfr^{B129} UAS-dsdfr/+* embryos. The absence of Dfr labeling in the RP neurons is indicated by the arrows. Dfr expression remains in the Slit-expressing midline glia (arrowhead) as well as in lateral neuronal clusters. (C) Two neuromeres of a *Lim3B-Gal4;UAS-taumycEGFP* VNC labeled with GFP (green) and the Lim3 antibody (magenta). Lim3 expression is co-localized with the reporter GFP expression in the RP neurons (lower arrow) and the TN neurons (arrowhead). The process of the TMNp neuron is also visualized in this image (upper arrow).

Misexpression of Drifter in TN neurons results in a redirection of TN motor axons to the ISNb muscle target field

If Dfr functions as part of a LIM/POU combinatorial code to specify ISNb motoneurons, then ectopically expressing Dfr in the Isl/Lim3-expressing TN motoneurons would be predicted to alter TN axon pathfinding towards an ISNb-like behavior. To test this, we misexpressed Dfr in postmitotic Isl/Lim3-expressing TN neurons using the *Lim3B-Gal4* driver. In wild-type development, the transverse nerve forms from the fasciculation of a sensory nerve axon (the lateral bipolar dendrite or LBD neuron) and the TMNp neuron (Gorczyca et al., 1994; Thor and Thomas, 1997). At late stage 15/16, these growth cones contact each other on the ventral interior muscle surfaces. The TN fascicle is on a different focal plane and in

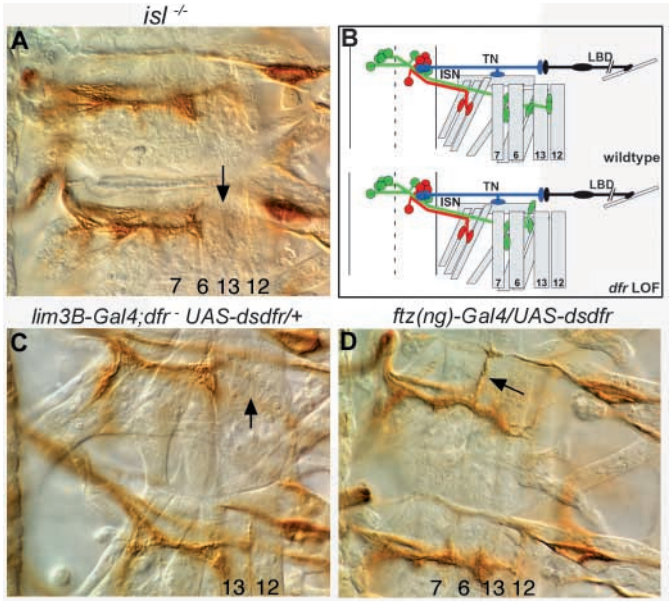

Fig. 4. Dfr is required for ISNb motoneuron specification. (A) In isl^{37Aa}/isl^{Df} embryos, the ISNb motor axons fail to correctly target the ventral muscles. The arrow indicates an absence of muscle cleft innervation at muscles 12 and 13. (B) Schematic summary of wild-type and *dfr* phenotypes. In the wild-type embryo, the ISNb (green) and TN (blue) neurons project axons to their appropriate target areas. In *dfr* mutants, the ISNb motor axons do not target the ventral muscles correctly and/or project ectopically in the TN fascicle area. (C) Two hemisegments of a stage 16 *Lim3B-Gal4; dfr*^{B129} *UAS-dsdfr/+* embryo labeled with anti-Fas2 antibodies. The arrow indicates a lack of innervation at muscles 12 and 13. (D) Removing Dfr function can also cause ISNb motor axons to target the TN fascicle (arrow) in ftz_{ng}*-Gal.20/dfr*^{B129} *UAS-dsdfr* embryos.

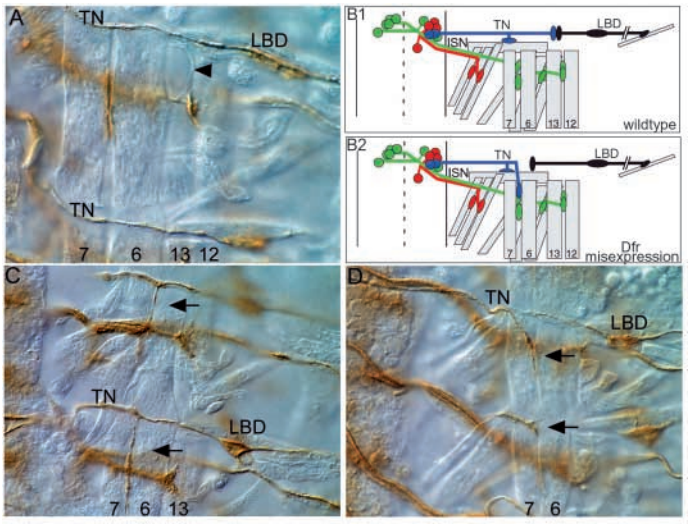

Fig. 5. Dfr misexpression in the TN neurons results in a redirection of TN motor axons to ISNb muscle targets. (A and B1) In the wild-type embryo, two TN motoneurons leave the VNC. One, TMN 25, innervates ventral muscle 25 (out of the plane of focus) and the other, TMNp, contacts the ventral process of the LBD neuron. The TN and ISNb fascicles do not ever meet and are on different focal planes (arrowhead). All embryos are stained with anti-Fas2 antibodies. (B) Schematic summary of wild-type and Dfr misexpression phenotypes in the TN neurons (blue). (B2) When Dfr is misexpressed in the TN neurons, the TMNp motor axons target the ISNb muscle field. (C,D) In *Lim3B-Gal4;UAS-dfr* embryo late stage 16 embryos, the TN axons have retargeted to either enter or innervate the ISNb muscle field (arrows).

wild-type embryos does not come in contact with the ISNb fascicle (Fig. 5A,B1).

Misexpression of Dfr protein in *Lim3B-Gal4;UAS-dfr* double transgenic embryos did not have any effect on TN axons exiting the ventral nerve cord or lead to random innervation in the periphery. Instead, adding Dfr function to the TN neurons caused the motor axons to target the ISNb muscle field in a significant number of hemisegments (52%, *n*=135). TN motor axons either send collaterals into ISNb muscle targets (Fig. 5B2,5D) or in some cases even innervated ISNb muscles (Fig. 5B2,C). This change in motor axon targeting appears to be specific to the TN motoneurons as misexpressing Dfr in most, if not all, motoneurons via the ftz_{ng}*-Gal4.20* (Thor et al., 1999) did not result in any targeting changes or defects in the SNc or ISN fascicles. It did, however, result in a similar percentage of TMNp targeting defects (not shown).

We also tested the ability of the TN motoneurons to be respecified by Dfr misexpression in *isl* mutant embryos. Without Isl function, the retargeting of TN motor axons did not occur (*n*=64), suggesting possible cooperative actions between these transcription factors. Our results indicate that it is the addition of Dfr specifically to the TN Isl/Lim3 LIM-HD code that allows this motoneuron subclass to exhibit ISNb motoneuron characteristics.

Drifter, Islet and Lim3 do not regulate each other

In *C. elegans* touch receptor neurons, the POU protein UNC-86 directly regulates the expression of the LIM-HD gene, *MEC-3* (Xue et al., 1992; Xue et al., 1993). And in vertebrates, abLIM is a transcriptional target of the POU factor, Brn3.2 (Erkman et al., 2000). To determine whether Dfr, Isl and/or Lim3 are possible transcriptional targets of each other, we analyzed the individual expression patterns in each mutant background. We first tested if Isl and Lim3 are required to initiate or maintain Dfr expression. In addition to its expression in the ISNb-projecting RP motoneurons located at the midline, Dfr is also expressed in midline glia (Anderson et al., 1995). To determine if Dfr expression remained in the ISNb neurons as well as the midline glia, we used *isl*- and *Lim3*-null mutants carrying the *islH-tau-myc* reporter construct. Dfr expression was unaffected in *isl* and *Lim3* single and double mutants (Fig. 1F; not shown), indicating that *dfr* is not a transcriptional target of these LIM-HD factors. We next examined Isl and Lim3 expression in *dfr* mutants. Neither *isl* nor *Lim3* reporter expression was affected, suggesting that Dfr does not regulate LIM-HD expression in ISNb neurons (Fig. 1G; not shown). Therefore, these results indicate Dfr, Isl and Lim3 must function at the same hierarchical level in ISNb motoneuron specification.

Islet and Lim3 genetically interact with the IgSF CAM, Beat Ic, in TN axon targeting and fasciculation

Studies in various model systems have led to the identification

of a number of transcription factors with highly restricted expression. These factors are important for different aspects of neuronal differentiation, including axon pathfinding. By contrast, many other molecules, such as cell adhesion molecules, receptor protein tyrosine phosphatases and semaphorins, also play a crucial role in axon guidance, yet these molecules are often, at least in *Drosophila*, more broadly expressed (Dickson, 2002; Huber et al., 2003; Shen, 2004). This apparent disjunction could indicate that the downstream genes regulated by highly restricted transcription factors such as Dfr and Isl are be thus far uncharacterized molecules. Therefore, to identify possible targets of the LIM/POU or LIM-HD combinatorial codes, we looked for specific molecules that are differentially expressed between the ISNb and TN motoneuron subclasses. A recent study identified and described the restricted expression of 14 *Drosophila* Beat-like members of the immunoglobulin superfamily (IgSF) of CAMs (Pipes et al., 2001). Members of the Beat family in *Drosophila* and the Zig family in *C. elegans* contain two Ig domains and most members have been shown to be highly restricted in their expression pattern, largely confined to subsets of neurons (Aurelio et al., 2003; Aurelio et al., 2002; Pipes et al., 2001).

We were particularly interested in *beat Ic*, because of its expression in a small number of embryonic neurons that include the TN motoneurons but not the ISNb neurons (Pipes et al., 2001). We used in situ hybridization and immunohistochemistry to verify the TN expression of *beat Ic*. *beat Ic* transcripts are expressed in lateral cell clusters that contain the TN neurons (arrow) but not in the ISNb RP neurons (arrowhead, Fig. 6A). To establish the identity of the lateral cluster cells, *Lim3B-Gal4;UAS-taumycEGFP* transgenic embryos were labeled for both GFP and *beat Ic* expression. We observe the GFP-expressing (brown) TN neurons also show blue (*beat Ic*) staining (Fig. 6B, arrows).

In addition to restricted TN expression, removal of *beat Ic* function through overlapping deficiencies results in TN phenotypes indistinguishable from those observed in *isl* and *Lim3* mutants. Embryos that lack *beat Ic* display errors in TN fasciculation. The TMNp axon fails to completely fasciculate with the LBD projection, resulting in bifurcation and aberrant ventral muscle exploration (Fig. 6C) (Pipes et al., 2001). This TN fasciculation in *beat Ic* mutants is improved by *elav-Gal4* driven *UAS-beat Ic* expression (Pipes et al., 2001). In *isl* mutants, the TN axons either do not exit the VNC or fail to fasciculate with the LBD projection, also leading to aberrant ventral muscle exploration (Fig. 6D) (Thor and Thomas, 1997). This failure of the TMNp and LBD axons to adhere is also observed in a significant number of *Lim3* mutant hemisegments (Table 2).

The similarity of TN mutant phenotypes suggests that Isl, Lim3 and Beat Ic are required to specify analogous aspects of motor axon targeting. To test this hypothesis, we analyzed the TN fascicle in trans-heterozygous combinations. Mutations in *beat Ic* are not available so defined deficiencies were used as previously described (Pipes et al., 2001). Embryos that lack one copy of *isl*, *Lim3* and *beat Ic* display a significant number of TN fasciculation defects (Fig. 6E,F; Table 2). As in individual mutants, the TMNp and LBD axons in several trans-heterozygous combinations fail to fasciculate, thus leading to bifurcation and/or aberrant axon outgrowth (Fig. 6E,F). Two chromosomal deficiencies removing *beat Ic*, *beat* Ic^{Df} and *beat* Ic^{Df1} (see Materials and methods) were used to demonstrate that other genes removed by an individual deficiency did not influence the TN phenotype. In addition, at least two different *isl* and *Lim3* alleles were used to verify that the observed genetic interactions were not due to specific chromosomes or alleles. Another Beat family member, *beat Ib*, is also

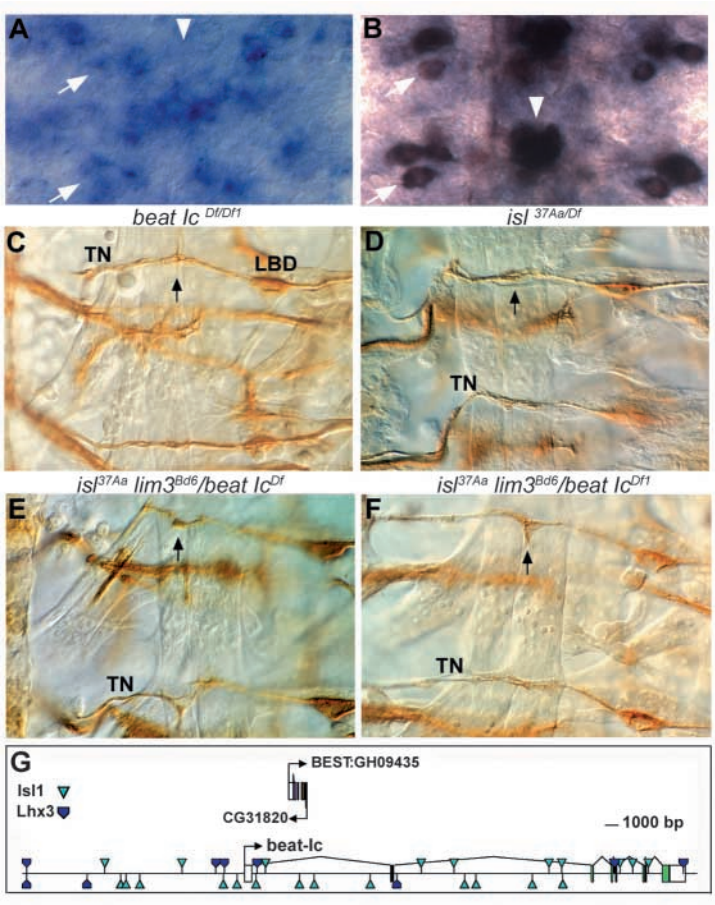

Fig. 6. *isl* and *Lim3* genetically interact with *beat Ic* in TN axon targeting and fasciculation. (A) In situ hybridization of *beat Ic* in a wild-type stage 15 VNC. Expression is evident in lateral clusters (arrows) but not in the RP neurons (arrowhead). (B) In situ hybridization and antibody labeling in a *Lim3B-Gal4;UAS-taumycEGFP* late stage 15 embryo. Both GFP (brown) and *beat Ic* (blue) expression is observed in the TN neurons (arrow). The cluster of RP neurons (arrowhead) stain very darkly for GFP expression but do not express *beat Ic* (see A). (C) Embryos that lack *beat Ic* were generated using the overlapping chromosomal deficiencies, *beat* Ic^{Df}/*beat* Ic^{Df1}(*Df(2L)TE35D-GW19/Df(2L)RM5*). In these embryos, the TMNp motor axon did not completely fasciculate with the LBD projection, resulting in bifurcation (arrow) and aberrant ventral muscle exploration. (D) In *isl* mutants, isl^{37Aa}/isl^{Df} embryos, the same failure of the TMNp motor axon to fasciculate with the LBD projection is observed (arrow). (E,F) TN targeting and fasciculation is also affected (arrows) in embryos in which one copy of *isl*, *Lim3* and *beat Ic* have been removed, isl^{37Aa} $Lim3^{BD6}$ *beat* Ic^{Df} (C) and isl^{37Aa} $Lim3^{BD6}$/*beat* Ic^{Df1} (E). (G) Schematic diagram of the *beat Ic* locus. Isl1 (CTAATG) and Lhx3 (AATTAATTA) consensus sites within this locus are graphically represented.

Table 2. Quantification of *isl*, *Lim3* and *beat Ic* genetic interactions

Genotype	No TN (%)	TN fasciculation defects (%)	Number of hemisegments examined
$isl^{-/-}$ (isl^{37Aa}/isl^{Df})	29.0	27.0	89
$Lim3^{-/-}$ ($Lim3^{BD6}/Lim3^{Df}$)	19.0	38.0	79
isl^{-}/beat Ic^{-} (isl^{37Aa}/beat Ic^{Df1})	11.5	42.0	95
$Lim3^{-}$/beat Ic^{-} ($Lim3^{BD6}$/beat Ic^{Df1})	17.0	25.5	47
isl^{-} $Lim3^{-}$/beat Ic^{-} (isl^{37Aa} $Lim3^{BD6}$/beat Ic^{Df1})	9.3	36.0	86
isl^{-} $Lim3^{-}$/beat Ic^{-} (isl^{37Aa} $Lim3^{BD6}$/beat Ic^{Df})	4.0	46.0	80

Quantification of TN phenotypes observed in transheterozygous embryos. For each chromosome, control crosses were done and in all cases any TN fasciculation defects were at or below wild-type levels (less than 6% total TN defects). For each genotype, abdominal hemisegments were scored at late stage 16. A description of each deficiency chromosome appears in the Materials and methods.

expressed in the CNS; however, a lack of Beat Ib does not result in any defects in neuronal development (Pipes et al., 2001). We could not test for genetic interactions with any other Beat family members because neither overlapping deficiencies nor individual mutations are available for these genes. However, to assess the specificity of these interactions among Ig-domain containing molecules, we tested for genetic interactions between *isl*, *Lim3* and the CAM *Fas2*, which contains five Ig-like domains (Grenningloh et al., 1991). We did not observe any fascicle defects in various trans-heterozygous combinations (not shown). These results raise the possibility that in TN motoneurons Isl and Lim3 may specify axon target selection through the actions of specific IgSF CAM members.

beat Ic expression can rescue the TN defects observed in *islet* and *Lim3* mutants

At least two possible explanations can be put forwards to explain the TN defects observed in *isl* and *Lim3* mutants. First, the defects observed are because the TMNp and the LBD axons cannot fasciculate or adhere to each other. A second hypothesis is that these two axons do not recognize each other and therefore do not grow close enough together to fasciculate properly. If the defect lies in fasciculation, then increasing the levels of Beat Ic, a promoter of motor axon adhesion, should reduce the TN defects in *isl* and *Lim3* mutants. To increase Beat Ic levels, the pan-neuronal driver *elav-Gal4* and *UAS-beat Ic* transgenes were crossed into *isl* and *Lim3* mutant backgrounds. As in previous experiments, strong *isl* and *Lim3* alleles were crossed to isl^{Df} and lim^{Df} deficiencies to create embryos null for *isl* and *Lim3*, respectively.

Increasing the levels of Beat Ic through one copy of *UAS-beat Ic* and the *elav-Gal4* driver significantly reduced the percentage of TN defects in *isl* mutants from 56% to 22% (n=66). In addition, the severity of TN adhesion defects decreased in the remaining 22% of affected hemisegments. Likewise, increasing Beat Ic levels in *Lim3* mutants also significantly decreased the occurrence of TN defects from 55% to 31% (n=67). These results suggest that the TN defects observed in *isl* and *Lim3* mutants are a result of a decrease in the adhesive properties between the TN motor axon and the LBD projection. This suggests that Beat Ic may be a direct transcriptional target of the LIM code.

At this time, we are unable to directly test this hypothesis because of the unavailability of a Beat Ic antibody. We tried analyzing *beat Ic* transcript accumulation in *isl* and *Lim3* double mutants but were unable to achieve cellular resolution in the mutant ventral nerve cords. However, DNA-binding site pattern searches using previously described LIM-HD binding motifs indicate that there are significant clusters of LIM-HD sites surrounding and within the *beat Ic* locus (Fig. 6G) (Freeman et al., 2003; Rebeiz and Posakony, 2004). The Isl1 consensus site (CTAATG) (Boam and Docherty, 1989; Karlsson et al., 1990) is found 15 times in the chromosomal region encompassing the *beat Ic* locus (Fig. 6G); the Lhx3-binding site (AATTAATTA) (Bridwell et al., 2001) is found nine times (Fig. 6G) and 21 Isl 2.2 sites (YTAAGTG) (data not shown) have been identified. Although functional analyses will be needed to determine if these sites are necessary and/or sufficient for *beat Ic* expression, searching the entire genome with the Isl 2.2 site places the *beat Ic* locus seven out of the first 10 identified. Furthermore, studies in *C. elegans* indicate that two LIM-HD genes, the *Lmx*-class gene *lim-6* and the *Lhx3*-class gene, *ceh-14*, are required for the expression of at least four IgSF *zig* genes (Aurelio et al., 2003).

Discussion

The generation of the large number of distinct cell types found in the nervous system presents an enormous challenge to an organism, especially as it is likely that the number of cell types exceeds the number of regulators found in the genome. Evolution has solved this problem, at least in part, by using a principle of combinatorial coding where the specific combinations of regulators, rather than any one regulator alone, dictates cell fate. Although this strategy represents a functional answer to the challenge of cellular diversity, the mechanisms that allow individual factors to be used repeatedly and yet direct different outcomes depending on their cellular context are still being addressed. However, insight into this question has come from recent studies on members of the LIM homeodomain family of transcriptional regulators.

LIM-HD proteins participate in a number of unique complexes through protein-protein interactions mediated by their LIM domains (Bach, 2000; Gill, 2003). For example, LIM domains can interact with the widely expressed co-factor, NLI(Ldb1/CLIM-2) in mice or Chip in *Drosophila* (Breen et al., 1998; Jurata and Gill, 1997; Jurata et al., 1998; van Meyel et al., 1999). The NLI/CHIP proteins homodimerize and generate a bridge between two LIM-HD proteins, thereby leading to the formation of tetrameric complexes (Jurata et al., 1998). Although this complex is functional in vivo, it has been found that other proteins also participate to generate further tissue specificity. In *Drosophila*, the newly identified Ssdp protein interacts with Chip to modify the activity of complexes comprising Chip and the LIM-HD protein Apterous in the wing (Chen et al., 2002; van Meyel et al., 2003). In vertebrates, the bHLH factors Ngn2 and NeuroM functionally interact with the NLI, Isl1 and Lhx3 complex to initiate motoneuron differentiation (Lee and Pfaff, 2003). These results suggest that the formation of further specialized combinations could be used to confer not only tissue but also cellular specificity.

In *Drosophila*, Isl and Lim3 are co-expressed in a subset of CNS neurons including two neuron subclasses, the TN and ISNb motoneurons (Thor et al., 1999). Although Isl and Lim3 are required in these distinct motoneurons to specific motor axon pathway choice neuronal identity (Thor et al., 1999; Thor and Thomas, 1997) these two factors alone cannot be responsible for the unique differentiation of each subclass. In this study, we provide evidence that the class III POU domain protein Dfr functions in combination with this Isl/Lim3 LIM code, to specify the ISNb motoneuron class. Loss-of-function analyses indicates each of these transcription factors is required in the ISNb neurons for the specification of motor axon target selection. Without Dfr, Isl or Lim3, these motor axons fail to correctly innervate their designated muscle targets. In addition, our genetic interaction studies suggest that this phenotype indicates a common aspect of motoneuron designation has been altered.

How might this LIM/POU code function in ISNb neurons? In *C. elegans* touch receptor neurons, the LIM-HD factor, MEC-3 and the POU protein UNC-86, physically interact to control specification (Rockelein et al., 2000; Xue et al., 1993). In the pituitary, the LIM domain of Lhx3 (P-Lim) specifically interacts with the Pit1 POU domain and is required for synergistic interactions with Pit1 (Bach et al., 1995). We have not determined whether Dfr, Isl and or Lim3 physically interact to regulate ISNb motor axon target selection. However, our misexpression experiments indicate that the re-specification of transverse motoneurons by the addition of Dfr does require functional Isl protein. Although, this result does not distinguish between the possibilities of direct interactions between these proteins or the binding of a common transcriptional target, it does indicate that a functioning 'LIM code' is required for the re-specification of the TN neurons.

A second finding of our Dfr misexpression studies is that we can robustly re-specify the target selection of postmitotic neurons. We used the *Lim3B-Gal4* line to add Dfr to the LIM-only transverse motoneurons. This *Gal4* line does not activate reporter construct expression until stage 14 – a post-mitotic stage even for the late developing transverse motoneurons. At this stage, the TN motor axons have exited the CNS and are navigating the periphery, although the TN motor axon and LBD fascicle have not come into contact. Misexpressing Dfr even at this relatively late stage of TN motoneuron differentiation can clearly alter axon pathfinding, and in a significant percentage of hemisegments, TN motor axons actually appear to ectopically innervate ISNb muscle targets. This result shows that these motoneurons remain plastic, even after becoming postmitotic and further indicates that the LIM/POU code may be acting directly on genes involved in axon targeting.

LIM-HD factors direct target specificity through the actions of an IgSF CAM

As described above, the combinatorial expression of LIM-HD transcription factors confers motoneuron subtypes with the ability to direct their axons to reach distinct muscle targets. If more than one subgroup of motoneurons use a LIM code, how does subtype-specific motor axon pathfinding occur? Presumably, it is the downstream targets of each LIM code that confer the ability of individual or groups of motor axons to find their correct innervation targets. What might be the target(s) of the LIM code in *Drosophila* transverse neurons?

Studies in vertebrates and invertebrates have demonstrated that members of the IgSF class of CAMs play important roles in cell-cell recognition and communication – processes that are crucial for nervous system wiring (reviewed by Brummendorf and Lemmon, 2001; Rougon and Hobert, 2003). The ability of Ig-domains to form linear rods when deployed in series, and their propensity to bind specifically to other proteins, has made these molecules ideal for functioning as cell-surface receptors and/or CAMs. Furthermore, IgSF molecules have dramatically increased the number of cell-cell recognition molecules through family expansion, the generation of multiple variants through alternative splicing, receptor multimerization and cross-talking intracellular signaling pathways (Brummendorf and Lemmon, 2001; Rougon and Hobert, 2003).

A recent genomic analysis indicates the *Drosophila* IgSF repertoire consists of about 150 proteins; in *C. elegans*, 80 IgSF molecules are predicted (Aurelio et al., 2002; Hutter et al., 2000; Hynes and Zhao, 2000; Vogel et al., 2003). Members of the Beat family in *Drosophila* and the *zig* family in *C. elegans* are located in gene clusters, have restricted expression patterns and share the same domain architecture (Aurelio et al., 2002; Fambrough and Goodman, 1996; Pipes et al., 2001; Vogel et al., 2003). Each protein is comprised exclusively of two Ig modules with either a transmembrane domain [*beat Ic*, *beat Ib*, *beat IIa*, *beat VI (GPI)*, *zig-1*] or secreted signals (10 Beat genes: *zig-2* to *zig-8*) (Aurelio et al., 2002; Hutter et al., 2000; Hynes and Zhao, 2000; Vogel et al., 2003). The Beat family members that have been functionally characterized appear to control fasciculation through anti- and pro-adhesive properties (Fambrough and Goodman, 1996; Pipes et al., 2001). Furthermore, unlike many previously described CAMs, several members of these families are expressed in subsets of CNS neurons.

beat Ic is transcribed in a small number of cells in the embryonic nerve cord, including the transverse motoneurons (Pipes et al., 2001) (this study). Loss-of-function experiments indicate Beat Ic is required in a pro-adhesive manner for the proper recognition/or fasciculation of the TMNp motor axon and the LBD fascicle. These TN defects are identical to the fasciculation defects we observe in *isl* and *Lim3* mutants. Our trans-heterozygous combinations reveal strong genetic interactions between *isl*, *Lim3* and *beat Ic*, and furthermore, we found that increasing Beat Ic expression in *isl* and *Lim3* mutants significantly rescues these TN axon fasciculation defects. Using in situ hybridization, we are unable to determine if *beat Ic* TN expression is dependent upon Isl and Lim3 function. However, DNA-binding site pattern searches (Freeman et al., 2003; Rebeiz and Posakony, 2004) using the described LIM-HD-binding motifs indicate that there are significant clusters of LIM-HD sites surrounding and within the *beat Ic* locus.

The downstream targets of combinatorial codes that control motor axon pathway selection in vertebrates and invertebrates, as well as the subtype-specific axon guidance cues remain poorly understood. Our experiments suggest that the LIM-HD code in *Drosophila* may specify a subset of axon target selection through the actions of IgSF CAMs. Previous studies have suggested that Beat Ic does not function as a homophilic adhesion molecule (Pipes et al., 2001). This leads to the hypothesis that the function of Beat Ic is mediated by heterophilic binding to an unknown Beat partner protein.

Therefore, the further characterization of both secreted and transmembrane IgSF molecules that exhibit restricted neuronal expression might provide a mechanism with which to refine subtype-specific signals through restricted cues or altering adhesive properties.

We are grateful to W. A. Johnson, G. C. T. Pipes, C. S. Goodman, J. B. Thomas, J. Roote, M. Ashburner and J. B. Skeath for flies and reagents. We thank the Bloomington Stock Center and the Developmental Hybridoma Center for antibodies. We are grateful to members of the Thor laboratory for comments and suggestions. We thank David Van Vactor, Dietmar Schmucker, Wayne Johnson and Kaan Certel for critically reading the manuscript. S.J.C. was supported by an NRSA Service Award (T32 NS07484-01) and this work was funded by grants from NIH (RO1 NS39875-01) and by the Freudenberger Scholarship Fund at Harvard Medical School (to S.T.).

References

Anderson, M. G., Perkins, G. L., Chittick, P., Shrigley, R. J. and Johnson, W. A. (1995). *drifter*, a *Drosophila* POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. *Genes Dev.* **9**, 123-137.

Aurelio, O., Hall, D. H., Hobert, O. and Boulin, T. (2002). Immunoglobulin-domain proteins required for maintenance of ventral nerve cord organization. Identification of spatial and temporal cues that regulate postembryonic expression of axon maintenance factors in the C. elegans ventral nerve cord. *Science* **295**, 686-690.

Aurelio, O., Boulin, T. and Hobert, O. (2003). Identification of spatial and temporal cues that regulate postembryonic expression of axon maintenance factors in the C. elegans ventral nerve cord. *Development* **130**, 599-610.

Bach, I. (2000). The LIM domain: regulation by association. *Mech. Dev.* **91**, 5-17.

Bach, I., Rhodes, S. J., Pearse, R. V., 2nd, Heinzel, T., Gloss, B., Scully, K. M., Sawchenko, P. E. and Rosenfeld, M. G. (1995). P-Lim, a LIM homeodomain factor, is expressed during pituitary organ and cell commitment and synergizes with Pit-1. *Proc. Natl. Acad. Sci. USA* **92**, 2720-2724.

Boam, D. S. and Docherty, K. (1989). A tissue-specific nuclear factor binds to multiple sites in the human insulin-gene enhancer. *Biochem. J.* **264**, 233-239.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Breen, J. J., Agulnick, A. D., Westphal, H. and Dawid, I. B. (1998). Interactions between LIM domains and the LIM domain-binding protein Ldb1. *J. Biol. Chem.* **273**, 4712-4717.

Bridwell, J. A., Price, J. R., Parker, G. E., McCutchan Schiller, A., Sloop, K. W. and Rhodes, S. J. (2001). Role of the LIM domains in DNA recognition by the Lhx3 neuroendocrine transcription factor. *Gene* **277**, 239-250.

Broihier, H. T. and Skeath, J. B. (2002). Drosophila homeodomain protein dHb9 directs neuronal fate via crossrepressive and cell-nonautonomous mechanisms. *Neuron* **35**, 39-50.

Brummendorf, T. and Lemmon, V. (2001). Immunoglobulin superfamily receptors: cis-interactions, intracellular adapters and alternative splicing regulate adhesion. *Curr. Opin. Cell Biol.* **13**, 611-618.

Campos-Ortega, J. A. and Hartenstein, V. (1985). *The Embryonic Development of Drosophila melanogaster*. Heidelberg, Germany: Springer-Verlag.

Certel, S. J. and Johnson, W. A. (1996). Disruption of mesectodermal lineages by temporal misexpression of the *Drosophila* POU-domain transcription factor, *Drifter*. *Dev. Genet.* **18**, 279-288.

Certel, K., Hudson, A., Carroll, S. B. and Johnson, W. A. (2000). Restricted patterning of vestigial expression in Drosophila wing imaginal discs requires synergistic activation by both Mad and the drifter POU domain transcription factor. *Development* **127**, 3173-3183.

Chen, L., Segal, D., Hukriede, N. A., Podtelejnikov, A. V., Bayarsaihan, D., Kennison, J. A., Ogryzko, V. V., Dawid, I. B. and Westphal, H. (2002). Ssdp proteins interact with the LIM-domain-binding protein Ldb1 to regulate development. *Proc. Natl. Acad. Sci. USA* **99**, 14320-14325.

Crowner, D., Madden, K., Goeke, S. and Giniger, E. (2002). Lola regulates midline crossing of CNS axons in Drosophila. *Development* **129**, 1317-1325.

Dickson, B. J. (2002). Molecular mechanisms of axon guidance. *Science* **298**, 1959-1964.

Dittrich, R., Bossing, T., Gould, A. P., Technau, G. M. and Urban, J. (1997). The differentiation of the serotonergic neurons in the Drosophila ventral nerve cord depends on the combined function of the zinc finger proteins Eagle and Huckebein. *Development* **124**, 2515-2525.

Duggan, A., Ma, C. and Chalfie, M. (1998). Regulation of touch receptor differentiation by the Caenorhabditis elegans mec-3 and unc-86 genes. *Development* **125**, 4107-4119.

Erkman, L., Yates, P. A., McLaughlin, T., McEvilly, R. J., Whisenhunt, T., O'Connell, S. M., Krones, A. I., Kirby, M. A., Rapaport, D. H., Bermingham, J. R. et al. (2000). A POU domain transcription factor-dependent program regulates axon pathfinding in the vertebrate visual system. *Neuron* **28**, 779-792.

Fambrough, D. and Goodman, C. S. (1996). The Drosophila beaten path gene encodes a novel secreted protein that regulates defasciculation at motor axon choice points. *Cell* **87**, 1049-1058.

Freeman, M. R., Delrow, J., Kim, J., Johnson, E. and Doe, C. Q. (2003). Unwrapping glial biology: Gcm target genes regulating glial development, diversification, and function. *Neuron* **38**, 567-580.

Gill, G. N. (2003). Decoding the LIM development code. *Trans. Am. Clin. Climatol. Assoc.* **114**, 179-189.

Gorczyca, M. G., Phillis, R. W. and Budnik, V. (1994). The role of tinman, a mesodermal cell fate gene, in axon pathfinding during the development of the transverse nerve in Drosophila. *Development* **120**, 2143-2152.

Grenningloh, G., Rehm, E. J. and Goodman, C. S. (1991). Genetic analysis of growth cone guidance in Drosophila: fasciclin II functions as a neuronal recognition molecule. *Cell* **67**, 45-57.

Higashijima, S., Shishido, E., Matsuzaki, M. and Saigo, K. (1996). eagle, a member of the steroid receptor gene superfamily, is expressed in a subset of neuroblasts and regulates the fate of their putative progeny in the Drosophila CNS. *Development* **122**, 527-536.

Huber, A. B., Kolodkin, A. L., Ginty, D. D. and Cloutier, J. F. (2003). Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annu. Rev. Neurosci.* **26**, 509-563.

Hutter, H., Vogel, B. E., Plenefisch, J. D., Norris, C. R., Proenca, R. B., Spieth, J., Guo, C., Mastwal, S., Zhu, X., Scheel, J. et al. (2000). Conservation and novelty in the evolution of cell adhesion and extracellular matrix genes. *Science* **287**, 989-994.

Hynes, R. O. and Zhao, Q. (2000). The evolution of cell adhesion. *J. Cell Biol.* **150**, F89-F96.

Inbal, A., Levanon, D. and Salzberg, A. (2003). Multiple roles for u-turn/ventral veinless in the development of Drosophila PNS. *Development* **130**, 2467-2478.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.

Jurata, L. W. and Gill, G. N. (1997). Functional analysis of the nuclear LIM domain interactor NLI. *Mol. Cell. Biol.* **17**, 5688-5698.

Jurata, L. W., Pfaff, S. L. and Gill, G. N. (1998). The nuclear LIM domain interactor NLI mediates homo- and heterodimerization of LIM domain transcription factors. *J. Biol. Chem.* **273**, 3152-3157.

Kania, A. and Jessell, T. M. (2003). Topographic motor projections in the limb imposed by LIM homeodomain protein regulation of ephrin-A: EphA interactions. *Neuron* **38**, 581-596.

Karlsson, O., Thor, S., Norberg, T., Ohlsson, H. and Edlund, T. (1990). Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. *Nature* **344**, 879-882.

Komiyama, T., Johnson, W. A., Luo, L. and Jefferis, G. S. (2003). From lineage to wiring specificity. POU domain transcription factors control precise connections of Drosophila olfactory projection neurons. *Cell* **112**, 157-167.

Landgraf, M., Bossing, T., Technau, G. M. and Bate, M. (1997). The origin, location, and projections of the embryonic abdominal motorneurons of *Drosophila*. *J. Neurosci.* **17**, 9642-9655.

Lee, S. K. and Pfaff, S. L. (2003). Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors. *Neuron* **38**, 731-745.

Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of Drosophila melanogaster*. San Diego, CA: Academic Press.

McEvilly, R. J., de Diaz, M. O., Schonemann, M. D., Hooshmand, F. and

Rosenfeld, M. G. (2002). Transcriptional regulation of cortical neuron migration by POU domain factors. *Science* **295**, 1528-1532.
O'Neill, J. W. and Bier, E. (1994). Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes. *Biotechniques* **17**, 874-875.
Odden, J. P., Holbrook, S. and Doe, C. Q. (2002). Drosophila HB9 is expressed in a subset of motoneurons and interneurons, where it regulates gene expression and axon pathfinding. *J. Neurosci.* **22**, 9143-9149.
Olson, P. F., Fessler, L. I., Nelson, R. E., Sterne, R. E., Campbell, A. G. and Fessler, J. H. (1990). Glutactin, a novel Drosophila basement membrane-related glycoprotein with sequence similarity to serine esterases. *EMBO J.* **9**, 1219-1227.
Pipes, G. C., Lin, Q., Riley, S. E. and Goodman, C. S. (2001). The Beat generation: a multigene family encoding IgSF proteins related to the Beat axon guidance molecule in Drosophila. *Development* **128**, 4545-4552.
Rebeiz, M. and Posakony, J. W. (2004). GenePalette: a universal software tool for genome sequence visualization and analysis. *Dev. Biol.* **271**, 431-438.
Rockelein, I., Rohrig, S., Donhauser, R., Eimer, S. and Baumeister, R. (2000). Identification of amino acid residues in the Caenorhabditis elegans POU protein UNC-86 that mediate UNC-86-MEC-3-DNA ternary complex formation. *Mol. Cell. Biol.* **20**, 4806-4813.
Rothberg, J. M., Jacobs, J. R., Goodman, C. S. and Artavanis-Tsakonas, S. (1990). slit: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains. *Genes Dev.* **4**, 2169-2187.
Rougon, G. and Hobert, O. (2003). New insights into the diversity and function of neuronal immunoglobulin superfamily molecules. *Annu. Rev. Neurosci.* **26**, 207-238.
Schmid, A., Chiba, A. and Doe, C. Q. (1999). Clonal analysis of Drosophila embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* **126**, 4653-4689.
Shen, K. (2004). Molecular mechanisms of target specificity during synapse formation. *Curr. Opin. Neurobiol.* **14**, 83-88.
Shirasaki, R. and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. *Annu. Rev. Neurosci.* **25**, 251-281.
Skeath, J. B. and Thor, S. (2003). Genetic control of Drosophila nerve cord development. *Curr. Opin. Neurobiol.* **13**, 8-15.
Spradling, A. C. (1986). P element-mediated transformation. In Drosophila, *A Practical Approach* (ed. D. B. Roberts), pp. 175-197. Oxford, UK: IRL Press.
Sugitani, Y., Nakai, S., Minowa, O., Nishi, M., Jishage, K., Kawano, H., Mori, K., Ogawa, M. and Noda, T. (2002). Brn-1 and Brn-2 share crucial roles in the production and positioning of mouse neocortical neurons. *Genes Dev.* **16**, 1760-1765.
Sze, J. Y., Zhang, S., Li, J. and Ruvkun, G. (2002). The C. elegans POU-domain transcription factor UNC-86 regulates the tph-1 tryptophan hydroxylase gene and neurite outgrowth in specific serotonergic neurons. *Development* **129**, 3901-3911.
Thaler, J. P., Lee, S. K., Jurata, L. W., Gill, G. N. and Pfaff, S. L. (2002). LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions. *Cell* **110**, 237-249.
Thor, S. and Thomas, J. B. (1997). The Drosophila islet gene governs axon pathfinding and neurotransmitter identity. *Neuron* **18**, 397-409.
Thor, S., Andersson, S. G., Tomlinson, A. and Thomas, J. B. (1999). A LIM-homeodomain combinatorial code for motor-neuron pathway selection. *Nature* **397**, 76-80.
Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.
van Meyel, D. J., O'Keefe, D. D., Jurata, L. W., Thor, S., Gill, G. N. and Thomas, J. B. (1999). Chip and apterous physically interact to form a functional complex during Drosophila development. *Mol. Cell* **4**, 259-265.
van Meyel, D. J., Thomas, J. B. and Agulnick, A. D. (2003). Ssdp proteins bind to LIM-interacting co-factors and regulate the activity of LIM-homeodomain protein complexes in vivo. *Development* **130**, 1915-1925.
Vogel, C., Teichmann, S. A. and Chothia, C. (2003). The immunoglobulin superfamily in Drosophila melanogaster and Caenorhabditis elegans and the evolution of complexity. *Development* **130**, 6317-6328.
Wright, T. R., Hodgetts, R. B. and Sherald, A. F. (1976). The genetics of dopa decarboxylase in Drosophila melanogaster. I. Isolation and characterization of deficiencies that delete the dopa-decarboxylase-dosage-sensitive region and the alpha-methyl-dopa-hypersensitive locus. *Genetics* **84**, 267-285.
Xue, D., Finney, M., Ruvkun, G. and Chalfie, M. (1992). Regulation of the mec-3 gene by the C. elegans homeoproteins UNC-86 and MEC-3. *EMBO J.* **11**, 4969-4979.
Xue, D., Tu, Y. and Chalfie, M. (1993). Cooperative interactions between the Caenorhabditis elegans homeoproteins UNC-86 and MEC-3. *Science* **261**, 1324-1328.