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

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**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 cell-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 versus 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 motorneuron 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 motorneuron subclasses. Dfr is co-expressed with Isl and Lim3 only in the ISNb motorneurons; 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 into 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 motorneuron subclasses. A recent report described the TN motorneuron expression of the cell-adhesion molecule (CAM) Beat Ic. Beat Ic belongs to a multigene family in Drosophila that encodes 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 motorneuron 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 standard stocks and balancer chromosomes not specifically mentioned in the text are standard. Stages of embryonic development are according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985).

Materials and methods

Immunohistochemistry

Staged embryos were labeled using a modification of protocols previously described (Certel and Johnson, 1996). The following primary antibodies were used: rabbit anti-FGF (1:500) (Molecular Probes), rabbit anti-Glutactin (1:300) (Olson et al., 1990), mAb 1D4 anti-Past2 (1:50), mAb 3A4 anti-Islet1/2 (1:20) (Tsuchida et al., 1994), mAb 9E10 anti-Myc (1:25) and mAb C556D 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 (Molecular Probes), Rhodamine-Red X-conjugated donkey anti-mouse, donkey anti-rabbit (Jackson ImmunoResearch Laboratories), Cy5-conjugated donkey anti-rabbit, 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-FGF 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 EcoRI/NrI 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-dsdr 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 EcoRI site at the 517 bp end, dfr5RI-GTCGAAATCTGATGGCTCACGGTTC and a SfiI site at the 1308 end, dfr3SfiI-GTCGGCAATCTTGGCCGTCTGTAACCTATCGGTCA. The second product had an XhoI site at the 517 end dfr5XhoI-GTCGAGAAGACGGTTGCCTCACGGTTC and a SfiI site at the 1308 end, dfr3SfiI(2)-GTCGAAATCTTGGCCGTCTGTAACCTATCGGTCA. Underlined sequences denote the central nonpalindromic core of each site. This 791 bp fragment does not include the POU domain. After digestion with SfiI, the two products were ligated together. Dimers were digested with EcoRI and XhoI 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, 12, 13, 14, 28 and 30. The TN and ISNd motoneuron subclasses both express a combination of Isl and Lim3, it is likely that other factors act to discriminate between these two subclasses.

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 egP289 lacZ enhancer trap line. β-Gal (green) expression is visible in the three EW neurons and the GW (ISNd) motoneuron. Dfr (red) is not expressed in the GW (ISNd) motoneuron (arrow). (F) Dfr expression (green) is unaffected in an isl279/islP 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 dfr254/dfrP Lim3A-tau-myc transgenic embryos.
Dfr expression is not detected in the pair of Isl- and Lim3-expressing TN neurons (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, egP289 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 targeting the TN fascicle (Fig. 2C, arrowhead). (D) In Isl3B/Bd6/+; dfrB129/+ embryos, the ISNb motor axons fail to project to and innervate their specific target muscles, including muscles 12 and 13 (arrow). (C) Muscle innervation is also affected in islb3N/+; dfrB129/+ embryos (arrow) and in some hemisegments, the ISNb motor axons target the TN fascicle (arrowhead). (D) Lim3B/Bd6/+; dfrB129/+ embryos also show severe defects in motor axon targeting and muscle innervation (arrow).

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 islb3N/Lim3B/Bd6/+; dfrB129/+ 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 islb3N/+; dfrB129/+ embryos (arrow) and in some hemisegments, the ISNb motor axons target the TN fascicle (arrowhead). (D) Lim3B/Bd6/+; dfrB129/+ embryos also show severe defects in motor axon targeting and muscle innervation (arrow).

To further confirm 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 exec/hb9 gene (Broihier and Skeath, 2002; Odden et al., 2002). However, 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 exec/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 (dfrB129/+; hb9kk36; 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
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, dfrB129. Although the protein produced by this allele is detected by the Dfr antisera, 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 (ftzGal4,20) 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.

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Failure to innervate muscles 12 and 13 (%)</th>
<th>ISNb axons crossing to TN (%)</th>
<th>Number of hemisegments examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(2L)E71/+</td>
<td>1.8</td>
<td>3.5</td>
<td>57</td>
</tr>
<tr>
<td>Df(2L)E71/+; dfrB129/+</td>
<td>22.0</td>
<td>16.5</td>
<td>79</td>
</tr>
<tr>
<td>isl377Aa/+</td>
<td>2.0</td>
<td>3.0</td>
<td>82</td>
</tr>
<tr>
<td>isl377Aa/+; dfrB129/+</td>
<td>21.0</td>
<td>16.0</td>
<td>129</td>
</tr>
<tr>
<td>Lim3BD5/+</td>
<td>1.5</td>
<td>2.8</td>
<td>87</td>
</tr>
<tr>
<td>Lim3BD5/+; dfrB129/+</td>
<td>29.0</td>
<td>24.0</td>
<td>99</td>
</tr>
<tr>
<td>dfrB129/+</td>
<td>1.5</td>
<td>4.0</td>
<td>136</td>
</tr>
</tbody>
</table>

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.

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

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 this 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, MECl-3 (Xue et al., 1992; Xue et al., 1993). And in vertebrates, abLIM is a transcriptional target of the POU factor, Brn3.2 (Erickman 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 1c, 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-tau/mycEGFP 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 fascication 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 IcP9 and beat IcP3 (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-tau/mycEGFP 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 IcP9/beat IcP3(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, islP9P9/islP3P3 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, islP9P9/Lim38R0P8 beat IcP9 (C) and islP9P9/Lim38R0P8 beat IcP3P3 (E). (G) Schematic diagram of the beat Ic locus. Isl1 (CTAAATG) and Lhx3 (AATTAATTA) consensus sites within this locus are graphically represented.](image-url)
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 Beat family members because neither overlapping deficiencies nor individual mutations are available for these genes.

At least two possible explanations can be put forward to explain the TN defects observed in islet 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 islet and Lim3 mutants. To increase Beat Ic levels, the pan-neuronal driver elav-Gal4 and UAS-beat Ic transgenes were crossed into islet and Lim3 mutant backgrounds. As in previous experiments, strong islet and Lim3 alleles were crossed to isletP1 and Lim3P2 deficiencies to create embryos null for islet and Lim3, respectively.

Increasing the levels of Beat Ic through a single copy of UAS-beat Ic and the elav-Gal4 driver significantly reduced the percentage of TN defects in islet 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 islet 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 islet 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 Is1 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 Is1 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 Is1 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, Is11 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.

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No TN (%)</th>
<th>TN fasciculation defects (%)</th>
<th>Number of hemisegments examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>isl–/– (isl17A,isl1Df)</td>
<td>29.0</td>
<td>27.0</td>
<td>89</td>
</tr>
<tr>
<td>Lim3–/– (Lim3,isl1Df)</td>
<td>19.0</td>
<td>38.0</td>
<td>79</td>
</tr>
<tr>
<td>isl /beat Ic–/– (isl17A,isl1Df)</td>
<td>11.5</td>
<td>42.0</td>
<td>95</td>
</tr>
<tr>
<td>Lim3–/–beat Ic–/– (isl17A,isl1Df)</td>
<td>17.0</td>
<td>25.5</td>
<td>47</td>
</tr>
<tr>
<td>isl–/–beat Ic–/– (isl17A,isl1Df,beat Ic)</td>
<td>9.3</td>
<td>36.0</td>
<td>86</td>
</tr>
<tr>
<td>isl–/–Lim3–/–beat Ic–/– (isl17A,isl1Df,beat Ic)</td>
<td>4.0</td>
<td>46.0</td>
<td>80</td>
</tr>
</tbody>
</table>

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.

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*References and further reading are included in the original text.*

*Note: The above content is a representation of the text from the document, formatted to remove any unnecessary or irrelevant details, and presented in a readable and understandable manner.*
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, MECP-3 and the POU protein UNC-86, physically interact to control specification (Rockekein 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 or a transmembrane domain [beat Ic, beat Ib, beat Ia, 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 TMSNp 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.

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